Please check our wiki for help on navigating the form.

Horizon 2020

Call: H2020-ICT-2018-20

(Information and Communication Technologies)

Topic: ICT-36-2020

Type of action: RIA

Proposal number: 101017180

Proposal acronym: NanoVIB

Deadline Id: H2020-ICT-2020-2

Table of contents

How to fill in the forms

The administrative forms must be filled in for each proposal using the templates available in the submission system. Some data fields in the administrative forms are pre-filled based on the steps in the submission wizard.

1 - General information

Topic	ICT-36-2020	Type of Action	RIA
Call Identifier	H2020-ICT-2018-20	Deadline Id	H2020-ICT-2020-2

Acronym	NanoVIB
Proposal title	NANO-scale VIsualization to understand Bacterial virulence and invasiveness - based on fluorescence NANOscopy and VIBrational microscopy

Note that for technical reasons, the following characters are not accepted in the Proposal Title and will be removed: < > " &

Duration in months	*48*
Fixed keyword 1	Photonics
Free keywords	*super-resolution microscopy, MINFLUX, fluorescence, Stimulated Raman scattering, pneumococci, virulence, invasiveness*

Abstract

In an interdisciplinary project, we will prototype a next-generation super-resolution microscope (SRM) and demonstrate its capability to bring about a major leap forward in our understanding of inter- and intracellular processes, and thus the cellular origin of diseases. Based on the recently invented MINFLUX concept, which pushes spatial resolution an order of magnitude beyond any other SRM technique, and by concerted development of detector technologies, lasers and image acquisition procedures, we will be able to retrieve information, not within reach by any other photonics-based technique. By extending operation to the near infrared, a hitherto un-accessible spectral range for SRM, we will strongly reduce phototoxicity and scattering, increase penetration depth and provide an additional spectral window for multiplexing. The developed prototype will allow nanometer-scale protein localization patterns to be resolved and to be placed in a cellular context by overlaid morphological, biochemical and metabolic images generated by label-free stimulated Raman scattering (SRS) and two-photon excitation (TPE).
In a lead application, we will use the unique capabilities of the to-be-developed technology to study the molecular mechanisms underlying pneumococcal disease, largely attributed to localization patterns of specific bacterial surface proteins, and their intricate interactions with immune and host target cells. Pneumococci are a major contributor to morbidity and mortality worldwide and we aim to provide vital information which can lead to new treatments and vaccines. We will also offer hands-on access to the technology to researchers from both academia and industry in an open demonstration facility. Together with the lead application, this will generate demand for microscopes, lasers and detectors, which the industrial partners will develop subsequent to this project based on the prototypes, further strengthening Europe´s industrial position in the microscopy field.

Remaining characters 1

Has this proposal (or a very similar one) been submitted in the past 2 years in response to a call for proposals under Horizon 2020 or any other EU programme(s)? ○ Yes ◉ No

Please give the proposal reference or contract number.	
xxxxxx-x	

Declarations

1) The coordinator declares to have the explicit consent of all applicants on their participation and on the content of this proposal.	☒
2) The information contained in this proposal is correct and complete.	☒
3) This proposal complies with ethical principles (including the highest standards of research integrity — as set out, for instance, in the European Code of Conduct for Research Integrity — and including, in particular, avoiding fabrication, falsification, plagiarism or other research misconduct).	☒

4) The coordinator confirms:

- to have carried out the self-check of the financial capacity of the organisation on http://ec.europa.eu/research/participants/portal/desktop/en/organisations/lfv.html or to be covered by a financial viability check in an EU project for the last closed financial year. Where the result was "weak" or "insufficient", the coordinator confirms being aware of the measures that may be imposed in accordance with the H2020 Grants Manual (Chapter on Financial capacity check); or	◉
- is exempt from the financial capacity check being a public body including international organisations, higher or secondary education establishment or a legal entity, whose viability is guaranteed by a Member State or associated country, as defined in the H2020 Grants Manual (Chapter on Financial capacity check); or	○
- as sole participant in the proposal is exempt from the financial capacity check.	○

5) The coordinator hereby declares that each applicant has confirmed:

- they are fully eligible in accordance with the criteria set out in the specific call for proposals; and	☒
- they have the financial and operational capacity to carry out the proposed action.	☒

The coordinator is only responsible for the correctness of the information relating to his/her own organisation. Each applicant remains responsible for the correctness of the information related to him and declared above. Where the proposal to be retained for EU funding, the coordinator and each beneficiary applicant will be required to present a formal declaration in this respect.

According to Article 131 of the Financial Regulation of 25 October 2012 on the financial rules applicable to the general budget of the Union (Official Journal L 298 of 26.10.2012, p. 1) and Article 145 of its Rules of Application (Official Journal L 362, 31.12.2012, p.1) applicants found guilty of misrepresentation may be subject to administrative and financial penalties under certain conditions.

Personal data protection

The assessment of your grant application will involve the collection and processing of personal data (such as your name, address and CV), which will be performed pursuant to Regulation (EC) No 45/2001 on the protection of individuals with regard to the processing of personal data by the Community institutions and bodies and on the free movement of such data. Unless indicated otherwise, your replies to the questions in this form and any personal data requested are required to assess your grant application in accordance with the specifications of the call for proposals and will be processed solely for that purpose. Details concerning the purposes and means of the processing of your personal data as well as information on how to exercise your rights are available in the privacy statement. Applicants may lodge a complaint about the processing of their personal data with the European Data Protection Supervisor at any time.

Your personal data may be registered in the Early Detection and Exclusion system of the European Commission (EDES), the new system established by the Commission to reinforce the protection of the Union's financial interests and to ensure sound financial management, in accordance with the provisions of articles 105a and 108 of the revised EU Financial Regulation (FR) (Regulation (EU, EURATOM) 2015/1929 of the European Parliament and of the Council of 28 October 2015 amending Regulation (EU, EURATOM) No 966/2012) and articles 143 - 144 of the corresponding Rules of Application (RAP) (COMMISSION DELEGATED REGULATION (EU) 2015/2462 of 30 October 2015 amending Delegated Regulation (EU) No 1268/2012) for more information see the Privacy statement for the EDES Database.

2 - Participants & contacts

#	Participant Legal Name	Country	Action
1	KUNGLIGA TEKNISKA HOEGSKOLAN	SE	
2	KAROLINSKA INSTITUTET	Sweden	
3	Abberior Instruments GmbH	DE	
4	Laser-Laboratorium Göttingen e.V.	Germany	
5	APE ANGEWANDTE PHYSIK UND ELEKTRONIK GMBH	DE	
6	Pi Imaging Technology SA	Switzerland	

2 - Administrative data of participating organisations

PIC	***Legal name***
999990946	*KUNGLIGA TEKNISKA HOEGSKOLAN*

Short name: KTH

Address of the organisation

Street BRINELLVAGEN 8

Town STOCKHOLM

Postcode 100 44

Country Sweden

Webpage www.kth.se

Legal Status of your organisation

Research and Innovation legal statuses

Public body .....yes

Non-profit .....yes

International organisation .....no

International organisation of European interest .....no

Secondary or Higher education establishment .....yes

Research organisation .....no

Legal person .....yes

Industry (private for profit).....no

Enterprise Data

SME self-declared status.....23/09/2008 - no

SME self-assessment ..... unknown

SME validation sme.....31/12/2011 - no

Based on the above details of the Beneficiary Registry the organisation is not an SME (small- and medium-sized enterprise) for the call.

Department(s) carrying out the proposed work

Department 1

Department name: Applied Physics/Exp Biomol Physics ☐ not applicable

☐ Same as proposing organisation's address

Street: Albanova Univ Center, Roslagstullsb. 21

Town: Stockholm

Postcode: 106 91

Country: Sweden

Dependencies with other proposal participants

Character of dependence	***Participant***	

Person in charge of the proposal

The name and e-mail of contact persons are read-only in the administrative form, only additional details can be edited here. To give access rights and basic contact details of contact persons, please go back to Step 4 of the submission wizard and save the changes.

Title: Prof.

Sex: (•) Male ( ) Female

First name: **Jerker**

Last name: **WIDENGREN**

E-Mail: **jwideng@kth.se**

Position in org.: Professor, unit head

Department: Applied Physics/ QBP/ Exp Biomol Physics

☐ Same as organisation name

☐ Same as proposing organisation's address

Street: Albanova Univ Center, Roslagstullb 21

Town: Stockholm

Post code: 106 91

Country: Sweden

Website: www.aphys.kth.se

Phone: +46-8-7907813

Phone 2: +46-73-2703768

Fax: *+xxx xxxxxxxxx*

PIC	***Legal name***
999978530	*KAROLINSKA INSTITUTET*

Short name: KI

Address of the organisation

Street Nobels Vag 5

Town STOCKHOLM

Postcode 17177

Country Sweden

Webpage www.ki.se

Legal Status of your organisation

Research and Innovation legal statuses

Public body .....yes

Non-profit .....yes

International organisation .....no

International organisation of European interest .....no

Secondary or Higher education establishment .....yes

Research organisation .....no

Legal person .....yes

Industry (private for profit).....no

Enterprise Data

SME self-declared status..... unknown

SME self-assessment ..... unknown

SME validation sme..... unknown

Based on the above details of the Beneficiary Registry the organisation is not an SME (small- and medium-sized enterprise) for the call.

Department(s) carrying out the proposed work

Department 1

Department name: C1 Department of Microbiology, Tumor and Cell Biology ☐ not applicable

☐ Same as proposing organisation's address

Street: Nobelsväg 16

Town: Stockholm

Postcode: 17177

Country: Sweden

Dependencies with other proposal participants

Character of dependence	***Participant***	

Person in charge of the proposal

The name and e-mail of contact persons are read-only in the administrative form, only additional details can be edited here. To give access rights and basic contact details of contact persons, please go back to Step 4 of the submission wizard and save the changes.

Title: Prof.

Sex: ○ Male ◉ Female

First name: **Birgitta**

Last name: **Henriques-Normark**

E-Mail: **birgitta.henriques@ki.se**

Position in org.: Professor of Clinical Microbiology

Department: C1 Department of Microbiology, Tumor and Cell Biology ☐ Same as organisation name

☐ Same as proposing organisation's address

Street: Nobelsväg 16

Town: Stockholm Post code: 17177

Country: Sweden

Website: https://ki.se/en/mtc/birgitta-henriques-normark-group?_ga=2.22355

Phone: +46-(0)8-517 712 16 Phone 2: *+xxx xxxxxxxxx* Fax: *+xxx xxxxxxxxx*

PIC	***Legal name***
922833751	*Abberior Instruments GmbH*

Short name: Abberior Instruments

Address of the organisation

Street Hans-Adolf-Krebs-Weg 1

Town Göttingen

Postcode 37077

Country Germany

Webpage www.abberior-instruments.com

Legal Status of your organisation

Research and Innovation legal statuses

Public body .....no

Non-profit .....no

International organisation .....no

International organisation of European interest .....no

Secondary or Higher education establishment .....no

Research organisation .....no

Legal person .....yes

Industry (private for profit).....yes

Enterprise Data

SME self-declared status.....06/03/2019 - yes

SME self-assessment ..... unknown

SME validation sme..... unknown

Based on the above details of the Beneficiary Registry the organisation is an SME (small- and medium-sized enterprise) for the call.

Department(s) carrying out the proposed work

No department involved

Department name: *Name of the department/institute carrying out the work.* ☒ not applicable

☐ Same as proposing organisation's address

Street: *Please enter street name and number.*

Town: *Please enter the name of the town.*

Postcode: *Area code.*

Country: *Please select a country*

Dependencies with other proposal participants

Character of dependence	***Participant***	

Person in charge of the proposal

The name and e-mail of contact persons are read-only in the administrative form, only additional details can be edited here. To give access rights and basic contact details of contact persons, please go back to Step 4 of the submission wizard and save the changes.

Title: Dr. | Sex: (•) Male ( ) Female

First name: **Andreas** | Last name: **Schönle**

E-Mail: **a.schoenle@abberior-instruments.com**

Position in org.: Head of Innovation and Intelligence

Department: Abberior Instruments GmbH ☒ Same as organisation name

☒ Same as proposing organisation's address

Street: Hans-Adolf-Krebs-Weg 1

Town: Göttingen | Post code: 37077

Country: Germany

Website: https://abberior-instruments.com

Phone: +49 551 30724 176 | Phone 2: +49 160 8409585 | Fax: +49 551 30724 171

PIC	***Legal name***
953856970	*Laser-Laboratorium Göttingen e.V.*

Short name: Laser-Laboratorium Göttingen e.V.

Address of the organisation

Street Hans-Adolf-Krebs Weg 1

Town Göttingen

Postcode 37077

Country Germany

Webpage www.llg-ev.de

Legal Status of your organisation

Research and Innovation legal statuses

Public body .....yes

Non-profit .....yes

International organisation .....no

International organisation of European interest .....no

Secondary or Higher education establishment .....no

Research organisation .....yes

Legal person .....yes

Industry (private for profit).....no

Enterprise Data

SME self-declared status..... unknown

SME self-assessment ..... unknown

SME validation sme..... unknown

Based on the above details of the Beneficiary Registry the organisation is not an SME (small- and medium-sized enterprise) for the call.

Department(s) carrying out the proposed work

Department 1

Department name: Optical Nanoscopy ☐ not applicable

☒ Same as proposing organisation's address

Street: Hans-Adolf-Krebs Weg 1

Town: Göttingen

Postcode: 37077

Country: Germany

Dependencies with other proposal participants

Character of dependence	***Participant***	

Person in charge of the proposal

The name and e-mail of contact persons are read-only in the administrative form, only additional details can be edited here. To give access rights and basic contact details of contact persons, please go back to Step 4 of the submission wizard and save the changes.

Title: Prof.

Sex: (•) Male ( ) Female

First name: **Alexander**

Last name: **Egner**

E-Mail: **alexander.egner@llg-ev.de**

Position in org.: Director of institute and head of department

Department: Optical Nanoscopy

☐ Same as organisation name

☒ Same as proposing organisation's address

Street: Hans-Adolf-Krebs Weg 1

Town: Göttingen

Post code: 37077

Country: Germany

Website: https://www.llg-ev.de

Phone: +49 (0)551 5035 35

Phone 2: *+xxx xxxxxxxxx*

Fax: +49 (0)551 5035 99

PIC	***Legal name***
989563834	*APE ANGEWANDTE PHYSIK UND ELEKTRONIK GMBH*

Short name: APE

Address of the organisation

Street PLAUENER STRASSE 163 165

Town BERLIN

Postcode 13053

Country Germany

Webpage www.ape-berlin.de

Legal Status of your organisation

Research and Innovation legal statuses

Public body .....no

Non-profit .....no

International organisation .....no

International organisation of European interest .....no

Secondary or Higher education establishment .....no

Research organisation .....no

Legal person .....yes

Industry (private for profit).....yes

Enterprise Data

SME self-declared status.....23/03/1993 - yes

SME self-assessment ..... unknown

SME validation sme.....23/03/1993 - yes

Based on the above details of the Beneficiary Registry the organisation is an SME (small- and medium-sized enterprise) for the call.

Department(s) carrying out the proposed work

No department involved

Department name: *Name of the department/institute carrying out the work.* ☒ not applicable

☐ Same as proposing organisation's address

Street: *Please enter street name and number.*

Town: *Please enter the name of the town.*

Postcode: *Area code.*

Country: *Please select a country*

Dependencies with other proposal participants

Character of dependence	***Participant***	

Person in charge of the proposal

The name and e-mail of contact persons are read-only in the administrative form, only additional details can be edited here. To give access rights and basic contact details of contact persons, please go back to Step 4 of the submission wizard and save the changes.

Title: Dr. Sex: (•) Male ( ) Female

First name: **Ingo** Last name: **Rimke**

E-Mail: **ingo_rimke@ape-berlin.de**

Position in org.: Director of R&D

Department: APE ANGEWANDTE PHYSIK UND ELEKTRONIK GMBH ☒ Same as organisation name

☒ Same as proposing organisation's address

Street: PLAUENER STRASSE 163 165

Town: BERLIN Post code: 13053

Country: Germany

Website: www.ape-berlin.de

Phone: +493098601130 Phone 2: +xxx xxxxxxxxx Fax: +4930986011333

PIC	***Legal name***
895315336	*Pi Imaging Technology SA*

Short name: Pi Imaging Technology SA

Address of the organisation

Street Rue de la Pierre-â-Mazel 39

Town Neuchâtel

Postcode 2000

Country Switzerland

Webpage www.piimaging.com

Legal Status of your organisation

Research and Innovation legal statuses

Public body .....no

Non-profit .....no

International organisation .....no

International organisation of European interest .....no

Secondary or Higher education establishment .....no

Research organisation .....no

Legal person .....yes

Industry (private for profit).....yes

Enterprise Data

SME self-declared status.....13/09/2018 - yes

SME self-assessment .....13/09/2018 - yes

SME validation sme..... unknown

Based on the above details of the Beneficiary Registry the organisation is an SME (small- and medium-sized enterprise) for the call.

Department(s) carrying out the proposed work

No department involved

Department name: *Name of the department/institute carrying out the work.* ☒ not applicable

☐ Same as proposing organisation's address

Street: *Please enter street name and number.*

Town: *Please enter the name of the town.*

Postcode: *Area code.*

Country: *Please select a country*

Dependencies with other proposal participants

Character of dependence	***Participant***	

Person in charge of the proposal

The name and e-mail of contact persons are read-only in the administrative form, only additional details can be edited here. To give access rights and basic contact details of contact persons, please go back to Step 4 of the submission wizard and save the changes.

Title: Dr. Sex: (•) Male ( ) Female

First name: **Michel** Last name: **Antolovic**

E-Mail: **michel.antolovic@piimaging.com**

Position in org.: CEO

Department: Pi Imaging Technology SA ☒ Same as organisation name

☒ Same as proposing organisation's address

Street: Rue de la Pierre-â-Mazel 39

Town: Neuchâtel Post code: 2000

Country: Switzerland

Website: https://piimaging.com/

Phone: +31649790417 Phone 2: *+xxx xxxxxxxxx* Fax: *+xxx xxxxxxxxx*

3 - Budget

No	Participant	Country	(A) Direct personnel costs/€	(B) Other direct costs/€	(C) Direct costs of sub-contracting/€	(D) Direct costs of providing financial support to third parties/€	(E) Costs of inkind contributions not used on the beneficiary's premises/€	(F) Indirect Costs / € (=0.25(A+B-E))	(G) Special unit costs covering direct & indirect costs / €	(H) Total estimated eligible costs / € (=A+B+C+D+F+G)	(I) Reimburse-ment rate (%)	(J) Max.EU Contribution / € (=H*I)	(K) Requested EU Contribution/ €
1	Kungliga Tekniska Hoegskolan	SE	790400	118000	0	0	0	227100,00	0	1135500,00	100	1135500,00	1135500,00
2	Karolinska Institutet	SE	454080	68000	0	0	0	130520,00	0	652600,00	100	652600,00	652600,00
3	Abberior Instruments Gmbh	DE	530799	711500	0	0	0	310574,75	0	1552873,75	100	1552873,75	1552873,00
4	Laser-laboratorium Göttingen E.v.	DE	451125	286600	0	0	0	184431,25	0	922156,25	100	922156,25	922156,00
5	Ape Angewandte Physik Und	DE	353420	209000	0	0	0	140605,00	0	703025,00	100	703025,00	703025,00
6	Pi Imaging Technology Sa	CH	336000	199500	0	0	0	133875,00	0	669375,00	100	669375,00	669375,00
	Total		2915824	1592600	0	0	0	1127106,00	0	5635530,00		5635530,00	5635529,00

4 - Ethics

1. HUMAN EMBRYOS/FOETUSES		Page
Does your research involve Human Embryonic Stem Cells (hESCs)?	○ Yes ◉ No	
Does your research involve the use of human embryos?	○ Yes ◉ No	
Does your research involve the use of human foetal tissues / cells?	○ Yes ◉ No	
2. HUMANS		**Page**
Does your research involve human participants?	○ Yes ◉ No	
Does your research involve physical interventions on the study participants?	○ Yes ◉ No	
3. HUMAN CELLS / TISSUES		**Page**
Does your research involve human cells or tissues (other than from Human Embryos/ Foetuses, i.e. section 1)?	◉ Yes ○ No	96-97
Are they available commercially?	◉ Yes ○ No	96-97
Are they obtained within this project?	○ Yes ◉ No	
Are they obtained from another project, laboratory or institution?	○ Yes ◉ No	
Are they obtained from biobank?	○ Yes ◉ No	
4. PERSONAL DATA		**Page**
Does your research involve personal data collection and/or processing?	○ Yes ◉ No	
Does your research involve further processing of previously collected personal data (secondary use)?	○ Yes ◉ No	
5. ANIMALS		**Page**
Does your research involve animals?	◉ Yes ○ No	96-97
Are they vertebrates?	◉ Yes ○ No	96-97
Are they non-human primates?	○ Yes ◉ No	
Are they genetically modified?	◉ Yes ○ No	96-97
Are they cloned farm animals?	○ Yes ◉ No	
Are they endangered species?	○ Yes ◉ No	

wild-type and genetically modified mice		
6. THIRD COUNTRIES		Page
In case non-EU countries are involved, do the research related activities undertaken in these countries raise potential ethics issues?	○ Yes ◉ No	
Do you plan to use local resources (e.g. animal and/or human tissue samples, genetic material, live animals, human remains, materials of historical value, endangered fauna or flora samples, etc.)?	○ Yes ◉ No	
Do you plan to import any material - including personal data - from non-EU countries into the EU?	○ Yes ◉ No	
Do you plan to export any material - including personal data - from the EU to non-EU countries?	○ Yes ◉ No	
In case your research involves low and/or lower middle income countries, are any benefits-sharing actions planned?	○ Yes ◉ No	
Could the situation in the country put the individuals taking part in the research at risk?	○ Yes ◉ No	
7. ENVIRONMENT & HEALTH and SAFETY		Page
Does your research involve the use of elements that may cause harm to the environment, to animals or plants?	◉ Yes ○ No	96-97
Does your research deal with endangered fauna and/or flora and/or protected areas?	○ Yes ◉ No	
Does your research involve the use of elements that may cause harm to humans, including research staff?	◉ Yes ○ No	96-97
8. DUAL USE		Page
Does your research involve dual-use items in the sense of Regulation 428/2009, or other items for which an authorisation is required?	○ Yes ◉ No	
9. EXCLUSIVE FOCUS ON CIVIL APPLICATIONS		Page
Could your research raise concerns regarding the exclusive focus on civil applications?	○ Yes ◉ No	
10. MISUSE		Page
Does your research have the potential for misuse of research results?	○ Yes ◉ No	
11. OTHER ETHICS ISSUES		Page

Are there any other ethics issues that should be taken into consideration? Please specify	○ Yes ◉ No	

I confirm that I have taken into account all ethics issues described above and that, if any ethics issues apply, I will complete the ethics self-assessment and attach the required documents. ☒

How to Complete your Ethics Self-Assessment

5 - Call-specific questions

Extended Open Research Data Pilot in Horizon 2020

If selected, applicants will by default participate in the Pilot on Open Research Data in Horizon 2020[1] , which aims to improve and maximise access to and re-use of research data generated by actions.

However, participation in the Pilot is flexible in the sense that it does not mean that all research data needs to be open. After the action has started, participants will formulate a Data Management Plan (DMP), which should address the relevant aspects of making data FAIR – findable, accessible, interoperable and re-usable, including what data the project will generate, whether and how it will be made accessible for verification and re-use, and how it will be curated and preserved. Through this DMP projects can define certain datasets to remain closed according to the principle "as open as possible, as closed as necessary". A Data Management Plan does not have to be submitted at the proposal stage.

Furthermore, applicants also have the possibility to opt out of this Pilot completely at any stage (before or after the grant signature). In this case, applicants must indicate a reason for this choice (see options below).

Please note that participation in this Pilot does not constitute part of the evaluation process. Proposals will not be penalised for opting out.

We wish to opt out of the Pilot on Open Research Data in Horizon 2020.	○ Yes	◉ No

Further guidance on open access and research data management is available on the participant portal: http://ec.europa.eu/research/participants/docs/h2020-funding-guide/cross-cutting-issues/open-access-dissemination_en.htm and in general annex L of the Work Programme.

[1] *According to article 43.2 of Regulation (EU) No 1290/2013 of the European Parliament and of the Council, of 11 December 2013, laying down the rules for participation and dissemination in "Horizon 2020 - the Framework Programme for Research and Innovation (2014-2020)" and repealing Regulation (EC) No 1906/2006.*

NANOSCALE LOCALIZATION AND VIBRATIONAL IMAGING OF BACTERIA - NANOVIB

List of participants

Participant No.	Participant organisation name	Country
1 (Coordinator)	Kungliga Tekniska Högskolan (KTH)	Sweden
2	Karolinska Institutet (KI)	Sweden
3	Abberior Instruments GmbH (AI)	Germany
4	Laser-Laboratorium Göttingen e.V. (LLG)	Germany
5	APE Angewandte Physik und Elektronik GmbH (APE)	Germany
6	Pi Imaging Technology SA (PII)	Switzerland

1. Excellence

1.1 Objectives

Not without reason, fluorescence is the by far most widely used modality for cellular imaging, offering a unique combination of spatial and temporal resolution, sensitivity and specificity. Following the remarkable development of fluorescence-based super-resolution microscopy techniques in the last decade, now MINFLUX, a next generation super-resolution concept, has recently been demonstrated, offering yet another order of magnitude higher spatial resolution.

In a coherent and interdisciplinary project, we will prototype a next-generation super-resolution microscope system based on this concept, capable to reveal intricate, detailed molecular mechanisms underlying inter- and intracellular processes and disease. As a lead application, we will demonstrate its unique capabilities to understand virulence and invasiveness of pathogenic bacteria, more precisely pneumococci. By concerted development of laser and detector technologies, microscopy devices and image acquisition procedures, we will be able to retrieve information, which is not within reach by any other microscopic or photonics-based technique. We will demonstrate how cellular nanoscale protein localization patterns can be resolved, which will not only help us revealing mechanisms of bacterial disease, but which also are likely to be of large relevance for many other diseases. Thus, the microscope system prototype will prove its broad applicability in biomedical research, as a tool to understand intra- and intercellular processes and the cellular origin of diseases.

The socio-economic and societal health relevance of our lead application is huge. In general, bacterial infections and the emerging antibiotic resistance pose major threats to mankind. *Streptococcus pneumoniae* is one of the major contributors to morbidity and mortality due to infectious diseases worldwide, causing millions of deaths annually. Pneumococci are the major cause of otitis media, sinusitis, and community-acquired pneumonia, but also a major cause of sepsis and meningitis (invasive pneumococcal disease, IPD). More than 50.000 individuals in Europe, and about 1,6 million individuals in the world, acquire IPD annually. Pneumococcal meningitis is a severe disease with a mortality of about 30%, and with high risk for sequelae. Despite that pneumococci can be devastating pathogens, carriage studies have shown that as many as up to 60% of healthy children in some European countries may harbor pneumococci in their noses.

What makes some bacteria virulent and invasive and others not, is largely attributed to detailed localization patterns of specific bacterial surface proteins, and their intricate interactions with

immune and host target cells. However, there are currently no techniques at hand, which can resolve these patterns with high enough resolution.

The overall objectives of this project are technological, but are mainly driven by biomedical needs:

On the biomedical side, the overall need is to significantly increase our understanding of diseases, on a cellular, as well as sub- and intra-cellular level. In this context, research on the inter- and intra-cellular processes behind pneumococcal virulence and invasiveness serves as a lead application. However, the knowledge gained will also form a basis for pathogenesis studies of other microbial pathogens as well as other diseases. To reach this goal, we will develop a MINFLUX-based prototype, as a next-generation super-resolution imaging system, to localize bacterial surface proteins implicated in pneumococcal virulence and invasiveness, with a ten-fold higher resolution compared to current state-of-the-art super-resolution microscopes. To fully discern the role of the proteins and their spatial distribution patterns, we will also resolve their interactions with different host cells and their proteins, and develop imaging procedures for simultaneous stimulated Raman scattering (SRS) and two photon excitation (TPE) imaging, which allows to place the protein patterns in a cell morphological and micro-chemical context, and to follow the metabolic states of the cells. The aim is to get a better understanding of mechanisms that influence how pneumococci cause disease. Data gained in this project will provide a basis for novel diagnostic, preventive, and therapeutic approaches to curb the extensive morbidity and mortality caused by these bacteria.

On the technology side, the overall objective is the successful development of a prototype of such a combined SRS/TPE and MINFLUX imaging system. More generally, nanoscale protein localization patterns on cells are likely to be of large relevance for many diseases, including microbial diseases and cancer development. The aim of this project is therefore that the prototype to be developed is suitable for a broad range of biomedical applications, and as a means to do this we will extend the spectral range of the microscope system into the near infrared (NIR), for the benefits of a lower background and higher penetration depths than attainable in biological samples. The aim is a truly disruptive photonics technology, allowing cellular protein localization patterns to be resolved, allowing the disentanglement of intra- and inter-cellular processes underlying a broad range of diseases. The successful development of laser and single-photon detector technologies, and the demonstration of their integration into the prototype of a combined SRS and super-resolution imaging system with unparalleled resolution, will undoubtedly strengthen the market position of the partner companies, and Europe's position on the market for microscopes, lasers and detectors as a whole.

The detailed objectives of the proposal are:

I. To construct prototypes of a next-generation fluorescence super-resolution microscopy platform for biomedical research and development, offering one order of magnitude higher spatial resolution than current state-of-the-art super-resolution microscopes.

II. To broaden the wavelength range of super-resolution microscopy into the near-infrared (NIR), for improved multiplexing, reduced background and better sample penetration.

III. To develop single-photon avalanche detector (SPAD) arrays, with >10 detectors, with at least equal performance to individual state-of-the-art SPADs and with enhanced sensitivity in the NIR.

IV. To develop a pulsed, narrow-linewidth, multi-line laser for SRS, which is quickly wavelength tunable and with rapid pulse length switching from ps to fs for TPE operation

V. To integrate the developed lasers and SPAD arrays into the prototypes of the super-resolution microscopy platform, for faster image acquisition, lower background, and with demonstration of combined SRS/TPE imaging.

VI. To demonstrate that the prototypes of objective V can resolve nanometer scale localization patterns of specific proteins in bacteria and host cells, provide overlaid morphological and

chemical images of the bacteria, and investigate correlations of these patterns with virulence and invasiveness of the bacteria.

VII. To make the prototypes of lasers, SPAD arrays and microscope platforms developed in the project attractive for researchers and stakeholders outside of the project.

VIII. To provide key information regarding specific pneumococcal surface proteins and their spatial distribution patterns correlated to biological relevance and disease outcome, leading to a better understanding of why some pneumococcal strains are more prone to cause IPD than other strains, in turn taking a decisive step towards better diagnostics, effective treatments and prevention of IPD.

1.2 Relation to the work programme

This project aims to develop a next generation super-resolution imaging system for studies of molecular mechanisms underlying the cellular origin of diseases. This proposal addresses the call "Disruptive photonics technologies" (ICT-36-2020), and the work proposed will be disruptive in several aspects.

First, the imaging system to be developed will be based on the MINFLUX principle, offering an order of magnitude higher resolution than any other super-resolution microscopy technique available today. For most bacteria with a virulent potential, increasing evidence suggests that certain surface proteins critically influence to what extent the bacteria are virulent and invasive and to what extent they can evade the immune defense of the host and cause disease. Critical is not only the amount of certain proteins present on the bacteria, but in particular the precise, nanometer-scale, localization of these proteins on the bacteria. With the next generation super-resolution imaging system to be developed in this project, we will be able to determine the localization patterns of fluorescently marked bacterial surface proteins down to such length scales, far beyond what is possible with current state-of-the-art super-resolution microscopy techniques.

Second, by extending the wavelength range of super-resolution imaging into the NIR and by enabling SRS/TPE imaging in parallel to super-resolution imaging, we will allow multiple specific proteins to be precisely localized on bacteria and host cells, and their (co)localizations to be overlaid on images of the cellular morphology and the local chemical environment. In general, functions of proteins strongly depend on their local environment, and their interaction with other proteins. The other way around, how specific bacterial surface proteins localize or co-localize on the nano-scale level influences how they can exert harmful and inflammatory effects on host cells, changing local environmental and metabolic conditions in these cells. The hitherto unparalleled ability aimed for in this project to resolve the nano-scale distribution patterns of several specific proteins in parallel, and to correlate them to local environmental parameters on the bacteria or in infected host cells, can thus generate a major leap in our understanding of the mechanisms behind bacterial virulence, invasiveness, and pathogenesis.

Indeed, the super-resolution technology to be developed in the project has the potential to revolutionize bacterial diagnostics and to spur the development of new treatment and vaccination strategies. This will create a completely new market for super-resolution microscopy systems. Knowledge of the disease mechanisms reflected in the nano-scale distribution patterns of specific bacterial surface proteins, can likely guide the design of new treatment schemes and strategies for vaccinations. Similarly, being able to distinguish different bacterial mutants/clones from each other by their detailed surface protein distribution patterns can open up for new diagnostic approaches. In the project, we will also for this reason explore how to scale down and simplify our imaging approach, opening for the additional use of the technology in clinical diagnostics and drug development.

In the project, we will develop prototypes of new detector-array and laser technologies, which in turn will be implemented into the super-resolution microscope prototype. The single-photon detector arrays will have an increased sensitivity in the NIR and will allow significantly increased image acquisition speeds. To take full benefit of the new array detector technology, real time data

handling will also be addressed in the project. Operation of the microscope in the NIR will allow reduced background, increased penetration depths, and will broaden the spectral detection window allowing for increased multiplexing with less spectral crosstalk and thus higher specificity. Implementation of new laser technology into the prototype of the microscope system will, for the first time, demonstrate correlative imaging of nano-scale protein patterns with morphological and chemical SRS images, as well as TPE images of auto fluorescent co-enzymes reflecting cellular metabolic states. This will decisively contribute to an increased specificity in the assessment of cellular originated diseases, which will be done in collaboration with medical doctors and their research laboratories. Apart from identifying an important application of the developed laser and detector array technologies in the super-resolution technology to be developed, these laser and detector technologies will also by their own merits be of interest to many other microscopy and biophotonics based applications.

Finally, from a biomedical application point of view, it is important to point out that the way specific proteins distribute themselves on or inside cells is not only of relevance for bacterial infections. For instance, the different mechanical, proliferative or adhesive properties of cancer cells can be reflected in the spatial distribution patterns of e.g. membrane, cytoskeletal and cell cycle regulating proteins, which are difficult to resolve by state-of-the-art fluorescence microscopy methods (see e.g. Blom and Widengren[1] for a review). Taking full benefit of the diagnostic information contained in such protein localization patterns in cancer cells has the potential to bring about a paradigm shift in cancer diagnostics. The decisive demonstration of resolution improvement with the super-resolution microscope prototype to be developed in this project, together with the demonstration that the correlation of nano-scale protein distribution patterns with metabolic, morphological and environmental parameters in the cells is feasible, is thus likely to open up new possibilities in cellular biology and disease diagnostics in general. It can thus be further iterated, as one of the cross-cutting priorities of the call addressed by this proposal, that the socio-economic and societal health-related impact of such progress would indeed be tremendous.

With respect to the other two cross-cutting priorities of this call, photonics and public-private-partnerships (PPPs), we'd like to point out that the aims of our proposal, as well as the constellation of the consortium (itself in a true sense a PPP), are very well in line with the major objectives expressed in the roadmap for the European PPP Photonics21[2]: It is an application-oriented and market-needs-driven project, with effective translation of a disruptive technology (MINFLUX) offering breakthrough advances in nanophotonics. It brings this technology into prototypes and demonstrates photonics solutions based on this technology to a true key societal challenge (to curb the devastating effects of pneumococcal and other bacterial diseases). It provides new ways to detect, possibly treat and even prevent a major disease (IPD), improving patient survivability and drastically reducing care. It drives the technological development and innovation in a strategic application area, where Europe is strong; biophotonics for medical and biomedical applications. It encompasses a broad cooperation across the whole value chain, also including end-users. This project will be coordinated by KTH in Stockholm, a member of PhotonicSweden, a national technology platform closely linked to Photonics21, and we will advertise our activities via this platform, via their workshops and conferences, as well as via corresponding organizations on a European level.

1.3 Concept and methodology

(a) Concept

This is a highly interdisciplinary project, covering areas from fluorescence and vibrational spectroscopy/imaging, physical and organic chemistry, optics, photonics, semiconductor physics, computer algorithm development and data handling/processing over to clinical bacteriology,

[1] Blom H and Widengren J "Stimulated Emission Depletion Microscopy" *Chem. Rev.* 117(11), 7377-7427, 2017

[2] https://www.photonics21.org/download/about-us/structure/workgroups/photonics_roadmap.pdf?m=1513613877&

molecular microbiology and pathogenesis. As a consequence of the interdisciplinary width of the project and its application-driven character, the main ideas, models and assumptions involved emanate from both biomedical/bacteriological, as well as photonics and microscopy technology related starting points. Naturally, the ideas and assumptions for these two starting points are mutually influencing each other: With world-leading expertise in clinical bacteriology, we can formulate the starting point from the biomedical side, from the societal needs and current bottle-necks in bacteriology research. At the same time, with likewise world-leading competence in next generation super-resolution and related biophotonic technology we can clearly identify where there is potential for the necessary further development. We can then focus our efforts on the identified specific features of these technologies, where we see that a further development would make a significant difference in biomedical applications. Likewise, from the biomedical side, we can subsequently direct our focus towards major bottle-necks and knowledge gaps where these newly developed devices have the potential to make a significant difference. From the lead application in bacteriology, we can then also extrapolate the developed microscope technology and procedures to be used on a much broader scale for cellular research and diagnostics.

From the biomedical/bacteriological side, the project thus takes as a starting point that the virulence and invasiveness of bacteria in general strongly depend on the properties of certain proteins on their surface. Of particular importance is how these proteins distribute themselves on the surface, and how their localization patterns are related to e.g. the cell cycle and local environment of the proteins. Currently used technologies in microbiology have proven insufficient to resolve these issues. The project aims to overcome these methodological barriers and is based on the idea that if we can resolve these aspects, we can also significantly increase our understanding of the intra- and inter-cellular processes underlying bacterial virulence and invasiveness.

While bacterial surface proteins and their localization patterns are likely key parameters of the virulence and invasiveness of a range of different bacteria, we will in this project focus on the major pathogen *Streptococcus pneumoniae* (pneumococci). Following a successful project, we then foresee that the developed procedures and techniques can be applied to understand the disease mechanisms also of other bacteria. *Streptococcus pneumoniae* is a major contributor to morbidity and mortality due to infectious diseases worldwide, causing millions of deaths annually. Pneumococci are the major cause of otitis media, sinusitis, and community-acquired pneumonia, but also a major cause of sepsis and meningitis (invasive pneumococcal disease, IPD). Pneumococcal meningitis is a severe disease with 30% mortality and with a high risk for sequelae. More than 50.000 individuals in Europe, and about 1,6 million individuals globally acquire IPD annually. Risk groups for IPD include young children and the elderly, as well as individuals with underlying diseases. A prior influenza virus infection also sensitizes for a pneumococcal infection. Pneumococcal infections are treated with antibiotics, but resistance rates to common antibiotics are increasing. Pneumococcal conjugated vaccines (PCVs) have been introduced in childhood vaccination programs in most countries in Europe, and have led to a dramatic decrease of IPD in vaccinated children. But concomitantly there has been an increase of IPD caused by non-vaccine types especially in the elderly, hampering the effectiveness of the PCVs. Importantly, even though pneumococci are devastating pathogens, they also frequently colonize the nasopharynx of healthy children. Hence, in carriage studies in Europe it was found that up to 60% of children harbor pneumococci in the nose without symptoms (Portugal and the Netherlands), and in Sweden we have found carriage rates of ca 30% in children post PCV introduction. Why pneumococci usually only colonize healthy individuals, but sometimes cause severe disease, remains to be clarified, and both bacterial and host factors contribute. Clearly, we need more knowledge on which factors, both on the bacterial and host side, contribute to disease development. Thus, in order to find better diagnostics, effective treatment and prevention (vaccines), we need to better understand why some pneumococcal strains are more prone to cause IPD than other strains, the interplay between the bacteria and the host, and which underlying mechanisms lead to disease development.

Bacterial surface proteins: The answers to what makes some bacteria virulent and invasive and others not, are largely to be found in the detailed display and dynamics of bacterial surface proteins, and their intricate interactions with immune and host target cells. In pneumococci several bacterial factors have been identified as virulence factors, such as

- the surface protein PspC, that interacts with factor H on host cells, an important negative regulator of the immune response (the alternative complement pathway),
- pili proteins, that build pili protruding from the bacterial surface, and are important for adhesion to host cells and carriage,
- the cytotoxin pneumolysin, suggested to be intracellular but recently indicated also to be surface exposed, and which can kill host cells,
- the autolysin amidase LytA, which can cleave the peptidoglycan cell wall of bacteria and is activated upon penicillin treatment.

In recent work by the research groups of partner 1 (KTH) and 2 (KI) it was demonstrated that by super-resolution STED microscopy it is possible to resolve specific spatial distribution patterns of bacterial surface proteins, and that important information about underlying mechanisms for disease and invasiveness of the bacteria could be revealed from these patterns. [3,4] As an example, the pneumococcal surface proteins PspC1 and PspC2 were studied with STED and found to localize differently (Fig. 1.3a), thereby rendering these proteins different immune-protective functions, something which would not have been possible to resolve with state-of-the-art confocal microscopy. However, while demonstrating the importance of localization patterns of specific proteins, for the virulence, invasiveness, as well as for the ability of the bacteria to evade immune responses of the host, these studies also clearly indicate that with another ten-fold increase in resolution (as previously between confocal and STED imaging) an additional major leap in the understanding of these mechanisms can be anticipated. This additional resolution increase, coming even closer to the actual spatial scale of the protein interactions, is going to be demonstrated, and exploited for bacterial studies in the NanoVIB project. This will allow us to study pneumococcal surface proteins and their localization and interactions with host factors and cells at a new level of detail, and relate this to their function and disease-causing capabilities:

On bacteria in different stages of division, we will study how different variants of the surface protein PspC distribute on the bacterial surfaces, to what extent they bind factor H, and if and how this can be affected by additives having potential antibiotic or bacteriostatic effects. The autolysin LytA will be studied in a similar way, and from how it distributes on and between bacteria, we aim to better understand the function of this protein, which still remains controversial.

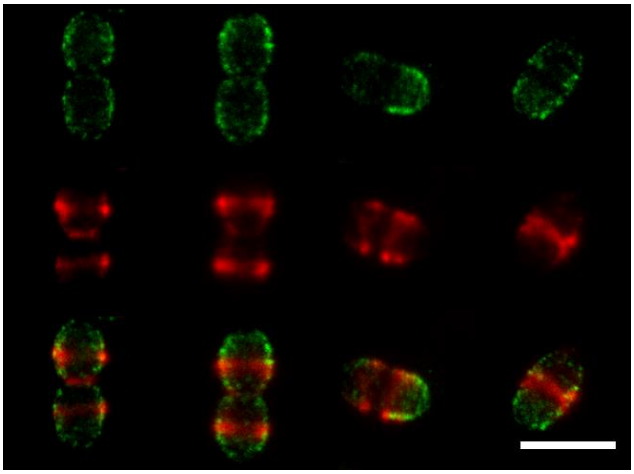

Figure 1.3a: Super-resolution STED microscopy images of different pneumococci in different growth phases, showing the distribution patterns for two variants of PspC, PspC1 (green) and PspC2 (red), during cell division and growth. Scale bar: 1 micrometer (from Pathak et al.[3]).

[3] Pathak A et al "Factor H binding proteins protect division septa on encapsulated Streptococcus pneumoniae against complement C3b deposition and amplification" *Nature Comm.* 9, 3398, 2018

[4] Iovino F et al "pIgR and PECAM-1 bind to pneumococcal adhesins RrgA and PspC mediating bacterial brain invasion" *J. Exp. Med.*, 214(6), 1619-1630, 2017

In biopsies from mice and from humans who died of pneumococcal meningitis (available via external collaboration with clinical researchers in the Netherlands), we will perform co-localization studies between pili proteins (RrgA, RrgB and RrgC) and receptor proteins on endothelial cells being part of the blood-brain barrier (BBB), to elucidate how these proteins bind and mediate invasion into the brain, and how the binding may be inhibited by antibodies competing for binding to the same sites as the pili proteins.

Nano-scale protein patterns correlated to cellular morphology, environment and metabolism: To take full benefit of the information contained in the imaged nano-scale localization patterns of the proteins above, we aim in this project also to put them in their cellular context, by monitoring their morphological, chemical and metabolic microenvironment in parallel. For this purpose, we will implement two imaging modalities. First, we will explore the possibility to correlate the nano-scale distribution patterns of specific bacterial proteins with the inflammatory response of host cells, as monitored by label-free two photon excitation (TPE) imaging of auto fluorescent NAD(P)H and flavins in the cells (described below). Second, we will use the chemical selectivity of stimulated Raman scattering (SRS) imaging[5], and then correlate the protein localization patterns to cytoskeletal structures as well as to lipid membranes, and even to specific lipid species in the membranes. [6] In this way, morphological images, e.g. from biopsies and cells as mentioned above, can be overlaid onto the protein distribution patterns, can put these patterns into context, and thereby enhance the biological significance of these patterns.

Bacterial exosomes, their possible role in disease and as a basis for vaccines: Exosomes are produced from the plasma-membrane of most eukaryotic cells and are currently intensively studied for practical use in medicine. However, also bacteria produce membrane vesicles. Most studies this far have been focused on outer membrane vesicles (OMVs) isolated from Gram-negative bacteria. Such OMVs contain most components that are expressed in the outer membrane, including endotoxin (lipopolysaccharide), and a cargo that primarily includes constituents of the periplasmic space. OMVs have attracted much interest in the vaccine industry as they may induce protective immune responses and are currently being used as antigens in a vaccine to prevent invasive meningococcal infections. Gram-positive bacteria such as pneumococci lack an outer membrane and have a thick peptidoglycan cell wall often decorated by capsular polysaccharides outside its plasma membrane. Therefore, formation of vesicles from Gram-positive bacteria was for long not expected. However, it was recently discovered that also Gram-positive bacteria indeed form extracellular vesicles. They are likely derived from the plasma membrane and seem similar to eukaryotic exosomes. Partner 2 (KI) has shown that membrane vesicles released by pneumococci can be internalized into host cells and potentially deliver the cytotoxin pneumolysin into the cells.[7] This will in turn affect their survival and lead to cytokine induction. Importantly, preliminary unpublished data from partner KI also suggest that immunization with membrane vesicles from pneumococci shows serotype-independent protection in mice. KI has profound expertise in microbial pathogenesis, focusing on Gram-positive bacteria, and has purified and characterized pneumococcal vesicles.[7] However, further studies are needed to understand how they are formed, as well as to understand the role of pneumococcal vesicles in disease development, and as potential candidates for new vaccines.

Using mass spectrometry, we know that these vesicles carry many pneumococcal surface proteins. However, we do not know whether these surface proteins are inside or on the membrane surface, how the proteins are distributed within and among the vesicles, and how this may depend on which strain they come from and on the local lipid composition in the vesicles. Furthermore, we need

[5] Prince RC et al "Stimulated Raman Scattering: From Bulk to Nano" *Chem. Rev.* 117, 5070-5094, 2017

[6] Cao C et al "Label-free digital quantification of lipid droplets in single cells by stimulated Raman microscopy on a microfluidic platform" *Anal. Chem.* 88, 4931-4939, 2016

[7] Codemo M et al "Immuno-modulatory effects of pneumococcal extracellular vesicles on cellular and humoral host defences." *MBIO* 9(2), e00559-18, 2018

more knowledge on mechanisms for how vesicles interact with host cells and consequences of such interactions.

In this project, we will make use of the ability to resolve the localizations of specific proteins by nano-meter scale resolution to address these questions. We will also overlay the protein localizations onto SRS images showing morphological cytoskeleton and lipid membrane maps of bacteria, host cells and vesicles, as a way to capture the role of the proteins in vesicle formation and in the subsequent vesicle interactions. By use of deuterium and other isotope labeling, we will use SRS imaging to track the lipogenesis from e.g. deuterated glucose added to the bacteria, and this will be specifically exploited to follow the generation and fate of membrane vesicles from specific bacteria.

When membrane vesicles are released by pneumococci and then internalized into host cells, they may deliver the cytotoxin pneumolysin into the host cells. This can result in metabolic activity changes and inflammatory reactions in the host cells. This is also a tightly coupled outcome to bacterial infections in general. To study this further, we will monitor the metabolic and inflammatory status of host cells by use of TPE transient state (TRAST) imaging of auto fluorescent co-enzymes.[8] Applying TPE TRAST in parallel with next generation super-resolution imaging will allow us to correlate local redox status and oxygenation in the cells to nano-scale localization patters of central bacterial and host cell proteins, as outlined above. We will study such correlations for bacteria-host cell interactions, adding whole bacteria, pneumococcal vesicles, or specific bacterial antigens (e.g. pneumolysin). Given that the inflammatory response of the host is a major component in the defense as well as a major reason for the detrimental effects of bacterial infections, it will be highly relevant to investigate these correlations.

Pneumococcal-viral-coinfection: A major risk factor for development of IPD is preceding infections with influenza A virus (IAV), and a significant part of the disease burden attributed to IAV is the result of superinfections with bacteria. Thus, during influenza pandemics, such as the Spanish flu during 1918 –1919, many of those that died, died due to superinfections with bacteria, in particular pneumococci. Why influenza A virus infections sensitize for pneumococcal infections is largely unknown. Our recent unpublished findings in mice suggest that influenza infections lead to capillary leakage and a nutrient rich milieu in the lower respiratory tract, promoting pneumococcal growth in the lower airways and pneumonia. Supply of antioxidants via this leakage to the lower airways was found to sustain bacterial growth, but pneumococcal adaptation to this oxidative environment also seems to entail induction of surface proteins, in particular the pneumococcal protease HtrA. By using the next generation super-resolution microscopy of this proposal, we envision that we can unravel in detail the distribution patterns of bacterial surface and membrane bound proteins including HtrA, how such patterns correlate with the microenvironment of the host, the presence of antioxidants, and the ability of the bacteria to evade immunological attack by imaging the degree of complement deposition on their surface. In this way, we expect to unravel important metabolic aspects in bacterial-viral co-infections, which seem to be a major driving force in such infections, and thereby also find better strategies to curb these infections.

From the photonics and microscopy technology side, this project will develop and implement a next generation super-resolution imaging system, to meet the specific needs for understanding the cellular origin of diseases, e.g. bacterial infections as lead application. This project will also drive the development of laser and SPAD array technologies to meet these needs, which are then implemented into the imaging system:

The next generation super-resolution concept on which the technology development in this project is based uses fluorescence as the main readout modality, by far the most widely used modality for cellular imaging, in biomedical research, drug development and diagnostics. This development builds further on the remarkable development of fluorescence-based imaging technology in the last

[8] Tornmalm J et al "Local redox conditions in cells imaged via non-fluorescent transient states of NAD(P)H" *Sci. Rep.* 9:15070, 2019

decade, allowing detection and characterization of single molecules, and offering a resolution far beyond the diffraction resolution limit (Nobel Prize in 2014)[9]. It also builds further on two previous EU projects (described further below), where we have successfully developed concepts and procedures for super-resolution imaging and their application for biomedical research, diagnostics and disease monitoring. In particular however, the development in this project takes as a starting point the very recent demonstration of a next generation super-resolution microscopy (SRM) concept launched by the Nobel prize winner Stefan W. Hell and his research group, called MINFLUX (Fig. 1.3b).

MINFLUX enables imaging of specific proteins or even epitopes within proteins with a resolution of up to 1-3 nm, in 3D, over large fields-of-view (FOV).[10] Thus, MINFLUX realizes the ultimate goal of biomedical imaging: to provide three-dimensional resolution in live cells, at length scales comparable to that of individual functional units within biological macro-molecules. In this sense, the MINFLUX concept has the potential to meet highly set demands from the biomedical field, to resolve the localization patterns of specific proteins down to the scale of nanometers, as we aim for in this project.

Apart from offering almost an order of magnitude higher spatial resolution than current fluorescence-based state-of-the-art super-resolution imaging techniques, such as stimulated emission depletion (STED) microscopy or single molecule localization based techniques using cameras, such as photo-activation light microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), MINFLUX offers additional advantages from an application point of view, which we will specifically take advantage of in this project:

i) It does not require high excitation and/or depletion irradiances and is thus inherently live cell compatible and does also not rely on exceptional photo stability of the dye molecules.

ii) It relies on far fewer detected photons than standard localization based super-resolution techniques and is thus not limited to the use of extraordinarily bright fluorophores.

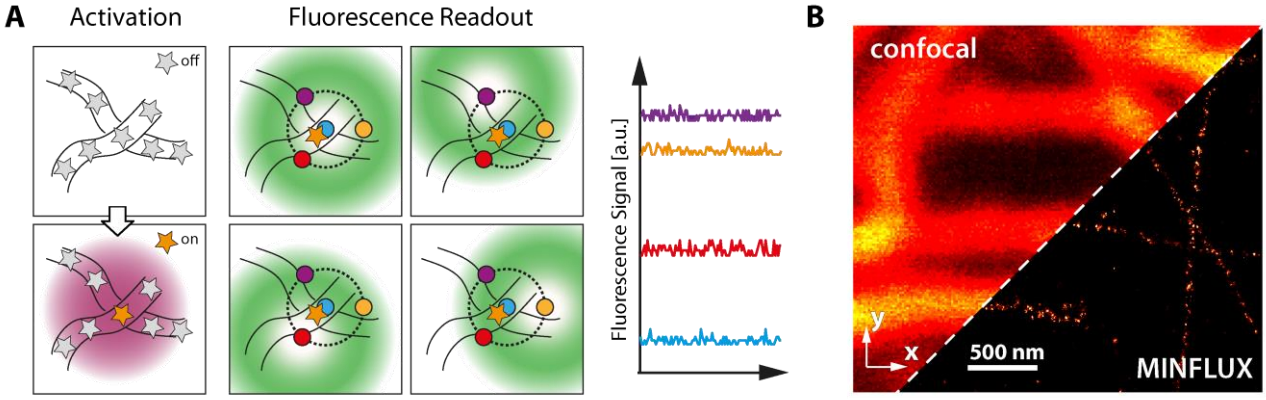

Figure 1.3b: Basic concept of MINFLUX microscopy. (A) Fluorescent molecules are sparsely driven from their non-fluorescent off-state into their on-state by a low intensity activation beam. Afterwards, fluorescence is probed with a low-intensity doughnut-shaped excitation laser. While the activated molecule resides within the center of the doughnut, no fluorescence is detected. Otherwise, fluorescence proportional to the excitation intensity is emitted. By placing the center of the excitation doughnut at four positions (cf. blue, purple, red, yellow dots) and detecting the respective fluorescence (see diagram), the molecule position can be calculated. (B) Imaging example of the vimentin network in Vero cells labelled with Alexa 647. Whereas in the confocal image the filaments look blurred, a clear separation of structures is apparent in the MINFLUX image. Measurement data are courtesy of Abberior Instruments, Germany.

[9] Ehrenberg M "Scientific background on the Nobel Prize in Chemistry 2014." *Royal Swedish Acad Sci*, 2014

[10] Gwosch KC et al "MINFLUX nanoscopy delivers 3D multicolor nanometer resolution in cells" *Nature Meth.* 17, 217-224, 2020

Hence, with a resolution one order of magnitude beyond existing fluorescence based imaging techniques and with important limiting factors of other SRM techniques overcome, the MINFLUX concept is poised to open a new chapter in biomedical imaging within fixed as well as living samples:

Extend MINFLUX into the near infrared (NIR) – a hitherto unexplored spectral range in super-resolution fluorescence microscopy: In general, taking fluorescence-based cellular *microscopy* into the near-infrared (NIR) spectral range (700nm-850nm) promises several distinct advantages:

a) strongly reduced scattering of both the excitation and fluorescence light by the surrounding tissue,
b) much lower absorption of the signal and significantly reduced autofluorescence from the sample,
c) lower phototoxicity,
d) deeper penetration depths,
e) provision of an additional spectral window, allowing for the simultaneous imaging over multiple, spectrally separated color channels.

Together, these advantages provide a good basis for multiplexed cellular imaging with higher specificity/lower spectral crosstalk and improved signal-to-background ratio. However, despite the potential merits, there are some bottlenecks which to-date have limited the exploitation of fluorescence-based cellular microscopy into the NIR spectral range. In particular, NIR organic fluorophores essentially all show lower fluorescence quantum yields (a few %), shorter fluorescence lifetimes (~0,5ns or less), and limited photostability. Specifically, single-molecule and super-resolution microscopy techniques critically rely on fluorophores capable to generate high numbers of detected photons per marker molecule and time. Therefore, the implementation of such techniques into the NIR range has been especially hampered. Now, since MINFLUX neither relies on the use of fluorophores with very high molecular brightness, nor on a very high photostability of the fluorophores, the use of NIR dyes is no longer excluded. Taking MINFLUX into the NIR can also give specific advantages as compared to the visible range. In particular, a minimized background level is critical for the performance of MINFLUX and can be far better achieved in the NIR.

In this project, the idea is thus to take advantage of the attractive features of NIR fluorescence in MINFLUX. While the general features of NIR dyes do not exclude them from use with MINFLUX, there is the requirement on dyes used in MINFLUX that they must be photo-switchable (Fig. 1.3b). The red-emitting cyanine dye Alexa 647 has been found to be very suitable for MINFLUX.[10] From this point of view, we will in the first place focus on the use of NIR cyanine dyes in MINFLUX, with likely similar, but red-shifted, switching properties, and characterize them photo-physically with respect to their switching properties as well as for multicolor readout approaches.

Developments in single photon detector array technology to further improve MINFLUX performance: By replacing single-photon avalanche photo diodes (APDs), the current gold standard in single-molecule detection and super-resolution imaging, with new SPAD array detector technology in MINFLUX we expect to improve the MINFLUX system significantly, to overcome current application barriers, and to further adapt the technique to meet the requirements for the bacterial studies in this project. While we aim to develop SPAD arrays offering improved quantum efficiency and dark count rates, which are comparable to or better than single detectors, we also expect the additional spatial information in the SPAD arrays to give distinct advantages for the MINFLUX instrument to be developed and used for bacterial studies. The spatial information can be used to speed up MINFLUX and reduce possible artifacts caused by the complex cellular environment: Emitters ready for localization (Fig. 1.3b) can be found more rapidly because a larger area is simultaneously probed due to the larger detection area. During the localization or tracking phase, more sophisticated background compensation algorithms can be implemented, because the area surrounding the molecule being imaged can be observed simultaneously. Such algorithms will

be developed in this project, and will give us a decisive edge in less controlled, cellular environments, such as the bacteria-host cell interactions to be studied in this project The approach is not feasible with readily available sCMOS or EMCCD cameras as they lack the necessary timing information. The timing information is needed both for MINFLUX itself (it requires to correlate detection with the position of the moving donut with precision better than 100ns) and for the time gating, which is used to reject both (auto-)fluorescence not coming from short-lifetime NIR fluorophores, as well as from scattering. This requires timing resolutions of <300ps, far beyond what is possible to get with sCMOS and EMCCD cameras.

Laser technology development to combine MINFLUX with stimulated Raman scattering (SRS) and two photon excitation (TPE) microscopy: Together with the nm-localization precision patterns of specific proteins in bacteria and host cells, it is valuable to overlay these patterns onto morphological images. To do this, without adding additional fluorophore markers that would sterically or spectrally interfere with the fluorophore reporters used for MINFLUX, we will demonstrate that two modalities can be run in parallel with the MINFLUX readout: Stimulated Raman scattering (SRS) microscopy and two photon excitation transient state (TPE-TRAST) imaging of auto fluorescent co-enzymes (NAD(P)H and flavins).

SRS is currently under strong development, and morphological as well as chemical imaging of cells is now progressing towards resolutions beyond the diffraction limit (Fig. 1.3c).[5] For the purpose of combining MINFLUX with SRS microscopy, a laser system providing three pulsed, narrow linewidth emission lines, two of them precisely tunable in their emission wavelengths, will be combined with the MINFLUX prototype to be developed in the project. Such laser systems represent the key technology, which has enabled the strong development of SRS microscopy in the last few years.[5] APE (partner 5) is in the absolute forefront of the development of such lasers and has been involved in the development of light sources for Coherent Raman Imaging for 15 years by now. Coherent Raman Imaging requires two ultrafast laser pulses of different wavelength, which are overlapping in space and time at the sample site. The energy difference of these two pulses needs to be tuned to the vibrational band of the sample under investigation. APE has developed synchronously pumped picosecond optical parametric oscillators (OPOs) which overcome many obstacles connected to coherent Raman light sources at the beginning, by providing ease of use,

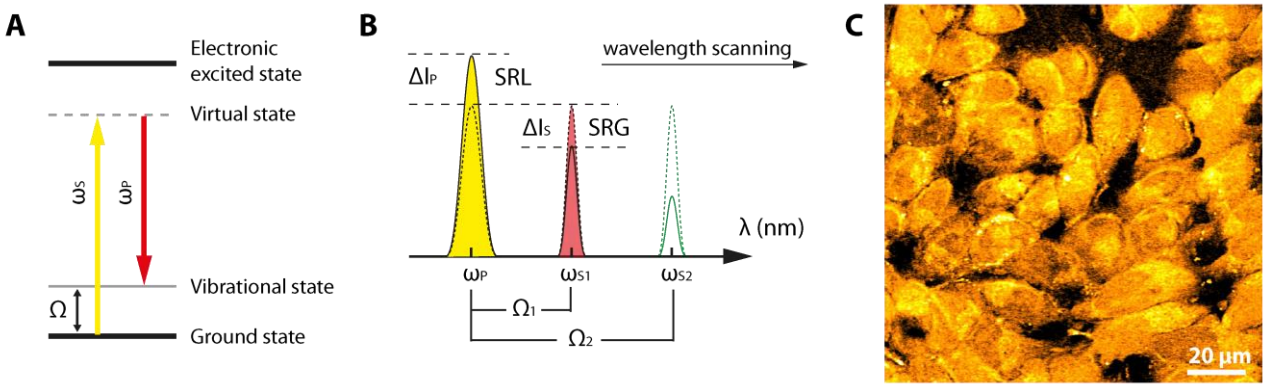

Figure 1.3c: Basic concept of stimulated Raman scattering (SRS). (A) A molecule is driven into a virtual excited state by the absorption of a photon from a pump laser with the frequency ω_P. A second, frequency shifted laser (Stokes-laser ω_S, with $\Omega = \omega_P - \omega_S$) is used to deplete this virtual state by stimulated emission. (B) Depending on the measurement scheme either the loss of the pump laser intensity (stimulated Raman loss, SRL) or the increase of the Stokes-laser intensity (stimulated Raman gain, SRG) is detected. By scanning the wavelength of the Stokes-laser, different vibrational levels can be probed. (C) SRS-imaging example of the fingerprint region at 1445cm^{-1} in living HeLa cells. The image was acquired using a modified Leica TCS SP8 microscope equipped with an APE SRS-detection set. A picoEmerald (APE) with 2ps pulses was used to pump the sample at 897nm with 50mW and deplete the excited state at 1032nm with 200mW. Data are courtesy of G. Hehl and A. Volkmer, Third Institute of Physics, University of Stuttgart.

robustness, narrow linewidth for selective excitation, no jitter between the two pulses, multi-MHz operation for fast image acquisition and shot noise limited operation necessary for stimulated Raman imaging. With its current product picoEmerald, APE is the leading light source provider for this application. Over 100 peer-reviewed articles were published using these light sources, including several Nature publications.[11,12] By further optimization of the pulse characteristics and spectral tuning, and by time-gating and spectral separation of the SRS signal from the fluorescence signal, we will demonstrate that this laser system can be simultaneously used for SRS and for operations required for MINFLUX, in particular for two photon activation. This parallel use of excitation will reduce light exposure (and sample photo-bleaching/photo-toxicity) and speed up image acquisition. SRS microscopy allows us to demonstrate that nanometer-scale MINFLUX protein localization patterns can be correlated with the morphology of the cell. For more complex problems, specific isotope labels can be introduced into the cells that allow generation of chemical images with SRS. This type of correlative imaging, putting the protein localization patterns generated by MINFLUX into a morphological and local chemical/environmental context, will significantly enhance the value of the bacteria-host cell interaction studies in the project.

Moreover, the ability to tune the pulse lengths below ps, will also allow TPE of auto fluorescent coenzymes (NAD(P)H, flavins). By TPE-TRAST, long-lived, dark, highly environment sensitive states of fluorescent molecules are monitored via the response in the fluorescence to spatio-temporal modulation of the excitation source.[8] These long-lived dark states of the fluorescent molecules, including triplet and photo-ionized states, are highly environment sensitive, not the least to parameters strongly influenced by cellular metabolism, such as local oxygen concentrations and redox status in the cells (Fig. 1.3d). TPE-TRAST imaging thus offers the possibility to monitor cellular metabolic states, in a widely applicable manner, and can reflect cellular environmental changes that are difficult, if possible at all, to detect by regular fluorescence parameters. Implementing TPE-TRAST will open the possibility to image also metabolic and micro-environmental conditions in cells in parallel with MINFLUX.

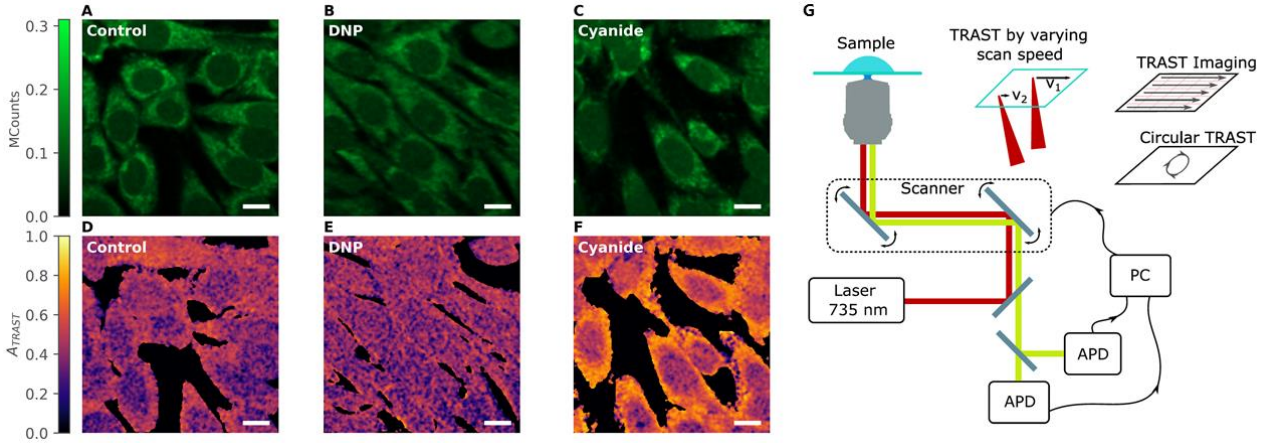

Figure 1.3d: TPE NAD(P)H autofluorescence images (A-C) and TPE-TRAST images (D-F) taken from mouse myoblast cells, subject to a mitochondrial un-coupler (dinitrophenol, DNP) (B and E), and a blocker (cyanide) (C and F). TPE-TRAST images show the photo-oxidative blinking of NAD(P)H, reflecting differences in oxidative environment, not possible to monitor via regular fluorescence parameters. (G) TPE-TRAST is performed following the NAD(P)H autofluorescence variation upon systematically varying the duration of the spotwise TPE excitation, by using different scanning speed on the sample (from Tornmalm et al.[8])

[11] Chau YY et al "Visceral and subcutaneous fat have different origins and evidence supports a mesothelial source" *Nat. Cell Biol.*16, 367-375, 2014

[12] Hu F et al "Supermultiplexed optical imaging and barcoding with engineered polyynes" *Nat. Methods* 15, 194-200, 2018

Positioning of the project, from ideas to applications, and from lab to market

Several different positions in this respect can be found in the project, depending on what aspect of the project that is considered.

From the biomedical/bacteriological side, the activities are centred on fundamental research, with the aim to understand the underlying molecular mechanisms of pneumococcal disease, as outlined above. However, findings regarding these mechanisms can also be expected to be quite directly funnelled into efforts to develop new vaccines, antibiotics and diagnostics. The transition of results from the bacteriological side of the project into such efforts will be promoted by the fact that the clinical partner of the project (KI) already has close contacts with pharmaceutical companies and other companies directly involved in e.g. vaccine development.

Also from the biophysics/bioimaging side, much focus is on fundamental research to demonstrate the principles and use of MINFLUX as a next generation super-resolution microscopy technique to reveal nano-scale protein patterns and their role in bacterial virulence, develop and refine acquisition and analyses of nano-scale protein patterns, in a multiplexed manner, extended into the NIR, and with SRS and TPE TRAST imaging providing overlaid morphological, chemical and metabolic images. Here, the plans/ideas will be brought into first demonstrations/applications, which will be reported scientifically, and where the principles for acquisition and analyses can then be implemented by other researchers more broadly, to understand fundamental intra- and inter-cellular processes, as well as how they may be altered upon disease.

Last but not least, **from a technological point of view**, there are three SMEs as partners in the project, which all of them have concrete plans for the development of their products and for how they eventually will be taken to the market. The positioning of the project activities from their points of view are shortly described below:

Partner 3 (AI): Abberior Instruments is an innovation leader in optical nanoscopy and hold exclusive rights to the MINFLUX concept developed by Nobel Laureate Stefan W. Hell at the MPI for biophysical Chemistry. After successfully claiming a significant market share for STED microscopes, AI now intends to make MINFLUX available to the biomedical imaging community as a user-friendly imaging tool offering ten times better resolution than any other optical super-resolution technique available. This project allows AI to partner up with both component developers on one side, and researchers on the other side, to ensure timely development of this next generation imaging system and the demonstration of its potential for biomedical research and development in a ground-breaking lead application. For the developed microscope system, we expect TRL9 and production transfer no later than one year after the end of the project.

Partner 6 (APE): Requests from users, such as fast tuning for quick spectrum acquisition and the flexibility in pulse length to do both, coherent Raman imaging as well as multiphoton fluorescence imaging, cannot be met with the current product. Within this project APE wants to solve these issues. To achieve this, new physical concepts for optical parametric oscillators and faster electronics are needed. This work starts at a technology readiness level TRL3, with first experimental results indicating the viability of this goal. Within the project we want to achieve at least TRL7 for the fast tuning of the device and TRL6 for the integration of pulse length flexibility from about 300fs to 2ps.

Partner 5 (PII): Pi Imaging Technology's Gen I SPAD array is a 23-pixel detector optimized for emission spectra around 520nm (green). It enables techniques like image scanning microscopy (ISM) to improve the spatial resolution and increasing the number of collected photons. The SPAD array detection electronics output time-tags with 10ns timing resolution. This array has been used by third parties both in research and industry and has TRL7 to TRL8. Within this project, we plan to push the TRL of the Gen I SPAD array by integrating it into a MINFLUX microscope. We will further improve the timing resolution to 20ps to allow finer time filtering. In parallel to the further development of Gen I SPAD array detection electronics, we will develop a SPAD array with enhanced red and NIR sensitivity. Red and NIR sensitivity is important due to the lower photo-

toxicity, lower background, and deeper penetration depth for optical microscopy in this wavelength range, compared to in the visible. Two arrays with the improved NIR sensitivity are planned. These arrays have a TRL of 4, and Pi Imaging Technology will improve the TRL of these arrays to 8 within the project.

Measures for public/societal engagement and use of stakeholders' knowledge

Pneumococcal infections pose major threats to society and there is an urgent need for better preventive and therapeutic options. In the project, the detailed knowledge of pneumococcal surface proteins and their role for pneumococcal virulence and invasiveness will promote both the diagnostic, preventive, and therapeutic abilities, to the benefit of societal health and wellbeing and of large interest to healthcare providers. If we can identify key bacterial proteins and their interaction partners on host cells that affect the ability to develop disease, we may find novel paths for interventions and how to combat the disease. To guide the activities in the project, we will keep a continuous dialogue with clinical bacteriology laboratories, regarding how this knowledge can come into use in their daily activities and about which aspects are likely to be of largest clinical significance. One of the partners, Birgitta Henriques-Normark, is physician and is highly connected to a clinical bacteriology laboratory in one of the biggest hospitals in Sweden (Karolinska University Hospital), as well as to the Public Health Agency of Sweden that is responsible for the national surveillance of contagious diseases in Sweden. We will also seek feedback from these laboratories on how the microscopy system to be developed in the project should be designed to allow a swift transfer and regular use in clinical bacteriology.

The major concept of this project is to develop a microscope system, capable to resolve nano-scale localization patterns of pneumococcal surface proteins on the bacteria, and with overlaid morphological and chemical/environmental images, thereby providing unique means to understand critical intra- and inter-cellular mechanisms of invasive pneumococcal disease (IPD). However, we expect that this concept will not only be applicable on pneumococcal disease. The same kind of information we aim to retrieve from pneumococci is likely to be critical also for the understanding of how and why other bacteria become virulent and invasive. Even more generally, highly resolved spatial distribution patterns of proteins on cells can likely provide fingerprints and suggest treatment strategies of a range of diseases. As demonstrated in our previous EU project (the FP7 project FLUODIAMON), and in research following after that project,[13] super-resolution STED imaging of cancer cells or even of platelets co-incubated with cancer cells, can reveal specific protein distribution patterns, as a possible basis for cellular cancer diagnostics and treatment monitoring. In this project, by combining the 10-fold higher resolution of MINFLUX, with additional multiplexing capabilities and overlaid morphological and chemical images of the cells, we will further demonstrate the validity of this strategy and that it can be further extended. As the project progresses, we will communicate the results to researchers active in bacteriology, virology, cancer research, or with other disease categories where our strategy may find use (see section 2.2b on communication activities for more details). As we predict that the microscope system to be developed in the project will be useful for a large end-user group of biomedical researchers,, we will arrange several workshops in the second half of the project (D7.4), to inform about the capabilities and potential of the microscopy technique. In addition, we will implement a group of potential end-users for feed-back on the development (D7.3). Thereby, we will receive input from biomedical researchers, on what they judge as the critical major features and performance, so that the company partners of this project can steer the development of the microscope system, as well as their lasers and detectors, towards those critical aspects. To further benefit from the knowledge of this important group of stakeholders, we will in the project also establish one of the developed microscope systems as a test facility open to biomedical researchers (D6.1), thereby providing valuable feedback for further improvements of the microscope system by hands-on experiments by

[13] Bergstrand J et al "Super-resolution microscopy can identify specific protein distribution patterns in platelets incubated with cancer cells" *Nanoscale* 11(20), 10023-10033, 2019

intended end-users. For the three companies in this consortium, the development of the microscope system, the SPAD arrays and the lasers in this project naturally goes hand-in-hand with the regular development and promotion of products to the market, including also feedback from present and potential customers and tuned to their needs and demands (see section 2 on impact).

Other national and international research and innovation activities linked with the project

Previous EU projects: The project will build on long-term experience from two previous EU projects. In these projects Jerker Widengren (KTH), coordinator of this proposal, and Stefan W. Hell (MPIBPC, Göttingen) cooperated, and concepts and procedures for super-resolution imaging and their application for biomedical diagnostics and disease monitoring were successfully developed. In the first of these projects (SPOTLITE, SW Hell coordinator, https://cordis.europa.eu/project/id/5049, 2005 – 2008), fundamental concepts for switching the fluorescence of fluorophore marker molecules on and off at low excitation light levels were investigated, and demonstrated in fluorescence imaging to provide useful mechanisms to break the diffraction resolution limit. Similar photo switching mechanisms will be investigated for NIR dyes in this project, as a basis for their use in MINFLUX imaging (D5.1). In the second project (FLUODIAMON, J Widengren coordinator, https://cordis.europa.eu/project/id/201837, 2008 – 2012), advanced fluorescence microscope techniques were developed for early diagnosis of breast and prostate cancer based on individual cell analyses. In particular, super-resolution STED imaging was successfully pioneered for diagnostic use. Specifically, STED imaging was demonstrated to identify spatial distribution patterns of specific proteins, not resolvable by other microscopic techniques, and was then used as a diagnostic strategy to identify cancer cells from suspect breast and prostate cancer lesions. Two years after the end of the FLUODIAMON project, which has been stated as a highly successful project by the Commission [14,15], Stefan W Hell was awarded the Nobel Prize for the invention of STED and for his seminal contributions to fluorescence-based super-resolution microscopy in general.[9]

Following the FLUODIAMON project, and in the context of several interdisciplinary projects on a national Swedish level, the Widengren research group at KTH has successfully applied STED imaging and analyses of spatial distribution patterns of specific proteins in platelets to understand their role in early cancer development (collaboration with Gert Auer, Karolinska Inst, Stockholm, funded by the Swedish Cancer Foundation and the Stockholm County council).[13] Furthermore, the Widengren group has also a long-term collaboration with the group of Birgitta Henriques-Normark, KI (partner 2) in which STED imaging and analyses of spatial distribution patterns of proteins on bacteria and host cells are a central part.[3,4] This research, funded by Stockholm County council, The Swedish Research Council, and the Swedish Foundation for Strategic research, forms an important starting point and directly links further into the activities in this proposal. In the joint research of the Henriques-Normark and Widengren groups this far, spatial distribution patterns of several pneumococcal surface proteins have been resolved to a resolution of 30nm. Thereby, mechanisms have been elucidated for how pneumococci can evade attacks from the host immune system and how they can bind to host cells with possible invasive pneumococcal disease as a consequence. These mechanisms would not have been possible to resolve without the use of STED imaging, and shows the potential of these analyses. With the 10-fold higher resolution of the protein localization patterns we aim for in this project, correlated with maps of the morphology and chemical environment at the sites of the proteins, we expect to be able to take a very decisive next step towards a more complete understanding of the underlying mechanisms for pneumococcal virulence and invasiveness.

Partner 2 (LLG) has been collaborating in several projects with Stefan W. Hell's department at the Max Planck Institute for Biophysical Chemistry (MPIBPC) as well as with the company Abberior

[14] https://horizon-magazine.eu/article/shedding-light-nanoworld.html,
[15] https://ec.europa.eu/programmes/horizon2020/en/news/fluorescence-adds-new-dimension-diagnosing-cancer

Instruments. Within the framework of the Collaborative Research Centre CRC 755 "Nanoscale Photonic Imaging" funded by the German Research Association, the LLG together with the MPIBPC has advanced STED microscopy for imaging within tissue. In two research alliances within the German Cluster of Excellence "Nanoscale Microscopy and Molecular Physiology of the Brain", the LLG and the MPIBPC collaborated on the development of new microscopy techniques and their quantitative application. Jointly with AI, the LLG has succeeded in developing a system for the correction of sample-induced aberrations in a project funded by the German Federal Ministry of Economics and Energy (see below). Currently, LLG and Abberior, a partner company of AI, are developing a computer-aided molecular design approach as well as a validation platform for live cell compatible fluorescent probes.

Partner 3 (AI) has been active in several interdisciplinary projects on a German national level. In particular, the BMBF Verbundprojekt "Dreidimensionale Lebendzell-Nanoskopie" (LiveCell3DNanoscopy), financed by the German ministry of education and research 2016 – 2019, AI together with MPIBPC, led to the development of a first MINFLUX microscope targeting early adopters and scientists who want to conduct research on the method as such.
AI currently applies for an extension of the project for an additional 36 months. The objective of this project extension will be to speed up image acquisition in MINFLUX systems by a factor of 10 to 50. To this end the synthesis of new, cell-compatible fluorophores, the re-design of the acquisition techniques and largescale application studies are envisaged.
While targeted at different aspects of MINFLUX microscopy, NanoVIB and LiveCell3DNanoscopy will complement one another and together result in an imaging system that is applicable to a wide range of cells and tissues and fluorescent dyes. Moreover, together with partner 4 (LLG), partner 3 (AI) also carried out a project financed by the Central innovation program for small and medium sized businesses of the German ministry of economic affairs and energy, run 2015 – 2017 and called "STED-Mikroskop mit aktiver Aberrationskorrektur & automatischer Justage' (perfectSTED)". This project resulted in: 1) the adaptive optics option for deep tissue imaging now part of Abberior Instruments Expert and Facility Line microscopes[16] and 2) in the automatic alignment option for Expert and Facility Line microscopes[17], which was an integral feature to make our superior STED technology available to technically less-savvy biomedical researchers using STED microscopes in a facility-like setting.
The main engineers and scientists participating in this project are still employed by the LLG and Abberior Instruments GmbH and the adaptive optics alignment automation technology developed during the project was the basis for integral parts of our current 3D MINFLUX microscopes and will be instrumental for the expansion to cellular environments and especially to the correlative SRS-MINFLUX approach.

(b) Methodology

Overall methodology of the project

As outlined in the implementation part, section 3.1, this project consists of six WPs and one WP for coordination. A new super-resolution microscope system will be developed in WP1-4, then established and used for a lead pilot application and offered as an open facility (WP5-6). In the lead application (WP6), with the unique resolution and imaging capabilities of the developed microscope system, inter- and intracellular processes underlying pneumococcal virulence and invasiveness will be revealed. Together with the open facility to be established (WP6), this will trigger the interest for the developed technologies and procedures in cell biology and biomedicine, and pave the way for the dissemination, exploitation and use of the developed microscopy system. Most of the activities in the project are heading towards deliverables in the form of demonstrators, but with somewhat

[16] https://www.abberior-instruments.com/products/expert-line/adaptive-optics/
[17] https://www.abberior-instruments.com/products/expert-line/autoalignment/

different purposes (Fig. 1.3e). Below, we give a brief outline of the overall methodology, the interrelationships of the overall activities in the WPs, their purposes and chronology:
This project has a very strong representation of SMEs, with three companies all having a leading role in their respective fields. Their market leading products in super-resolution microscopy (AI), tunable lasers (APE), and photon detectors (PII) will form an important starting point in the project. In WP3 and WP4, the photon detectors and the tunable lasers will be further developed to meet the demands of the new microscope system to be developed. However, the developed detectors and lasers will on their own merits be attractive for other applications in photonics and biomedicine, and thus the developed detector systems and tunable lasers in WP3 and WP4 will also serve as templates for stand-alone first market replications. In WP1, two super-resolution MINFLUX microscope systems will be constructed, and modified to accommodate the new detector system, while optical integration, including the new tunable lasers, will be done in WP2. With feedback from the pilot applications in WP5 and WP6 for the design of image acquisition and analysis algorithms, the developed microscope platform, with the tunable laser offered separately, will also be ready as a commercially available product within at most a year upon the end of the project. Apart from the central role of LLG, the main responsible partner for the optical integration of the lasers and detectors into the microscope platform, the activities in WP1-4 by the SMEs will not only lead to demonstrators providing the prerequisites for the cellular studies in the project (WP5 and 6), but will also pave the way to products which will be commercially available no later than 12 months after the end of the project.
In WP5 and WP6, with their main activities building upon the microscope prototypes developed in WP1-4, a main goal is to demonstrate the capability of this system to resolve intra- and intercellular processes underlying pneumococcal disease. First, the prerequisites in the form of sample preparation, excitation schemes and labeling strategies will be established (WP5), after which the actual bacteriological studies will take place (WP6). The two academic partners (KTH and KI), with very strong records in bioimaging and clinical bacteriology, will have the major roles in these WPs, and research of highest international standard is expected here. However, we expect such research as an outcome already from the optical integration (WP2) stage, shifting in subject from photonics, bioimaging over to clinical bacteriology and cell biology, within the course of the project, and towards WP6.
By generating research of highest international standard, we will also fulfill another important purpose of the activities in WP5 and WP6: to provide a lead example of the capabilities of the developed microscope system to resolve intra- and intercellular processes of large biomedical relevance. Such lead example will spur interest in the technique and its use and promote the demand of the instruments to become commercially available one year after the end of the project. As a complementary strategy for dissemination, a microscope system will also be made available as a

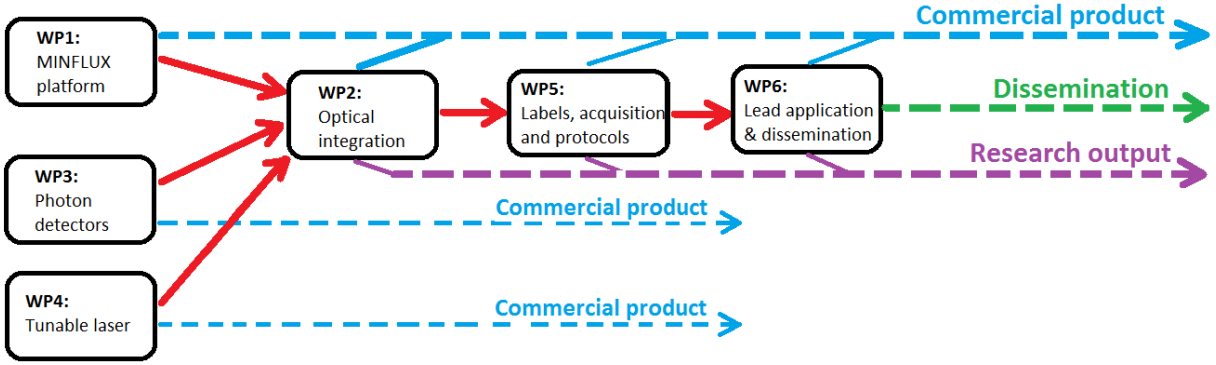

Figure 1.3e: Chart illustrating how the flow of activities in the project (illustrated by red lines) is heading towards outputs in the form of dissemination, research and commercial products. See also section 3 and the pert chart contained therein for more details.

facility, open to a broad group of researchers from academia as well as from companies, to further spur the interest in the microscope system, lasers and detector systems developed in the project and to promote their exploitation.

Gender aspects

From the photonics and microscopy technology side of the project, we have not been able to identify any particular role played by sex and gender with regard to the technology to be developed in this project. However, although we don't see any need to adapt the technology development at this point, we will still keep a constant attention to sex and gender aspects as the development progresses in the project. The technique development in our project will take benefit from feedback given by lead users in the project, as well as from potential end-users, who will test the instrument in the open facility to be established in the project. In the feedback from these groups of users (which we will strive to make diverse), we will pay attention to any gender related aspects, such as if the instrument would not be optimally designed for any of the genders from an ergonomic point of view, if there would be any need to otherwise gender-tailor the instruments in any aspect, and if so, if such measures should better be included as early on as possible, or could be inexpensively adapted at a post-development stage.

From the biomedical/bacteriological side, one may note for some infectious diseases, e.g. in the ongoing Covid-19 pandemic, that men and women are struck somewhat differently by the infection. Pneumococcal infections, to be studied here, affect all age groups and both sexes. However, the youngest children and the elderly population are the most prone to get a severe infection. Other risk groups include immunocompromised individuals and HIV infected patients and those that have been splenectomized. Also, a prior influenza infection sensitizes for a pneumococcal infection. In some studies, it has been shown that among children the proportion of boys that get an invasive pneumococcal infection has been somewhat higher than for girls. In this project, the studies will be performed on a (sub)cellular level, where sex effects are either not relevant or difficult to analyze. Nonetheless, for the studies on cells from mice and human biopsies, we will include the sex of the mice and donors as a parameter to correlate our data against whenever possible in order to identify any existing significant differences in this aspect.

From a project management point of view, a female researcher is leading the tasks of partner KI, one of the two CEOs of APE is female and the department of Optical Nanoscopy of partner LLG, which is responsible for WP2, has a share of over 60% female scientists. In the recruitment of new personnel to the project, the selection will in the first place be based on professional qualifications, but an equal representation of women and men in project will also be a high priority. Overall, in the efforts to adequately include relevant gender aspects into this project, we will take benefit from the significant priority and competence build-up regarding these issues that has taken place at the organization of the coordinator (KTH). As an example, in all recruitment advertisements from KTH, it is explicitly stated that gender equality, diversity and zero tolerance against discrimination and harassment are a natural part of KTH's core values and considered an issue of quality. It goes without saying that the same will apply to this consortium.

1.4 Ambition

This proposal is ambitious in many ways. Below, we describe the state-of-the-art from the bacteriology/biomedical side and from different aspects of the technology development side, and the advances that we foresee in this project:

From the biology/bacteriology side, we have previously used STED super-resolution microscopy for our pathogenesis studies, and then demonstrated the ability to resolve and analyse spatial distribution patterns of specific bacterial surface proteins, and have also shown their relevance for pneumococcal virulence and invasiveness.[3] With these studies, we are indeed in the research front

line in the application of super-resolution microscopy in bacteriology research. In this proposal we aim at taking the research to the next level, well above the state-of-the-art.
In this project, we will extend the capabilities of MINFLUX, a next generation super-resolution microscopy technique with ten-fold higher resolution than super-resolution STED imaging, and establish this technique for bacterial studies. Just as STED imaging, with its ten-fold higher resolution than state-of-the-art confocal microscopy, made it possible to reveal the role of specific proteins and their spatial distribution patterns on bacteria, we now foresee to be able to take another major leap in our understanding of bacteria and their virulence, by the additional ten-fold resolution increase of the MINFLUX technique. This will bring us very close to the actual interaction distance of proteins, which is needed when we aim at really understanding the role played by different proteins, their different localization patterns and their interaction sites with the host. Hence, the development of this new technique, which enables a resolution down to a few nm, will revolutionize our view of pathogen-host interactions and will give the possibility to exploit new territories previously not possible to study due to technological limitations. From a bacteriological point of view, the proposal is very ambitious, yet realistic, and will give results well above the state-of-the-art. The aim is not only a significantly increased understanding of disease mechanisms, but also that this understanding will open up new strategies for prevention (vaccines) and treatment (antibiotics). On a yet broader biomedical perspective, nano-scale spatial distribution patterns of proteins are likely to reflect and underpin a whole range of cellular processes. The ambition of the project is therefore also to establish a general approach of acquiring and analyzing such patterns on cells, which will be applicable far outside of the bacteriology field.

On the technology development side, the recent, very exciting progress in super-resolution microscopy is in the forefront of this application. The emerging state-of-the-art is represented by the MINFLUX concept, recently invented by the lab of Stefan W Hell, and then transferred to Abberior Instruments (partner 3) for launching on the market. With the ten-fold higher resolution offered by this concept compared to current state-of-the-art super-resolution microscopes available today, and with additional development potential to be addressed not the least in this project, MINFLUX indeed will be a cornerstone in the next generation of super-resolution imaging systems. In the project, we will address several aspects, which will further enhance and extend the capacity of MINFLUX:

Extension into the NIR: Up to now (state-of-the-art), super-resolution microscopy in the NIR spectral range has been essentially excluded. A major reason for this is that fluorophores in the NIR have too low brightness and photostability for such applications. A second reason is the lower quantum yields of detectors in this spectral range. In contrast to other super-resolution microscopy techniques however, MINFLUX does not rely on the fluorophore's molecular brightness, and a limited photostability can be tolerated. Rather, a minimized background level is critical for the performance of MINFLUX and can even be better achieved in the NIR than in the visible. We thus see an opportunity to, for the first time, bring (next-generation) super-resolution microscopy into the NIR. This will bring several distinct advantages: i) strongly reduced scattering of both the excitation and fluorescence light by the surrounding tissue, ii) much lower absorption of the signal and significantly reduced sample disturbing autofluorescence, iii) lower phototoxicity, and iv) deeper penetration depths. This provides a good basis for cellular imaging with improved signal-to-background ratio and opens up an additional spectral window, allowing for the simultaneous imaging over multiple, spectrally separated, color channels. In the project, we will identify NIR fluorophores, as well as excitation and photo switching conditions appropriate for MINFLUX. We will also develop photon detector arrays with increased sensitivity in the NIR. The advances foreseen are fully realistic and will represent a major extension of the application range of super-resolution microscopy.

Increased imaging speed and lowered background: In the project, we will implement new array detector technology into MINFLUX to further overcome current application barriers. The additional

spatial information of detector arrays compared to single detectors will be used to speed up MINFLUX and reduce possible artifacts caused by a complex cellular environment. Emitters ready for localization can be found more rapidly because a larger area is simultaneously probed due to the larger detection area. During the localization or tracking phase, more sophisticated background compensation algorithms can be implemented, because the area surrounding the molecule being imaged can be observed simultaneously. This will give a decisive edge in less controlled, cellular environments. The approach is not feasible with readily available state-of-the-art sCMOS or EMCCD cameras as they lack the necessary timing information, both for MINFLUX itself (requiring correlation of fluorescence photon detection with the position of the moving excitation beam with a precision better than 100ns), as well as for time gating, used to reject both (auto-)fluorescence not coming from short-lifetime NIR fluorophores and scattering.

Correlative imaging: Although MINFLUX offers multiplexed mapping of particular protein localizations and co-localizations in cells, the biological significance of this information will be significantly increased if this mapping can be put into a morphological, environmental, or microchemical context. Preferably, this should be made possible without adding additional fluorophore markers that would sterically or spectrally interfere with the fluorophore reporters used for MINFLUX. Therefore, we will demonstrate the implementation of label-free stimulated Raman scattering (SRS) imaging into the MINFLUX prototype, in order to generate overlaid vibrational images of specific lipid- or cytoskeleton-structures in the cells, or of specific metabolites. In addition, label-free two photon excitation TRAST imaging of auto fluorescent co-enzymes will be implemented for provision of metabolic state images of cells overlaid on the MINFLUX images. In this project, we will take benefit of the state-of-the art in SRS[5] and TPE TRAST imaging[8], and rather than progressing these techniques by themselves, we aim for a substantial progress beyond the state-of-the-art, by demonstrating correlative imaging with these imaging techniques together with MINFLUX.

These major lines of development of the MINFLUX technique are in the forefront of this project and form the basis for the subsequent studies on bacteria. However, there will also be significant advances beyond state-of-the-art for both the detector array and laser technologies in their own sense:

Detector arrays with enhanced red and NIR sensitivity: While the majority of scanning confocal microscopes still use point detectors, representing the state-of-the-art, there is a growing trend, not the least for novel microscopy techniques such as image scanning microscopy (ISM) and MINFLUX, to take benefit from array detectors. Currently used array detectors in microscopy are fiber-coupled PMT arrays, with SPAD arrays as an emerging new technology. However, none of these implementations has an optimized red and NIR sensitivity. A main ambition of PII in this project is to design a SPAD array with enhanced red and NIR sensitivity (Fig. 1.3f), which will represent a useful and significant advance beyond state-of-the-art, not only for MINFLUX, but also for other imaging applications.

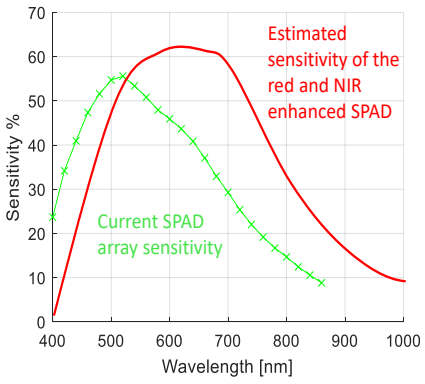

Figure 1.3f: Current (green) and estimated (red) detection quantum yields of the SPAD arrays to be developed in the NanoVIB project.

Tunable lasers for SRS and TPE: State-of-the-art in stimulated Raman imaging is represented by two main approaches by fast and sensitive acquisition of 3D images from specific vibrational bonds, made possible by narrowband picosecond light sources. Second, by setups for simultaneous multispectral stimulated Raman imaging, which however lack in sensitivity. As a third option, spectral acquisition or addressing of multiple vibrational lines is also the stronghold of classical Raman microscopy, but here the acquisition times are extremely long and the 3D resolution is far inferior. To turn the first approach into a useful multispectral readout, fast switching of the

wavelength from the light source is necessary. Currently the switching takes about 60 to 120 seconds. In this project, our aim is to reduce this time by more than a factor of 10, aiming for tuning times of a few seconds and thus speeding up multispectral imaging by an order of magnitude.
Regarding the pulse duration, the light sources for coherent Raman imaging, multiphoton imaging and fluorophore activation require different time regimes, from the picosecond down to femtosecond time range, to work efficiently. Currently, to achieve this, either two laser systems are required, or complex opto-mechanical setups, which only could be handled by optics and laser experts. The cost of an additional laser for an extra modality is in the range of 100 to 250k€ depending on the requirement. The aim in this project is to combine both modalities in one laser. This will drastically simplify the setup and reduce system cost, thereby opening up this application to the biomedical community. Both points are ambitious tasks and well beyond the statet-of-the-art, pushing the accessibility and usability of stimulated Raman imaging by a significant improvement of the light source.

Innovation potential

This project harbours a significant innovation potential, in particular on the technology development side, with new products of the three SMEs addressing market needs expected to grow strongly in the next coming years. However, also on the bacteriological side, the outcome of the studies planned in this project is likely to spark the development of new treatments (antibiotics) and vaccine strategies preventing pneumococcal disease.

On the bacteriological side, we expect to be able to identify novel mechanisms for pneumococcal interactions with host cells and how pneumococci cause disease, and the importance of protein localization for their function. We expect the knowledge gained to elucidate new pathways that could be used for intervention, thereby affecting disease development. Furthermore, we envision that the results from this project will form a basis for vaccine development based on for example bacterial exosomes and bacterial protein interactions with the host.

On the technology development side, all three companies in our consortium can identify obvious innovation potential, with new products that are likely to be generated as a result of this project:

MINFUX imaging system as standard microscopy tool for biomedical research (AI): AI's current focus is STED microscopy and its microscopes currently offer the best resolution performance on the market and feature numerous innovations developed by AI, many of them directed towards live-cell imaging. With the invention of MINFLUX which pushes resolution another order of magnitude, it was a logical step for AI to secure exclusive rights to this technology and the company succeeded to acquire such a license. A first generation MINFLUX system operating in the visible range and targeting early adopting users focused on method development and with a high level of technical skills has been developed and will be delivered to 3-4 customers within the year. However, AI aims at wide-spread adoption of the method in the biomedical imaging community, a potential this ground-breaking technology certainly has. Using new detector technology and by demonstrating correlative label-free imaging, the prototype developed as part of this project can be used by biomedical researchers as a robust imaging tool and the lead application will demonstrate this in a convincing way. This will be an essential contribution helping AI to reach acceptance of the method by the imaging community. At the same time the partnership with PII and APE will allow AI to add innovative features based on array detection and label-free contrasts to its existing line-up of STED microscopes, strengthening its profile as innovation leader and allowing the company to expand its share in the high-end microscopy market. In fact, AI has filed patents in anticipation of array detectors with sufficient sensitivity becoming readily available and conducted patent searches to ensure FTO for these plans. The FTO analysis for adding Stimulated Raman imaging to AI's microscope platform is under way and the details of our preliminary findings are outlined in section 2. In the case of MINFLUX, AI holds all necessary licenses for existing patents and established FTO before the launch of the first-generation platform.

SPAD arrays with red/NIR enhanced sensitivity (PII): Confocal microscopes are the most used high-end microscopes in the world with around 2000 confocal microscopes sold per year. It is predicted that at least 50% of confocal microscopes will feature a detector array in 3 years. This development is primarily driven by the innovation of image scanning microscopy. Secondly, specialized techniques, such as FLIM, FRET and MINFLUX will further drive the introduction of detector arrays. In these applications, detector arrays enable imaging of the single-molecule surrounding and increase imaging speed by parallelization. SPAD arrays developed by Pi Imaging Technology are specially designed to meet the needs of the mentioned applications. A SPAD array with red/NIR enhanced sensitivity, as planned to be developed during this project, will further strengthen Pi's competitive advantage. Pi Imaging Technology has (exclusive) rights for 5 patent applications.

Coherent light sources with fast tuning and flexible fs-ps operation (APE): APE is currently market leader for coherent Raman light sources with its product picoEmerald, However it is a narrowband picosecond only system optimized for coherent Raman imaging (CARS and SRS). Further, the tuning time between 60 to 120 seconds is too slow for imaging multiple vibrational lines within an acceptable time. Even though it is possible to image second harmonic generation (SHG, for instance in collagen), or multi-photon excited (MPE) fluorescence with picosecond pulses, this process is much less effective than using femtosecond pulses. In this project, the aim is to develop a new, ease-of-use, fully integrated light source, which is fast tunable, and offers flexible fs-ps operation. To achieve this goal, novel tuning concepts and nonlinear interactions in optical parametric oscillators will be investigated and implemented as described in section 3. The new light source will make the accessibility of stimulated Raman microscopy and the combination with other techniques, such as photo-activation and multiphoton excitation microscopy much easier than before. This combination will give new insight for instance into bacterial research as aimed for as a lead application and being a main objective of the NanoVIB project.

Regarding state-of-the-art, one competitor, MKS/Newport/Spectra-Physics, offers a stimulated Raman solution for its femtosecond laser Insight X3, which is primarily designed to be used for MPE and photo-activation. The concept they follow, called "spectral focussing", is realized in the product SF-TRU. In theory, this setup is capable of similar tasks as we propose. However, the underlying concept is extremely complex and requires laser physicists to operate it, thus preventing the practical use in a biological or medical environment.

An extensive patent search showed that the ideas APE pursues in this project are free of conflicting patents and APE has the freedom to develop the light source described.

2. Impact

2.1 Expected impacts

There are two major parts of this project: First, the development of a next generation super-resolution imaging system, the required detector and laser components resulting in an end-user ready system by the end of the project. Second, the successful application of this microscope system to a pioneering lead application within a highly relevant field of medical biology. Concurrently the system will be operated in an end-user facility during the final year to promote dissemination and further exploitation. All project activities are centered around MINFLUX nanoscopy, a fluorescence imaging concept for structural analysis of biological specimen, which closes the resolution gap between conventional super-resolution microscopy techniques like STED and single molecule localization based approaches (PALM/STORM) on one hand, and electron microscopy and complex spectroscopic techniques like Förster Resonance Energy Transfer (FRET) on the other hand.

Consequently, this project can be expected to generate significant impact in two major areas: (1) the consolidation and extension of Europe's leading position in the development and manufacturing of

innovative biomedical imaging devices and (2) the acceleration of fundamental biomedical research.
While the first MINFLUX instruments, operating in the visible wavelength range, will soon be delivered by partner 3 (AI) as a commercial product to laboratories focused on method development, this system is not fit for routine application without support by researchers with a deep understanding of the method, and it is limited to a small subset of available fluorophores and applicable only to certain samples. As it was the case with STED, PALM and STORM nanoscopy a decade ago, the MINFLUX technology has been proven to work but is not yet ready for widespread acceptance. This early phase presents unique opportunities: The sudden availability of a new class of fluorescence images with resolutions an order of magnitude better than existing techniques will potentially lead to important discoveries in biology and to significant business opportunities for highly innovative SMEs, if the most important prerequisite steps towards broad acceptance of a biomedical imaging technique can be swiftly completed: (1) Ensure and demonstrate compatibility of the method with a wide range of fluorescent dyes and labeling techniques, and its fitness to routinely image live cell samples. (2) Interface it with complementary imaging techniques that yield important, additional information and (3) demonstrate its capability to deliver fundamentally new results, in a groundbreaking lead application of significant biomedical and clinical relevance.
By doing just that, this project will ensure that a major part of the expected benefits, both on the industrial side and within the research community, will be reaped within the EU. To meet these expectations, NanoVIB has an ideal constellation of partners: NanoVIB will bring together three highly innovative SMEs with business models already targeting early adopters in the biomedical imaging research community (AI, PII and APE), an institution focusing on the interface between academic research and industry (LLG) and two academic institutions with an impressive track record of applying novel imaging techniques successfully to real-world biological problems (KTH and the KI).
In WPs 5-6, by establishing the use of this super-resolution imaging system for bacterial studies (WP5), and then showing that it can resolve features underlying bacterial virulence and invasiveness not within reach by other microscopic techniques (WP6), we will demonstrate by a striking example the capacity of this microscopic technique for cellular research. Enabled by the technical development in WPs 1-4, we will thus gain new significant understanding of the inter- and intracellular processes underlying pneumococcal disease. Pneumococci are the cause of worldwide morbidity and many thousands of deaths in Europe alone, and further understanding of these processes can likely pave the way for new antibiotics and vaccines. Thus, fulfilling this objective will directly benefit clinicians and patients and eventually have a huge positive influence on quality of life for all of society. In turn, this reflects on the commercial side, where there is an enormous potential in the development of new vaccines. Huge resources are spent by the pharmaceutical and biotech industry, where not only development of viral vaccines, but also of bacterial vaccines, e.g. against pneumococci, is high on the agenda.
Importantly, resolving cellular proteins on the nanoscale is not only necessary to understand pneumococcal disease, as aimed for in our lead application, but also for cell biology in general and for a broad range of diseases. Bringing this application to a successful end will thus also broadly demonstrate the importance of our new imaging technique, contribute to the commercial success of correlative MINFLUX microscopy, and thus strengthen Europe's position as the prime hub for leading manufacturers of high-end microscopes. Moreover, this project will result in affordable SPAD array detectors with high quantum efficiencies becoming available for the whole visible to NIR range. The development of detection electronics will result in an interface standard with the control logic of the microscope, allowing it to control other parameters based on spatial and temporal information of each detected photon in real time. This is not only a prerequisite for array detection in MINFLUX but will allow array detection to be used in many other advanced imaging techniques and allow research labs to envision and develop innovative new microscopy approaches. The project will also result in the development and commercial availability of a single, robust, and

compact laser, compatible with both label-free stimulated Raman based imaging techniques and two photon excitation and activation. For both research labs and system integrators, this project will make all these techniques more accessible, which will inevitably result in their accelerated development and acceptance. This technological development will generate IP which will be secured by the project partners, who will then spearhead their adoption into fluorescence microscopes in general.
Our project, with three market-leading EU companies aligning their development efforts to establish a multi-functional MINFLUX microscope system with ground-breaking performance, and guided by a lead application paving the way for a broad applicability of the microscope system in cellular research, will inevitably strengthen Europe´s industrial position in the biophotonics related market for microscopes and research and development tools.
Below, we describe in more detail how our project will contribute to the expected impacts and how the activities in the project make them link to and support each other:

Significant understanding of inter- and intracellular processes. Here, the deliverables in WP5 and 6 are themselves defined to meet this impact. As a strategy accompanying the activities in WP5 and 6, project partners KTH and KI will act as lead users of the instruments developed in WP1-WP4. By convincingly demonstrating that distribution patterns of proteins can be mapped on bacteria with a ten-fold higher spatial resolution than with any other state-of-the-art fluorescence-based super-resolution imaging technique, and where overlaid SRS and TPE-TRAST images can place the proteins distribution patterns in a morphological, chemical or microenvironmental context (Deliverable D5.5), we expect to trigger a demand from other research groups in biophotonics and biomedicine to use this imaging technology for fundamental cellular research. By showing in WP6 (D6.2) that this information also makes it possible to significantly further our understanding of the disease mechanisms of pneumococci, we will also raise the interest in the bacteriology research community and pharmaceutical industry for using the concepts and instrumentation developed in this project. This will eventually lead to a better understanding of fundamental bacterial disease mechanisms, and subsequent development of new diagnostics, antibiotics and vaccines.
We also envision that the bacterial and host-pathogen interaction data generated as part of our specific lead application will be of commercial interest. New paths and knowledge influencing pneumococcal pathogenesis and disease development can be explored for interventions including novel treatment and prevention. Partner 2, KI, already has promising data for bacterial exosomes as vaccine candidates, something that will be further investigated in this project (Task 6.3). KI has regular contacts with possible industrial partners that will facilitate commercial exploitation of such new inventions, including anti-virulence drugs, antibiotics, and vaccines.
Finally, how specific proteins distribute themselves on or inside cells is not only a key to understand bacterial infections. For instance, the different mechanical, proliferative or adhesive properties of cancer cells can be reflected in the spatial distribution patterns of e.g. membrane, cytoskeletal and cell cycle regulating proteins, which are difficult to resolve by state-of-the-art fluorescence microscopy methods (see e.g. Blom and Widengren[1] for a review). The super-resolution microscope system to be developed in the project is thus likely to find applications not only in fundamental cell biology, or for bacterial diagnostics, vaccine and antibiotics development, but will progress understanding of intra- and intercellular processes, underlying a much broader group of cellular states and diseases.
Therefore, while the project specifically targets a significant gain in understanding of inter- and intracellular processes in the context of pneumococcal disease, we also set as an aim of the project to promote the understanding of such processes and the awareness of the enabling technology more broadly.
As complementary strategies to the lead application, two important dissemination activities are also included in the project: First, one of the microscope systems to be developed will be available in an open facility during the last year of the project. Thereby, biomedical researchers from academia as

well as from biotech and pharma companies can use the unique possibilities of the device to address a broad range of intra- and intercellular processes (D6.1).
Second, a group of potential end-users will be engaged early in the project (D7.4). Their feedback will help us take the needs and concerns of a broad range of potential end users into account when developing the final system and procedures.
By demonstrating a lead application of high biomedical relevance in bacteriology (D6.2), with the open microscope facility (D6.1) and by forming the end-user group (D7.4), we expect to minimize the threshold of acceptance, and shorten the time until the microscopy technique and the developed procedures have found a broader use in academia and industry.

Strengthen Europe´s industrial position in the biophotonics-related market for microscopes and research and development tools. Europe's industrial leadership in high end optical microscopy methods is already remarkable (Confocal, Structured Illumination, Spinning Disk, Single molecule localization based (PALM/STORM), Total internal reflection (TIRF), STED, MINFLUX, Two Photon Excitation (TPE) Microscopy, etc.). Two (Zeiss & Leica) out of the four major optical nanoscopy players (the others are Nikon & Olympus) are European. The total market is estimated to about 1000-1500 units per year, with a total volume of more than 1 billion EURO. These "big four" generate most of their revenue in this segment from traditional high-end microscopes, like confocal laser scanning and camera-based wide field setups.
However, the emergence of "super-resolution techniques" over the last 20 years has transformed the market. As of 2018, an estimated 10% of the units sold feature technology to overcome the traditional resolution limit in optical microscopy. This percentage has been growing steadily over the past years and is expected to grow in accelerated fashion in the future.
The "big four" have adapted to this development by licensing techniques like STED, PALM and STORM, by integrating them with their established system framework, and by marketing them through their traditional channels. This has created an opening for highly innovative SMEs. Among these SMEs there are both system integrators and component manufacturers. The system integrators are focusing on bringing the full potential of these techniques to the end user as fast as possible and on designing systems that can be continuously upgraded as these novel techniques evolve. Component manufacturers often start out targeting the research community. However, they are also ideal OME partners for system integrators with quick innovation cycles as they can "keep up" with this process. Such partnerships are an opportunity to scale up their business.
To take advantage of this opening, Abberior Instruments was established in 2012 as a spin-off from the Max-Planck department of Prof. Stefan W. Hell, one of the Nobel laureates in Chemistry in 2014 and the inventor of STED and MINFLUX microscopy. Stefan W. Hell is strongly supporting this project and has agreed to be on its advisory board. By focusing on quick adoption of novel concepts into commercial products, by closely cooperating both with researchers in instrument and application development, and with its customers in the research community, the company has managed to conquer a significant market share in optical nanoscopy. At the same time, Europe's research community remains very active in the development of innovative imaging techniques, which has resulted in other, comparable success stories. Such development efforts critically depend on light sources and photonic detectors, along with acquisition and control logic being made available to the scientists. This is something APE has been doing for many years and PII has begun recently in the context of several collaboration projects.
This successful niche of highly innovative SMEs has already led to the creation of many new jobs in Europe, and there is potential for further expansion:
AI alone has not only created more than 50 high-tech jobs within the EU in the past years. By continuously improving its STED microscopes, often based on feedback from lead applications in academia, AI has accelerated their adoption in routine biomedical research. This has increased AI´s market share in comparison with other approaches manufactured outside the EU. While guaranteeing high-tech jobs in the EU, this has also made STED microscopes more affordable and

more usable for researchers within and outside the EU. The wide-spread adoption of MINFLUX as an imaging tool, as a result of this project, is expected to create at least 20-30 additional jobs at AI.
Out of the 65 people working at **APE**, more than 20 highly qualified jobs are directly linked to coherent Raman microscopy light sources, developed throughout the last 15 years. Competition from especially American companies, such as MKS/Spectra Physics and Invenio Imaging offering different technologies, has arisen in the last few years. However, they are still behind APE regarding ease of use and flexibility. This project will help APE to bring its coherent Raman light sources to the next level and widen its application to MINFLUX and multiphoton microscopy, thus strengthening and expanding their market position. With this project APE can secure these jobs and expects to create 10 additional high-tech jobs.
PII currently hosts 3 high-tech employees and one full-time consultant and expects to absorb great talent from EU universities working in the domain of SPAD detector arrays. PII focuses on close collaborations within the EU and believes they can elevate the competitive position of their EU partners.
The experiences during the emergence of STED and PALM/STORM a decade ago now offer a blueprint for the development, dissemination, and exploitation of the next generation super-resolution microscope techniques. The MINFLUX technique offers another order of magnitude improvement in resolution and significant potential to become a wide-spread, ground-breaking imaging technique in the biomedical community. The speed with which this can be achieved, ultimately putting a powerful technique into the hands of biomedical and biopharmaceutical researchers, and where the jobs will be created, will depend on how fast and by whom the challenges – which are similar to those faced by STED and PALM/STORM – will be overcome. Several points are critical to make this happen:

- Systematic research into the spectroscopy of label candidates, at wavelengths compatible with specific applications, like live-cell imaging or high throughput screening applications.
- Identification of the most suitable and cost-effective laser and detector technologies, their targeted development, with the requirements for the imaging technique in mind and integration into the microscope.
- Providing a preliminary implementation that allows routine application by biomedical researchers and later by medical staff.
- Successful high-profile projects using the technology and publication of their results to generate acceptance of the technique in the research community.

Essentially, the aim of this project is to ensure that these points are addressed as fast as possible, within the EU, and with all relevant partners for the further exploitation of all findings already in play when the project ends. This is in perfect agreement with the aims of H2020: Our project will achieve a significant gain in understanding of inter- and intracellular processes through a groundbreaking lead application and by putting the imaging technology into the hands of third parties working on promising projects. At the same time it will strengthen Europe´s industrial position in the biophotonics-related market for microscopes and research and development tools by giving AI, APE and PII the possibility to combine their specific strengths to trigger a directed, well guided and concerted technology development. Thereby, we will create an innovative imaging platform for which all major components are manufactured in the EU. This will strengthen AI's position as innovation leader in modern optical nanoscopy, while displaying APE's innovative laser sources and PII's SPAD arrays with intelligent readout electronics. Joint technology development in this project will help promoting these products for a much wider range of applications.
Making the technology more accessible by improving ease of use and saving costs, we will also strive to open this technology to a much wider customer base. Thereby, the technology can better reach out to medical researchers and biologists, who currently refrain from using it due to its current complexity and price. One example in this direction would be a fully automated microscope with

laser integration and with less lasers being used, thus saving costs as well as allowing for a much faster data acquisition.

Barriers/obstacles and framework conditions affecting the achievement of the expected impacts

We have not identified any significant barriers that would stop the project partners from achieving the expected high impacts. As with all plans relying on the successful completion of an ambitious development and research project, the most obvious obstacle derives from the implementation risks that are outlined in section 3. There is a medium to low probability that certain parts of the implementation plan (laser / detector / electronics development, identification of suitable dyes, establishing proper labeling and sample preparation protocols) are delayed while additional mitigating actions are taken. However, none of those individual aspects of the development processes has the potential to halt work in other work packages. We are therefore certain that the technological objective of developing a new MINFLUX platform, with a focus on usability and applicability to biomedical imaging, and demonstrating its fitness as a tool for routine research, will be achieved even in a worst case scenario.

Also, while not part of this project, the synergetic integration of SPAD array technology developed in WP1 and WP3 to STED and other imaging methods, as outlined below, will be a major avenue to exploit technology developed here, independently of the work in WP5 and WP6. Similarly, the new fast-tunable fs-ps light source developed by APE will find application and commercial success beyond MINFLUX imaging.

AI has thoroughly analyzed the IP situation for MINFLUX and concluded that we have FTO for the new system development. However, to be able to commercially offer correlated STED- and SRS-MINFLUX microscopes in Germany, France, Great Britain and the US, AI will have to acquire licenses for at least one patent family owned by HARVARD COLLEGE, US. AI has received requests to integrate Raman based imaging with its microscopes in the past. Therefore, the necessary work related to IP has already been started, both at AI and at the LLG, and we plan to enter negotiations with Harvard as soon as possible. However, an agreement cannot be expected until the project is under way. There is thus some risk that licenses will not be granted, or that licensing conditions are unacceptable, which would delay commercial exploitation of the correlated imaging approach in the afore mentioned markets, in worst case until 2029 (max. 5 years after the end of the project), when the patent runs out. On the other hand, APE has successfully marketed SRS imaging packages to end users of confocal microscopes for many years. This strategy can be applied to STED and MINFLUX owners as well and ensure exploitation of Raman-Imaging related results. There are also no IPR-related restrictions in the use of SRS-MINFLUX in the lead application, as planned in WP5 and WP6 in this project. Also, use of APE's lasers for two-photon activation in MINFLUX would not be limited by the current IP situation.

Finally, even with all system and method development on schedule, the lead application in WP6 could prove more challenging than expected, delaying its conclusion and prominent publication, and thereby also the push this would generate during commercial exploitation of the new developments. We are, however, confident that even if this were the case, this project would still achieve its impacts by placing a powerful new tool into the hands of the bioimaging community, eventually leading to important new insights. Similarly, the new technology developed will increase the competitiveness of the three SMEs involved and thus strengthen the Europe's position in the biophotonics market. And as the partners have agreed to leave the two new systems in place after the project is finished and continue to work on MINFLUX applications, the positive effect expected from a high-impact lead application would not be voided but merely delayed.

2.2 Measures to maximize impact

To ensure and maximize the impacts aimed for in this project, KTH, KI, LLG, together with AI will focus on *direct interaction with potential end-users* of the technology and with the target audience

for their findings in bacteriology. The lead application conducted in WP5 and WP6 of this project will result in a significant gain in understanding of intra- and intercellular processes. These findings will be disseminated as outlined below. However, by demonstrating the capacity of the microscope developed in this project to reveal intra- and intercellular processes underlying pneumococcal virulence and invasiveness, we also pave the way for a broader end-use, which will spur the interest for this technology both in academia, in the clinics and in pharmaceutical and biotech industry.
To maximize this effect, we will also establish a microscope facility (D6.1), open to potential end-users of the microscope technology. This facility will not exclude any category of potential end-users and allows researchers from all fields of application to familiarize themselves with the technique and its possibilities. Likewise, we will, for the same reason, recruit people to the end-user group formed in the beginning of the project (D7.4), from as many different relevant categories as possible (researchers in cell biology, biophysics, bacteriology, cancer researchers, representatives from large pharma companies, smaller spin-off companies,…). In this way, we can promote the demand for the microscope systems to be developed, and open new markets for the microscopes, as well as for the lasers and detector systems themselves. In addition, all three companies (AI, PII, APE) will *accompany and follow up development within NanoVIB with synergetic development projects building on the results. These efforts and product marketing through their established channels will be* financed from their regular revenue. This project will therefore establish PII as a manufacturer of next generation SPAD arrays for biomedical imaging, strengthen APE's position as a leading provider of light sources for innovative imaging applications and strengthen AI's position as the innovation leader in super-resolution microscopy. Extension of MINFLUX to the NIR

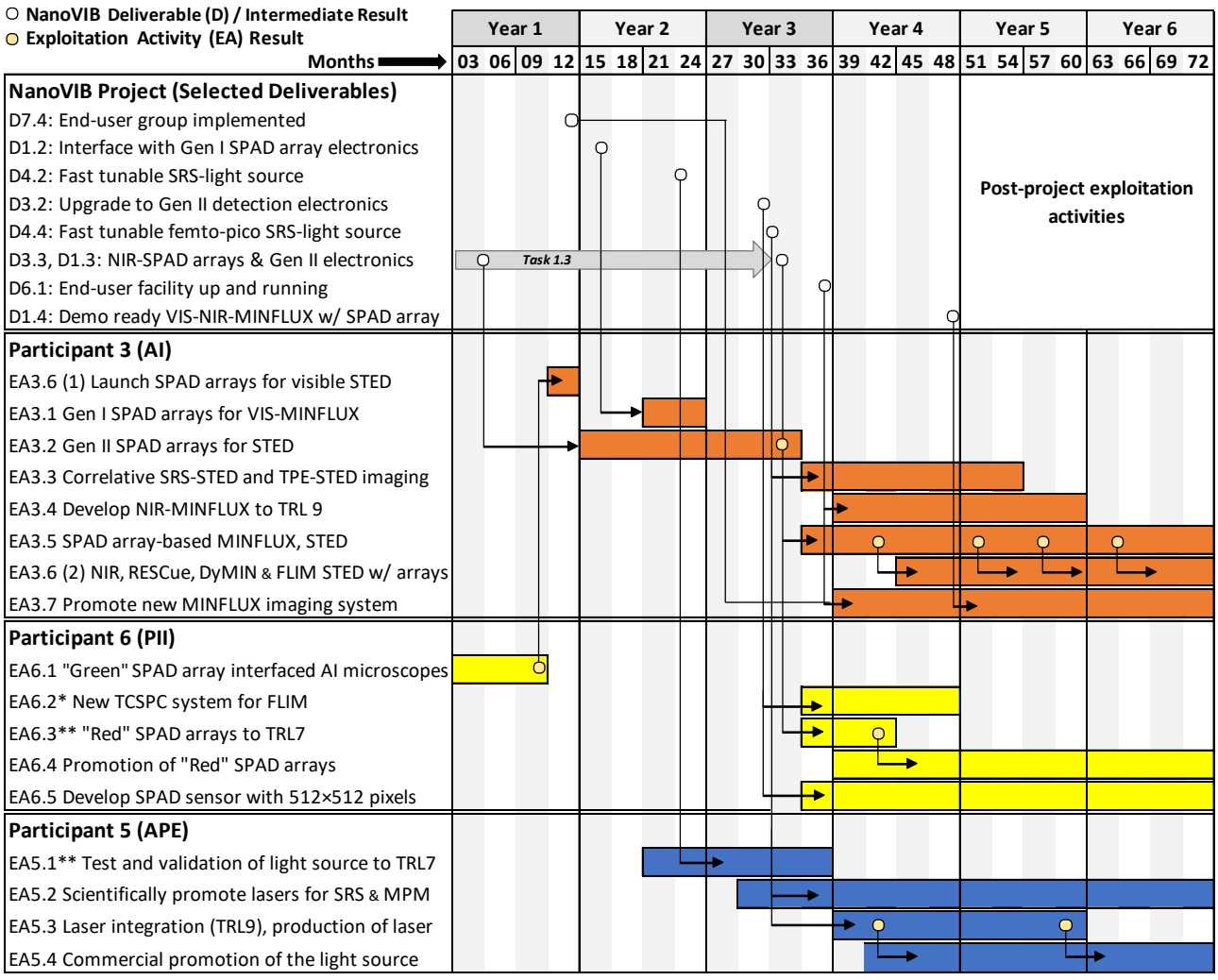

Figure 2.1a: SME exploitation activities (EA) during and after completion of the NanoVIB project.

wavelength range and its combination with complementary imaging techniques will help grow the market share of this technique within the super-resolution segment.
For the SMEs involved, the interaction with potential end-users, as planned in this project, will result in better visibility of both the products developed during this project and their dedication to advancing imaging technology in general. We expect this message to be heard by a broad audience, including opinion leaders in the international bioimaging community. This will bring further energy into the photonics branch in Europe in general, will strengthen the competitiveness of the companies in the project, and will allow for their further growth.

a) Dissemination and exploitation of results

The three SME partners have coordinated their plans for exploitation of the newly developed technology, both within the project and as part of synergetic development and subsequent exploitation efforts, in related fields of their business. The planned activities are outlined in Fig. 2.1a, and there also put in context with the deliverables defined in section 3 below. These plans for additional development beyond the scope of NanoVIB, exploiting its results to related imaging techniques, rely on the strong relations formed within our project and on the documented commitment to achieve important technological goals within the four years the project will be active. The R&D personnel hired for and trained during this project will form the backbone for these additional developments. As the primary focus within the project is to deploy prototypes rapidly to our partners, in order to ensure the success of WP5 and WP6, all three companies will also finance the final development steps to TRL9 from their regular revenue and use their established channels for marketing and sales activities. Documenting the SMEs commitment to translate the projects result into commercial success, all other activities listed here are also financed independently of NanoVIB except for those that are prerequisite to later project work and thus partly (marked with *) or entirely (marked with **) financed through the project budget.

The paragraphs below highlight the perspective of each of the SMEs involved and give a short description of the individual exploitation activity (EA) planned by each project partner:

Participant 3 (Abberior Instruments GmbH, AI)

The project will result in a MINFLUX imaging prototype demonstrating the applicability of the technology as a standard research tool in biomedical and biopharmaceutical research, with its fitness demonstrated during the lead application in WP5 and WP6.
In a recent joint research project together with the Max-Planck-Institute for Biophysical Chemistry, AI could develop the current, first MINFLUX generation, targeting technically savvy researchers familiar with its technical intricacies and interested in exploring the potential of the method early on. With this achievement and experience as a starting point, and together with the project partners, AI is now well prepared to develop a TRL 5 platform within the project and to advance the prototype to a market-ready device (TRL 9) that can serve as a standard research tool, no later than a year upon completion of the project.
Facility-like operation of the prototypes created during the project, as envisioned as part of the dissemination efforts, will generate even larger interest and allow us to interest potential customers very early.
The money applied for in this project only covers integration efforts and improvement over the prototypes that can be deployed to our project partners in time, to maximize their productivity in WP5 and WP6. Production transfer, certification and marketing efforts beyond this scope can be covered through AI's regular revenue.
Based on the interest the research community has already shown for the MINFLUX technology we estimate that an instrument, compatible with routine facility operation, will result in 10 - 25 sales within 1-2 years after introduction. This would result in 10-25 M€ additional revenue translating into the creation of an estimated 20-30 jobs at Abberior Instruments in management, marketing, sales, production and service.

In the likely case that the FTO concerns outlined above are mitigated, the know-how resulting from the development of correlative SRS-MINFLUX microscopy in this project would allow Abberior Instruments to immediately develop a similar combination of SRS and STED microscopy in parallel using minimal resources. An SRS channel will then be offered optionally for all Expert Line configurations and will also be available as an upgrade to all current Expert Line customers. This will also make combining STED with two photon excitation (TPE) more attractive, since the same light source allows later integration of coherent Raman imaging without significantly adding to the system price.
Abberior Instruments has received requests to integrate Raman based imaging with its microscopes in the past. Based on the frequency of such requests and its experience with past upgrades and imaging options, like two-photon excitation and fluorescence lifetime imaging, AI expect to sell at least 5 upgrades within 2 years after the initial offering is available and that 10 to 20 percent of future Expert Line microscopes will feature the combined TPE/SRS option. During the first 2 years of introduction this would result in at least 4Mio€ additional revenue and 4 additional jobs being created.
The development of next generation SPAD arrays with high sensitivity in the NIR makes them an ideal candidate for adding image scanning capabilities to STED microscopes. Automatic background subtraction is an obvious application and AI has also filed patents for technology that will bring about significant improvement in 3D STED microscopy based on this approach. The efforts of this project to integrate the next generation of SPAD arrays more deeply with AI´s detection electronics will allow their combination with both fluorescence lifetime imaging and several technologies, offered exclusively by Abberior Instruments (e.g. RESCue STED, DyMIN STED), and that focus on reducing light exposure of the sample in live cell imaging applications. With this integration and NIR sensitive versions of the SPAD available, array detectors could become the standard detector in all of AI's microscopes, thus leveraging the full potential of the optical setup and giving AI a significant edge over its competitors. This will allow AI to further increase its market share in the super-resolution microscopy segment and proportionally create additional jobs not only in our head-quarter in Göttingen, but also in our development and production facilities in Heidelberg and Basel.
The activities planned by AI during the project and the following two years are listed below. These projects will build on the know-how and work force of the personnel hired and trained as part of NanoVIB and will be conducted in close cooperation with AI's R&D department using AI's established development and exploitation strategy: Upon proof of principle for a new technique or application, it will be integrated into AI's flexible hardware and software platforms. This will essentially guarantee initial commercial exploitation, since it immediately becomes available to all existing customers as an upgrade, and strengthens our product offering as an optional add-on. This reliably generates several initial sales and these success stories support the accompanying marketing and sales efforts, which will be carried out by AI's respective departments using the same approach as for our existing advanced imaging devices. From AI´s side, the individual activities planned are:
EA3.1 Interface modified Gen I SPAD array electronics with visible MINFLUX (M19-M24): Using the preliminary solution developed as part of the project, the modifications to Gen I array electronics will allow AI to also explore the benefits of array detection in the visible range, complementing the work in WP2 and WP5. This will improve MINFLUX as a whole and generate IP within the company securing its future technological leadership beyond the duration of the exclusive license currently held for the technique.
EA3.2 Interface Gen II SPAD array electronics with STED acquisition (M13-M33): As Gen II array electronics becomes available, AI will also re-design its current electronic platform for the Facility-Line and Expert-Line STED-microscopes. This will eventually allow SPAD array detection to play well with its RESCue, DyMIN and FLIM STED products. We will also ensure compatibility of array detection with our flexible rainbow detector unit.

EA3.3 Explore correlative SRS-STED and TPE-STED imaging (M34-M54): AI aims at testing the new APE light source as soon as possible in-house for projects other than NIR-MINFLUX. Provided FTO can be achieved, the expertise gained from NanoVIB will allow us to develop label-free imaging as an add-on for existing STED installations. Independently of FTO concerns, the new light source will also allow us to promote already available options for TPE microscopy and two-photon STED microscopy more aggressively.
EA3.4 Develop NIR-MINFLUX to TRL 9 (M37-M60): After deployment of the two prototype platforms, our optics engineer working on NanoVIB will be tasked to monitor integration progress and address any problems during integration and application. This will allow us to (1) re-deploy solutions to our project partners and (2) start collecting data for the development of the final platform early on. Based on these data and the added experience from facility operation during year 4, AI will then make NIR-MINFLUX part of its overall R&D strategy and we expect TRL9 and production transfer no later than one year after the end of the project.
EA3.5 Develop new SPAD array-based MINFLUX, STED and confocal imaging techniques (M34-M72): Based on NIR- and VIS-sensitive SPAD arrays deeply integrated into our acquisition electronics, AI will focus on leveraging the added information to improve all its microscopes. Thus, as soon as both VIS- and NIR-sensitive arrays with Gen II electronics and high timing resolution become available, PII and AI will expand their cooperation to finding novel applications of array detection in STED imaging. In addition to the more obvious uses like enabling background reduction and improved 3D deconvolution, AI has already filed patents for potentially game-changing applications in this context.
EA3.6 Launch SPAD arrays as an option for visible STED and confocal imaging (M10 – M12) **and for NIR, RESCue, DyMIN and FLIM STED** (M43-M72): Existing VIS-SPAD arrays can be used with STED with minor modification. Following EA6.1 (PII) will create an upgrade option for all Expert Line customers. Later, building on the results of EA3.5 improved, array-based versions of existing imaging modalities will be launched as Expert and Facility-Line options and offered as upgrades to existing customers as soon as they reach sufficient maturity level. This will give AI an edge over competition in the STED market and help consolidate and grow its market share.
EA3.7 Promote new MINFLUX imaging system (M37-M72): As soon as the open facility (D6.1) is up and running, AI will actively encourage potential customers to evaluate the new NIR-MINFLUX system. AI will guarantee upgradability for MINFLUX systems sold from this point forward and our sales and marketing team will remain in contact with all these potential customers and start advertising through established channels using preliminary results from WP5 and WP6.

Participant 6 (Pi Imaging Technology, PII)

PII's focus is to create new technology that enables microscopy innovations: new imaging and spectroscopy modalities, higher resolution, faster imaging speed, live cell imaging. Confocal microscopes are the most used high-end microscopes in the world. It is predicted that at least 50% of confocal microscopes will feature a SPAD detector array in 3 years. This development is primarily driven by the innovation of image scanning microscopy. Secondly, specialized techniques, such as FLIM, FRET, MINFLUX and STED will further drive the introduction of SPAD detector arrays. In these applications, detector arrays enable imaging of the single molecule surrounding and increase imaging speed by parallelization. SPAD detector arrays developed by Pi Imaging Technology are specially designed to meet the needs of the mentioned applications. With the results from this project, PII will improve its position in the biophotonics related market for research and development tools. By introducing a SPAD array with enhanced red and NIR sensitivity ("red" SPAD), PII will supplement its "green" SPAD arrays and cover the complete visible to NIR spectrum. This enables color/dye specificity without compromising the sensitivity.
To maximize impact of the project results, we plan actions targeted at identified communities:

- Lead users (early adapters in research and industrial community; instrumentation laboratories and innovative companies),

- End users (late adapters in research and industrial community; biologists and large corporations),
- Investors (business angels and venture capital funds).

We will communicate results and offer IP to lead users for early adaptation. Further, we will organize workshops with demo microscopes showcasing the plethora of new microscopy applications enabled by SPAD detector arrays. This will lead to the identification of the most promising applications and targeted marketing to end users. Through participation in a high-profile project like NanoVIB, PII will improve its investment attractiveness. Additional start-up investment will, in turn, fuel faster growth.

PII will, at the end of the project, translate the building block of this project to other applications in spectroscopy, quantum physics, random number generation, material science and laser ranging.

PII's current exploitation plan entails the following activities:

EA6.1 Interface "green" SPAD array with AI's STED microscopes (M1-M9): PII and AI will collaborate to integrate PII's current generation of SPAD arrays into AI's STED microscopes. The focus is going to be placed on hosting multiple SPAD arrays in a compact microscope area. This will improve image quality through background compensation and advanced deconvolution techniques for "green" STED.

EA6.2* Develop new time-correlated single photon counting (TCSPC) system for fluorescence lifetime imaging (FLIM) (M34-M48): PII will develop and market its SPAD array with TCSPC capability. The TCSPC will be implemented on an FPGA hosting an array of time-tagging electronics with 20 ps resolution. This development will be synergic with the development of time-tagging Gen II detection electronics planned for this project. Precise time-tagging enables reduction of auto-fluorescence, gated-STED and FLIM. These features will enrich AI's application possibilities. Time-tagging detection electronics will be also offered as standalone units capable of hosting a plethora of other photon counting detectors.

EA6.3 Test and validation of SPAD arrays with enhanced red and NIR sensitivity in relevant environment (AI) to TRL7** (M34-M42): We will tailor our new SPAD array in a relevant application environment. AI, KTH and LLG will provide valuable feedback on geometry, functionality, and on the electronic interface of the detector. This will lead to TRL7 and pave the way to commercial exploitation of the new SPAD array.

EA6.4 Promotion of SPAD arrays with enhanced red and NIR sensitivity (M37-M72): PII will start marketing the new SPAD array at the end of the third project year. With TRL7 and first application results in MINFLUX microscopy, PII will expand the application field of the new SPADs beyond microscopy. The new applications will include spectroscopy, quantum physics, material science and laser ranging.

EA6.5 Develop SPAD image sensor with 512×512 pixels using the new "red" SPAD as building block (M34-M72): Using the newly developed "red" SPADs as building blocks, PII will develop and exploit large SPAD image sensors with 512×512 pixels. The main benefit of SPAD image sensors is the elimination of readout noise and the high frame rate. These features pave the way to high-speed photon-counting imaging. A SPAD image sensor will be offered as a replacement for scientific EMCCD cameras.

Participant 5 (Angewandte Physik und Elektronik GmbH, APE)

APE is a SME company and manufacturer with a strong focus on producing light sources for the biophotonic microscopy market, where APE's lasers are used for multi photon excitation (MPE) microscopy and coherent Raman imaging (CARS and SRS). APE is the market leader in coherent Raman light sources, but this application forms only a small fraction of the total MPE market, which is currently dominated by the two American companies Coherent Inc. and MKS/Newport/Spectra-Physics with their tunable fs-lasers. We estimate about 300 MPE laser sales every year, of which about 10% make up for coherent Raman applications and an APE-market share of 50% for the latter. However, MPE lasers are expensive, with prices ranging from 100k€ to

250k€, making it difficult for users to buy two lasers to do both coherent Raman and classical MPE microscopy.
By enhancing the capability of the proposed new light source, to do both efficient MPE and coherent Raman microscopy, APE will considerably strengthen its position in this market. Within this project, APE will develop a version of the light source that is easily integrated by our partners into MINFLUX microscopes, both hardware and software-wise (and, outside the project, into STED microscopes as well). Dissemination to the general market and production transfer is not covered by the money applied for in this grant application. However, to bring this project to a commercial success this step is vital and it will be covered from APE's regular revenue.
APE has the infrastructure necessary to conduct the tasks required for the project and to bring the new prototype to TRL9. TRL9 should be achieved one year after the end of the project at the latest. Investments in infrastructure of 200k€ to 300k€ and in people will then be necessary for scaling up the production, something which APE can cover through its regular revenue. We expect that the new offering will double the sales of coherent Raman light sources and raise the revenue from these products to about 4M€ per year. This will secure 15 highly qualified jobs at APE and generate 10 more within 5 years after product launch. Together with the other industrial partners in this consortium we will thus strengthen the European position in the bio-photonic technology market.
Specific activities to maximize impact are:
EA5.1 Test and validation of light source in relevant environment (LLG and KTH) to TRL7** (M22-M36): In parallel to the two prototypes at LLG and KTH, APE will build a third prototype, to be used in direct interaction with the two partners. The interaction with the users is essential for improving hardware and software, for best performance and ease of use with the SRS- and MINFLUX microscope. This process will bring the prototype to TRL7.
EA5.2 Scientific promotion of light source for SRS and multiphoton microscopy (M28-M72): With the first results from the partners LLG and KTH, APE will start marketing the new light sources on scientific conferences, as a combined light source for SRS and femtosecond multiphoton microscopy. This will be done together with the consortium partners by presenting microscope and light source data.
EA5.3 Laser qualified with microscope, bringing it to TRL9, production of laser (M37-M60): Together with AI we will qualify and integrate the laser for NIR-MINFLUX and coherent Raman imaging, thus bringing the laser to TRL9. Production transfer will be finalized no later than 12 months after the end of the project. This does include investment into production infrastructure in the order of 200 to 300 k€. Small-scale prototype production for early adopter customers is aimed for M46.
EA5.4 Commercial promotion of the light source (M40-M72): APE will present the new light source on trade shows and through media / social media activities. It will use its international sales and distribution network for promotion. Other microscope companies doing coherent Raman imaging will be contacted. Several of them are already customers of APE. APE is the premier supplier for coherent Raman light sources and well established in the multi-photon microscopy community. The reputation of APE and its scientific and commercial contacts will help to promote the new light source.

Data management

Within the scope of this project, three types of data are in principle generated: 1) software routines for hardware control, 2) software routines for data evaluation and 3) measurement raw data.
Concerning the storage requirements, most of the data is generated by measurements: Typical acquisition times in MINFLUX-based nanoscopy are 5-60 minutes. During this time some 10,000 molecules are localized. The resulting memory requirement for the raw data is currently approx. 1 GB per 60 minutes measurement time. Consequently, a maximum of 2-4 TB of raw data per year will be generated, which is within the range of commercially available hard drives. For SRS recordings the requirements are even one order of magnitude lower. Therefore, short-term and long-

term data storage solutions already in place at the partners are sufficient. In addition, all research data associated with a publication will be subject to specific long-term storage in accordance with the rules of good scientific practice and current data guidelines.
To provide access to all data and routines for the entire consortium (shared domain), we will establish a cloud at KTH to which the partners will have access under the consortium agreement. We will make a distinction with regard to access of the general public (public domain) to this data: Software routines for hardware control represent proprietary IP and know-how of the companies of the consortium and are only made publicly available in compiled form in connection with the corresponding products. Software routines for data evaluation and raw measurement data will be freely accessible. However, a general disclosure will only be made after a thorough assessment whether the data and routines have to be embargoed for a certain period of time due to legitimate interests of the scientists (planned publication) or the research institutions (planned patenting). Any lifting of an embargo requires the approval of the project management committee (PMC).
A corresponding **data management plan** (DMP) which meets with H2020 requirements for Open Research Data will be developed and implemented in the scope of Task 7.1 within WP7 during the first six months of the project (D.7.2).

Knowledge management and protection

Results and procedures of potential interest to the project partners and the public will be deposited in a repository as part of our standard publishing procedure in the project. As the SMEs involved are R&D centered and have extensive experience working on emerging technology, policies to ensure proper documentation and archiving of development and research activities are in place. This is of course also the case for the leading research institutions partnering up in this project.
We expect significant IP to emerge from our development efforts, given that the project focuses on a new imaging method and combines array detection and coherent Raman imaging with MINFLUX (array detection and STED as part of EAs) for the first time. During development of the lead application, the project partners will be pursuing optimal solutions to practical problems arising, which routinely results in protectable intellectual property.
Fortunately, several of the researchers involved are inventors of many successfully filed patent families in the past, and therefore the necessary awareness and know-how to secure IPR is guaranteed within the NanoVIB consortium. If needed, the partners from KI, KTH and the LLG are supported by their respective local university patent offices, and AI, APE and PII have IP experts on their staff and procedures in place. As we anticipate a major part of the IP to result from our intensive cooperation, the consortium agreement will contain a non-disclosure agreement (NDA) and a detailed plan how to ensure proper handling of IP in this situation. The partners will also agree on the extent to which they will grant each other automatic licenses for patents filed as a direct result from the joint research conducted as part of the project.
An explicit aim of this project is to publish our scientific results in peer-reviewed, high impact journals, and to maximize outreach to the scientific community. All scientific publications emanating from the project will be published following the “gold” model, i.e. in online open access journals, such that the publications are immediately freely disseminated by the publisher upon acceptance. If appropriate, the partners will also consider publishing preprints e.g. on arXiv to accelerate dissemination of the scientific results. Open access publication is already an established routine at the academic partners (KTH, KI, LLG), and will be used as a standard also in this project. Prior to submission of manuscripts for publication, due considerations will be made to secure any IPR. For all publications planned in the project, prior notice of one month before submission to all partners in the project is required. During this time, any partner with concerns must respond. Planned publications and their handling with respect to IPR matters will be a standing point on the agenda at the meetings with the project management committee and will be regulated in detail in the consortium agreement.

b) Communication activities

Communication will be set high on the agenda to promote the project actions and its results. In line with this, we have raised the communication activities into a dedicated task (Task 7.2) of WP 7, on the same level as the coordination of the project as a whole, and at the regular meetings of the project management committee, communication activities will be a standing point on the agenda.
Given the highly multidisciplinary character of the project, its potentially huge implications on societal health and well-being, and its significant exploitation potential, there are many relevant target audiences for our communication activities.
On the exploitation side, the project primarily aims to promote the as to developed super-resolution microscopy, photon detector and tunable laser technologies, but the outcome of the project may also pave the way for exploitation on the biomedical as well as the biopharmaceutical side, for the development of new antibiotics and vaccines. This further emphasizes the need to reach out to a broad target group for the communication activities, and where the communication also has to be tailored to the different groups. Below, we have listed the main targets groups for our communication activities, the objectives of the communication to each of these groups, and in what form it will take place. All major communication activities are defined as explicit deliverables in the work package 7 (Project management and communication), as referred to in the listing below:

Communication with the general public

In the communication with the public, we want to bring forward three major aspects:

1. This project is a transnational cooperation in a European consortium, funded by the EU, with a scope that is impossible to take on at a national level, but requiring the larger critical mass found on a European level.
2. This project will bring about scientific results in the fields of bioimaging, cell-biology and bacteriology as well as the development of microscopy and photonic products which are world leading.
3. The relevance of the outcome of this project for society, its industrial competitiveness, and how it may contribute to better health and well-being for the society at large. The latter aspect will also be concretized from an individual citizen perspective, emphasizing that cellular originated diseases, such as pneumococcal disease, are a major cause of illness and death, and what a better understanding of the underlying mechanisms can mean for the individual in terms of lowered risk of severe illness, access to new antibiotics and prevention by new vaccines.

Already at the beginning of the project, we will establish a project web page (D7.3). On this web page, a summary of the aims and planned activities will be given, and updates will be done on a regular basis reporting on ongoing studies and achievements. This web page will have two levels, one addressing the public, and one intended for specialists. Second, we will arrange several open lectures to the public (D7.6). These lectures will both be given at the beginning of the project, then mainly communicating the aims, plans and how the outcome is of relevance to the public, and then during the last year of the project, communicating the achievements of our cooperation. These lectures will be publicly announced, given in Stockholm within the regular open lecture series arranged by the academic partner organizations (KTH and KI), as well as in Göttingen, arranged by LLG in collaboration with the Georg-August University, the University Medical Center as well as the Cluster of Excellence in Göttingen. We will also use the communication office at KTH and the LLG for press releases, to announce the start of the project, and whenever called for, e.g. when exciting new results have been generated in the project.

Communication with potential end-users

By addressing potential end-users of the microscope system to be developed in the project, we want to inform them about what this new technology can add, beyond what is possible with current state-of-the-art microscopy techniques in their different fields of activity. As we see it, this group of end-users is quite broad, and includes not only researchers in bacteriology, cell biology, or cellular biomedicine in general. Also included in this group are users outside of academia, in biotechnology and pharmaceutical companies, in healthcare and at hospitals.

Reaching out to this group serves several purposes; First, we expect that the scientific results and established procedures from the lead application on clinical bacteriology in WP6 can be transferred to several different fields of biomedical and biopharmaceutical research, and help furthering the understanding of the cellular origin of diseases. Second, with a broad group of potential end-users realizing the potential of the developed microscopic technique for their activities, a large surge in the commercial demand can be expected.

To reach out to this group, we will arrange several end-user workshops (D7.5). In conjunction to two of the consortium meetings, a one-day workshop will be arranged, taking place in Stockholm in year two of the project, and in Berlin during year three. In addition, two hands-on workshops for end-users will be arranged in Göttingen during the last year of the project, which in addition to lectures and tutorials also contain practical training at the MINFLUX facility. These workshops will be announced broadly over Europe, and among potential end-users in several different fields and communities. The facility (D6.1), to be established and to be in operation during the last year of the project (and beyond), will thus support both the dissemination and communication outcomes, and will as well promote the exploitation of the developed microscope system, lasers and detectors. For the companies of our consortium an important communication channel to potential end-users is also the participation in fairs and conferences. Here, results from the project will be used and advertised in the form of e.g. demonstrations and application notes. To identify additional end-users, and to adapt the scope and activities of our project efforts for best possible impact, we will establish and then take advice from an end-user group (EUG) (D7.4), representing a broad scope of potential stake-holders.

Outreach to scientists:

With the academic partners of our consortium, and with the three companies having long experience in working very closely with academia, an important communication channel about the outcomes of this project is via scientific publications, lectures, conferences and meetings. We will publish our scientific results in open access, high-impact journals, for maximal spread of our results in the photonics, bioimaging, bacteriology, cell-biology and biopharmaceutical communities and beyond, allowing also others to implement the results and progress science further. PhD students and postdocs in the project will be given the opportunity to present their results at least at one yearly scientific conference or meeting, in addition to the workshops arranged as part of our agenda (see above).

3. Implementation

3.1 Work plan — Work packages, deliverables

The NanoVIB brings together a highly inter-disciplinary consortium, to work closely together in a work plan comprising seven WPs. A next-generation super-resolution microscope system will be developed in WP1-WP4, then established and used for a lead pilot application and offered as an open facility in WP5-WP6. The time frame of all WPs is shown in Fig. 3.1a and the inter-dependence of the scientific tasks is visualized in Fig. 3.1b. At the project's core is the development and realization of a prototype of a super-resolution microscopy platform (WP1 & WP2), to which prototypes of new lasers and photon detectors developed within the project (WP3 & WP4) will be added. During the course of the project, the super-resolution platform is continuously further

developed (see Tasks 2.1 – 2.5) and upgraded with new and/or improved components (see Tasks 1.2, 1.3, 3.1 – 3.4, 4.1, 4.2), rendering it faster as well as with new correlative and NIR imaging capabilities. The identification of suitable acquisition strategies as well as sample preparation protocols (see Tasks 2.2, 5.1 – 5.3) gives valuable feedback for the platform development and allows proof-of-concept measurements on different biological samples (see Tasks 5.4 – 5.5). These experiments in turn provide the basis for the biological studies in WP6. In this WP, the performance of the developed microscope system will be demonstrated in a lead application, to reveal detailed molecular mechanisms underlying virulence and invasiveness of pathogenic bacteria with pneumococci as model organism (Tasks 6.1 – 6.4). Furthermore, a user facility will be established to make the system available for testing by researchers outside the project (see Tasks 1.4 & 6.5). WP6 thus provides two complementary means for dissemination and for promoting exploitation of the developed microscope system, its lasers and detectors, but also provides feedback to optimize

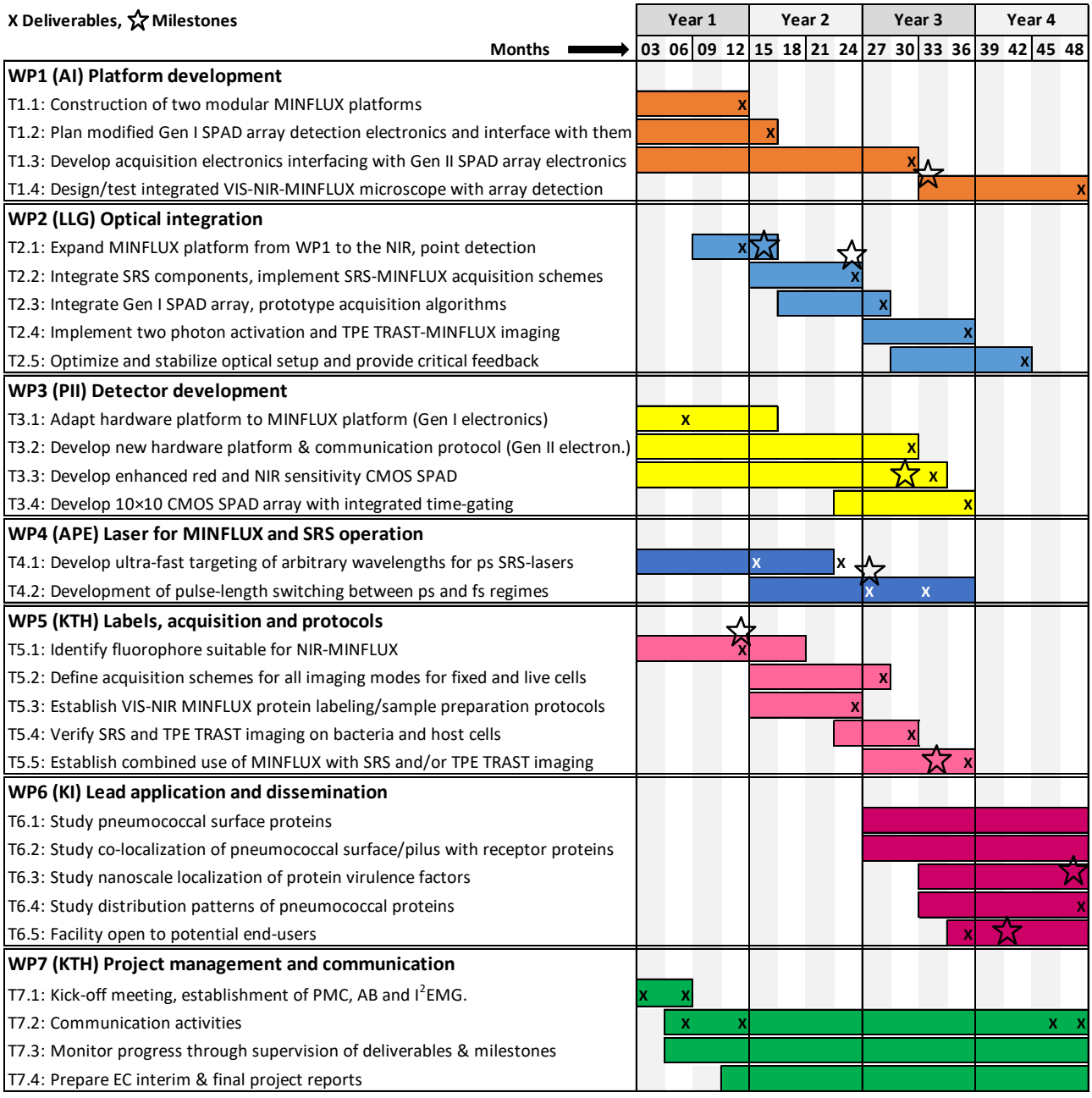

Figure 3.1a Gantt-chart of NanoVIB showing the time-line of the WPs and their different tasks, and the time point of the related deliverables (crosses) and milestones (stars).

the platform design and user interface, thereby supporting commercialization also in this way (Task 1.4).

The project is coordinated and steered according to a dedicated management plan (WP 7), which will ensure timely achievements of tasks in the project, and that the WPs are run efficiently and reach the expected results (Tasks 7.1, 7.3-7.4). Furthermore, by dedicated communication and dissemination activities (Task 7.2), it will be ensured that the project results are translated into successful exploitation and leads to scientific outcomes of highest international standard.. Each WP has measurable deliverables (Table 3.1c) and intermediate milestones (Table 3.2c) that will be assessed to ensure project progress, and there are robust measures at hand to mitigate critical risks (Table 3.2b).

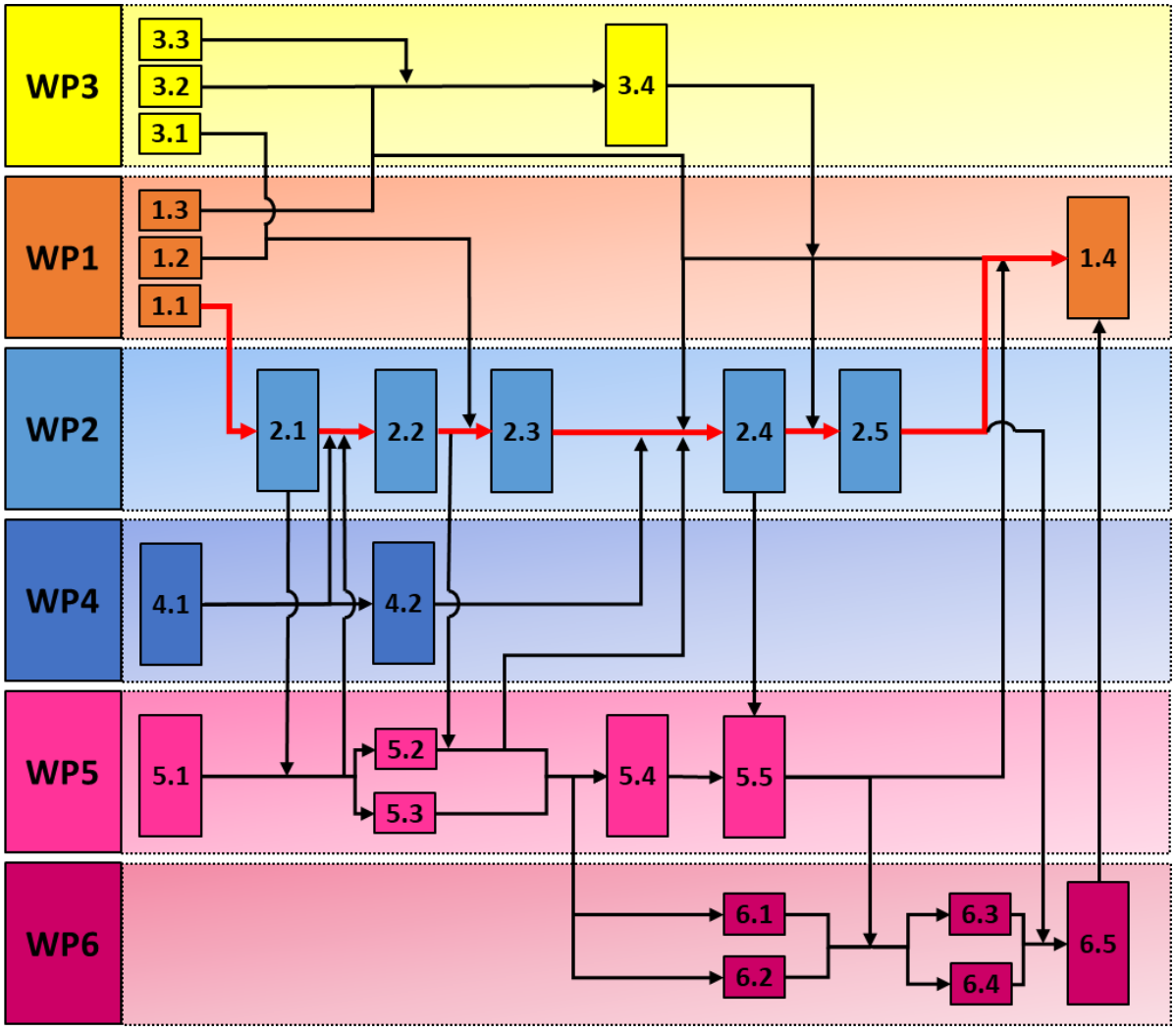

Figure 3.1b: Pert chart, illustrating the overall workflow of NanoVIB, how the different WPs with their main tasks link together, and with the main path in prototyping the next-generation super-resolution microscope system indicated with red arrows.

Table 3.1a: List of work packages

WP No	Work Package Title	Lead Part. No	Lead Part. Short Name	Person-Months	Start Month	End month
1	Platform development	3	AI	81	1	48
2	Optical integration	4	LLG	81	7	42
3	Detector development	6	PII	43	1	36
4	Laser for MINFLUX and SRS operation	5	APE	57	1	36
5	Labels, acquisition and protocols	1	KTH	95	1	36
6	Lead application and dissemination	2	KI	93	25	48
7	Project management and communication	1	KTH	30	1	48
				480		

WP1: Platform Development

The initial aim of work package **WP1** is to provide LLG with two MINFLUX platforms that are fully functional for imaging in the visible wavelength range but feature an open optical design and modular, extensible application software. These platforms are to be equipped with the additional light sources, optics, detectors and control algorithms in order to extend them both to MINFLUX in the NIR wavelength range, to demonstrate correlative TPE TRAST and SRS imaging, as well as to evaluate two photon activation in MINFLUX microscopy (**Task 1.1**). As the project moves forward, AI will then focus on integrating novel SPAD arrays into the platform, developed by PII, that provide additional positional and timing information about each detected photon, and which have the potential to increase the quality of MINFLUX images greatly (**Task 1.2, 1.3**). Towards the end of the project, the platform will undergo a third transition, to incorporate all insights gained during implementation, testing and successful application of the new technology to challenging biological problems in WP6, into a second generation MINFLUX microscope (**Task 1.4**). The timing of the different tasks is visualized in a Gantt chart (Fig. 3.1a).

The first task (**Task 1.1**) is expected to be completed within one year after starting the joint project, with the first platform being delivered after M6. With the first proof of principle of MINFLUX published in 2017 it is still a novel approach and AI's current implementation, launched as an end-user product in Feb 2019, accommodates this by being flexible both in its optical design and the details of data acquisition and analysis. This makes it an ideal starting point to add optical ports and software interfaces and allows smooth integration of the additional components in WP2**.**

To this end, a dedicated member of our optical engineering team will focus on assembling the platforms and AI will cooperate closely with LLG from the start. This will ensure an efficient deployment of MINFLUX platforms which allows NIR MINFLUX imaging at the KTH and KI site and ensures that a working prototype is available for further integration work at the LLG site.

When both platforms are on place, the optics engineer responsible for platform assembly will have a part of her/his work hours reserved to contribute to WPs 2, 5 and 6 and will collect feedback about optical stability and alignment protocols.
From the start, AI will also begin working on the two-step integration of array detectors into its acquisition logic. For an initial proof of concept, PII will be modifying its Gen I detection electronics to provide a configurable number of electronic pulse outputs, corresponding to photon detection events in different areas of the array. This will allow AI to integrate these novel detectors into the existing acquisition electronics (**Task 1.2),** with minimal changes to the FPGA implementation and electronics as the detectors can essentially be handled in the same way as single photon detectors currently used in MINFLUX microscopy. Nevertheless, by providing the additional spatial information to the acquisition sequencer and the data analysis algorithms, we expect a measurable improvement of the image quality. Through AIs flexible software interface, the partners within the consortium will be able to prototype and test these approaches and provide valuable feedback for the second implementation step.
In a second step, AI will enable the partners within the consortium to leverage all spatial and temporal information provided by PII's Gen II detection electronics. A jointly defined communication protocol will allow the transfer of the arrival time and detector element for each detected photon to the MINFLUX electronics in real time during the acquisition process. However, AI's current platform is not equipped to receive and analyze this 'photon stream'. Therefore, AI will redesign and modularize the detection and acquisition control electronics and implement a new FPGA based platform capable of receiving the data from PII's Gen II detection electronics.
This requires close cooperation with PII who will be developing this Gen II detection electronics for their SPAD arrays simultaneously. To ensure that partners KTH, KI and LLG have enough time in WP5 and WP6 to fully explore and exploit all possibilities offered by this improvement, both AI and PII will start their respective tasks as soon as NanoVIB is kicked off. Both companies are already engaged in a successful cooperation aimed at leveraging SPAD arrays for STED microscopy. Thus, with all the key personnel already acquainted, AI and PII will be able to design and finalize the specification of a communication protocol for detector data within 3 months after the starting date of the task. During the remaining part of the task PII and AI will continue to cooperate closely providing the respective partner with test hardware and consulting each other.
On AI's side, an electronics engineer will be hired as soon as possible and exclusively tasked with this project. As AI's current STED and MINFLUX platforms all employ similar techniques, the engineer will be provided with all necessary resources from the start and the project will be overseen by the lead developers of the STED and MINFLUX FPGA platforms. After the design phase, a test implementation will be realized on a commercially available FPGA evaluation board and verified with simulated detector input based on the specifications of the communication protocol.
This test implementation will be the basis to task a C/C++ developer with providing an interface that allows integration in the instrument control software, while the electronics engineer proceeds to the design, layout and production of required components in order to provide a module that is also compatible with traditional single photon detectors and the Gen I SPAD array detection electronics. In this way, testing can commence independently of the WP3 timeline. This module will replace the previous generation electronics in both deployed MINFLUX platforms by M32 making them ready for the integration of Gen II SPAD arrays.
At this time, the platforms will already have been in operation since M7, application development and regular imaging operation will have been under way since M9 and M25 respectively. With the AI optics engineer and the AI project coordinator closely tied into WP2, WP5 and WP6, sufficient feedback on improving both details of the optical design and the user interface will have been collected to start making design changes aimed at getting the technology ready to be demonstrated to and tested by researchers outside the project (**Task 1.4**). Those changes which can be

incorporated into the existing platforms will be implemented directly and others will be used to design the final product.
Operation of the prototypes at both sites, within a lead application and as an open facility, will attract early adopters that both give additional feedback and form a pool of potential customers for the new MINFLUX generation. By the end of our project, AI will be in position to demonstrate NIR-MINFLUX in collaboration with KTH/KI and LLG.

WP2: Optical integration

The aim of work package **WP2** is, on the one hand, the demonstration of a multimodal super-resolution platform for correlative MINFLUX, SRS and TRAST imaging (**Tasks 2.2**, **2.4**) and on the other hand, the further development of the MINFLUX method as such (**Tasks 2.1**, **2.3**). The focus lies on the optical and mechanical integration of the components provided by the partners AI, PII and APE into the platform and on the development of acquisition protocols, which are the basis for specialized strategies for bacteria and host cell studies within WP5 and WP6. To ensure know-how transfer between Göttingen and Stockholm, several research visits of scientists from KTH are planned. Optimization of the platform in **Task 2.5** will generate valuable feedback for the design of the partners' prototypes. The timing of the different tasks is visualized in a Gantt chart (Fig. 3.1a).
Already in the construction of the modular base MINFLUX platforms by AI (in Task 1.1), the requirements of the later device integration steps must be considered. This is especially true with regard to the wavelength-dependent optical properties of the components, since the final microscope platform will cover a wide wavelength range from UV to NIR. Therefore, the LLG will be involved in the platform design from the start of the project and supports AI in Task 1.1. Further platform development will be performed at LLG, with each step being accompanied by proof-of-concept measurements on test samples. During the course of the project, the platform is repeatedly upgraded with (improved) components provided by APE, PII and AI. After successful verification of each development step, a second identical platform at KTH will be upgraded to the new standard in order to allow for the parallel establishment of best practices and new labeling protocols (WP5).
In detail, as a first step (**Task 2.1**), the MINFLUX method will be extended to the NIR by upgrading two modular MINFLUX platforms with suitable excitation lasers and point detection modules. Platform I will be provided by AI at the beginning of the work package; platform II will follow at the end of year 1. A PhD student from KTH will support the LLG in Göttingen and familiarize himself/herself with the microscope and its usage. Upon successful implementation, platform I will be delivered to KTH and installed there by the end of year 1. The complex and sophisticated MINFLUX acquisition scheme, whose central part is an elaborate fluorophore localization procedure in which the fluorescent signal of a single fluorophore is iteratively analyzed for different positions of a doughnut-shaped excitation focus, is sensitive towards various parameters, like focus quality, background fluorescence and fluorophore photophysics. Therefore, taking into account the results of Task 5.1 and the therein identified suitable NIR fluorophore, the acquisition protocol will be adapted and optimized using the Python interface which allows the acquisition parameters, as well as the FPGA-controlled acquisition sequences to be accessed and modified.
Platform II at LLG will be used for initial research on combining MINFLUX with SRS (**Task 2.2**). This requires the implementation of an SRS-light source, which will be developed and then provided by APE within WP4, taking benefit from APE's expertise in SRS imaging. For correlative imaging, the mutual influence of both imaging modalities needs to be carefully analyzed in order to prevent artifacts, e.g. due to pre-bleaching of the fluorophores by the SRS acquisition. Therefore, proof-of-principle measurements for correlated SRS-MINFLUX imaging will be combined with the development of suitable acquisition strategies, maximizing the useful information content while minimizing the light dose and thus sample damage.
MINFLUX itself can be conceptually improved by using an array detector instead of a point detector (**Task 2.3**). LLG will therefore integrate a SPAD array into the setup in close collaboration

with PII, who modified the Gen I detection electronics in Task 3.1, and AI, who integrated the array into their acquisition electronics in Task 1.2. Taking the spatial information into account, new data analysis algorithms for background suppression and faster molecule localization will be developed using the Python interface of AI's application software.
With the upgrade of the SRS-light source with the femto-pico conversion add-on (from Task 4.2), the SRS-laser can be switched between picosecond and femtosecond pulse length operation. Using the latter allows to exploit the intrinsic optical sectioning capability of two photon processes in **Task 2.4**. Therefore, MINFLUX will be combined with two photon activation of the fluorophores, which spatially restricts fluorophore on-switching to the focal plane and thus potentially reduces the resolution-limiting background signal. As well, label-free two photon excitation TRAST metabolic imaging will be implemented for correlative TRAST-MINFLUX imaging, as performed within Tasks 5.5 and 6.2. Again, viable acquisition schemes are developed which take into account possible adverse mutual influences of the correlative imaging modalities.
The optimization of the platform in **Task 2.5** includes mechanical stabilization and workflow improvements. Further, the newly designed CMOS SPAD arrays will be evaluated and the photon stream-data made available by the Gen II detection electronics and AI's new FPGA platform will be used for extended data analysis. Our feedback on handling and performance of the components provided by the partners will give valuable insight for a commercial prototype design by AI in Task 1.4.
The WP is headed by Prof. Alexander Egner who is supported by Dr. Claudia Geisler, an experienced research assistant. The work in connection with optical and mechanical implementation and acquisition strategies will be performed by a PhD student in close cooperation with a postdoc and the visiting scientists from KTH.

WP3: Detector Development

The aim of WP3 is, on the one hand, to enable scalable integration of SPAD arrays into MINFLUX microscopes, and, on the other hand, to develop complementary metal–oxide–semiconductor (CMOS) SPAD arrays with enhanced red and NIR sensitivity. The main specifications to be achieved are as follows:

- Time-tagging electronics with 20ps timing resolution
- A peak quantum efficiency above 50% at 640nm
- Timing jitter of the SPAD with less than 250ps FWMH at 640nm
- On-chip time-gating with 1ns gate windows

PII will first modify existing detection electronics to integrate Gen I SPAD arrays in MINFLUX microscopes. Further, to utilize all 23 channels of the current SPAD array, PII will develop Gen II detection electronics and in parallel design new SPAD arrays with enhanced red and NIR sensitivity. Lastly, PII will develop larger SPAD arrays with chip-level time-gating. The timing of the different tasks is visualized in a Gantt chart (Fig. 3.1a).
In **Task 3.1** PII will modify its existing detection electronics (field programmable gate array and printed circuit board, i.e. FPGA and PCB) to allow seamless integration of its green-detection-optimized 23-pixel SPAD arrays into AI's MINFLUX microscopes. The Gen I detection electronics will forward software-selectable SPAD array channels with intrinsic jitter through a reconfigurable FPGA. SPAD channels are going to be forwarded to 4 input channels available in the MINFLUX system.
Further in **Task 3.2**, PII will develop the Gen II detection electronics together with AI. Because the MINFLUX technique requires feedback loops (scan positioning depends on detector output), the firmware functionalities of photon counting/time-tagging and microscope control will be merged through a dedicated communication protocol. This will enable utilization of all 23 pixels of the SPAD array with full timing resolution. PII and AI will explore scalable electronics platforms with both parallel and serial protocols for communication. PII will further develop time-tagging

functionalities with 20ps timing resolution and explore non-uniformities in terms of differential and integral non-linearity, as well as power versus area trade-offs.
To supplement its current SPAD array optimized for detection around the green spectra, PII will develop SPAD arrays with enhanced red and NIR sensitivity, targeting a peak quantum efficiency (QE) above 50% at 640nm and specially enhanced QE spectra between 700nm and 850nm (**Task 3.3**). The array will be optimized for off-chip time-gating, with a timing jitter of less than 250ps at 640nm. The new SPAD array will be made platform compatible with Gen II detection electronics. PII will analyze fill factor (ratio between detection area and pixel area) versus noise trade-offs.
As an outcome of these activities, PII will develop larger (10×10 pixels) SPAD arrays (small image sensors with 1Mframes per second) and develop a chip architecture to dynamically pre-select relevant photon events by chip-level time-gating in **Task 3.4**. Both chips will be supplemented with microlenses to utilize the intrinsic sensitivity of the SPAD array. PII will analyze and optimize additive and imprint microlens fabrication procedures. The larger SPAD array will be developed by integrating our partners' feedback on the deliverable of **Task 3.1**. Chips will be fully electro-optically characterized.
PII will hire a chip and FPGA designer to supplement the PII's experienced team. The designer will work in a close collaborative setup throughout **Task 3.1** and **Task 3.2**, visiting our partners AI, LLG and KTH for the development of Gen I and Gen II detection electronics. She/he will further continue designing novel SPAD arrays tailored for the MINFLUX application. Special emphasis is going to be placed on early data processing on the FPGA, utilizing the parallelized computation power of the FPGAs.

WP4: Laser for MINFLUX and SRS operation

The aim of **WP4** is to develop a new, easy to use one-box light source, which provides picosecond pulses for SRS imaging and femtosecond pulses for two-photo-activation in MINFLUX imaging and thus makes the usage of two separate complex and expensive laser systems obsolete. The light source should be completely computer controlled allowing for integration into microscope setups. Further, the tuning time of the current SRS light source picoEmerald should be improved by more than a factor of 10, from typically 60 to 120 seconds to less than 5 seconds in order to enable fast multispectral SRS imaging. The main parameters to be achieved are as follows:

- Automatic temporal and spectral overlap of pump and stokes beam for SRS
- Noise of the pump beam around 160dBc at 20MHz; modulation of the stokes beam at 20 MHz
- Wavelength tuning at least 700 to 990nm / 400 – 4600cm^{-1} wavelength difference for SRS, tuning time <5s
- Bandwidth of ps-mode ~10cm^{-1}, pulse length about 2ps, pulse length of fs-mode ~300fs.
- Several hundred mW output power in each beam in the femto- and the picosecond mode

To decouple the two major development steps, we have divided the work into two tasks. **Task 4.1** will concentrate on the fast tuning and **Task 4.2** on pulse length switching between femto- and picosecond operation. The timing of the two tasks is visualized in a Gantt chart (Fig. 3.1a).
The light source is based on an optical parametric oscillator (OPO) pumped by a ps-fiber laser. To achieve the goals described, a different tuning concept needs to be developed and the OPO needs to be completely redesigned. Further, APE plans to implement a modern and flexible electronic and software platform. A laser and optics physicist will be assigned to the work package throughout the project to develop optical schemes, perform optical tests, define requirements on electronics, mechanical design and software requirements and validate these results.
First, APE will focus on developing ultra-fast targeting of arbitrary wavelengths for picosecond lasers in **Task 4.1**. The current OPO tuning concept in APE's SRS light source is based on temperature tuning of nonlinear crystals. Even though the heating elements are optimized for speed, the limitations are tuning times of one to two minutes. To overcome this, different nonlinear interactions will be evaluated to tune the OPO by mechanical shift or rotation of nonlinear crystals

as well as fast cavity length adjustments. Next to the specifications mentioned above, the following parameters need to be verified: nonlinear crystal degradation, conversion efficiency, beam quality and beam pointing over tuning. Mechanical actuators need to be validated regarding the required tuning speed and accuracy. A first assessment of the femto-pico conversion based on spectral broadening in the cavity will be performed. After this evaluation phase mechanical design of the OPO cavity and the overall light source will start. A mechanical design engineer at APE will be assigned to this task for about 6 months. To minimize the risk, the current picoEmerald platform has to be used as a starting point for the mechanical design. The electronic and software architecture used in the picoEmerald is not flexible to adapt to new tuning schemes and new features. Therefore, the electronic hardware will be switched to a modular architecture based on a mixture of CAN and I2C bus communication. The platform architecture described does already exist at APE, which does allow for efficient implementation and requires only 3 months work from an electronics engineer. For software development, a similar modular approach will be followed. APE will use a LabVIEW based modular software architecture, established at APE. Moreover, a software architect will be assigned at APE at the beginning to allow for basic, scripting based tuning tests and later for the full software integration of the laser (7 months).
LLG plans to start Task 2.2 already in M13. APE will only be able to deliver a first prototype of a fast tunable SRS-light source in M22 (D4.2). To bridge the time and enable LLG to start working, APE will provide a loan SRS laser (picoEmerald) to LLG with D4.1 in M13.
APE will work together with LLG to demonstrate that the prototype can be integrated into the microscope setup and support them to demonstrate correlative SRS imaging with its expertise. To support LLG efficiently, APE needs to build a second prototype to verify and reproduce problems and fix possible issues. This prototype will also be the basis for the following task.
Task 4.2 will concentrate on switching the pulse length between about 2ps and 300fs to enable narrowband SRS imaging as well as MINFLUX with the same light source.
The work will start with the evaluation of spectral broadening of the pulses inside the optical parametric oscillator. Proof-of-concept work is already done in **Task 4.1** during the evaluation of new oscillator and tuning concepts. APE will study the concept in detail and implement it into software tuning and stabilization routines. Since the spectrally broadened pulses from the OPO will still have picosecond pulse lengths, it will be needed to develop pulse compressor designs based on diffractive gratings. The challenge here is to cover the whole tuning range of the picoEmerald with a high compressor efficiency of at least 50% and maintain beam quality and beam pointing. The coupling between spectral broadening and optimized compressor settings will be evaluated. APE will deliver a prototype femto-pico conversion upgrade of SRS-light source (D4.3) as an add-on for the prototype light source to LLG in M25. This is milestone M4 of the NanoVIB project.
A second compressor will be built to do software development and more close integration of the compressor and the light source. The feedback from LLG is taken to further improve software and optics. LLG will receive regular software updates for the light source and the compressor as well as hardware upgrades if necessary. For this work package APE will allocate 3 months of software engineering, 2 months of mechanical design and one month of electronic engineering resources in addition to the leading physicist. A matured prototype fast tuneable femto-pico SRS light source will be delivered to KTH in M31 (D4.4). APE will also produce a third prototype light source and compressor to be able to support KTH and LLG and finalize software and mechanical design within 6 months until end of M36. APE will allocate 4 months of software engineering and 1 month of mechanical design in addition to the laser physicist to this task.

WP5: Labels, acquisition and protocols

WP5 has two major aims: First, to identify fluorophore labels in the NIR suitable for MINFLUX imaging (**Tasks 5.1, 5.2**), second, to establish and optimize acquisition and sample protocols for the bacterial studies in WP6, allowing operation of MINFLUX (in the visible as well as in the NIR),

SRS and TPE TRAST imaging by their own or in combination (**Tasks 5.3** – **5.5**). The timing of the different tasks is visualized in a Gantt chart (Fig. 3.1a).

Although MINFLUX does not require exceptional photo-stability of the NIR fluorophores, they need to display stable, controlled photo-switching properties to qualify for MINFLUX imaging, which adds other requirements on the fluorophores and on the sample preparation. In **Task 5.1**, KTH will therefore investigate a range of NIR fluorophores with respect to their photophysical properties in order to identify dyes with switching properties which lend them suitable for MINFLUX imaging, and KTH will study under which sample and excitation conditions such switching can be induced. In the first place, KTH will look for suitable NIR dyes within the same category of dyes as currently used for MINFLUX in the visible wavelength range. In the red visible range, the cyanine dye Alexa 647 (excitation at 640nm, photo-switching at 405nm) has been found quite suitable for MINFLUX imaging, and KTH will thus explore if the suitability can be extrapolated to other cyanine dyes in the NIR, and if so, verify their usefulness for cellular measurements, and under what sample and excitation conditions they work optimally.

Apart from taking MINFLUX into the NIR (with the benefits of an extended spectral range, opening for further multiplexing, lowered photo-toxicity, autofluorescence as well as scattering background), a major methodological progress aimed for in this project is to combine MINFLUX with SRS and TPE TRAST for correlative imaging. In **Task 5.2**, KTH will explore the prerequisites for combined two photon activation for MINFLUX (of NIR as well as visible dyes) and label-free TPE TRAST imaging based on NADH/Flavin autofluorescence. Specifically, KTH wants to achieve parallel use of the excitation source, to minimize photo-bleaching/toxicity and to speed up image acquisition. Also, KTH will investigate the possible combined use of the laser developed in WP4 for simultaneous SRS and NIR dye excitation. For SRS the emission wavelength of the laser beams can be set over a broader range. As an option, the wavelength of one of them can then be set to match the excitation maximum of the NIR dye. Similarly, also here the benefit would be a lowered photo-bleaching/toxicity and a faster image acquisition. The outcome of the studies in **Task 5.1** and **Task 5.2** will provide useful feedback to the activities in WP4 and WP2.

Following the photophysical investigations above, the second major task of **WP5** is to establish and optimize the use of the MINFLUX instrument developed in WP2 for bacterial and host cell studies (to be carried out in WP6). The bacteria to be used here are mainly suitable mutants of pneumococci, and the host cells mainly cultured lung epithelial cells and immune cells. With the acquisition procedures determined in WP2 and in **Task 5.1/5.2** as a starting point, and using the MINFLUX instrument provided to KTH, KTH will first establish labeling protocols and sample handling/preparation of bacteria and host cells for VIS- and NIR-MINFLUX imaging (**Task 5.3**). For the most suitable VIS and NIR dyes, as identified in **Task 5.1**, we will then establish labeling procedures to specific relevant bacterial and host cell proteins and optimize the sample preparation to maximize the fluorescence and switching performance of the dyes in the samples. As studies will be performed on both fixed and live cells, different labeling strategies will be used, based on dye-labeled antibodies, as well as on modified dyes binding to genetically tagged proteins, following recently developed procedures for cellular studies by MINFLUX.[10] Likewise, the sample preparation and optimization of dye switching performance will be adapted depending on whether the imaging is to be performed in live cells or not, and will be largely based on standard switching buffers, as used in single-molecule localization super-resolution.[18] Next, with support from visiting scientists from LLG, we will verify SRS and TPE TRAST imaging on bacteria and host cells (**Task 5.4**). SRS imaging, as established in WP2 and using a laser established in WP4, will be verified on bacteria and host cells, by analyzing major lipid vibrational bands in these cells (e.g. the 3015cm^{-1} band associated with C=C-H stretching modes in unsaturated fatty acids, or the CH_2 stretching mode of lipids at 2845cm^{-1}). Moreover, the cells will be grown in deuterated glucose, and SRS

[18] van de Linde S et al "Direct stochastic optical reconstruction microscopy with standard fluorescent probes" *Nat. Protocols* 6, 991 – 1009, 2011

imaging of isotopically shifted bands will be verified, as an approach to be further used for isotopic labeling and the exosome studies in WP6. For TPE TRAST imaging, KTH will take its recently developed procedures[8] as a starting point, to establish and optimize this imaging on bacteria and host cells. To start with, KTH will use the same instrumentation as in Tornmalm et al.[8] for these studies, and then switch over to the fs-pulsed laser to be developed in WP4. Finally, within the constraints given by the dye photophysics, acquisition procedures and sample preparation, KTH will demonstrate the combined use of MINFLUX with SRS and/or TPE TRAST imaging (**Task 5.5**). This demonstration will be based on several preceding steps in the project, in particular on the integration and procedures established in WP2 and the preceding steps of this WP, and will establish best practices and the protocols for the bacterial studies in WP6. The gained knowledge will be transferred to AI in order to be considered in the commercial prototype design.
The WP is headed by Prof. Jerker Widengren who is supported by Dr. Joachim Piguet, an experienced researcher. The work in connection with tasks 5.1 – 5.5 will be performed by a PhD student in close cooperation with a postdoc. They will be supported for tasks 5.4 and 5.5 by visiting scientists from LLG and for task 5.3 by a postdoc from KI.

WP6: Lead application and dissemination

WP6 will effectuate two important final outcomes of the NanoVIB project: First, as a lead application of the VIS-NIR-MINFLUX system developed in this project (WP1-4), utilizing the unique resolution and imaging capabilities of this microscope system, KI will reveal inter- and intracellular processes underlying pneumococcal virulence and invasiveness. Second, KI will establish an open end-user facility to a broad group of potential end-users, to trigger the interest for the developed technologies and procedures in cell biology and biomedicine, and pave the way for the dissemination, exploitation and use of the developed microscopy system.

Lead application (**Tasks 6.1** – **6.4**): Pneumococcal virulence and invasiveness strongly depend on the properties of certain proteins on their surface, how they distribute themselves on the surface, and how their localization patterns are related to e.g. the cell cycle and the local cellular and outer cellular environment. In recent work by partners KTH and KI it was demonstrated that specific spatial distribution patterns of pneumococcal surface proteins can be resolved by super-resolution STED microscopy, revealing important information about underlying mechanisms for disease and invasiveness of the bacteria.[3,4] In this WP, with the ten-fold higher resolution offered by the MINFLUX microscope system as compared to other super-resolution microscopes, KI will demonstrate that an additional major leap in the understanding of these mechanisms can be achieved. With this resolution increase, coming even closer to the actual spatial scale of the protein interactions, with correlative morphological and environmental images by SRS and TPE TRAST, and with protocols and procedures for bacteria-host cell studies established in WP5, KI will address several central aspects of pneumococcal proteins and their role in disease:
Bacterial virulence and host defense evasion (**Task 6.1**): KI will study surface-associated pneumococcal proteins and focus on two proteins with central, but yet incompletely understood roles in virulence and host defense evasion. The studies will be done on pneumococci in different states of cell division, and on different strains with deleted or modified forms of these proteins. First, KI will study the surface protein PspC, known to bind factor H, in turn preventing immunological attacks by complement binding. KI will study how different variants of the surface protein PspC distribute on the bacterial surfaces, to what extent they bind factor H, cover particularly vulnerable division zones of the bacteria, and if and how this can be affected by additives having potential antibiotic or bacteriostatic effects. LytA, a pneumococcal protein that mediates autolysis of the cell wall, will be studied in a similar way: how it is brought to the surface from the cytosol and distributes on and between bacteria. KI aims to better understand how it is activated upon penicillin treatment, as well as the function of this protein, which still remains controversial. These studies will be performed on fixed cells, following similar labeling protocols as

for previous STED studies,[3] but now with the ten-fold higher resolution of MINFLUX, and with the protein distribution patterns correlated to the cellular morphology by SRS imaging.

Invasiveness (**Task 6.2**): Pneumococci, the main cause of bacterial meningitis globally, pass the blood-brain-barrier (BBB) with help of pilus proteins binding to receptor proteins on epithelial cells of the BBB. In a recent work by KTH and KI,[4] based on protein co-localization studies using STED, KI could identify two receptor proteins on the epithelial cells to which the pneumococcal adhesion proteins RrgA (a pilus protein) and PspC bind. In this WP, KI will use MINFLUX, with its ten-fold higher resolution, to take these studies to a next level. KI will study the co-localization of pneumococcal surface and pilus proteins with receptor proteins on epithelial cells of the BBB, in brain biopsies from patients who have died from pneumococcal meningitis and from mice models. Samples will be prepared following established protocols, with possible modifications for MINFLUX imaging based on the outcome in WP5. With MINFLUX extended into the NIR, these studies can be performed with significantly lower background, scattering, deeper penetration, and with less cross-talk. With overlaid SRS images, the protein (co)localization patterns can also be correlated to the morphology of the bacteria and the cells of the BBB. In samples from mice, KI will study effects of adding anti-bodies competing for binding to the BBB epithelial cell receptors as a potential approach to prevent pneumococcal meningitis.

Bacterial exosomes, their possible role in disease and as a basis for vaccines (**Task 6.3**): Recent studies by KI have shown that pneumococci can form extracellular vesicles (bacterial exosomes), that they can be internalized into host cells and elicit cytolysis and inflammatory reactions,[7] but can also generate immunization with serotype-independent protection in mice (unpublished). To better understand the role of pneumococcal exosomes in disease development, how they are formed, and their potential as candidates for new vaccines, KI will use the MINFLUX microscopy system to study the nanoscale localization of protein virulence factors, including the cytotoxin pneumolysin, PspC, LytA, RrgA etc, within the exosomes. KI will investigate if there are differences between different pneumococcal strains and how the vesicles bind and internalize with host cells (epithelial and immune cells). By overlaying the protein localizations onto SRS images showing morphological cytoskeleton and lipid membrane maps of bacteria, host cells and vesicles, KI will further elucidate the role of the proteins in vesicle formation and in subsequent host cell interactions. Inflammatory reactions on host cells will be studied by TPE TRAST imaging, adding whole bacteria, pneumococcal vesicles, or specific bacterial antigens (e.g. pneumolysin). These images, reflecting local redox status and oxygenation in the cells, will then be correlated to nano-scale localization patterns of protein virulence factors.

Pneumococcal-viral coinfection (**Task 6.4**): By using the next generation super-resolution microscopy of this proposal, KI will study distribution patterns of pneumococcal surface proteins, coupled to sustained bacterial growth in lungs of pneumococcal-influenza virus co-infected mice with a focus on the pneumococcal protease HtrA. HtrA is central for bacterial growth during inflammatory conditions and protects and removes misfolded (oxidized) proteins. KI will study how the distribution patterns and localization of HtrA correlate with the microenvironment of the host, presence of antioxidants, and with the ability of the bacteria to evade immunological attack by imaging the degree of complement deposition on their surface. In this way, we expect to unravel important metabolic aspects in bacterial-viral co-infections, which seem to be a major driving force in such infections, and thereby also find better strategies to curb these infections.

End-user facility (**Task 6.5**): During the last year of the project, one of the next-generation super-resolution MINFLUX microscope systems established in the project will be set up by LLG as an end-user facility, open to a broad group of researchers from academia as well as from companies. Thereby, potential end-users will get the possibility to evaluate the capabilities of the MINFLUX microscope system for their applications, via guided, hands-on pilot experiments. Together with the lead application (**Tasks 6.1** – **6.4**), this will additionally spur the interest for the microscope system, lasers and detector systems developed in the project, and pave the way for the further dissemination, exploitation and use of the developed microscopy system.

Finally, the experience from both the lead application above and from the pilot end-user experiments in the open facility will be brought back to the SMEs in this project (AI, PII, APE), providing them with important feedback for further refinement on their technologies.
The WP is headed by Prof. Birgitta Henriques-Normark who is supported by Dr. Anuj Pathak, an experienced researcher. The work in connection with **Tasks 6.1** – **6.4** will be performed by two postdocs from KI and one postdoc from KTH. Work in connection with the end-user facility will be performed by one postdoc from KI and one postdoc from LLG.

WP7: Project management and communication

Activities within this WP are outlined in detail in section 3.2.

Tables 3.1b: Work package description

Work package number	WP1		**Lead beneficiary**	AI		
Work package title	Platform Development					
Participant number	1	2	3	4	5	6
Short name of participant	KTH	KI	AI	LLG	APE	PII
Person months per participant	4	-	64	9	2	2
Start month	1	**End month**		48		

Objectives

Provide two modular MINFLUX platforms for fluorescence super-resolution imaging that are fully functional in the VIS range and easily extensible by additional light sources and detectors and new acquisition algorithms. (Objective I)

Modify acquisition electronics to allow preliminary integration of existing SPAD arrays with multiple counter outputs.

Develop a new acquisition electronics platform that allows leveraging the full information from Gen II SPAD array electronics with fluorescence lifetime information. (Objective V)

Integrate hardware control of additional detectors/light sources and newly developed acquisition control algorithms in a ready-to-use application software for dissemination. (Objective VII)

Description of work

Using its existing infrastructure and experience, AI will build two fully functional MINFLUX microscopes for the VIS range. The platforms' optical layout will be modified to allow both the integration of additional light sources and detectors for NIR-MINFLUX imaging, SRS and TPE TRAST imaging and two photon activation in WP2. The platforms will be equipped with an open and modular version of our operating software that allows seamless integration of hardware control modules for the additional hardware. It also allows fine-grained control of image acquisition sequences through python scripts and modification of the FPGA based localization and tracking algorithms through the same python interface.

Closely cooperating with PII, AI will redesign the acquisition electronics platform to be able to handle a real-time data stream from their Gen II SPAD array detection electronics. This will provide the MINFLUX acquisition control with positional and lifetime information of each photon detected. The software will allow our partners to design algorithms to control acquisition sequences and localization algorithms based on this data in real time and test them on their platform.

Integration of these new algorithms in our regular MINFLUX user software will be handled by AI's software department based on the feedback gained from WPs 2, 5 and 6.

Task 1.1: Construction of two modular MINFLUX platforms (**AI**, LLG).

Task 1.2: Plan modifications to existing Gen I SPAD array detection electronics with PII (Task 3.1) and interface the modified version with current AI acquisition electronics (**AI**, PII).

Task 1.3: Develop next generation acquisition electronics interfacing with Gen II SPAD array detection electronics from Task 3.2 (**AI**, PII).

Task 1.4: Based on feedback from user interaction in the demo centers established in Task 6.3, design and test an integrated VIS-NIR-MINFLUX microscope with array detection (**AI**, APE, LLG, KTH). Make correlated label-free imaging and two photon activation more user friendly.

Deliverables

D1.1: Provide two extensible VIS-MINFLUX platforms to LLG for integration work (M12); **D1.2:** Platforms can interface with Gen I SPAD array electronics output (M15); **D1.3:** Replace acquisition electronics with Gen II SPAD array electronics compatible version (M32); **D1.4:** Demo ready integrated SPAD array based VIS-NIR-MINFLUX (M48)

Work package number	WP2		Lead beneficiary		LLG	
Work package title	Optical integration					
Participant number	1	2	3	4	5	6
Short name of participant	KTH	KI	AI	LLG	APE	PII
Person months per participant	14	3	10	49	3	2
Start month	7	**End month**		42		

Objectives

Realization of a MINFLUX platform for fluorescence super-resolution imaging (Objective I)

- in the near infrared wavelength range (Objective II)
- with faster image acquisition and lower background (Objective V)
- correlated with SRS and TPE TRAST imaging (Objective V)

Development and implementation of advanced localization algorithms for SPAD array-based MINFLUX and acquisition strategies for NIR-MINFLUX and correlative imaging.

Description of work

Two modular MINFLUX platforms, which are provided by the partner AI, will be further developed into multimodal super-resolution platforms, one at the LLG for continuous improvement and the other being transferred to KTH and regularly upgraded. The focus lies on optical and mechanical integration of necessary components partly provided and newly developed by our partners (detectors (PII), lasers (APE) and electronics (AI)). Further, image acquisition strategies and algorithms will be designed and implemented in close collaboration with KTH (photophysical dye properties) and AI (electronics and acquisition control).

Task 2.1: Expand MINFLUX platform from WP1 to the NIR by integrating additional excitation laser and point detectors (**LLG**, AI, KTH).

Task 2.2: Integrate SRS light source from Task 4.1 and SRS detector and implement acquisition strategies for correlated SRS-MINFLUX imaging (**LLG**, APE).

Task 2.3: Integrate Gen I SPAD array and prototype background suppression and molecule finding algorithms based on spatial detection (**LLG**, PII, AI).

Task 2.4: Implement two photon activation for MINFLUX and establish acquisition strategies for label-free TPE TRAST metabolic imaging for correlative TRAST-MINFLUX imaging (**LLG**, APE, AI, KTH).

Task 2.5: Optimize and stabilize optical setup and provide critical feedback (**LLG**, AI, APE, PII, KI).

Deliverables

D2.1: One platform for VIS-NIR-MINFLUX imaging installed at KTH (M12); **D2.2:** SRS-MINFLUX modality integrated (M24); **D2.3:** Platforms equipped with Gen I SPAD arrays (M27); **D2.4:** Two photon activation and TPE TRAST modality integrated (M36); **D2.5:** Report on prototype design (M42)

Work package number	WP3		**Lead beneficiary**		PII	
Work package title	Detector development					
Participant number	1	2	3	4	5	6
Short name of participant	KTH	KI	AI	LLG	APE	PII
Person months per participant	1	-	4	2	-	36
Start month	1	**End month**		36		

Objectives

Develop single-photon avalanche detector (SPAD) arrays, with >10 detectors, with at least equal performance to individual state-of-the-art SPADs, and with enhanced sensitivity in the NIR (Objectives III and VII) by

- tailoring the existing SPAD array for MINFLUX microscopes
- developing novel SPAD arrays with enhanced red and NIR sensitivity
- developing a 10×10 SPAD array with time-gating

Description of work

We will modify existing detection electronics to integrate Gen I SPAD arrays in MINFLUX microscopes. Further, we will develop Gen II detection electronics with time-tagging and 20ps timing resolution in close collaboration with AI. Gen II detection electronics will be used to communicate the information of all 23 pixels to AI's MINFLUX platforms, which will use photon-counts for iterative positioning within the sample.

We will develop a new CMOS SPAD array with enhanced red and NIR sensitivity, a peak quantum efficiency (QE) above 50% at 640 nm and specially enhanced QE spectra between 600nm and 900nm. This will finally lead to larger SPAD arrays (small image sensor) with dynamically pre-selected relevant photon events by chip-level time-gating and a frame rate of more than 1Mfps.

Task 3.1: Gen I detection electronics: Hardware platform adaptation to MINFLUX platform (**PII**, AI, LLG, KTH).

Task 3.2: Gen II detection electronics: Develop a new hardware platform following a newly defined communication protocol that passes time-resolved information to AI's MINFLUX platform (**PII**, AI)

Task 3.3: Develop enhanced red and NIR sensitivity CMOS SPAD (**PII**).

Task 3.4: Develop 10×10 CMOS SPAD array with integrated time-gating (**PII**, AI, LLG, KTH).

Deliverables

D3.1: Two SPAD arrays with Gen I detection electronics integrated, tested and delivered to AI for integration (M6); **D3.2:** Upgrade to Gen II detection electronics with time-tagging and 20ps timing resolution (M30); **D3.3:** Replace SPAD arrays with newly designed CMOS SPAD array with enhanced red and NIR sensitivity (M32); **D3.4:** Characterization of 10×10 CMOS SPAD array (M36)

Work package number	WP4		Lead beneficiary		APE	
Work package title	Laser for MINFLUX and SRS operation					
Participant number	1	2	3	4	5	6
Short name of participant	KTH	KI	AI	LLG	APE	PII
Person months per participant	1	-	-	2	54	-
Start month	1	End month		36		

Objectives

Development of new tuning concepts and implementation into a one-box laser source for SRS imaging to allow fast wavelength tuning to target several Raman lines per image and to allow pulse length tuning to the fs regime for two photon activation and TPE TRAST imaging (Objectives IV and VII).

Description of work

Using our core technologies, synchronously pumped OPOs and nonlinear optics, we will develop a new, easy to use one-box light source for two photon activation and TPE TRAST and SRS imaging.

We will validate and verify new OPO tuning concepts, which will allow to improve the tuning time by more than a factor of 10 to less than 5 seconds. The spectral broadening of the pulses inside the OPO cavity will be evaluated and pulse compressor designs will be developed, in order to achieve pulse length shortening into the femtosecond regime (~300fs). A new mechanical design of the OPO cavity will be made in order to realize both ultra-fast tuning and pulse length switching in a one-box light source.

Further, we will implement modern and flexible electronic hardware and software and implement software tuning and stabilization routines. This will allow for complete computer control of the light source and integration into microscope setups.

The prototypes are to be delivered to LLG and KTH for integration into their MINFLUX platforms and for validation of the technology.

Task 4.1: Develop ultra-fast targeting of arbitrary wavelengths (< 5s) for picosecond SRS-lasers and building of prototype light source (**APE**, LLG).

Task 4.2: Development of pulse-length switching between ps and fs regimes for the new laser platform and building of prototype light source (**APE**, LLG, KTH).

Deliverables

D4.1: Loan picoEmerald to bridge the gap until the prototype is finished to LLG for Task 2.2 (M13); **D4.2:** Fast tunable SRS-light source to LLG (M22); **D4.3:** Femto-pico conversion upgrade of SRS-light source to LLG (M25); **D4.4:** Fast tunable femto-pico SRS-light source to KTH (M31)

Work package number	WP5		Lead beneficiary		KTH	
Work package title	Labels, acquisition and protocols					
Participant number	1	2	3	4	5	6
Short name of participant	KTH	KI	AI	LLG	APE	PII
Person months per participant	72	9	2	11	1	-
Start month	1	End month		36		

Objectives

Identify NIR fluorophores for MINFLUX, as a means to take super-resolution microscopy into the NIR, for improved multiplexing, reduced background and better sample penetration (Objective II).

Establish and optimize acquisition and sample preparation protocols for VIS-NIR-MINFLUX, SRS and TPE TRAST imaging, as well as the combined use of these techniques, for bacteria and host cell studies (Objectives V and VI).

Description of work

NIR fluorophores will be evaluated regarding their photo-switching abilities in order to identify dyes suitable for MINFLUX in the NIR and for combined readout with SRS and/or TPE TRAST imaging. Excitation and acquisition schemes, use of additives and sample preparation to optimize the above properties will be established and evaluated for compatibility with live cell imaging.

Based on the instrument integration established in WP2 and with lasers established in WP4, VIS-NIR-MINFLUX, SRS and TPE TRAST imaging, as well as their combined use for correlative imaging, will be established and optimized for bacterial and host cell studies. The best practices and protocols regarding labeling, sample preparation, and acquisition will provide the starting point for the bacterial studies in WP6.

Task 5.1: Identify at least one NIR fluorophore suitable for MINFLUX**,** allowing an extended spectral range and increased multiplexing capabilities. Define additives and sample preparation to optimize its fluorescence and switching properties in fixed and live cells (**KTH**, LLG, KI).

Task 5.2: Define excitation, photo-activation and illumination schemes for combined VIS-NIR-MINFLUX, SRS and/or TPE TRAST imaging of fixed and live cells (**KTH**, LLG, AI, APE).

Task 5.3: Establish protein labeling and sample preparation protocols for VIS-NIR-MINFLUX on fixed and live bacteria and/or host cells (**KI**, KTH).

Task 5.4: Verify SRS and TPE TRAST imaging on bacteria and host cells, providing relevant morphological, chemical and/or metabolic information about the cells (**KTH**, KI, LLG, APE).

Task 5.5: Within the constraints given by dye photophysics, acquisition procedures and sample preparation, establish combined use of MINFLUX together with SRS and/or TPE TRAST imaging (**KTH**, LLG, AI, KI).

Deliverables

D5.1: At least one fluorophore verified for NIR-MINFLUX imaging (M12); **D5.2:** Excitation, photo-activation and illumination schemes allowing combined VIS-NIR-MINFLUX, SRS and/or TPE TRAST imaging established (M27); **D5.3:** Protein labeling and sample preparation for VIS-NIR MINFLUX imaging for bacterial studies (M24); **D5.4:** SRS and TPE TRAST imaging on bacteria and host cells established (M30); **D5.5:** Combined use of MINFLUX together with SRS and/or TPE TRAST on bacteria and host cells (M36)

Work package number	WP6		Lead beneficiary		KI	
Work package title	Lead application and dissemination					
Participant number	1	2	3	4	5	6
Short name of participant	KTH	KI	AI	LLG	APE	PII
Person months per participant	16	70	1	6	-	-
Start month	25	End month		48		

Objectives

Provide key information about the role of pneumococcal surface proteins in pneumococcal disease using the MINFLUX microscope system developed in WP1 – WP4, and established for pneumococcal-host cell studies in WP5 (Objective VIII).

Promote dissemination and exploitation of the developed microscopy, laser and detector technologies in the project by a lead application and via an open end-user facility, and make them available to researchers and stakeholders outside of the project (Objective VII).

Description of work

With the next generation super-resolution MINFLUX microscope system developed in this project, we will address several central aspects of pneumococcal proteins and their role in disease, specifically exploiting the unique nanometer resolution and possibilities of correlative morphological and environmental images by SRS and TPE TRAST imaging.

Task 6.1: Study pneumococcal surface proteins with central, but yet incompletely understood roles in pneumococcal virulence and host defense evasion, and how they are affected by additives having potential antibiotic or bacteriostatic effects (**KI**, KTH).

Task 6.2: Study possible co-localization of pneumococcal surface and pilus proteins with receptor proteins on epithelial cells of the BBB, in brain biopsies from patients who died from pneumococcal meningitis and from mice models. Identify protein interactions promoting adhesion and investigate if they can be prevented by competitive binding by antibodies (**KI**, KTH).

Task 6.3: Study nanoscale localization of protein virulence factors in exosomes, their role in vesicle formation and in subsequent host cell interactions (**KI**, KTH).

Task 6.4: Study distribution patterns of pneumococcal proteins coupled to sustained bacterial growth in lungs of pneumococcal-influenza virus co-infected mice, and how such patterns correlate with presence of anti-oxidants and with the ability of the bacteria to evade immunological attack (**KI**, KTH).

As a result of the lead application studies in Tasks 6.1 – 6.4, we will be able to demonstrate nanometer precision localization patterns of at least two specific bacterial surface proteins and their interaction partners on host cells, show their correlation to bacterial virulence and invasiveness, and identify possible interfering strategies.

Task 6.5: Establish a facility open to potential end-users of the MINFLUX microscope system developed in the project (**LLG**, KTH, KI).

Deliverables

D6.1: End-user facility up and running (M36); **D6.2:** Finding nanometer precision localization patterns of at least two specific pneumococcal surface proteins, their interaction partners on host cells, showing their correlation to bacterial virulence and invasiveness, and identifying possible interfering strategies (M48)

Work package number	WP7		Lead beneficiary		KTH	
Work package title	Project management and communication					
Participant number	1	2	3	4	5	6
Short name of participant	KTH	KI	AI	LLG	APE	PII
Person months per participant	20	2	2	2	2	2
Start month	1	End month		48		

Objectives

Ensure effective management of NanoVIB and cohesiveness between WPs; establish effective communication activities, distribute EC grant & ensure effective coordination of legal, financial and administrative work; ensure that contractual obligations, such as reports and deliverables, are delivered to EC on time; coordinate contact between the Commission and the consortium; management of IPR.

Description of work

The project will be led by the project coordinator (PC) and managed by the project management committee (PMC). Each work package will be led by a work package coordinator (WPC). The PC will be assisted in the daily administrative work by a Technical Project Assistant (TPA). Major support will be provided by the central administration of KTH. Before the start of the project all IPR matters will be discussed and agreed on and confirmed in a consortium agreement. During the project IPR matters will be a standing item on the agenda for meetings with PMC. Patentable results and the partners responsible for protecting them will be identified.

Project meetings are held every 6 months, always alternating between video conferences and face-to-face meetings, covering reports on work during the last period, monitoring progress in accordance with the grant agreement and planning for the following period.

Lectures and seminars will be organized to present the progress and outcomes of the project to consortium members as well as to a general audience.

Internal project communication on a daily basis will be through an internal web page with password access. An external web page will be set up for dissemination of project results and dialogue with civil society.

For further information see Section 3.2.

Task 7.1: Kick-off meeting. Formal establishment of project management committee (PMC), advisory board (AB) and Innovation, IPR and Exploitation Management Group (I^2EMG). Preparation of gender action plan (KTH, All).

Task 7.2: Communication activities, to the public, to potential end-users and scientific community; Public web page, open public lectures, end-user workshops, formal establishment of end-user group (EUG), seminars, international scientific exchange (KTH, All).

Task 7.3: Monitor progress through supervision of deliverables & intermediate targets (milestones) and mitigation of risk. Scientific coordination is overseen by the coordinator and WP leaders within technical WPs. Plan & facilitate project meetings and research visits.

Task 7.4: Prepare EC interim & final project reports (KTH, All).

Deliverables

D7.1: Internal web page (M1); **D7.2:** Data management plan (M6); **D7.3:** Public web page (M6); **D7.4:** End-user group established (M12); **D7.5:** End-user workshops (M45); **D7.6:** Public lectures on the activities and outcome of the project (M48)

Table 3.1c: List of Deliverables

Del. (No)	Deliverable name	WP No	Lead part.	Type	Dissem. level	Date
D7.1	Internal web page	7	KTH	DEC	CO	M1
D3.1	Two SPAD arrays with Gen I detection electronics integrated, tested and delivered to AI for integration	3	PII	DEM	PU	M6
D7.2	Data management plan	7	KTH	R	PU	M6
D7.3	Public web page	7	KTH	DEC	PU	M6
D1.1	Provide two extensible VIS-MINFLUX platforms to LLG for integration work	1	AI	DEM	CO	M12
D2.1	One platform for VIS-NIR-MINFLUX imaging installed at KTH	2	LLG	DEM	PU	M12
D5.1	At least one fluorophore verified for NIR-MINFLUX imaging	5	KTH	DEM	PU	M12
D7.4	End-user group established	7	KTH	R	PU	M12
D4.1	Loan picoEmerald to bridge the gap until the prototype is finished to LLG for Task 2.2	4	APE	DEM	PU	M13
D1.2	Platforms can interface with Gen I SPAD array electronics output	1	AI	DEM	CO	M15
D4.2	Fast tunable SRS-light source to LLG	4	APE	DEM	PU	M22
D2.2	SRS-MINFLUX modality integrated	2	LLG	DEM	PU	M24
D5.3	Protein labeling and sample preparation for VIS-NIR-MINFLUX imaging for bacterial studies	5	KTH	DEM	PU	M24
D4.3	Femto-pico conversion upgrade of SRS-light source to LLG	4	APE	DEM	PU	M25
D2.3	Platforms equipped with Gen I SPAD arrays	2	LLG	DEM	PU	M27
D5.2	Excitation, photo-activation and illumination schemes allowing combined VIS-NIR-MINFLUX, SRS and/or TPE TRAST imaging established	5	KTH	DEM	PU	M27
D3.2	Upgrade to Gen II detection electronics with time-tagging and 20ps timing resolution	3	PII	DEM	PU	M30
D5.4	SRS and TPE TRAST imaging on bacteria and host cells established	5	KTH	DEM	PU	M30
D4.4	Fast tunable femto-pico SRS-light source to KTH	4	APE	DEM	PU	M31
D1.3	Replace acquisition electronics with Gen II SPAD array electronics compatible version	1	AI	DEM	CO	M32

D3.3	Replace SPAD arrays with newly designed CMOS SPAD array with enhanced red and NIR sensitivity	3	PII	DEM	CO	M32
D2.4	Two photon activation and TPE TRAST modality integrated	2	LLG	DEM	PU	M36
D3.4	Characterization of 10×10 CMOS SPAD array	3	PII	R	CO	M36
D5.5	Combined use of MINFLUX together with SRS and/or TPE TRAST on bacteria and host cells	5	KTH	DEM	PU	M36
D6.1	End-user facility up and running	6	KI	DEM	PU	M36
D2.5	Report on prototype design	2	LLG	R	CO	M42
D7.5	End-user workshops	7	KTH	DEC	PU	M45
D1.4	Demo ready integrated SPAD array based VIS-NIR-MINFLUX	1	AI	DEM	PU	M48
D6.2	Finding nanometer precision localization patterns of at least two specific pneumococcal surface proteins, their interaction partners on host cells, showing their correlation to bacterial virulence and invasiveness, and identifying possible interfering strategies	6	KI	DEM	PU	M48
D7.6	Public lectures on the activities and outcome of the project	7	KTH	DEC	PU	M48

3.2 Management structure, milestones and procedures

NanoVIB is an ambitious multidisciplinary project and will need to be managed intelligently to overcome technical hurdles, mitigate risks, and achieve its scientific targets. Best-practice management approaches will be used, aligned with the Grant Agreement and the pre-signed Consortium Agreement. The project will be led by the **Project Coordinator (PC)** and managed by the **Project Management Committee (PMC)**. Each Work Package will be led by a **Work Package Coordinator (WPC)**. The PC will be assisted in the daily administrative work by a **Technical Project Assistant (TPA)**.

The **Project Coordinator (PC), Prof. Jerker Widengren**, will be responsible for administrative and financial co-ordination as well as for monitoring the overall progress of the project. He will steer the scientific and innovative direction of the project, ensuring cross-fertilization and synergies, and manage overall project activities and liaising with the EC. He will chair the meetings of the PMC, composed of one executive representative of each of the participants, take on all tasks and responsibilities of the coordinator as identified in the Commission grant agreement and work closely with the WPCs to ensure excellence in research and technology development, timely progress and reporting of deliverables and milestones (see tables 3.1c and 3.2a). The Coordinator will assemble all reports required by the EC and execute action points raised by the PMC. Project reporting periods are M13 (PR1), M31 (PR2) and M49 (PR3).

Prof. Widengren has extensive previous experience of project management from several national, bilateral, as well as multi-national research projects, funded by EU or by other sources. He was the coordinator of the highly successful EU FP7 project FLUODIAMON, which pioneered the use of super-resolution STED imaging for diagnostic applications, and from which this proposal in several ways is starting off. He is presently heading a successful research unit of more than 30 persons and has organized and chaired many larger international conferences and meetings, in Sweden and elsewhere. Both in research, education and innovation, Prof. Widengren has taken on several tasks as a coordinator to promote cross-disciplinary activities, in particular between KTH and KI, Stockholm. Being both a physicist and a physician, Prof. Widengren combines in-depth knowledge in ultrasensitive and ultrahigh resolution fluorescence spectroscopy/imaging, physics and clinical medicine in a unique way, which in particular for this multi-disciplinary project is a strong asset.

For financial and legal aspects Prof. Widengren will have major support from the administrative functions at KTH. KTH is one of the leading research organizations in Sweden taking part in the EU framework programmes and has so far been involved in more than 200 and managed more than 15 H2020 projects.

The **Project Management Committee (PMC)** will have one permanent representative from each participating organization. Each organization will also be asked to nominate a proxy. The PMC will meet at least every 6 months, alternating between video conferences and face-to-face meetings, at milestone and key decision points, or more often if needed. The PMC will support the coordinator in monitoring project progress and ensuring that deliverables are completed in line with the commitments of each partner. The PMC has responsibility for the success of the project, and all the technical, legal, ethical and regulatory aspects. The PMC will also take decisions concerning patenting, dissemination and exploitation of the results. Key items on the agenda will furthermore be the identification of potential problems and the possible need for revisions of the project plan. The PMC will log and assess risks and give recommendations for solutions in case critical risks would occur (see table 3.2b). The PMC will seek consensus when making decisions. Through the PMC, the Coordinator will facilitate the resolution of disagreements through fair and transparent decision-making processes. Each organization represented will have 1 vote with the coordinator having a casting vote in case of a tie. The aim is however to have consensus in all major decisions.

The **Work Package Coordinators (WPCs)** will be responsible for coordinating the work of all partners in the respective work package, overseeing that deliverables and milestones are reached on time, reporting progress within the work package in project meetings and in the contractual reports

to the Commission and having regular contacts with the PC concerning progress within their work package. The WPCs are Schönle (WP 1), Egner (WP 2), Antolovic (WP 3), Rimke (WP 4), Widengren (WP 5, 7) and Henriques-Normark (WP 6).
An **Advisory Board (AB)** consisting of a scientific representative (**Nobel Laureate, Stefan W. Hell**), a representative of the optical industry (**former CEO of Leica Microsystems, Martin Haase**) and the biopharmaceutical industry (**Science Relations Director AstraZeneca, Anna Sandström**) will closely follow the progress of the project. The AB members will provide valuable feedback and advice from their different expertise, experiences and perspectives. The AB members will take part at yearly meetings with the PMC, where topics such as project progress, management, and supervision of innovations as well as measures on exploitation and dissemination will be discussed.
An **Innovation, IPR and Exploitation Management Group (I^2EMG)** led by the PC will promote an effective management of the intended innovations, the protection of intellectual property and the sustainable exploitation of results. Core task of **innovation management** is the regular and systematic screening of the current scientific and market development in the targeted fields with the objective to open up new opportunities and competitive advantages for the consortium. Potential challenges will also be addressed in this context. Regarding **IPR**, recommendations will be compiled as to how the acquired intellectual property can be optimally protected with respect to the project's overall objective, while simultaneously considering the individual interests of all partners. Furthermore it is reviewed whether 3rd party components have to be licensed in. While **commercial exploitation** (Fig. 2.1a) will be mainly driven by the industrial partners at their own expense, the I^2EMG will monitor that the actions in the project to promote exploitation remain effective and optimize the final outcome. **Scientific exploitation** will be achieved by the academic partners through publications and conference contributions. I^2EMG will be responsible for the coordination of these activities.
Before the start of the project a **consortium agreement** will be set up, containing legally enforceable provisions relating to dispute resolution and notice periods. It will specify especially **IPR** aspects of the project, but also aspects concerning project coordination, governance structure, responsibilities and financial provisions. The Research Support Office of KTH, together with its innovation office (KTH Innovation) have extensive experience of handling IPR matters and formulation of consortium agreements for EU projects, and will provide active expertise assistance in the preparation of the consortium agreement. Such an agreement will be in place before the start of the project. The overall responsibility of the IPR management within the project will fall onto the PC and the PMC.
Consortium wide annual NanoVIB meetings, alternating between the facilities of the consortium partners, will be held to ensure cohesiveness between different WPs. The main part of the meetings will cover scientific project work and will be open to everybody working actively in the project, also the PhD students. Before each of the following meetings the WPCs will compile reports on progress and submit these to the PC, who will circulate them to all PMC members.
The **PMC will meet directly after each project meeting**. Standard items on the agenda will be 1) minutes from last meeting 2) assessment of project work in relation to deliverables and milestones 3) if necessary, decisions on corrective measures based on project progress or on findings of the I^2EMG 4) planned activities during the next period 5) communication activities 6) scientific publications and IPR matters, including identification of potentially patentable results 7) financial matters 8) ethical and gender issues 9) other relevant issues.
A **project web page** will be set up directly at the start of the project. It will contain one part open to the public (D7.3), providing general information about scope, objectives and results, and one part with password access only for project participants (D7.1). The closed page will be the main route for internal communication and will contain all project key documents, such as the grant agreement with all annexes, especially the description of work. There will be information and templates for reporting and cost statements, and guidelines for administrative matters. Continuously updated

information about progress made in the respective work packages will be available, as well as all information in relation to different meetings, such as agendas and minutes. A well-functioning web page will be the responsibility of the co-ordinator.
As already explained in section 2.2, a **data management plan (DMP)** which meets with H2020 requirements for Open Research Data will also be developed and implemented (D7.2) during the first six months of the project. This enables access to all data and routines for the entire consortium (shared domain). Due to IPR considerations we will have to make a distinction with regard to access of the general public (public domain) to this data: Software routines for hardware control represent proprietary IP and know-how of the companies of the consortium and are only made publicly available in compiled form in connection with the corresponding products. Software routines for data evaluation and raw measurement data will be freely accessible. However, a general disclosure will only be made after a thorough assessment whether the data and routines have to be embargoed for a certain period of time due to legitimate interests of the scientists (planned publication) or the research institutions (planned patenting). Any lifting of an embargo requires the approval of the project management committee (PMC).
The **consortium** is set up as a **highly multidisciplinary co-operation and a public-private partnership**. To facilitate the necessary communication over the disciplines, and better mutual understanding of the possibilities, needs and prerequisites within the other fields and sectors involved, means have been allocated in the budget for **frequent exchange and visits of staff between the different partners**. In addition, the opportunity will be taken in connection to the project meetings to arrange internal as well as open training sessions and seminars (D.7.6), as well as site visits to the different facilities of the project partners.

Implementation risks and mitigating actions

While every ambitious research project has the inherent risk of not producing the expected outcome, we feel that the risks associated with the technology development part are minimal except for the specific aspects outlined below which do not threaten the core technological objective of the project: Extending MINFLUX to the NIR wavelength range and to the image live cells thus making the technique a potential standard imaging tool in biomedical research. This goal will require complex research into the spectroscopy of dye candidates and into novel strategies for data acquisition and analysis and will indeed pose challenges. However, these challenges are not greater than those that have been overcome by the project partners AI and LLG together with the MPI for Biophysical Chemistry during the development of the current STED-microscopes, and mitigation strategies successful then will help us achieve the expected results.
Developing suitable protocols for sample preparation and imaging of the targeted bacteria may prove more challenging than we expect, giving our lead application not enough time to produce excellent, convincing results within the project. However, concerning fluorophores, sample preparation, as well as excitation and photo-activation procedures, there are numerous established protocols used in current imaging applications, bearing major similarities to the ones planned in this project. The probability that no one of them would be viable under the conditions used in this project is small. Moreover, the two MINFLUX prototypes that will be constructed in WPs 1-4 will remain at KTH and LLG after the project is finished, as permanent loans. In worst case, if there would not be time to produce the expected results in the lead application, this will ensure that the study proposed herein, projects started by third parties during the demonstration phase, and all possible, promising follow-up studies, can continue without interruption even after the four-year period of the project. Again in the worst case, we anticipate that this would merely result in a delay of our exploitation plans would result while the main objectives of our proposal would still be accomplished.
The objective of combining MINFLUX in the NIR with coherent Raman based imaging methods may limit the number of usable dyes if both methods are to be applied simultaneously or iteratively due to interaction of certain dye molecules with wavelengths used for Raman imaging. In this case

we would focus on selecting the best possible MINFLUX dyes, even if it will initially limit us to Raman imaging only after the MINFLUX analysis is complete. Even in this combination, the complementary structural and chemical information will be very appreciable and the search for suitable dye candidates for correlative imaging without restrictions can be postponed. Similarly, two-photon activation (TPA) for MINFLUX with the novel laser developed as part of this project, could turn out to put too much strain on some of our dye candidates. Weighing this against the potential benefits (reduced background, limitation of activation to a single plane in 3D samples, live cell compatibility) is part of WP5. Even if TPA will turn out to be unusable for all dyes considered, addition of the fs pulse feature to the coherent Raman laser would still add significant value, enabling two-photon excitation (TPE) microscopy and other nonlinear imaging techniques to be performed on the system.

Some specific risks have been identified by APE and PII in their development of the red sensitive SPAD arrays and the fast tuning fs-ps laser. Both companies have identified several possible mitigation strategies and the overall risk of significant delay is low. The project can move forward even if deployment of the light source is delayed: As outlined above, TPA is not vital to the overall success of our project, and even if fast tuning in SRS imaging were available later than anticipated, proof of principle could still be achieved with the loaned state-of-the art light source. If production of "red" SPAD arrays is delayed, we will use the "green" optimized version to verify our strategies for exploiting spatial information and have the option to either use single APDs until the new array becomes available, or temporarily compromise on quantum efficiency using the "green" version.

To achieve better MINFLUX images by exploiting the large amount of additional information about each detected photon (provided by the next generation SPAD arrays) requires the development of novel approaches to data acquisition, both on the electronics and the software side. To avoid a possible delay of WP2, WP3 and WP5, PII and AI have agreed on the following strategies to mitigate the implementation risks a re-development of this scale poses: (1) We will provide a preliminary solution, only delivering partial spatial information through a minor modification of the existing technology, so implementation and testing of the analysis tools needed can commence as soon as possible (Task 3.1 and 1.2). (2) The new electronics will rely on programmable FPGA hardware that allows fast development cycles without the need of physically changing hardware in the prototypes and (3) we will use ready-to-use demonstration/OEM boards wherever possible to avoid undue delay. As the focus during the project is on providing usable prototypes as fast as possible, achieving commercial grade stability of operation and subsequent production transfer may prove more challenging than we currently anticipate. In case of such a delay AI will start exploitation using the preliminary solution, as described above, and will guarantee an upgrade as soon as the development of the new electronic components is finished. It is AI's experience that this is acceptable for a significant number of early customers who appreciate the head start with the new technology. Also, because MINFLUX is exclusively licensed to AI and currently unrivalled in resolution, we only anticipate a delayed generation of revenue as the worst-case scenario.

Finally, there is a low risk that we over-estimate the significance of the nanoscale protein distribution patterns of pneumococcal surface proteins. Our previous data using STED microscopy support that localization and distribution patterns of pneumococcal surface proteins are closely associated with their function. However, even conclusively showing that spatial organization of several pneumococcal surface proteins down to the molecular level is not correlated to the virulence of pneumococci would constitute a major scientific contribution and lay the ground for further investigation into alternative proteins or mechanisms beyond the timeline of this project. The goal of showcasing the new MINFLUX system would still be achieved. In addition, due to our early engagement with the end user group and direct contact to the biomedical research community, we expect to identify worthwhile projects to be started during open facility operation. This would help us identify other early applications that will help establishing the method.

Communication and dissemination activities

Communication will be set high on the agenda to promote the project actions and its results. Given the highly multidisciplinary character of the project, its potentially huge implications on societal health and well-being, and its significant exploitation potential, there are many relevant target audiences for the communication activities in this project. The plans for the communication activities and the main target groups are detailed in section 2.2b. As part of the communication activities, and to promote dissemination and exploitation, an **end-user group (EUG)** will be formed at the beginning of the project (D7.3). The members of the EUG will represent a broad scope of potential stake-holders of this project, and a major purpose of this group is to identify end-users, and to provide advice on how to adapt the scope and activities of our communication efforts to reach these, and for best possible impact.

Dissemination is also a major priority in the project. As a major strategy for dissemination, a microscope system in the project will be made available as a facility, open to a broad group of researchers from academia as well as from companies. This will spur the interest in the microscope system, lasers and detector systems developed in the project and promote their exploitation. This strategy will be complemented by a second strategy: By generating research of highest international standard in the project, providing a lead example of the capabilities of the developed microscope system to resolve intra- and intercellular processes of large biomedical relevance, we will further promote interest in the technique and its use, and thereby increase the demand for the instruments to become commercially available by the end of the project.

Table 3.2a: List of milestones

No	Milestone name	WP(s)	Due date	Means of verification
1	Identify NIR dye and suitable embedding medium	**5**	M12	MINFLUX test sample with NIR dye and embedding is ready; Report
2	Proof-of-principle of NIR-MINFLUX	**2**, 1, 5	M15	Generate super-resolved image of samples using NIR dye; Publication
3	Proof-of-principle of SRS-MINFLUX	**2**, 1, 4	M24	Generate correlative SRS-MINFLUX image of samples; Publication
4	Perform pulse-length switching between ps and fs regimes	**4**	M25	Measure pulse lengths of 2ps and of about 300fs over whole tuning range; Prototype up and running
5	SPAD with optimized NIR sensitivity	**3**	M30	Show improved NIR sensitivity and use Gen II electronics; Prototype up and running
6	Operate Gen II SPAD arrays with new FPGA platform	**1**, 3	M32	Handle real-time data stream from Gen II SPAD array; Prototype up and running
7	First SRS-MINFLUX measurement on bacteria	**5**, 2, 4	M33	Generate SRS-MINFLUX images of bacteria with 5 nm resolution in MINFLUX channel; Report
8	Installation of SRS-MINFLUX platform in end-user facility	**6**, 2, 7	M40	First workshop performed
9	Localization patterns of bacterial surface proteins and their interaction partners on host cells demonstrated	**6**, 5	M48	Presentation of scientific data on international conference

Table 3.2b: Critical risks for implementation

Description of risk (indicate level of likelihood: Low/Medium/High)	WP(s) involved	Proposed risk-mitigation measures
NIR-MINFLUX: best NIR dye shows significant background due to residual fluorescence from switched off fluorophores (low contrast between on- and off-state) (Low)	2, 5, 6	By testing a broad range of fluorophores, specifically for good contrast between on- and off-state this risk will be minimal; as an additional measure the imaging buffer can be adjusted.
Correlative SRS-MINFLUX imaging: pre-bleaching of fluorophore due to SRS imaging (Low)	2, 5, 6	Perform SRS imaging after MINFLUX imaging, alternatively use fluorophore that does not show spectral absorption at SRS wavelengths for MINFLUX imaging, or select laser beam wavelengths for SRS to avoid the absorption band of the fluorophores.
Two photon activation for MINFLUX imaging: two photon process does not activate fluorophore or leads to bleaching (Medium)	2, 1, 5	Specific screening of NIR fluorophores with respect to both MINFLUX and two photon activation suitability. Alternatively, if no activation, use another laser and a one-photon activation process.
SPAD array: long design and manufacturing cycles of 1 year (Medium)	3, 1, 2, 5, 6	Store chips without finished metallization for potential changes (metal fix procedure).
Laser: nonlinear interaction scheme for fast tuning is not working (Low)	4, 2, 5	Three different tuning schemes will be evaluated.
Laser: conversion to femtosecond pulses leads to longer pulses than expected / femtosecond pulse generation leads to lower than expected power (Medium to High)	4, 2, 5	Use of alternative optical components for compression scheme; use of higher power pump lasers.
Association of nanometer scale protein distribution patterns of pneumococcal surface proteins for pneumococcal disease cannot be verified (Low)	6	Several different proteins and phenomena will be studied, and it is unlikely that all hypotheses would miss out. If we do not find a correlation, the data will be important anyway since it will give us information on how these proteins are localized and will give us new hypotheses that need to be tested.
Completion of next generation MINFLUX acquisition electronics delayed significantly due to technical difficulties or manufacturing delays (Low to Medium)	1, 3	Extend modifications to first generation electronics and use Gen I PII detection electronics to allow WPs 2, 5 and 6 to proceed independently.
A WP leader leaves the NanoVIB (Low)	All	Another supervisor within the partner organisation concerned, or within the consortium as a 2nd option, will be appointed.

3.3 Consortium as a whole

The call addressed in this application is quite multidisciplinary in its character, and it is thus a challenge to cover the full disciplinary span of expertise needed for a consortium in this call. Also, the expected impacts the project will contribute to are ambitious and multi-faceted, including fundamental insights in biology and medicine (significant gain in understanding of inter- and intra-cellular processes) as well as impact on the industrial exploitation side in biophotonics (strengthen Europe's industrial position in the biophotonics-related market for microscopes and research and development tools). However, our consortium has a profile of expertise well adapted to the objectives of the project, and the necessary interdisciplinary width, spanning from solid state physics, detector technology, optics, laser technology, microscopy, data acquisition and processing, fluorescence and Raman spectroscopy, over fluorophore chemistry and photo physics, molecular and cell biology, all the way to clinical bacteriology. Moreover, the consortium includes three SMEs active in biophotonics (AI, APE, PII) with long successful track records of close interaction with academia, a research institute placed in the border zone between private industry and academia (LLG), and two academic partners from large renowned universities (KTH, KI), such that we have the competence, experience and prerequisites to meet the expected impacts.

In the forefront of this application is the recent, very exciting progress in super-resolution microscopy, where the MINFLUX imaging technique has raised the level even further. With the ten-fold higher resolution offered by this technique compared to state-of-the-art super-resolution microscopes available today, and with additional development potential to be addressed e.g. in this project, MINFLUX indeed will be a cornerstone in the next generation of super-resolution microscopes. **Abberior Instruments GmbH (AI, partner 3)** is a spin-off company from the research group of Stefan W. Hell (Nobel laureate 2014), where also the MINFLUX technique was invented. AI is the unquestionable market leader in super-resolution microscopy. With numerous strong recruitments of postdocs and senior researchers from the research group of S. W. Hell, AI has by no comparison stronger competence in super-resolution microscopy than any other microscopy company worldwide. With the MINFLUX technique in the forefront of this project, AI adds considerable strength to this consortium, and the contributions from AI will be instrumental, in particular to the detailed objectives I, V and VII as set out for this project. AI will be main responsible partner for WP1.

Yet, while a ten-fold increase in spatial resolution compared to current super-resolution microscopy techniques already gives a substantial potential for the MINFLUX technique to enable prominent gain in the understanding of inter- and intra-cellular processes, we aim in this project to raise this potential even further:

First, we will expand the spectral range of MINFLUX into the NIR. This will make it possible to perform imaging with lower background, increased penetration, and with extended multiplexing, with less cross-talk and higher specificity. To make this expansion into the NIR possible, **Pi Imaging SA (PII, partner 5)** will develop photon detector arrays with enhanced sensitivity in the NIR. PII is world-leading in the development of single-photon sensitive detector arrays, and the expertise of PII will be critical to establish such detector arrays into MINFLUX. Together with AI, PII will implement their detector arrays into the MINFLUX microscope platform, which will result in lower background, faster image acquisition and access to the NIR spectral range for imaging and increased multiplexing. PII's contribution to this activity is an absolute requirement, and so is its contribution to the detailed objectives II, III, V and VII. PII will be the main responsible partner for WP3.

Second, although MINFLUX offers multiplexed mapping of particular protein localizations and co-localizations in cells, the biological significance of this information will be considerably increased if this mapping can be put into a morphological, environmental, or microchemical context. To make this possible, we will implement stimulated Raman scattering (SRS) imaging into the MINFLUX prototype platform, to provide vibrational imaging of specific lipid- or cytoskeleton-structures in the cells, or of their metabolites. **Angewandte Physik und Elektronik GmbH (APE, partner 6)** is

a world-leading provider of pulsed, narrow line-width lasers for SRS, and in their use for SRS microscope imaging. In the project, APE will provide laser prototypes, precisely tunable not only spectrally but also with respect to the pulse widths. These laser prototypes will be implemented into the MINFLUX platform for multifunctional use, for SRS imaging, dye photo-activation (as required for MINFLUX operation) as well as for two photon excitation TRAST imaging for provision of metabolic state images of cells overlaid with the MINFLUX images. The laser prototypes developed by APE for this project will be necessary for the addition of the SRS and TRAST imaging capabilities into the MINFLUX platform, which will require a close interplay between in particular AI and LLG in the project. The contributions from APE are required for several of the detailed objectives (IV, V, VI and VII), and APE will be the main responsible partner of WP4.

The central task of optical integration of lasers and NIR detector arrays rendering a multi-functional MINFLUX instrument lies on **Laser-Laboratorium Göttingen e. V. (LLG, partner 4)**. As a research institute with strong resources and world-leading competence in super-resolution microscopy and laser technology, LLG has the capacity to take on this task, which would have been difficult to do by any of the SMEs, which by necessity have to focus their activities towards development of their core technologies. Also, the technical and engineering effort required for this task lies a bit outside of what an academic research group at a university would be able to handle within their research activities. The contributions of LLG in many ways link the overall instrument development in the project together and prepare for the lead application and dissemination parts to follow and are crucial for the project. LLGs contributions are key for the project objectives I, II, V and VII. LLG is also main responsible for WP2 and has a key role for the dissemination by establishing and operating the open MINFLUX facility in the project.

Following the establishment of a multi-functional MINFLUX microscope platform extended into the NIR, the next step is to implement it for cellular studies, and more specifically for studying bacteria-host cell interactions and revealing mechanisms underlying pneumococcal disease. To render this possible, considerable effort must first be paid on optimizing the sample preparation, explore and identify fluorophores in the NIR that are compatible with MINFLUX and identify the proper excitation, photo-activation and acquisition conditions. The partner group from the **Royal Institute of Technology (KTH, partner 1)** has a very strong record in fluorescence-based single molecule spectroscopy, not the least in the photophysical aspects. Moreover, KTH also has long experience in super-resolution imaging and pioneered its use for subcellular diagnostics, first in the cancer field (e.g. as the coordinator of the EU FP7 project FLUODIAMON), then in bacteriology (together with the KI partner in this project). Based on this experience, KTH will be ideally suited to establish the multifunctional MINFLUX instrument for bacteria-host cell studies, and then together with the KI partner, take the full benefits out of this instrument in the studies of the inter- and intra-cellular processes underlying pneumococcal disease. KTH's contributions will be absolutely necessary for the objectives V, VI and VIII. KTH is the main responsible partner for WP5, and with Jerker Widengren having a background both in engineering physics and clinical medicine, and with a successful track record as coordinator of a larger EU project on a similar topic (FLUODIAMON) KTH will have the proper experience and competence to take on also the task as coordinator of this project (WP7).

The partner group from **Karolinska Institutet (KI, partner 2)** has a very strong research record in clinical bacteriology, in particular on pneumococci, where the group is internationally leading. Since the research of the group goes all the way from studies on the molecular mechanism underlying pneumococcal disease, to studies on mice, patient samples and even epidemiology, the studies in this project can be efficiently guided towards retrieving the clinically most relevant information. KI also already has all the contacts, resources and competence necessary to provide the samples needed for these studies. Thereby, and in close interaction with KTH, and with the unique information that can be acquired by the MINFLUX system, we will indeed be well settled to achieve a significant gain in the understanding of inter- and intra-cellular processes underlying

pneumococcal disease. In the project KI is main responsible for WP6, and for the fulfillment of the objectives VI and VIII, the participation of KI is absolutely required.
As indicated above, the consortium covers a broad interdisciplinary expertise. All the consortium members adequately complement each other and are all required to take the chain of activities all the way, from the start to the end. Since the project builds on the development and subsequent use of a prototype microscope system, harnessed with new laser and photon detector technology, a significant part of the project budget had to be allocated to equipment costs at the SMEs (AI, PII, APE), where the prototypes of microscope system, lasers and detector arrays will be developed and made available to the project, and at LLG, where the optical integration of the microscope will take place. The equipment costs (as specified in section 3.4) and the person months of each partner are adequate and proportionate to their contributions in the project. In the project, some competence overlaps do exist among the partners. For instance, partners AI, LLG and KTH all have a strong record in super-resolution imaging, however, from different points of view (AI, LLG: optics, instrument development, KTH: implementation, sub-cellular diagnostics). Also, APE and LLG both have a strong competence in laser technology, but again of a complementary character (APE: specialist in pulsed, tunable, narrow-band lasers, LLG: implementation of lasers in microscopy/spectroscopy). Overall, this competence overlap will be necessary to bridge the different activities in the project.
The two academic partners (KTH and KI), with very strong records in bioimaging and clinical bacteriology, will have the major roles in the last two WPs, and research of highest international standard is expected here. However, we expect such research as an outcome already from the optical integration (WP2) stage, shifting in subject from photonics, bioimaging over to clinical bacteriology and cell biology, within the course of the project, and towards WP6.
By generating research of highest international standard in WP5 and WP6, we will provide a lead example of the capabilities of the prototyped microscope system to resolve intra- and intercellular processes of large biomedical relevance. Such lead example will spur interest in the technique and its use and promote the demand of the instruments to become commercially available after the end of the project. As a complementary strategy to promote the interest in the microscope system, lasers and detector systems developed in the project and to promote their exploitation, one of the microscope prototypes will also be made available as a facility, open to a broad group of researchers, from academia as well as from companies. This facility will be located at LLG, which has the resources and competence to maintain its operation, and as a partly industry-financed entity, also has the channels to act as a link between end-users and the SMEs.

Industrial/commercial involvement in the project

The strong representation of SMEs in this consortium, with each of the three SMEs having ample experience from close collaborations with academia, and of how to take benefit of such collaborations to promote their products, will further facilitate the industrial exploitation outcome of this project:
APE: The aim of APE is to develop a prototype of an easy to use and robust light source, which is capable to do SRS, two photon activation as well as TPE. To ensure the commercial success of such a product in the end, a system integrator and lead users of the overall system are absolutely necessary for practical validation. Only within this framework APE is able to test and optimize the light source for the given application. Even though such product will not be exclusive for the microscope setup being prototyped in this project, or the lead application aimed for in this project, a common marketing and sales based on the project will strengthen the market position of APE, as well as of all the three commercial partners.
PII: PII has a strong commercial interest to develop SPAD arrays with enhanced red and NIR sensitivity. There is an industrial need for it, driven by eye safety regulations in the automotive market and by the need to reduce photo-toxicity and expand the spectral detection range in life-sciences. The consortium will give PII the opportunity to develop the new SPAD technology in an

application driven environment. This will bring the technology closer to lead users (instrumentation laboratories and innovative companies), the key community for the exploitation of SPAD arrays.
AI: Rapid commercialization of novel and groundbreaking nanoscopy methods, typically customized, is at the core of Abberior Instruments' business model. The short development cycles necessary to claim these new markets can only be achieved by very close and trusting collaboration with both, component manufacturers and researchers developing, applying and validating those new methods. This consortium brings together the ideal mix of partners to establish MINFLUX as an essential addition to the toolbox of biomedical and biopharmaceutical research.

3.4 Resources to be committed

We have ensured sufficient resources to deliver the work plan, based on experience of the partners in executing complex projects. Total budget is € 5.635.530 with 480 person months (PM), which is distributed across the WPs according to Table 3.4a. The PMs correspond to 64.7% of the total budget. The travel budget covers the necessary meetings between individual partners, the whole consortium as well as conferences. Within this context, longer research visits of KTH project staff at LLG, and vice versa, are included for joint efforts and to ensure substantial know-how transfer. The expenses for other direct costs exceed 15% of the personnel costs for the partners mainly working on prototype and method development (AI, LLG, PII and APE). This is a consequence of the extensive instrument development for building the prototypes of the next-generation fluorescence super-resolution platform (main objective I), the SPAD arrays with enhanced NIR sensitivity (main objective III) and the pulsed – narrow linewidth – multi-line laser (main objective IV). These costs cover mainly the purchase of components needed to build those prototypes (equipment in table 3.4b) and have been carefully planned. It is important to note that the overall costs for all prototypes (€ 1.2 million) are significantly lower than the current market price of a single high-end microscope (€ 1.5 million). Personnel costs are standardised to 2020 rates for each individual partner and verified by the partner organisations.

Table 3.4a: Summary of staff effort

	WP1	**WP2**	**WP3**	**WP4**	**WP5**	**WP6**	**WP7**	**Total Person-Months per Participant**
1/KTH	4	14	1	1	**72**	16	**20**	128
2/KI	0	3	0	0	9	**70**	2	84
3/AI	**64**	10	4	0	2	1	2	83
4/LLG	9	**49**	2	2	11	6	2	81
5/APE	2	3	0	**54**	1	0	2	62
6/PII	2	2	**36**	0	0	0	2	42
Total Person Months	81	81	43	57	95	93	30	480

Table 3.4b: 'Other direct cost' items

Participant 3/AI	Cost (€)	Justification
Travel	18.500	2 research visits at PII (1-2 persons), 2 research visits at KTH (1-2 persons), 4 annual project meetings (2 persons)
Equipment	688.000	Components for 2 MINFLUX Platform prototypes to remain at KTH and LLG: Microscope stand (2x € 80.000), lasers (2x € 40.000), optomechanics (2x € 62.000), electronics (2x € 30.000), optics (2x € 36.500), detectors (2x € 12.000), scanners (2x € 36.000), custom mirror coatings (€ 60.000). Components for Gen2 electronics board prototypes (€ 35.000)
Other goods and services	5.000	Audit
Total	711.500	

Participant 4/LLG	Cost (€)	Justification
Travel	45.000	4 research visits at KTH (1-2 persons for 3 weeks), 4 international conferences (1 person), 4 annual project meetings (3-4 persons)
Equipment	172.600	Components for integration of IR, SRS and SPAD into the 2 MINFLUX Platform prototypes which remain at KTH and LLG: NIR laser (2x € 20.000), NIR detector (2x € 5.000), NIR spatial light detector (2x € 15.000), optics (€ 38.300), mechanics (€ 30.300), filters and dichroics (24.000)
Other goods and services	69.000	Consumables: Chemicals (1.000 p.a.), dyes (1.500 p.a.), cell culture supplies (€ 2.500 p.a.), optical supplies (€ 3.500 p.a.), mechanical supplies (€ 2.500 p.a.) and electronic supplies (€ 1.000 p.a.) Three open access publications (€ 2.000 each), organisation of two workshops (invitations, flyers, consumables, catering, € 10,000) Audit (€ 5.000)
Total	286.600	

Participant 5/APE	Cost (€)	Justification
Travel	14.000	2 research visits at LLG (1-2 persons), 1 research visit at KTH (1-2 persons), 4 annual project meetings (2 persons), installation of picoEmerald at KTH and LLG
Equipment	190.000	Components for 2 prototype light sources to remain at KTH and LLG, 1 reference for APE. Pump Laser for light source (3x € 50.000), optics (€ 20.000), mechanics (€ 12.500), electronics (€ 7.500)
Other goods and services	5.000	Audit
Total	209.000	

Participant 6/PII	Cost (€)	Justification
Travel	12.500	2 research visits at AI/LLG (1-2 persons), 4 annual project meetings (2 persons)
Equipment	182.000	Fabrication of SPAD array prototypes: Chip fabrication of 5x5 mm2 (€ 137.000), FPGA electronics (€ 5.000), Chip bonding (€ 3.000), microlenses (€ 35.000), chip packages (€ 2000)
Other goods and services	5.000	Audit
Total	199.500	

Section 4: Members of the consortium

4.1. Participants (applicants)

Participant 1 - Royal Institute of Technology (KTH), Sweden

Royal Institute of Technology (KTH), founded in 1827, is one of Europe's leading technical and engineering universities and the largest technical research and learning institution in Sweden, with 13,000 full-time students, over 1,700 PhD students and approximately 3,600 full-time employees (about 1/3 are women and 2/3 men, in all the three categories). KTH is addressing world leading, high-impact research and education in natural sciences and all branches of engineering, and is part of extensive international research collaborations and participate in a large number of educational exchange or joint programs with universities and colleges in Europe and worldwide. Almost two-thirds of the total turnover of KTH (500 million Euro) relates to research. Life Science Technology (LST) is a strong area of research at KTH, and is appointed as one of its six focus areas, converging engineering, natural and mathematical sciences with life sciences, and with numerous examples of world-leading research. KTH maintains strong interaction with industry, and provides competent innovation support to its researchers via the KTH Innovation office, with a long and successful track record in taking research and ideas from KTH into exploitation outside of academia.

The Experimental Biomolecular Physics research group, headed by Prof. Jerker Widengren, consists of 10 persons (1 professor, 3 researchers, 2 postdocs, 4 PhD students), and contributes to the strong status of LST research at KTH. Most of the members have an engineering physics background, but also educational backgrounds in chemistry, molecular biology and medicine, together forming a solid multi-disciplinary research competence of the group. The Widengren research group belongs to the pioneers in Fluorescence Correlation Spectroscopy (FCS), and is still in the very forefront in the development, application and use of FCS and single-molecule fluorescence methods for studies of biomolecules, their dynamics and interactions. Over the years, Widengren and his research group have studied extensively the photophysical prerequisites for fluorescence-based single-molecule analyses, and the influence of excitation conditions and sample conditions on fluorophore brightness, blinking properties and photo-stability. These parameters are critical for ultrasensitive fluorescence spectroscopy/imaging. Moreover, they are also fundamental for all forms of fluorescence super-resolution imaging. In two EU projects (described above) performed in collaboration with the group of Stefan W. Hell, Göttingen, the Widengren group early on started to explore photophysical switching properties of fluorophores for super-resolution imaging (EU SPOTLITE project, coordinated by S. W. Hell), and then pioneered the use of super-resolution STED imaging for diagnostic applications, demonstrating cellular diagnostics of breast and prostate cancer, based on spatial distribution patterns of specific proteins in the cells (EU FLUODIAMON project, coordinated by J. Widengren). In recent years, the Widengren group has applied super-resolution STED imaging for studying the role of platelets in early cancer development, and together with the Henriques-Normark group at KI, to elucidate the role of pneumococcal surface proteins for the virulence and invasiveness of these bacteria. Finally, of relevance for this project, the Widengren group has also invented the TRAST imaging technique, which emanated from the strong photophysical track record of the group, and uses long-lived dark transient states of fluorophores as very sensitive reporters of several physiologically relevant environmental parameters (as described above).

Infrastructure:

The Widengren lab at KTH is well equipped, with several FCS, multi-parameter single-molecule spectroscopy, STED and TRAST instruments, and multiple laser sources, including tunable supercontinuum lasers and a laser for TPE, making it possible to get started with core activities in WP2 and WP5 from day one. There are also facilities for cell culturing and a biochemistry lab next to the fluorescence lab for use in the project, and the Applied Physics department of KTH offers workshop and research engineering resources and an overall very strong environment within the opto-bio-nano fields of physics.

Main tasks in this project:

The particular expertise and experience of partner KTH is in several aspects very well aligned with the project needs. First, partner KTH will work on the evaluation/identification of NIR fluorophores (Task 5.1), optimization of excitation, photo-activation and illumination schemes (Task 5.2) and their integration into the MINFLUX platforms (Task 2.4). In these tasks, the profound experience of partner KTH in ultrasensitive and ultrahigh resolution fluorescence methods, and its leading expertise in fluorophore photophysics will come to its full right. Second, partner KTH will also take a major role in the establishment of VIS-NIR MINFLUX, SRS, TPE TRAST and combinations thereof, for bacterial and host cell studies (Tasks 5.3-5.5), and in the subsequent studies in WP6, to demonstrate how the developed imaging platform can diagnose bacteria, as well as to identify and characterize their intricate virulence and invasion mechanisms. These tasks will strongly benefit from the experience and expertise of KTH in both super-resolution imaging and TPE TRAST imaging, as well as in the fact that KTH pioneered the use of super-resolution imaging for cellular diagnostic and bacterial studies, specifically focusing on analyses of protein localization patterns in the cells. Finally, partner KTH will take on the role as coordinator of this project (WP7). Scientifically, Prof. Widengren has both the necessary expertise and interdisciplinary competence to take on this task, and has also a successful record as coordinator of the EU FP7 project FLUODIAMON (see below). For financial and legal aspects, Prof. Widengren will have major support from administrative functions at KTH. As one of the leading research organizations in Sweden, KTH has a long and successful track record as coordinator of EU projects. This will guarantee that all administrative support needed for this project will be provided.

Profile of Prof. Jerker Widengren:

Prof. Jerker Widengren (male) will be responsible for the coordination and planning of all project contributions of KTH, and will also take on the task as the coordinator of the project as a whole. Starting as a PhD student in 1990 at Karolinska Institutet, Stockholm (supervisor Rudolf Rigler), Jerker Widengren has a 30 year successful track record in fluorescence-based ultrasensitive and super-resolution based spectroscopy/microscopy. Jerker Widengren belongs to the pioneers of fluorescence-based single-molecule detection (SMD) and fluorescence correlation spectroscopy (FCS), and contributed already as a PhD student to the breakthrough of this technique for biomolecular and cellular studies, in academia as well as in biotechnology and pharma industry. As a postdoc (with C. A. M. Seidel at the Max Planck Institute for Biophysical Chemistry in Göttingen) Jerker Widengren with colleagues established so-called single-molecule multi-parameter detection (smMFD), demonstrated its diagnostic potential by detection and identification of single DNA molecules and its use for fundamental protein conformational studies. Jerker Widengren has also extensively studied the photophysical aspects of single-molecule and FCS measurements, with several highly cited papers describing the mechanisms and remedies for such measurements. Since 2003, Jerker Widengren leads a successful research group at KTH with a focus on development of fluorescence-based ultrasensitive and super-resolution spectroscopy/microscopy techniques, and their application for biomolecular and cellular studies. Jerker Widengren and his group were one of the first outside of the S. W. Hell lab in Göttingen to start with fluorescence-based super-resolution

STED imaging, and pioneered its use for sub-cellular characterization and diagnostics. With Birgitta Henriques-Normark, KI, Jerker Widengren has thereafter successfully applied STED imaging to resolve and characterize the role of pneumococcal surface proteins in the virulence and invasiveness of these bacteria. Jerker Widengren is also the inventor of the TRAST imaging technique, and Jerker Widengren and his group have in several articles demonstrated how this technique can transform the limiting photophysical aspects of single-molecule spectroscopy into highly informative imaging parameters.

Jerker Widengren has more than **100 publications** and **h-index of 31**, and his work has resulted in several patents and patent applications on fluorescence microscopy/spectroscopy techniques. He has supervised 12 PhDs and 16 postdocs. Currently there are 2 postdocs and 4 PhD students under his direct supervision. He has given more than 40 invited lectures and talks (Sweden and abroad) during the last 10 years and was chairman and organizer of several international workshops and meetings on single-molecule spectroscopy, FCS and related techniques.

As an engineering physicist (M.Sc.) as well as clinical physician (M.D.) by training, Jerker Widengren combines in-depth knowledge of ultrasensitive and super-resolution spectroscopy/microscopy and clinical medicine in a unique way. This will be particularly useful given his coordinator role in this project, and very well fits with the highly multi-disciplinary scope of the project.

Education:

- Jerker Widengren, born 1965, received his **M.Sc. in Engineering Physics** from KTH in 1989 and his **Ph.D. in Medical Biophysics** from Karolinska Institutet, Stockholm in 1996.
- After his graduation from medical school (**M.D./Physician**, Karolinska Institutet) in 1998, he was a postdoctoral researcher at the **Max Planck Institute for Biophysical Chemistry** in Göttingen as well as Docent in medical Biophysics at Karolinska Institutet.
- Since 2003 he heads the **Experimental Biomolecular Physics** research group at KTH.

Scientific Career (Detail):

- 1985 – 1989: M.Sc. in Engineering Physics, KTH
- 1990 – 1996: Ph.D., Medical Biophysics, Karolinska Institutet, Stockholm
- 1998: M.D./Physician, Karolinska Institutet (KI)
- 1998, 2001: Internship as Physician (AT) Karolinska Univ. Hospital
- 1999 – 2001: Postdoctoral researcher, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany
- 2001: Docent in medical Biophysics, Karolinska Institutet
- since May 2003: Professor in Experimental Biomolecular Physics, KTH
- 2006: Invited guest professor at EPFL, Lausanne
- 2008 – 2012: Head of the research studies at the department of Applied Physics, KTH
- 2008 – 2012: Coordinator of the EU FP7 project FLUODIAMON with 12 European partners
- 2009: Invited guest professor at Joseph Fourier University, Grenoble
- 2013 – 2015: Director of the Life Science Technology platform of KTH
- 2015-2017: Deputy Head (pro-prefekt) of the department of Applied Physics, KTH
- since 2018: Head of the Quantum and Biophotonics unit/Applied Physics, KTH, with >30 scientists

Memberships:

- Elected member of the Swedish Academy of Engineering Sciences (IVA), since 2017

Top 5 relevant achievements:

Publications:

1. Tornmalm, J.; Sandberg, E.: Rabasovic, M. & Widengren, J. "Local redox conditions in cells imaged via non-fluorescent transient states of NAD(P)H" *Scientific Reports* 9, 15070 (2019)
2. Bergstrand, J.; Xu, L.; Miao, X. Y.; Li, N. L.; Öktem, O.; Franzén, B.; Auer, G.; Lomnytska, M. & Widengren, J. **"**Super-resolution microscopy can identify specific protein distribution patterns in platelets incubated with cancer cells" *Nanoscale* 11(20), 10023-10033 (2019)
3. Pathak, A.; Bergstrand, J.; Sender, V.; Spelmink, L.; Aschtgen, M.-S.; Widengren, J. & Henriques-Normark, B. "Factor H binding proteins protect division septa on encapsulated Streptococcus pneumoniae against complement C3b deposition and amplification" *Nature Comm.* 9, 3398 (2018)
4. Blom, H. & Widengren, J. "Stimulated Emission Depletion Microscopy" *Chemical Reviews* 117(11), 7377-7427 (2017)
5. Iovino, F.; Engelen-Lee, J. Y.; Brouwer, M.; van de Beek, D.; van der Ende, A.; Valls Seron, M.; Mellroth, P.; Muschiol, S.; Bergstrand, J.; Widengren, J. & Henriques-Normark, B. "pIgR and PECAM-1 bind to pneumococcal adhesins RrgA and PspC mediating bacterial brain invasion" *The Journal of Experimental Medicine* 214(6), 1619-1630 (2017)

Up to 5 relevant projects or activities:

The NanoVIB project builds further several previous EU and national Swedish projects, in which the Widengren group at KTH has taken part, and have clear synergies (but no overlap) with one recently started national project:

- EU FP6 project SPOTLITE, 2004-2006 (Stefan W. Hell coord): Development of nanoscopy, based on blinking of long-lived dark transient states in fluorophores.
- EU FP7 project FLUODIAMON, 2007-2012 (Jerker Widengren coordinator): Development of super-resolution STED imaging, TRAST imaging and other advanced fluorescence-based imaging techniques for early subcellular diagnosis of breast and prostate cancer.
- Swedish Foundation for Strategic Research (SSF) project Immunomodulation of host-microbe interactions in infections caused by commensal pathogens - MOHICAN, 2014-2018 (B. Henriques-Normark coord): A multidisciplinary project targeting the molecular mechanisms through which the major global pathogen *Streptococcus pneumoniae* interacts with the host immune defense system. Localization and roles played by different pneumococcal surface proteins and their interactions with the immune defense system such as the complement system resolved by super-resolution STED microscopy were studied.
- Swedish Cancer Research Foundation (Cancerfonden) project, 2018-2020 (PI: Jerker Widengren): Super-resolution STED imaging analyses to elucidate the role of platelets in early cancer development, and the possible use of highly resolved protein distribution patterns on the platelets as a diagnostic marker.
- Swedish Foundation for Strategic Research (SSF) project Bacterial exosomes and their nano-mimics as vaccine - BENVAC, 2019-2023 (B. Henriques-Normark coord): With the main goal to generate new nano-vaccines based on bacterial exosomes, they will be studied in vitro and in vivo. For this, we will apply fluorescence-based ultrasensitive, super-resolution imaging and spectroscopy techniques to reveal underlying molecular mechanisms and host-bacterial exosome interactions.

Participant 2 - Karolinska Institutet (KI), Sweden

Karolinska Institutet (KI) is one of the world's leading medical universities that was founded by King Karl XIII in 1810. Since 1901, the Nobel Assembly at Karolinska Institutet selects the Nobel laureates in Physiology or Medicine. The vision of Karolinska Institutet is to significantly contribute to the improvement of human health. Karolinska Institutet is Sweden's single largest center of medical academic research and offers the country's widest range of medical courses and programs. About 40-50% of medical research in Sweden is placed at Karolinska Institutet, and the research spans the entire medical field, from basic experimental research to patient-oriented and nursing research. The research is conducted in 22 departments, most of which are situated or adjacent to Stockholm's teaching hospitals. This creates ample opportunities for translational research in which new experimental results are rapidly implemented for patient benefit, and where clinical observations provide a basis for new research ideas. Also, the close proximity of the Karolinska University Hospital and other teaching hospitals in the Stockholm area plays an important role during education. Karolinska Institutet offers the widest range of medical education under one roof in Sweden. Several of the programs include clinical training or other training within the healthcare system. Approximately 6,000 full-time students are taking educational and single subject courses at Bachelor and Master levels at Karolinska Institutet. Also, Karolinska Institutet carries out 12% of Swedish doctoral/third cycle education at universities or university colleges and it has collaboration agreements in research and education with a large number of universities all over the world, with companies in the biomed and biotech sectors and also with individual countries. In 2019, Karolinska Institutet had 5,088 full time employees. In addition to this a large number of people without formal employment, especially visiting scientists, fellows and unpaid docents were active at KI. Karolinska Institutet's turnover in 2019 was SEK 7,120 million.

Birgitta Henriques-Normark is professor at Karolinska Institutet (KI), senior consultant/head physician in clinical microbiology at Karolinska University hospital and associated to the Public Health Agency of Sweden (FOHM). Since about 25 years she has studied pneumococcal infections with a broad and translational approach going from clinical studies and epidemiological investigations also on the molecular level, to more basic understanding of mechanisms important for disease development. **The research group of Birgitta Henriques-Normark** is located at the Karolinska University hospital close to Karolinska Institutet and consists of about 35 researchers with a mix of different competences from clinicians to basic researchers including PhD students, Postdocs and Assistant professors, a Senior professor, and Senior researchers. Competences in the group include molecular epidemiology, infectious disease epidemiology, clinical microbiology, bacterial molecular biology, genomics, sequencing and data analyses, proteomics, RNA technologies, visualization, animal models, antibiotic resistance development, and vaccination studies in mice. Birgitta Henriques-Normark has supervised 18 PhD students as main and 10 as co-supervisor, and more than 50 postdocs. Collaborators add competences in biophysics, chemistry, structure biology, and visualization. Hence, all competences needed for the project are present within the group or by collaborators. At KI and at the Karolinska University hospital and at FOHM we have access to all core facilities needed, as stated below, and access to all clinical pneumococcal isolates including serotype data and patient samples, as well as huge collections of other bacterial strains. The research environment includes researchers at all level from Master students, and PhD students to assistant professors, associate professors and professors and clinicians, molecular biologists and microbiologists. We are responsible for a seminar series in infections and we have group meetings every week where the latest research findings are presented and discussed.

Infrastructure:

The research environment is outstanding with access to all equipment and infrastructure needed such as molecular biology tools, FACS, microscopy facilities, different omics approaches such as genomics, transcriptomics, proteomics, metabolomics etc., both in our laboratories, but also in the core facilities at the Karolinska hospital, at KI, and at Science for Life Laboratory. Also, there are excellent animal facilities with large collections of mutant mice. Strains, patient samples and diagnostic competence are all found in the clinical microbiology laboratory and at FOHM.

Main tasks in this project:

Birgitta Henriques-Normark and her group will be responsible for the microbiology part of the project and applications of the visualization tools that will be developed in the project. Birgitta Henriques-Normark will lead WP6 where the aims will be to demonstrate how the developed imaging platform with super-resolution can be used to distinguish pathogenic bacteria from commensal bacteria, and to identify and characterize their intricate virulence and invasion mechanisms. Her group has an extensive expertise in microbiological and molecular tools for pathogenesis studies both in vitro and in vivo mice. The group of Birgitta Henriques-Normark together with the partners in the project will investigate protein localization patterns on the bacterial surface and on membrane vesicles, not previously possible due to resolution issues. Moreover, they aim at studying the biological relevance of identified protein localization patterns and their role for virulence and disease outcome. This will be investigated using microbiological technologies that are already in place in the laboratory of Birgitta Henriques-Normark. The data generated are expected to form a basis for development of novel approaches for prevention and treatment of bacterial diseases.

Profile of Prof. Birgitta Henriques-Normark:

Birgitta Henriques-Normark (female) is professor and senior consultant/head physician (M.D.) in Clinical microbiology at the Karolinska Institutet (KI) and Karolinska University Hospital. She is also a visiting professor at Nanyang Technical University (NTU) in Singapore. Her research mainly targets respiratory tract and invasive infections, with a focus on pneumococcal infections. Her studies go from clinical and epidemiological research to studies on host-pathogen interactions, and more basic understanding on microbial and host immune factors that determine disease outcome. Birgitta Henriques-Normark has published over **200 publications** in the field, **h-index of 49**, and has been the supervisor of over 25 PhD students (18 as main supervisor) that have defended their thesis. She has evaluated research on numerous occasions and for many different organizations such as for the European Research Council, ERC, and the European commission, the Helmholtz association in Germany, Max Planck in Germany, and she has been a member of the steering board for Medicine and Health at the Swedish Research Council for 6 years. She has been the vice dean for recruitment of higher positions (professors, associated professor etc.) and the chair of the recruitment committee at KI for almost 6 years and is now Academic vice president for research at KI. She has participated in several EU networks/projects: 3 in the 5th framework (EURIS, DEAR, STREP-EURO), 7 in the 6th fp (Europathogenomics, GRACE, OmVac, INCA, EIMID, IMO-train, PREVIS), 3 in the 7th fp (Pneumopath, EIMID-IAPP, Lapaso), 2 JPIAMR projects, ERA-Infect project. She was the coordinator of the EU program PREVIS (Molecular mechanisms of resistance, virulence and epidemicity in Streptococcus pneumoniae) within the 6th framework. She has several international and national collaborators both in industry and academia. She also participated in networks governed by DG research and ECDC for the following pathogens: Diphtheria, H. influenzae, and S. pneumoniae. Birgitta Henriques-Normark is a member of the European Academy of Microbiology (EAM), and of the American Academy of Microbiology (AAM). She is also a member of the Royal Swedish Academy of Sciences and of the Nobel assembly at Karolinska Institutet, awarding the Nobel prize in Physiology or Medicine, as well as a member of EMBO (European Molecular Biology Organization).

Education:

- Birgitta Henriques-Normark, born in 1958, became **Medical Doctor (M.D.)** at the Karolinska Institutet (KI), Stockholm, Sweden in 1983. She was licensed as physician (Leg Läk) in 1987, and in 1994 she got here **specialization in Clinical Bacteriology**.
- In year 2000, she got her **Doctoral degree, Ph.D., in Infectious disease control**, and in 2004 she became Associate professor/Docent, at KI. In year 2000 she was appointed as Senior consultant/Head physician at Smittskyddsinstitutet (SMI, today Public Health Agency of Sweden), and in 2008 she became **Professor in Medical Microbial Pathogenesis**, Dep Microbiology, Tumor- and Cellbiology, MTC, at KI.
- In 2011 she became **Professor in Clinical microbiology at KI**, as well as Senior consultant/Head physician at the Karolinska University Hospital. Since 2016 she is also Guest professor at Nanyang Technological University, NTU, in Singapore.

Scientific Career (Detail):

- 1983: Medical Doctor (M.D.) at the Karolinska Institutet (KI), Stockholm, Sweden
- 1983 – 1985: Physician Södertälje/Huddinge hospital Ear-Nose- and Throat Department
- 1985 – 1987: Internship physician Huddinge hospital/Södertälje hospital
- 1988 – 1993: Physician at the National Bacteriological Laboratory
- 1993 – 1994: Physician at the Department of Infectious Diseases at Huddinge hospital
- 1993 – 2014: Physician at the Swedish Institute for Infectious Disease Control (Smittskydds-institutet, SMI)
- since 2000: Head physician, SMI, since 2014 Public Health Agency of Sweden (FOHM)
- 2001 – 2007: Member of the steering group for SMI
- 2001 – 2003: Head Department of Molecular Epidemiology and Biotechnology SMI (~50 employees)
- 2001 – 2010: Head Section Respiratory tract/Invasive infections at SMI (ca 25 employees)
- 2004 – 2007: Head Department of Bacteriology at SMI (~100 employees)
- 2010 – 2012: Chair evaluation committee for Infection and Global Health at the Swedish Research Council
- since 2010: Member of the reference group for Clinical Microbiology appointed by FKM (association for clinical microbiology in Sweden)
- since 2011: Chair of the Centre for Infectious Disease Control, CID, at KI
- 2012 – 2016, since 2018: Member of the steering group of Dep MTC at KI
- 2012 – 2017: Chair of the Research and education committee in Clinical microbiology Karolinska University hospital
- 2012 – 2019: Evaluator at the European Research Council, ERC
- 2013 – 2018: Member of the steering board of Medicine and Health, the Swedish Research Council (VR)
- 2014 – 2019: Vice dean for recruitment at KI responsible for recruitments of higher positions at KI (professors, lecturers, centrally financed positions such as Assistant professors and researchers at KI)
- 2014 – 2019: Chairman of the recruitment committee at KI
- since 2014: Editor of FEMS Microbiology Reviews (IF 13,231)
- 2017 – 2020: Chair of the Evaluation committee of the Helmholtz Centre for Infection Research, Braunschweig, Germany, Member of the Strategic Advisory Board for Helmholtz Health
- 2017 – 2021: Member of the board of Umeå Centre for Microbial Research (UCMR) and Molecular Infection Medicine Sweden (MIMS) at Umeå University
- 2019 – 2022: Academic Vice President for Research at KI, and Chairman of the Committee for Research at KI

Memberships:

- Elected Member of the European Academy of Microbiology (EAM), since 2013
- Elected Member of the American Academy of Microbiology (AAM), since 2015
- Appointed Wallenberg Clinical Scholars (one out of two in 2017) in national competition, since 2017
- Elected member of the Royal Swedish Academy of Sciences (KVA), since 2018
- Elected member of the Nobel Assembly at KI, awarding the Nobel Prize in Physiology or Medicine, since 2019
- Elected member of EMBO (European Molecular Biology organization), since 2019

Top 5 relevant achievements:

Publications:

1. Subramanian, K.; Neill, D.; Malak, H. A.; Spelmink, L.; Khandaker, S.; Dalla Libera Marchiori, G.; Dearing, E.; Kirby, A.; Yang, M.; Achour, A.; Nilvebrant, J.; Nygren, P. A.; Plant, L.; Kadioglu, A. & Henriques-Normark, B. "Pneumolysin binds to the Mannose-Receptor C type 1 (MRC-1) leading to anti-inflammatory responses and enhanced pneumococcal survival" *Nature Microbiology* 4(1), 62-70 (2019)
2. Pathak, A.; Bergstrand, J.; Sender, V.; Spelmink, L.; Aschtgen, M. S.; Muschiol, S.; Widengren, J. & Henriques-Normark, B. "Factor H binding proteins protect division septa on encapsulated Streptococcus pneumoniae against complement C3b deposition and amplification" *Nature Commun.* 9(1), 3398 (2018)
3. Iovino, F.; Engelen-Lee, J. Y.; Brouwer, M.; van de Beek, D.; van der Ende, A.; Valls Seron, M.; Mellroth, P.; Muschiol, S.; Bergstrand, J.; Widengren, J. & Henriques-Normark, B. "pIgR and PECAM-1 bind to pneumococcal adhesins RrgA and PspC mediating bacterial brain invasion" *The Journal of Experimental Medicine* 214(6), 1619-1630 (2017)
4. Iovino, F.; Hammarlöf, D. L.; Garriss, G.; Browall, S.; Nannapaneni, P. & Henriques-Normark, B. "Pneumococcal meningitis is promoted by single cocci expressing pilus adhesin RrgA" *J Clin Invest.* 126(8), 2821-2826 (2016)
5. Hentrich, K.; Löfling, J.; Pathak, A.; Nizet, V.; Varki, A. & Henriques-Normark, B. "Streptococcus pneumoniae senses a human-like sialic acid profile via the response regulator CiaR" *Cell Host&Microbe* 20(3), 307-317 (2016)

Up to 5 relevant projects or activities:

- Swedish Foundation for Strategic Research (SSF) project Immunomodulation of host-microbe interactions in infections caused by commensal pathogens - MOHICAN, 2014-2018 (B. Henriques-Normark main applicant and coordinator): A multidisciplinary project targeting the molecular mechanisms through which the major global pathogen *Streptococcus pneumoniae* interacts with the host immune defense system. Localization and roles played by different pneumococcal surface proteins and their interactions with the immune defense system such as the complement system resolved by super-resolution STED microscopy were studied.
- Swedish Foundation for Strategic Research (SSF) project Bacterial exosomes and their nano-mimics as vaccine - BENVAC, 2019-2023 (B. Henriques-Normark main applicant and coordinator): With the main goal to generate new nano-vaccines based on bacterial exosomes, they will be studied in vitro and in vivo. For this, we will apply fluorescence-based ultrasensitive, super-resolution imaging and spectroscopy techniques to reveal underlying molecular mechanisms and host-bacterial exosome interactions.

Participant 3 – Abberior Instruments GmbH (AI), Germany

Abberior Instruments GmbH (AI) was founded in April 2012 as a spin-off from the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany by Nobel prize laureate Prof. Stefan W. Hell and several senior scientists from his department. Around this core, AI has built the world's strongest development team in the area of fluorescence nanoscopy, with a proven track record of rapidly commercializing both, in house developments and innovations emerging from academic research. AI has thereby significantly advanced the performance of nanoscopy in biomedical research and its applicability to biomedical research.

Already in its first full fiscal year 2013, Abberior Instruments grossed 1.3 Mio€ yielding a positive result. Based on its technological leadership in fluorescence nanoscopy, the company has been profitable and exponentially growing ever since. Currently, Abberior Instruments has 55 employees. With its headquarters in Göttingen, Germany and business locations in Heidelberg, Germany and Basel, Switzerland, AI is in direct contact with many of Europe's leading researchers in both development and application of biomedical imaging techniques. AI is present on all important international markets.

To further strengthen this position and aggressively drive world-wide expansion, AI has founded its US subsidiary Abberior Instruments America (AIA) and plans to open a business location in Asia within the year. Based on the current order volume, AI expects to greatly increase its revenue in 2020 despite the effects caused by the outbreak of SARS-CoV-2.

Since its inception, AI has acquired exclusive licenses for groundbreaking super-resolution imaging techniques, and simultaneously built a strong portfolio of its own intellectual property. After acquiring exclusive rights to the MINFLUX technique developed in Stefan Hell's department at the MPI, the company developed a market-ready microscope within three years after the first published proof of concept. In STED microscopy – a mature super-resolution technique – AI has achieved times-to-market as short as half a year.

Being able to always offer the latest technology in STED, RESOLFT and MINFLUX imaging has helped AI to build a strong customer base especially among early adopters, method developers and, more generally speaking, researchers that drive scientific discovery by leveraging their instrument's full potential.

This has ensured that AI's new developments have always been used on real-life scientific problems within weeks after their implementation and has helped the company to build an exceptional understanding of how to design instruments that facilitate method development during the early adoption phase and to create an atmosphere of openness necessary for such collaborations.

The company employs highly skilled optical engineers, software, FPGA and electronics developers and application scientists, which have been involved in the development of three STED and one MINFLUX based super-resolution products, and has proven to possess one of the most innovative development teams in the industry. At the same time AI maintains close ties with the research community and is continuously involved in research activities through projects funded by the German Ministry of Education and Research. In addition, Abberior Instruments will hire one additional optics developer and one electronics- and FPGA-developer as part of NanoVIB project to address specific tasks related to the integration of detector arrays and an SRS imaging path into the microscope and to develop a market-ready microscope in the later stages of the project.

Infrastructure:

AI's development infrastructure is geared towards rapid development of novel microscopy techniques. The company staff includes 6 former postdoctoral researchers that worked with Stefan Hell during the development of STED and MINFLUX microscopy. Our R&D team consist of 10 optics, electronics and software engineers and routinely prototypes novel microscopy hardware based on standard and custom produced optical components, microprocessors, and FPGAs, with both hardware and firmware development conducted in-house. Integrating custom light sources, detectors and beam paths into our Expert Line microscopes is an important part of AI's business model. To this end AI runs 3 fully equipped optical laboratories and routinely uses optical simulation software, CAD for mechanical design and software for electronics design and simulation. Acquisition software and the firmware for our hardware control electronics is developed using C/C++, Python, LabVIEW, VHDL and JAVA and AIs software department employs expert users of all these tools. Abberior Instruments also considers IP management as an important part of its business and currently employs two specialists. In the past two years (2018 and 2019) AI has filed 13 original patents.

Main tasks in this project:

AI's main task in this project is to extend its current implementation (optics, electronics and software) of MINFLUX imaging to allow (1) integration of additional light sources for NIR-MINFLUX, two photon activation and label-free imaging (2) integration of position-sensitive detectors (SPAD arrays) and (3) implementation and test of advanced data acquisition and analysis schemes within the instrument control software. As the design and production of custom microscope configurations is one of AI's unique selling points to advanced microscope users and because AI's competitiveness is largely based on the company's ability to quickly adopt new technology into their existing line of products, all infrastructure and know-how required for these tasks is available and AI has demonstrated this ability repeatedly: Among others, the RESOLFT, Easy3D, RESCue STED, DyMIN STED and MINFLUX imaging techniques were all either developed completely in-house or licensed from research institutions long before they could be considered mature and developed to TRL9 and offered as a commercial product within 1-3 years. Similar FPGA technology as is necessary for the integration of SPAD arrays is already at the core of AI's current products and AI's R&D staff has extensive experience with all the tools and techniques needed for the task at hand. Providing optimal starting points for custom integration of additional hardware and optics into AI's microscopes is part of its business model and will not pose significant challenges.

Profile of Dr. Andreas Schönle:

Dr. Andreas Schönle (male) will be responsible for coordination and planning of all project contributions by Abberior Instruments. He will join the lead developers of AI's current MINFLUX product in conceiving the open MINFLUX platform, he will recruit a qualified FPGA developer and lead the development necessary to integrate SPAD arrays into the MINFLUX electronics and oversee all necessary software development at AI. He will also plan and oversee all modifications that might become necessary due to the critical feedback from other project partners.

After heading the software department and contributing to many of AI's important technology and product developments until fall 2019, Andreas Schönle is now the head of AI's newly established "Intelligence and Innovation" section. There he focusses on highly innovative long-term development projects and technology scouting.

During his academic career, Andreas Schönle has worked in the field of optical microscopy beyond the diffraction barrier with far-field optics since 1996. He has worked on theoretical modelling of novel super-resolution techniques based on photo-switching and nonlinear spectroscopy, experimental development and theoretical analysis of STED microscopy and related concepts. During his time at the Max Planck Institute for Biophysical Chemistry under Stefan Hell he also developed

imaging software that was used to operate and analyze the data of many of the 4Pi, STED and localization based super-resolution microscopes, making him part of a large number of successful research projects. He co-authored more than 40 scientific publications in the field of fluorescence nanoscopy and book chapters about the fundamentals of super-resolution. His work has also resulted in numerous inventions and several important patents in the field.

Education and professional career:

- Andreas Schönle, born 1973 in Munich, studied **Physics, Mathematics and Economics** at Osnabrück University, Germany, Kent State University, USA and Heidelberg University, Germany. From Heidelberg University he received his "Diplom" in Physics in 1998 and his Doctorate in Physics in 2003.
- He was subsequently postdoctoral researcher and later senior scientist in the **Department of NanoBiophotonics** at the **Max Planck Institute for Biophysical Chemistry**, Göttingen.
- In 2012 he became **co-founder of Abberior Instruments GmbH,** a spinoff from the Department of NanoBiophotonics and became head of software development. Since October 2019 he is head of the "Intelligence and Innovation" department at AI.

Top 5 relevant achievements:

Products:

1. Abberior Instruments VIS-MINFLUX microscope:
   Within 3 years after the initial proof of concept, Abberior Instruments has developed its current MINFLUX offering and received orders for this novel type of microscope from several leading research institutions, including the EMBL in Heidelberg. The platform has an open design to allow optimization due to feedback from applicants in this early phase and with some modifications will offer an ideal base platform for the NanoVIB project.
   (https://www.abberior-instruments.com/products/minflux/)
2. Imspector Software:
   The software was first created in Stefan Hell's group (now the Department of NanoBiophotonics) at the Max Planck Institute for Biophysical Chemistry as a flexible base for incorporation and synchronized operation of lasers, detectors, optomechanical components and acquisition and control electronics in order to quickly design and build microscopes based on novel super-resolution concepts and allow their test and operation by non-engineers. An early version has been licensed to LaVision Biotec to operate their two-photon microscopes and numerous research labs to operate their home-built STED microscopes by the Max Planck Society. At AI, Imspector was systematically streamlined, further modularized and improved and is now at the core of the Abberior Instruments Expert Line and Facility Line microscopes, and a major facilitator for both AI's rapid innovation cycles and the ability of AI's customers to find innovative applications of AI's technology beyond the current state of the art.

Publications:

3. Heine, J.; Wurm, C. A.; Keller-Findeisen, J.; Schönle, A.; Harke, B.; Reuss, M.; Winter, F. R. & Donnert, G. "Three dimensional live-cell STED microscopy at increased depth using a water immersion objective" *Review of Scientific Instruments* 89(5) (2018)
4. Heine, J.; Reuss, M.; Harke, B.; D'Este, E.; Sahl, S. & Hell, S. W. "Adaptive-illumination STED nanoscopy" *PNAS* 114(37), 9797-9802 (2017)
5. Schloetel, J.-G.; Heine, J.; Cowman, A. F. & Pasternak, M. "Guided STED nanoscopy enables super-resolution imaging of blood stage malaria parasites" *Nature Scientific Reports* 9(4674) (2019)

Up to 5 relevant projects or activities:

- Optical nanoscopy for three-dimensional imaging of live cells ('LiveCell3DNanoskop', German ministry of Education and Research, 2017-2020): Cooperation with Abberior GmbH, Göttingen and Max Planck Institute for Biophysical Chemistry, Göttingen. Project ongoing.
- STED microscope with active aberration correction & automatic adjustment: Integration of relevant optics into existing microscope and software-based integration of expert knowledge into image acquisition software. Cooperation with the LLG. Product launched in 2019.
- Automated STED-nanoscopy for high-throughput analysis in cell biology ('ScreeningSTED', German ministry for Education and Research): Cooperation with Bayer AG, Leverkusen.
- Development of Easy3D STED: AI has developed an adaptive optics solution to create and overlay two independent intensity patterns using a single beam path. The resulting stability is critical for routine 3D STED nanoscopy. The resulting Easy3D module is a core component of most Expert Line and Facility Line microscopes and one of AI's important USPs.
- Development of intelligent light dose management in confocal and STED microscopy: Cooperation with the Max Planck Institute for Biophysical Chemistry. Illumination intensities are dynamically adjusted during acquisition based on real-time analysis of detector count-rates to avoid unnecessary illumination of the sample with low information density. Corresponding products: RESCue STED, DyMIN STED.

Participant 4 - Laser-Laboratorium Göttingen e.V. (LLG), Germany

Laser-Laboratorium Göttingen (LLG) was founded in 1987 as a non-profit making special-purpose enterprise, being institutionally supported by the State of Lower Saxony. Promotion of optical technologies is successfully realized by applied pure research and knowledge transfer between research institutions and industry in the form of collaboration projects, research assignments, consultancy and training. Research and development results are usually marketed or distributed by companies under license.

The LLG divisions "**Short Pulses/Nanostructures**", "**Optics/Short Wavelengths**", "**Photonic Sensor Technology**" and "**Optical Nanoscopy**" have gained worldwide acceptance in various fields of photonics. Research activities range from development of non-contacting laser measurement technology, manufacturing of new products, laser based product processing and development of new laser systems to applications in medical technology and the life sciences.

The LLG receives important institutional sponsorship from the Federal State of Lower Saxony, substantial third party funding for contract research from the industry as well as from projects of the Federal Government and the DFG (German Research Association). The LLG employed 52 employees in 2019 and is well networked with small and medium-sized enterprises as well as conglomerates. In the education of Bachelor, Master as well as PhD students, the LLG collaborates closely with the Georg August University Göttingen and the University of Applied Sciences Hildesheim/Holzminden/Göttingen. Furthermore, it regularly offers insights into modern optical research to trainees.

The **Department of Optical Nanoscopy**, to be directly involved in the NanoVIB project, focuses on basic research and applications in the field of super-resolution fluorescence microscopy. For this purpose, switchable optical transitions are used to bypass restrictions imposed by the laws of diffraction, which are inherent to all optical far-field techniques. One of the main activities of the department is the development of new optics, tools and microscopes that can be routinely used by life scientists without the need for optical expertise. The department cooperates closely with the Department of NanoBiophotonics at the Max Planck Institute for Biophysical Chemistry led by Stefan W. Hell as well as the Department of Photonic Sensor Technology of the LLG, which among other things focuses on Raman scattering-based analytics.

Infrastructure:

The department has a 120 meter square lab space fully equipped for optical nanoscopy research purposes as well as an additional laboratory equipped for routine biological research. Among other things, the laboratories contain: two STED nanoscopes with single color imaging capability, three STED nanoscopes with multicolor imaging capability, two PALM/STORM nanoscopes with multicolor capability, an isoSTED microscope which can also be used as an interferometric PALM/STORM nanoscope, a scanning electron microscope and an atomic force microscope. The Department has access to the core light microscopy facility of the Max Planck Institute for Biophysical Chemistry and the DFG Clusters of Excellence "Multiscale Bioimaging: from Molecular Machines to Networks of Excitable Cells" (MBExC) as well as the cell culture of the department of NanoBiophotonics at the Max Planck Institute for Biophysical Chemistry.

Main tasks in this project:

LLG's main task in this project is the optical integration of several components, developed by the partners, in order to realize a NIR-MINFLUX system capable of correlative SRS-MINFLUX as well as correlative TRAST-MINFLUX imaging. The optical planning is completed by the implementation of acquisition strategies and the prototyping of e.g. molecule finding algorithms. The Department of Optical Nanoscopy, led by Alexander Egner, will therefore be responsible for the step-wise

development of the super-resolution system as specified in WP2 and will provide partner KTH with an update to the platform after each step. Alexander Egner and his group have extensive experience and expertise in the design and implementation of microscopy systems for various super-resolution techniques. The development of new tools as well as the integration of novel components and approaches is part of the department's main activities, alongside with the analysis and implementation of acquisition strategies for super-resolution imaging. Equipped with several optic laboratories as well as a laboratory dedicated to biological research and sample preparation, the department has all the knowledge and infrastructure necessary to start with the optical integration as well as to assist the partners from the very beginning.

Profile of Prof. Alexander Egner:

Prof. Alexander Egner (male) will be primarily responsible for carrying out the LLG based research and innovation activities in NanoVIB. He will lead WP2 "optical integration" and will work directly on the project tasks.

Prof. Egner is the Director of the LLG and also heads the Department of Optical Nanoscopy. His research is focused on: working on optical microscopy beyond the diffraction barrier with far-field optics since 1996; experimental development and theoretical analysis of 4Pi microscopy; development of STED microscopy and related concepts using photo-switching of fluorophores for far-field optical resolution on the nanoscale; and development of PALM/STORM microscopy and related concepts using photo-switching of fluorophores for far-field optical resolution on the nanoscale.

He has **53 publications** and **h-index of 37**, and his work has resulted in more than 8 patent applications in the field. He has supervised 17 PhDs. There are currently 4 postdocs, 3 PhD students, and 1 Master student in Egner's team, focusing on basic research and the resulting practical implementations for use in the field of super-resolution fluorescence microscopy. Egner's group has attracted over €8.000.000 in the past 5 years alone. He has a number of significant national and international collaborations, including: Tel Aviv University, "The mitochondrion-plasma membrane junction at super-resolution microscopy"; Ljubljana University, "super-resolution fluorescence microscopy (nanoscopy) for live imaging of subcellular organelles by fluorescence means in living cells"; Abberior Instruments GmbH, "automated STED-nanoscope"; Göttingen University, DFG Clusters of Excellence "Multiscale Bioimaging: from Molecular Machines to Networks of Excitable Cells".

Education:

- Alexander Egner, born 1970 in Mannheim, studied **Physics** at **Heidelberg University**, where he received his **Doctorate in Physics** in 2002.
- He was subsequently postdoctoral researcher and later senior scientist in the **Department of NanoBiophotonics** at the **Max Planck Institute for Biophysical Chemistry**.
- In 2010 he became **Director of the Laser-Laboratorium Göttingen** where he also heads the **Department of Optical Nanoscopy**.

Scientific Career (Detail):

- 1991 – 1997: Studied in Physics, University of Heidelberg
- 1997: Diploma in Physics, University of Heidelberg, Prof. Dr. S. W. Hell
- 1998: Visiting Scientist, Department of Applied Physics, University of Osaka, Prof. Dr. S. Kawate
- 1998 – 2002: Ph.D. (Dr. rer. nat.), Physics, University of Heidelberg, Prof. Dr. S. W. Hell
- 2002 – 2003: Postdoctoral Researcher, Max Planck Institute for Biophysical Chemistry, Göttingen, Prof. Dr. S. W. Hell

- 2003 – 2010: Senior scientist, Max Planck Institute for Biophysical Chemistry, Göttingen, Prof. Dr. S. W. Hell
- 2005 – 2010: Head of Central Light Microscopy Facility, Max Planck Institute for Biophysical Chemistry, Göttingen
- since 2010: Managing Director and Head of Department of Optical Nanoscopy, Laser-Laboratorium Göttingen e.V.
- 2014: Habilitation, Physics, University of Göttingen
- 2017: Adj. Prof., Physics, University of Göttingen

Memberships:

- Executive board member, "Microscopy at the Nanometer Range" section of the "Cluster of Excellence: Nanoscale Microscopy and Molecular Physiology of the Brain", 2011 – 2019
- Faculty member, Göttingen Graduate School for Neurosciences, Biophysics and Molecular Biosciences, since 2011
- Steering committee member, DFG Collaborative Research Center 755 "Nanoscale Photonic Imaging", 2015 – 2019
- Spokesperson of the northern regional group, German Industrial Research Association Konrad Zuse, since 2017
- Member, DFG Clusters of Excellence "Multiscale Bioimaging: from Molecular Machines to Networks of Excitable Cells", since 2019

Top 5 relevant achievements:

Publications:

1. Klar, T. A.; Jakobs, S.; Dyba, M.; Egner, A. & Hell, S. W. "Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission" *Proc. Natl. Acad. Sci. U. S. A.* 97, 8206-8210 (2000)
2. Egner, A.; Jakobs, S.; Hell, S. W. "Fast 100-nm resolution three-dimensional microscope reveals structural plasticity of mitochondria in live yeast" *Proc. Natl. Acad. Sci. U. S. A.* 99, 3370-3375 (2002)
3. Schmidt, R.; Wurm, C. A.; Jakobs, S.; Engelhardt, J.; Egner, A. & Hell, S. W. "Spherical nanosized focal spot unravels the interior of cells" *Nat. Methods* 5, 539-544 (2008)
4. Aquino, D.; Schönle, A.; Geisler, C.; von Middendorf, C.; Wurm, C. A.; Okamura, Y.; Lang, T.; Hell, S. W. & Egner, A. "Two-color nanoscopy of three-dimensional volumes by 4Pi detection of stochastically switched fluorophores" *Nat. Methods* 8, 353-359 (2011)
5. Vinçon, B.; Geisler, C.; Egner, A. "Pixel hopping enables fast STED nanoscopy at low light dose" *Opt. Express* 28, 4516-4528 (2020)

Up to 5 relevant projects or activities:

- Nanoscale analysis of the molecular interactions that control insertion and elimination of the complement C5b-9 complex (Volkswagen Foundation): Development of labelling strategies and acquisition protocols for super-resolution imaging based characterization of the induced translocation of mitochondria and mitochondrial proteins to the plasma membrane.
- isoSTED microscopy within tissue (DFG): Expand isoSTED microscopy to imaging under strongly aberrated imaging conditions, such as those introduced by biological tissue.
- STED microscope with active aberration correction & automatic adjustment (Federal Ministry of Economics and Energy): Integration of relevant optics into existing microscope and software-based integration of expert knowledge into image acquisition software. Cooperation with Abberior Instruments. Product launched in 2019.

- Fast monochromatic reflection nanoscopy by absorption modulation (German Research Council): Transfer of the STED principle to photochromic molecules and from the visible to the ultraviolet spectral range.
- Super-resolution microscopy with smart and sample-specific scanning patterns (German Research Council, within the framework of the German excellence initiative): Acceleration of STED and MINFLUX microscopy by using smart algorithms to allow the microscope to autonomously recognize the structure to be examined. In cooperation with Stefan W. Hell.

Participant 5 – Angewandte Physik und Elektronik GmbH (APE), Germany

Angewandte Physik & Elektronik GmbH (APE) was founded in 1992 and employs about 70 people at present. Headquartered in Berlin, APE is a worldwide leading supplier in the field of ultrashort laser pulse diagnostics and tunable wavelength conversion. With development expenditures of 30% of total revenues we constantly improve our products and develop new ones to serve the scientific community as well as the industrial ultrafast market.

The company is a hidden champion in the photonics industry: highly specialized, in the ultrashort pulse niche closely connected with users and customers, but for many rather unknown. However, due to its highly specialized focus on nonlinear optics, APE is a sought-after OEM development partner and supplier for laser manufacturers, microscopy companies and other well-known technology companies.

Today, the APE repertoire encompasses a large range of nonlinear optics equipment, from spectrometers to harmonic generators, from pulse compressors to quantum-dot single photon generation sources. Over the years, APE has built up an impressive array of more than 30 products entirely developed and produced within its headquarters in Berlin. APE's products are used worldwide in the leading universities and research institutes as well as by all large ultrafast laser manufacturers. They are sold and serviced by APE GmbH in Berlin and its partner company APE Inc. in Freemont, California, as well as through a worldwide distribution network.

In the field of microscopy APE has amassed experience over the last 20 years and offers several products.

- APE provided visible OPOs for second generation STED microscopes especially in the laboratory of Stefan W. Hell.
- Multi-photon microscopy is supported by APE with several products and APE has been the first on the market for many of them, such as light sources for two-photon excitation above 1000nm ("Chameleon compact OPO"), light sources for three-photon excitation ("AVUS-SP"), microscope autocorrelators ("Carpe") and precompressors to compensate the chirp of the microscopes ("femtocontrol").
- The company also provides infrared light sources for near field microscopy such as s-SNOM (scattering scanning nearfield optical microscopy) → "Carmina"

APE has been developing and building OPO-based light sources for coherent Raman microscopy (CARS and SRS) since 2005. In close cooperation with the leading scientists in this field, such as Sunney Xie, Andreas Zumbusch, Herve Rigneault, Jürgen Popp, Ji-Xin Cheng, Wei Min and many others we have developed easy to use and robust light sources and detectors for this type of microscopy. As of now, APE is the leading supplier of such light sources. The latest APE products for coherent Raman microscopy are the picoEmerald laser and the SRS detection set, with completely hands free and computer controlled operation to enable easy setup and operation of coherent Raman microscopy.

Infrastructure:

APEs infrastructure encompasses 13 fully equipped laser laboratories including 3 clean rooms for production and development as well as more than 2000m² of office and manufacturing space. With a 20 people strong R&D team, APE covers the expertise for development in optics, mechanical design, electronics and software. Tools such as optical simulation software, CAD for mechanical design and software for electronics design and simulation are used. The software department has the capability of programming FPGA and microcontrollers as well as high level control software in LabVIEW, Java, C and Python. APE has the in-house capability of small scale in-house prototyping of

mechanical and electronics parts. Furthermore, all products are designed and manufactured directly at APE in Berlin, ensuring short reaction times and direct means of communication.

Main tasks in this project:

The main task of APE in this project is to develop a laser for MINFLUX and SRS imaging operation. The two main goals are:

1. Increasing the tuning speed by more than an order of magnitude to <5s
2. Femto-picosecond switching for enabling SRS and photoactivation with a single light source

The expertise and product line of APE proves that APE understands the requirements for SRS and multiphoton microscopy and is able to develop hands free and easy to use microscopy light sources. The core technology of APE's light sources are optical parametric oscillators, which will be implemented in this development. With 25 years of experience in developing synchronously pumped OPOs in the femto- and picosecond regime, over 20 different OPO-based products and more than 800 installed systems within this time there is no other company in the world with this degree of expertise. This knowledge does help us to implement new nonlinear interactions and tuning regimes to speed up the tuning time and to implement the femto-picosecond switching. For the required pulse compression, APE has shown its expertise as well with its product "femtocontrol". Therefore, APE's profile is perfectly matching the tasks in this project.

Profile of Dr. Ingo Rimke:

Dr. Ingo Rimke (male) will be primarily responsible from APE for carrying out the proposed research and innovation activities in NanoVIB. He will lead WP4 "Laser for MINFLUX and SRS imaging operation".

Dr. Ingo Rimke is director of the research and development department at APE. He joined APE in 2001 and was since involved in all OPO related product developments at APE, either as the lead physics engineer, project manager or project supervisor.

He further coordinates APE's activities in the field of Coherent Raman microscopy and acts as a scientific advisor.

Education and professional career:

- Ingo Rimke, born 1970 in Berlin, studied **Physics** at Humboldt University Berlin, the University of Manchester and the Free University Berlin. He received his **Doctorate in Physics** from the Free University in 1999.
- In 2000 he joined **Bioptic Laser GmbH** to develop a diode pumped, frequency tripled ns-laser for mass spectrometer applications (MALDI-TOF).
- From 2001 Ingo Rimke was working as **Manager Product Development at APE**.
- In 2015 Ingo Rimke was appointed as the **Director of Research and Development at APE**.

Top 5 relevant achievements:

Products:

1. picoEmerald (one-box, hands free light source for coherent Raman microscopy) https://www.ape-berlin.de/en/cars-srs/
2. SRS detection set (detector and lock-in amplifier combination for easy SRS-detection) https://www.ape-berlin.de/content/uploads/2020/04/APE-SRS-Detection-Set-Rev-3-2-0.pdf

Publications:

3. Rimke, I.; Hehl, G.; Beutler, M.; Volz, P.; Volkmer, A. & Büttner, E. “Tunable dual-wavelength two-picosecond light source for coherent Raman scattering microscopy” *Proc SPIE* 894816 (2014)
4. Stiebing, C.; Meyer, T.; Rimke, I.; Matthäus, C.; Schmitt, M.; Lorkowski, S. & Popp, J. “Real-time Raman and SRS imaging of living human macrophages reveals cell-to-cell heterogeneity and dynamics of lipid uptake” *J. Biophoton.* 10, 1217-1226 (2017)
5. Audier, X.; Heuke, S.; Volz, P.; Rimke, I. & Rigneault, H. “Noise in stimulated Raman scattering measurement: From basics to practice” *APL Photonics* 5, 011101 (2020)

Up to 5 relevant projects or activities:

- Development of the Levante OPO in 2005, the first jitter free light source for CARS microscopy
- Development of the Levante Emerald OPO in 2007 for CARS microscopy, a completely redesigned ps-OPO with much larger tuning range and improved handling
- First one-box, fully integrated light source for CARS microscopy – picoEmerald, Prism award finalist in 2009
- BMBF funded project MicroQuant (2011-2014) in the call “optical technologies in life sciences” to develop a hands free and computer controlled light source for stimulated Raman microscopy. Partners were among others Leica Microsystems, Univ. Konstanz and Univ. Stuttgart. The results of this project were used to develop the second generation of picoEmerald lasers, now capable to do SRS, as well as a SRS detection unit.
- EU-Attract project SRS-Histology (2019-2020) together with Institute Fresnel, Marseille to develop building blocks to generate stimulated Raman based histopathology images for cancer research

Participant 6 – Pi Imaging Technology SA (PII), Switzerland

Pi Imaging Technology SA is a spin-off from EPFL, based on the know-how acquired during research work of Dr. Ivan Michel Antolovic, Dr. Claudio Bruschini and Prof. Edoardo Charbon on detectors counting single quanta of light, photons. The co-founders have an accumulated experience of more than 35 years in the field of photon-counting detectors. The company is headquartered in Neuchâtel and has offices in Ecublens.

Our company creates highly efficient photon-counting arrays in standard semiconductor technology, which enables unlimited number of pixels, and miniaturized shapes and architectures. The implemented photon-counting arrays directly transform photons to digital pulses. Our first product is a 23-pixel photon-counting array with excellent sensitivity, dynamic range and speed. In microscopy, this can improve the image quality by a factor of 2, reducing photo-toxicity of living cells. In cyber-security, a high dynamic range enables true random numbers at 1Gbps for data encryption. In machine vision, our arrays image in dark conditions at speeds faster than 1MHz.

Infrastructure:

Pi Imaging Technology is a spin-off from EPFL, with headquarters in Neuchâtel and offices in EPFL Innovation Park, Ecublens. Pi Imaging Technology's equipment consists of tools for electro-optical characterization and testing of photodetection systems. Pi Imaging Technology has access to fast electronic signal analyzers, fast picosecond-pulse lasers, temperature chambers and integrating spheres, and to the advanced nano-fabrication facilities at Center of MicroNanoTechnology, EPFL. This facility comprises photolithography, electron beam lithography, physical and chemical etching and material deposition machines. Moreover, Pi Imaging Technology has extensive experience in custom chip design with the emphasis on single-photon detectors and software packages Cadence (chip design), Xilinx ISE/Vivado (firmware design) and Altium Designer (electronics design).

Furthermore, Pi Imaging Technology has access to all infrastructure of EPFL Innovation Park, where more than 200 technological young companies with an impact on our economy and society create a unique ecosystem.

Main tasks in this project:

Pi Imaging Technology's main task is to design a new application specific SPAD array with special emphasis on increasing the sensitivity in the red spectrum. Pi Imaging will also adapt its current SPAD array detector to enable time filtering of autofluorescence and imaging of the fluorophore surrounding, both important features in MINFLUX searching algorithms. These tasks will strongly benefit from the experience and expertise of Pi Imaging in designing SPAD devices and semiconductor chips specifically for microscopy applications. Dr. Antolovic has both the necessary expertise and interdisciplinary competence to take on this task, gained through industrial and research projects.

Profile of Dr. Ivan Michel Antolovic and Dr. Harald Homulle:

Dr. Ivan Michel Antolovic (male)

Ivan Michel Antolovic obtained his Ph.D. in 2018 at TU Delft. His Ph.D. thesis on SPAD arrays for super resolution microscopy was awarded the 2018 Else Kooi Award for young researchers in the field of applied semiconductor research conducted in the Netherlands. After being a scientist at EPFL for two years, he co-founded Pi Imaging Technology with Prof. Edoardo Charbon, Dr. Claudio Bruschini and Dr. Ron Hoebe and is currently leading the company. Dr. Antolovic authored and co-authored over 15 articles in technical journals and conference proceedings and 5 patent applications.

Education and professional career:

- Ivan Michel Antolovic, born 1988, studied **Electrical Engineering and Computing** at University of Zagreb and TU Graz. He received his **Ph.D.** in SPAD arrays for super-resolution microscopy from TU Delft in 2018.
- He was subsequently postdoctoral researcher and scientist at **EPFL**.
- In 2018, he became **co-founder of Pi Imaging Technology,** a spinoff from EPFL and is currently CEO.

Dr. Harald Homulle (male)

Harald Homulle obtained his Ph.D. (*cum laude*) in 2019 at TU Delft. During his Ph.D., he worked on both quantum computing electronic interfaces and single-photon avalanche diode systems. At the same, he was employed as consultant at Fastree3D for the implementation of SPAD arrays for automotive light detection and ranging systems. After the Ph.D., he joined Pi Imaging Technology as a Senior Design Engineer. He authored and co-authored over 25 technical journal articles and conference papers.

Education and professional career:

- Harald Homulle, born 1990 in Den Haag, studied **Microelectronics** at TU Delft and EPFL. He received his **Ph.D.** in quantum electronic from TU Delft in 2019.
- During his Ph.D., he worked as **consultant at Fastree3D**, a SME focused on SPAD arrays for automotive light detection and ranging systems.
- He joined Pi Imaging Technology in 2020 as **Senior Design Engineer**.

Top 5 relevant achievements:

Products:

1. 23-pixel SPAD array optimized for green detection, designed in a standard CMOS process (scalable), matched with excellent sensor performance and low system complexity. The SPAD active area is 107µm^2. The SPAD pixels feature a QE of 45%, a median dark count rate of 140cps, and a FWHM timing jitter of less than 130ps. Afterpulsing and crosstalk are below 0.1%. This SPAD array represents a compact solution for advanced scanning techniques such as fluorescence lifetime imaging (FLIM), fluorescence correlation spectroscopy (FCS), image scanning microscopy (ISM) and stimulated emission depletion (STED).

Services:

2. Custom design of SPAD arrays and systems with SPAD arrays. Pi Imaging offers top-down (starting from specifications) services of SPAD array design and manufacturing. Design services are based on building blocks verified in previous Pi Imaging products. On the system level, Pi Imaging offers advanced photon time-tagging and counting modalities implemented on FPGA.

Publications:

3. Antolovic, I. M.; Burri, S.; Bruschini, C.; Hoebe, R. A. & Charbon, E. “SPAD imagers for super resolution localization microscopy enable analysis of fast fluorophore blinking” *Sci. Rep.* 7, 44108 (2017)
4. Bruschini, C.; Homulle, H.; Antolovic, I. M.; Burri, S. & Charbon, E. “Single-photon avalanche diode imagers in biophotonics: review and outlook” *Light Sci. Appl.* 8, 1–28 (2019)

5. Ulku, A.; Ardelean, A.; Antolovic, I. M.; Weiss, S.; Charbon, E.; Bruschini, C. & Michalet, X. “Wide-field time-gated SPAD imager for phasor-based FLIM applications” *Methods Appl. Fluoresc.* 8, 024002 (2020)

Up to 5 relevant projects or activities:

Pi Imaging Technology currently participates in multiple industrial projects aimed at utilizing SPAD arrays in microscopy, spectroscopy and random number generation. Within his scientific activity, Dr. Antolovic participated in scientific projects related to the activities in this proposal:

- NWO project “Ultra-fast GSDIM super resolution microscopy using a SPAD-array camera”
- NWO project “L3SPAD: A Single-Photon, Time-Resolved Image Sensor for Low-Light-Level Vision”
- SNSF project “Three-Dimensionally Integrated, Ultra-Fast Cameras for Time-Resolved Multi-Wavelength Fluorescence Imaging”

4.2. Third parties involved in the project

No third parties involved.

4.3. Letters of support – Advisory Board

Max Planck Institute for Biophysical Chemistry
Karl Friedrich Bonhoeffer Institute

MAX-PLANCK-GESELLSCHAFT

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Göttingen, 15 June 2020

To whom it may concern

Following the development of high-resolution fluorescence microscopy, a new paradigm shift in photonics-based in-vivo/in-vitro imaging is currently taking place. The latest technology developed in my laboratory, MINFLUX, enables for the first time ever the optical separation of molecules in a cell that are only a few nanometers apart. As a result, completely new applications in biomedical and pharmaceutical research and development are conceivable in the near future. Europe thus has the unique opportunity to be at the forefront of the (further) development of related markets and to play a decisive role in shaping them.

The NanoVIB project, which has now been applied for as part of H2020, will address this latest paradigm shift in superresolution fluorescence microscopy and will develop a next-generation fluorescence microscope that builds on the MINFLUX principle and is capable of routinely resolving spatial distribution patterns of specific proteins down to the nanometer range, even under difficult optical conditions. As a lead application, this microscope will then be used in the project to resolve mechanisms underlying pneumococcal virulence and invasiveness.

Being a pioneer in this field, I am highly interested in following the development of this new methodology within the project. It will also be very exciting to follow the lead application, where an increased understanding of bacterial virulence and invasiveness is crucial for the development of new antibiotics and vaccines, obviously an area of great interest for the biopharmaceutical industry.

Considering this project's potential and its relevance for the development of new optical technologies and health research, I would like to give this application my full support. I have been asked to be a **member of the advisory board** of the consortium and I gladly accept this request. In this role, I look forward to follow the project's progress and to provide my expertise, perspectives and feedback.

Stefan W. Hell

Nobel Laureate in Chemistry 2014

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To whom it may concern:

Photonics based tools, such as fluorescence microscopy, provide unique contrast for in-vivo as well as for in-vitro imaging. They are by their nature, non- or minimally-invasive, harmless, continuous and inexpensive. **Biophotonics** is therefore one of the **most vibrant and promising markets** especially when **component manufacturers** align their products with the requirements of **systems and whole solution providers** as well as **institutions for basic and applied research**, thus generating additional synergies in a **public–private partnership**.

The NanoVIB project now applied for within H2020 addresses the latest paradigm change in super-resolution fluorescence microscopy and aims to develop a **next-generation fluorescence microscope** that builds on the MINFLUX principle and is capable of routinely **resolving spatial distribution patterns** of specific proteins **down to the nanometer range.** As a lead application, this microscope will then be used in the project to resolve mechanisms underlying pneumococcal virulence and invasiveness.

As former CEO of Leica Microsystems CMS, Bruker AXS and Heraeus Kulzer I am highly familiar with the **opportunities**, but also with the **challenges**, associated with the **introduction of a completely new technology** for understanding the cellular origin of disease **into the market**. Therefore, I am very interested in following the development of the new components as well as the whole methodology within the project.

Considering this project's potential and its relevance for the development of new optical technologies and health care related research, I would like to give this application my **full support**. I have been asked to be a **member of the advisory board** of the consortium and I gladly accept this request. In this role, I look forward to follow the project's progress and to provide my expertise, perspectives and feedback.

Heidelberg, June 2020

Martin Haase

AstraZeneca

To whom it may concern:

Biophysical and bioimaging methods play an important role in pharmaceutical R&D. It belongs to my role as Director of Science Policy and Relations Europe at AstraZeneca to keep an overview on what biophysical and microscopic techniques are used in bioscience R&D and the evolving state-of-the-art to assess what needs in AstraZeneca R&D needs the latest methodologies may fill.

The proposed Horizon 2020 NanoVIB project is aimed at harnessing the latest paradigm in super-resolution fluorescence microscopy and develop a system capable of resolving spatial distribution patterns of specific proteins down to a nanometer scale. As a lead application, this microscope will then be used in the project to resolve mechanisms underlying pneumococcal virulence and invasiveness.

Fluorescence-based super-resolution microscopy has developed tremendously in the last few years, and the NanoVIB project will offer another major leap in resolution to microscopy users in biomedical and pharmaceutical research. It will be very interesting to follow the development of this new methodology in the project, potentially of large interest for AstraZeneca and other companies in Pharmaceutical R&D. It will also be very interesting to follow the lead application, where an increased understanding of bacterial virulence and invasiveness is crucial for the development of new antibiotics and vaccines, obviously an area of strong interest for AstraZeneca.

Given the potential of this project and its relevance for pharmaceutical research and drug development, I´d like to give this project application my full support. I have been asked to be part of the advisory board of this project, and I gladly accept this request. In this role, I look forward to follow the project and bring in perspectives and provide feed-back, based on the needs of a pharmaceutical company, and as a potential end-user of the developed techniques and procedures.

Kind regards

DocuSigned by:
1F47B2307533444

Anna Sandström

Director Science Policy and Relations Europe at AstraZeneca

Section 5: Ethics and Security

5.1 Ethics

How the proposal meets the national legal and ethical requirements: All animal experiments and studies on human tissue samples will take place in Stockholm, within WP6 of the project. They will be performed in accordance with all Swedish national regulations from the concerned authorities:

- Arbetsmiljöverket (the Swedish Work Environment Authority), regarding laboratory security, where handling of bacteria is regulated. In the project, all work using bacterial pathogens including mutants (BSL2 pathogens) will be taking place in laboratories classified at biosecurity level 2 (BSL2), all available to us, and approved by this authority.
- Jordbruksverket (the Swedish Board of Agriculture) and the local ethical committee for animal experiments (Stockholms Norra Djurförsöksetiska nämnd), regarding handling of animals in the research with ethical approval from the local ethical committee (Stockholms Norra Djurförsöksetiska nämnd).
- Etikprövningsmyndigheten (the Swedish Ethical Review Committee) and the local ethical committee for human experiments (KI Forskningsetik-kommitté Nord), regarding handling of human tissue material.

Research objectives, methodologies and potential impact of the research: In this project, all research involving animals (wild-type and genetically modified mice), human tissues and cells, as well as pathogenic bacteria will be performed within WP6. In WP5 we will develop sample preparation procedures for the use of the de developed microscopy techniques, and for the research in WP6, but this work will not be based on animal or human tissue samples. WP1-WP4 will entirely focus on microscope system development and will also not involve samples or activities raising any ethical issues. In WP6, two tasks concern the use of animals or human tissue:

In task 6.2, we will study possible co-localization of pneumococcal surface and pilus proteins with receptor proteins on epithelial cells of the blood-brain-barrier (BBB), in brain biopsies from patients who died from pneumococcal meningitis and in brain biopsies from mice models. The brain biopsies will either be obtained via collaboration with the Department of Neurology, Academic Medical Center, University of Amsterdam (Prof Diederik van de Beek), or locally from the clinics at Karolinska University Hospital, Stockholm, with ethical approval from the ethical committee when needed. The biopsies will come from deceased persons, who are fully anonymized; with no other information connected to the samples than that they are from individuals who died from pneumococcal meningitis, and there will be permission for autopsies by family and relatives of the deceased persons. The research will not involve any human participant with interventions, and no personal data collection and/or processing, or further processing of previously collected personal data. Tissue samples will only be used in accordance with obtained approvals, or if necessary, after additional application for approval to the appropriate ethical committee. Human cells that will be included are bought from commercial sources such as A549 epithelial cells, or THP-1 macrophages from American Type Culture Collection (ATCC). No human embryos will be used.

In samples from mice, we will study effects of adding antibodies competing for binding to the BBB epithelial cell receptors, as a potential approach to prevent pneumococcal meningitis. Both wild type mice and genetically modified mice are planned to be used. Mice samples will only be used in accordance with obtained approvals, or if necessary, after additional application for approval to the appropriate ethical committee.

In task 6.4, we will use the developed microscopy of this proposal to study distribution patterns of pneumococcal surface proteins, coupled to sustained bacterial growth, in lungs of pneumococcal-

influenza virus co-infected mice. The goal is to unravel important metabolic aspects in bacterial-viral co-infections, which seem to be a major driving force in such infections, to find better strategies to curb these infections. As for task 6.2, both wild type mice and genetically modified mice are planned to be used, and the mice and the samples from them will be handled in accordance with obtained ethical approvals, or if necessary, after additional application for approval to the appropriate ethical committee.

For both tasks 6.2 and 6.4, we see no detrimental impact as a consequence of the research planned in these work packages. The mice will be kept in a dedicated center for animals at Karolinska Insitutet, with well-established routines and extensive experience in these activities. Likewise, research on live pathogenic bacteria will be performed in BSL2 labs, with researchers and personnel well-trained and educated about any potential hazard in handling bacteria. No research will be performed in non-EU countries.

For the ethical approvals of the planned studies, we refer to the following approved ethical applications for the suggested mice experiments:

11367-2019: Study of pathogen and host responses in infections caused by Streptococcus pneumoniae, S. aureus and K. pneumoniae (approval dated 2015-12-30), pdf-file: N235-15_co-infection.pdf.

English summary: First page is the decision page from the local ethical committee for animal experiments (Stockholms Norra Djurförsöksetiska nämnd). “Concerning Your application(s) for ethical approval of animal experiments, Stockholms Norra Djurförsöksetiska nämnd have at this meeting on the 17th of December 2015 approved Your application(s) regarding ethical approval of animal experiments number N235/15”. On the second page, the head page of the application approved, Birgitta Henriques-Normark is named as the PI (försöksledaren), and the title of the application (as stated above) is given in box 1 at the bottom of the page

N235-15: Study of pathogen and host response in co-infections caused by influenza virus and Streptococcus pneumoniae (approval dated 2019-09-12), pdf-file: 11367-2019_general_permit.pdf

English summary: The first page is the decision page from the local ethical committee for animal experiments (Stockholms Norra Djurförsöksetiska nämnd). “Stockholms Djurförsöksetiska nämnd approves your application and your planned experiment from an ethical point of view, and you can hereby perform your experiment, following the conditions below: 1. PI is Birgitta Henriques-Normark, 2. Director is Elisabeth Andersson, 3. The experiments are performed on sites specified in supplementary part A, 4. The experiments are performed according to supplementary part A”. The title of the ethical application approved (as stated above) is given in section 1.5 in the application, p.5(45).

Ethical approvals needed for the studies using brain biopsied postmortem will be applied for and will not start until ethical approvals have been granted.

5.2 Security

This project will involve:

- activities or results raising security issues: NO
- 'EU-classified information' as background or results: NO

STOCKHOLMS
DJURFÖRSÖKSETISKA NÄMND
Avdelning 1

MISSIV
2015-12-30
Stockholm

Till: Birgitta Henrigues Nordmark

Angående Er ansökan/Era ansökningar om etisk prövning av djurförsök

Stockholms norra djurförsöksetiska nämnd har vid sammanträde den 17 december 2015 godkänt Er ansökan/Era ansökningar om etisk prövning av djurförsök nr

N 235/15

under de eventuella förutsättningar som framgår av det påtecknade beslutet/besluten i respektive ansökan. Kopia av ansökan/ansökningarna med påtecknat/påtecknade beslut bifogas. En anvisning om hur man överklagar nämndens beslut återfinns nedan. *Godkännandet kan återkallas om djurförsöket inte utförs i enlighet med godkännandet.*

Veronika Lundström
sekreterare

HUR MAN ÖVERKLAGAR

Den regionala nämndens beslut kan överklagas. Ett överklagande, ställt till den Centrala djurförsöksetiska nämnden, ska ha kommit in till den regionala nämnden inom tre veckor från den dag Ni fick del av beslutet. Om klaganden är en part som företräder det allmänna, ska överklagandet dock ha kommit in inom tre veckor från den dag då beslutet meddelades. Överklagandet ska ske skriftligen. I överklagandet ska Ni ange vilket beslut Ni överklagar (t.ex. genom att ange ansökans diarienummer), hur och varför beslutet ska ändras samt Ert namn, Er adress och Ert telefonnummer. Er e-postadress kan också vara lämplig att ange. Överklagandet ska undertecknas av Er.

När överklagandet kommer in, kommer den regionala nämnden att ompröva det överklagade beslutet, såvitt Ni inte begär att överklagandet ska sändas över till den Centrala djurförsöksetiska nämnden utan föregående omprövning. En omprövning beräknas kunna ske inom fem veckor från det att överklagandet har kommit in till nämnden. För det fall nämnden vid en omprövning ändrar beslutet enligt överklagandet är överklagandet förfallet och kommer inte att sändas över till den Centrala djurförsöksetiska nämnden.

Postadress:	Besöksadress:	Telefon:	E-post:
Box 8307	Stockholms tingsrätt	08–561 65 144	agnes.leijonhufvud@dom.se
104 20 Stockholm	Scheelegatan 7	08–561 65 209	veronika.lundstrom@dom.se

Jordbruks verket

KI - Komparativ medicin
Djurförsöksetisk ans./tillst.
Datum 2015-10-26
Centuri ET nr. 42144

ANSÖKAN
– Etisk prövning av djurförsök

Datum

Denna blankett är fastställd av Jordbruksverket den 20 december 2012. Vägledning för att fylla i blanketten finns att hämta på Jordbruksverkets webbplats (www.jordbruksverket.se). Information om var ansökan ska skickas finns på sista sidan.

Stockholms norra djurförsöksetiska nämnd
Ink. 28/10 2015
Dnr. N 235/15

☐ I ansökan bör med motivering anges vilka uppgifter som enligt sökande kräver sekretess

Uppgifter om sökande (försöksledaren)

Namn Birgitta Henriques Normark		
Institution, avdelning eller motsvarande Institutionen för Mikrobiologi, Tumör och Cellbiologi (MTC), Karolinska Institutet		
Telefonnummer (även riktnummer) 0851771216/0851771214	Faxnummer (även riktnummer)	E-postadress Birgitta.henriques@ki.se
Adress Nobels väg 16		Postadress SE-171 77 Stockholm

Betalningsmetod

Betalningsmetod ☐ Kort ☒ Faktura	Ordernr 47519	Belopp 6000 SEK

Fyll i vilken betalningsmetod du har använt när du betalade ansökan via Jordbruksverkets webbutik. Fyll även i det ordernummer du har fått från webbutiken. Ordernumret behöver nämnden för att kunna hantera din ansökan. Om du har valt att betala via faktura behöver inte denna vara betald när du skickar in ansökan men ordernumret måste alltid anges här. Under rutan belopp anger du den summa du har eller kommer att betala.

Ansökan är en fortsättning av tidigare prövade försök

Diarienummer

☐ Försöket avser framställning av genetiskt modifierade djur

☒ Försöket avser användning av genetiskt modifierade djur

Försöksledarens klassificering av försökets svårhetsgrad (endast ett alternativ ska anges)

☐ Ringa svårhet ☒ Måttlig svårhet ☐ Avsevärd svårhet ☐ Terminal ☐ Terminal/organ

Uppgifter om det planerade djurförsöket

Sidorna 1-6 i ansökningsblanketten samt bilaga 1 fylls i av den sökande. OBS! Vid uppgiftslämnandet ska ett enkelt språk användas. Viktigt är också att tillräckliga uppgifter lämnas för den djurförsöksetiska nämndens bedömning av försöket.

1. Projektets titel

Studie av patogen och värdsvar vid koinfektioner orsakade av influensa virus och *Streptococcus pneumoniae*.

2. Syftet med djurförsöket och eventuell redovisning av tidigare resultat

Pneumokockinfektioner är en av de vanligaste orsakerna till sjuklighet och dödlighet i världen. Varje år dör cirka 1-2 miljoner människor av pneumokockinfektioner och en stor andel av dessa är barn. Pneumokocker orsakar allt från milda luftvägsinfektioner, öroninflammation och bihåleinflammation till svårare infektioner såsom lunginflammation, blodförgiftning och hjärnhinneinflammation. På senare tid har studier visat att en tidigare influensa infektion gör värden mer känslig för att få en bakteriell infektion med pneumokocker. Vid influensa pandemin 1918 var bakteriella superinfektioner med pneumokocker framför allt anledningen till att så många dog. Mekanismerna bakom denna ökade känslighet är inte helt klarlagt. Därför behövs mer forskning för att i detalj förstå vad som ger denna ökade känslighet.
Idag finns det en risk att ett influensavirus framgångsrikt skulle kunna sprida sig över världen, med hög mortalitet som följd. Genom att studera de faktorer som påverkar dödligheten av influensa kan bättre behandlingsstrategier utarbetas. Detta ger oss bättre beredskap för hur man ska kunna hantera en eventuell pandemi.
Målet här är att studera influensa-inducerade förändringar av immunförsvaret och att studera interaktion mellan influensa virus

och pneumokocker. Immunförsvaret efter influensa infektionen kommer att analysera och koinfektionsmodellen kommer att jämföras med singelinfektion.

3. Andra metoder än den valda

Uppgifter ska kortfattat lämnas om det finns andra metoder, med eller utan användning av djur, för att uppnå syftet med försöket

Andra likvärdiga metoder saknas.

In vivo försöken kompletteras med cellbaserade (*in vitro*) försök för att minska antal djurförsök maximalt. Med dagens tekniker finns tyvärr ingen metod som t ex cellkulturer eller datorsimuleringar som kan ersätta ett intakt helkroppssystem. Vi finner att de nedan nämnda metoderna utgör olika sätt att optimalt ge svar på våra frågeställningar. Andra, alternativa, metoder till de nedan beskrivna har givetvis tagits i beaktande vid valet av vetenskapliga metoder.

4. Dokumentationskrav

Om nationella eller internationella dokumentationskrav genom djurförsök föreligger, ska uppgifter som styrker behovet av försöket anges

Inga dokumentationskravs.

5. Valet av djurart, ras och stam

Djurart	Totalt antal djur
Mus musculus	5000

Motivering för val av djurart med karaktärisering av djuren

Vi använder oss av etablerade metoder för att studera virus och bakterieinfektioner i möss.

Vi kommer att använda C57BL/6, MF1, C3H/He, eller liknande vildtypstammar.

Utöver vildtypmöss kommer vi att använda genetisk modifierade möss som saknar en eller flera gener viktiga för immunförsvaret. Då nya musstammar hela tiden utvecklas vill vi ha möjlighet att använda stammar som har förändringar i det medfödda och/eller det adaptiva immunsvaret alternativt förändringar av fysiologisk betydelse för det medfödda och/eller det adaptiva immunsvaret eller bakteriens förmåga att orsaka sjukdom. Förutsättningen för att mössen ska kunna användas är att de vid normaltillstånd inte har klinisk score som uppgår till eller överskrider 0.3.

Detta inkludera men begränsar oss inte till möss som är defekta med avseende på;

MHC klass II receptor; som är av vikt vid exempelvis presentation av antigen.

NOD och Toll-liknande receptorer: som är av vikt vid exempelvis igenkänning av patogen eller stressignaler.

Signaleringsvägar, såsom MyD88, TIRAP, Mal, RIG-I, IPS.1, TRIF: som blant annat är av vikt för att mediera signaler från NOD och Toll-liknande receptorer.

Interferonreceptorer såsom IFNR och IRF3: som är av vikt för att aktivera och styra immunsvaret.

Interleukiner/cytokiner och dess receptorer såsom IL-1, IL-6, IL-8, IL-17, IL-18, ICE; TNF receptorer, och G-CSF: som är av vikt för att aktivera, styra immunsvaret och bildning av immunceller.

PGRP; Scavenger receptorer, lektiner, såsom SIGN-R1 och integriner, såsom CR3: som är av vikt för igenkänning, gener som påverkar inflammasomen, såsom Caspase-1, ASC, NLRC4, NAIP5; eller inhibitorer av signaleringsvägar, såsom A20, IRAK-M, Tollip, IκB-α; collektiner, såsom SP-A eller möss med förändringar i Glykanstruktur såsom CMAH.

Platelet Ednothelial adhesion Molecule (pecam-1) och Poly Immunglobulin Receptor (pIgR) som påverkar bakteriens förmåga att binda till värdceller.

Vi vill aven kunna använda CD11c.DTR samt CD11c.DOG möss. Dessa möss som bär gener som gör att man, genom att injicera difteri-toxin (som ej orsakar sjukdom hos möss), tillfälligt kan slå ut dendritiska celler och makrofager (två typer av vita blodkroppar).

För CD11c.DTR resulterar behandlingen i att nästintill 100 % av de dendritiska cellerna i mjälten är borta under den första och andra dagen efter injektionen. Under dag 3-4 återkommer ungefär 50 % av de dendritiska cellerna. För CD11c.DOG mössen är 80% av de dendritiska cellerna i mjälten borta under första och andra dagen efter behandlingen. Vi vill kunna använda dessa möss för att studera de dendritiska cellernas betydelse vid infektion med influensa virus och/eller pneumokocker.

Fenotypen av musstammarna som är angivna ovan avviker inte från den hos vildtypmöss, men det kan hända de uppvisar ett annorlunda infektionsförlopp än möss med intakt immunförsvar och detta vill vi studera. De kommer därför att observeras noga enligt nedan (punkt 8).

Anledningen till att vi vill använda möss som saknar en eller flera gener eller en särskild typ av cell är för att få kunskap om dessa geners betydelse för immunförsvaret. Vi kommer även att studera influensa- och bakteriestammar med olika egenskaper samt delar av bakterier och ligander eller inhibitorer till olika receptorer för att lättare kunna förstå vilka faktorer som påverkar sjukdomsutvecklingen. På så sätt kan vi få kunskap som leder till utveckling av effektivare behandlingsstrategier mot bakteriella superinfektioner och därmed ge en högre beredskap vid en eventuell pandemi.

6. Försökets tids- och genomförandeplan

Försöket beräknas påbörjas	Försöket pågår t.o.m.
Snarast efter godkännande	5 år efter beslutet

Beskrivning av försökets uppläggning och genomförande ska göras med tyngdpunkt på de ingrepp som utförs, olika moments varaktighet m m

Mössen kommer att vara minst 4 veckor vid försökets start. Försöken kommer att pågår i maximalt 4 veckor efter bakterieinfektioner och upp till 3 månader efter virusinfektioner för att observera influensa-inducerade förändringar av immunförsvaret, men kan komma att avslutas tidigare.
Beroende på försökets upplägg kommer endast de försöksgärder, angivna under punkt 6, som behövs för att besvara frågeställningen att utföras (se översiktsschema sist under punkt 6).

Generellt för behandlingar angivna under punkt 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8 och 6.10 samt provtagningen enligt punkt 6.11
Vid intratrakeal och intranasal administrering kommer mössen att vara sövda, vid intraperitoneal och intravenös administrering kan mössen komma att sövas för att underlätta hanteringen samt för att minska infektionsrisken för personalen. Vid intravenös administrering kommer mössen att värmas med värmelampa, värmedyna eller liknande för att lättare kunna injicera lösningen i svanskärl. Mössen kan komma att sövas i samband med provtagning, detta för att underlätta provtagning samt för att minska infektionsrisken för personalen.
Behandlingen där substanser tillförs mössen (angivna i punkt 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.10 och 6.12) kan komma att utförs i anslutning till varandra (här definierat som 3 timmar innan eller efter annan behandling). Totalvolymen för behandlingarna under de angivna 6 timmarna kommer i så fall inte att överstiga följande volymer; intravenöst (5 ml/kg/behandling), intraperitonealt (maximalt 500 µl), intratrakealt (maximalt 100 µl) och intranasalt (maximalt 100 µl).

Försöksåtgärder

6.1 Identifiering och genotypning av möss
Individer kan komma att identifieras genom öronmärkning.
Då vi använder genetisk modifierade möss vill vi ha möjlighet att ta ett vävnadsprov för att säkerställa individers genotyp vid behov. Detta sker genom att ett vävnadsprov i form av ett blodprov (maximalt 20 µl), vävnadsprov från öra (några millimeter i diameter) eller att några millimeter stort vävnadsprov från svansspetsen tas, eventuellt sker detta under sövning. Vävnadsprov som tas är så små att det inte är troligt att djuren upplever något betydande obehag. Om lämpligt kommer vävnad som tas i samband med öronmärkning att användas för genotypning.

6.2 Infektion
I infektionsmodellen kan mössen inokuleras intranasalt (maximalt 50 µl), intratrakealt (maximalt 100 µl), intraperitonealt (maximalt 200 µl) eller intravenöst (maximalt 5 ml/kg) med nedan angivna substanser. Inokulation kan ske 1-2 gånger med minst tre dygn mellanrum. Lösningar 6.2a - 6.2d kan komma att administreras i en blandad suspension.

a. Infektion
De lösningar som används är virus, bakterie-/bakteriefragment/ligand- eller vesikelsuspension eller kontrollösning av medium, fosfatbuffrad saltlösning, isoton koksaltlösning eller liknande. Avdödade/inaktiverade mikroorganismer och fragment från mikroorganismer andra än *S. pneumoniae* kan även komma att användas. Mössen inokuleras först med virus (eller kontrollösning) och efter 3 dagar till 4 veckor kommer mössen att inokuleras med bakterie-/bakteriefragment/ligand- eller vesikelsuspension (eller kontrollösning). Vi kommer att även göra tvärsom och starta med bakteriell infektion och sedan med virusinfektion. Mössen i kontrollgrupp kommer endast att infekteras med antingen bakterier eller virus. Mössen kan även komma att lämnas obehandlade. Bioluminiscerande bakterier kan komma att användas.

b. Infärgning av lösning
För att utvärdera hur lösning fördelas i luftvägarna kan färglösning alternativt färgad infektionslösning administreras i likhet med vad som är beskrivet för intranasal och intratrakeal administrering ovan.

c. Fluorescerande inmärkning av lösning
För att kunna följa bakteriernas spridning i värden vill vi ha möjlighet att märka komponenterna i infektionslösningen med t.ex. fluorescerande substanser såsom fluorescein isothiocyanate (FITC), Rediject, pHrodo eller liknande substanser. Metoden finns beskriven sedan tidigare (Birjandi *et al.*, 2011, *J. Immunology* 186; 3441-51).

d. Administrering av partiklar i lösning
För att kunna följa bakteriernas spridning i värden vill vi ha möjlighet att administrera latex- eller polystyren-partiklar eller liknande, detta inkludera så så kallade micropheres och Qdots Nanocrystal Tracers (Nakajima *et al.*, 2005, *Cancer Sci.* 96; 353-6; Gravier *et al.*, 2011 *J. Biomed Opt.* 16;096013). Vi vill även ha möjlighet att konjugera partiklar med fluorescerande markörer och/eller biologiska ämnen såsom bakteriella fragment och membranstrukturer, serum, värdprotein etcetera.

6.3 Inmärkning av värdceller
a. Märkning in vivo
För att studera hur värdens immunceller svarar på och migrerar som en följd av virus- eller pneumokockinfektion, vill vi ha möjlighet att märka värdceller, främst fagocytiska immunceller. Märkningen sker med fluorescerande ämnen som binder till värdcellernas yta eller som tas upp genom fagocytos eller genom annan mekanism med motsvarande effekt. Denna typ av inmärkning har använts tidigare av andra forskargrupper (Sun and Metzger

2008, *Nature medicin* 14; 558-564). Kontrollgrupp kan istället ges medium lösning, isoton koksaltlösning, fosfatbuffrad lösning eller liknande, alternativt lämnas obehandlad.

Substanserna kan komma att administreras intravenöst (maximalt 100 µl), intraperitonealt (maximalt 200 µl), intratrakealt (maximalt 100 µl) eller intranasalt (maximalt 100 µl), innan, i samband med eller efter infektion. De volymer som används vid intratrakeal och intransal administrering är nödvändiga då lösning måste nå lungorna för att inmärka cellerna.

b. Cell-transfusion
Vi vill få möjlighet att isolera blodkoppar (erytrocyter), blodplättar (trombocyter) och immunceller exempelvis monocyter, makrofager och neutrofiler samt förstadier till dessa celler från möss. Cellerna stimuleras *in vitro* för att sedan återföras till mottagarmöss. Vi kan på så sätt förstå hur specifika typer av värdceller påverkar bakteriens möjlighet att sprida sig från olika vävnader och hur värden svarar för att bekämpa infektioner. Metoden kan även användas för att påvisa substanser som påverkar värden immunsvar, utan att behöva behandla värden med dessa. Vi kan även studera hur immunceller transporterar bakterier och bakterierfragment genom värden och hur värdceller förflyttar sig mellan olika vävnader.

Isolering av celler sker post mortem enligt avlivningsmetoder beskrivna under punkt 9. De celler som isoleras är antigen primära färdigutvecklade celler alternativt att celler från benmärg eller blod isoleras. Dessa celler kan sedan induceras in vitro (i cellkultur) för att differentiera sig till en viss celltyp. De isolerade cellerna stimuleras med cytokiner, levande virus eller bakterier, avdödade/behandlade virus eller bakterier, baktierierfragment/ligander/vesiklar. Avdödada/inaktiverade mirkoorganismer och fragment från mikroorganismer andra än *S. pneumoniae* kan även användas. De stimulerade cellerna återförs sedan till en mus av motsvarande stam. Cellerna återförs mottagarmöss genom de metoder som beskrivits under punkt 6.3a "Inmärkning av värdceller". Metoden i sig innebär således att djuren inte kommer att utsättas för nya typer av ingrepp.

6.4 Avlägsnande (depletion) av värdceller
För att studera immuncellers roll vid pneumokockinfektion kan immunceller, eller andra celler av betydelse vid infektion, komma att avlägsnas, hämmas i sin funktion eller i sin interaktion med bakterien. Effekten uppnås genom behandling med liposom/vesiklar eller med antikroppar, receptorligander eller liknande. Behandlingen påbörjas i regel innan mössen infekteras och pågår sedan under infektionsförsöket.

Behandling med antikroppar eller receptorligander: specifika populationer av immunceller avlägsnas eller hämmas i sin funktion genom att en lösning innehållande antikroppar eller receptorligander specifika för en eller flera cellpopulationer administreras. Kontrollgrupp kan istället ges isoton koksaltlösning, fosfatbuffrad lösning alternativt antikroppar med annan specificitet eller liknande, alternativt lämnas obehandlad. Metoden möjliggör en mer precis utarmning av specifika immuncellspopulationer än vad som är möjligt genom liposombehandling.

Liposombehandling: populationer av fagocyterande celler kommer att avlägsnas genom att liposomer innehållande den verksamma substansen klodronat administreras. Kontrollgrupp kan istället ges isoton koksaltlösning, fosfatbuffrad lösning alternativt liposomer som ej innehåller den versamma substansen klodronat eller motsvarande, alternativt lämnas obehandlad. Uppemot 90 % av alla makrofager i en population avlägsnas genom liposombehandlingen.

Liposom-/antikropps-/receptorligand-/kontrollösning kommer antigen att injiceras intravenöst (maximalt 200 µl), intraperitonealt (maximalt 200 µl), intratrakealt (maximalt 100 µl) eller intranasalt (maximalt 100 µl). Volymen som anges för intravenös injektion överstiger den tillåtna och får därför endast användas vid ett tillfälle och injiceras långsamt. De volymer som administreras vid respektive behandlingsmetod är nödvändiga för att effektivt kunna avlägsna immunceller, särskild Kupferceller i lever och makrofager i mjälte kräver en stor volym lösning för att utarmas. I samband med intratrakeal administrering av lösen intuberas mössen. Metoden att utarma populationer av fagocytiska celler med klodronat är etablerad sedan länge (van Roijen och Sanders 1994, *J. Immunol. Methods.* 174; 83-93). För att utvärdera hur lösningen fördelas i luftvägarna kan färglösning administreras i likhet med vad som är beskrivet för intranasal och intratrakeal administrering ovan. De volymer som används vid intratrakeal och intranasal administrering är nödvändiga då lösningen måste nå lungorna där den har effekt.

Då immunceller nybildas kan mössen komma att behandlas med liposomer, antikroppar, ligander alternativt motsvarande kontrollösning vid upprepade tillfällen för att säkerställa att cellpopulationen är utarmad. Behandlingen kan därför komma att ske upp till två gånger innan infektion samt upp till tre gånger efter infektion.

6.5 Avlägsnande av celler i DTR- och DOG-möss
CD11c.DTR-mössen ges en dos difteria-toxin (maximalt 4 ng/g kropssvikt) intraperitonealt (maximalt 100 µl) eller intranasalt (maximalt 50 µl), ca 6-24 timmar innan infektion. Metoden är beskriven sedan tidigare (Jung *et al.*, 2002, *Immunity* 17;211-220), och ger en övergående utamning av immunceller.

För CD11c.DOG möss används behandlas de cirka 24 timmar innan infektion med intraperitoneal injektion (maximalt 100 µl, 8 ng/g kroppsvikt) difteria-toxin. Mössen därefter komma att injiceras vid upprepade tillfällen med 1-2 dagars mellanrum. Detta kan pågå i upp till 12 dagar och är inte associerat med viktnedgång eller minskad överlevnad hos mössen. Vid intranasal administrering av lösning kommer volymen att uppgå till

maximalt 50 µl och administreras vid ett tillfälle, den maximala dosen av toxiner är 8 ng/g kroppsvikt. Metoden är beskriven sedan tidigare (Hochweller *et al.,* 2008, *Eur. J. Immunol.* 38;2776-2783).

Kontrollgrupp till CD11c.DTR och CD11c.DOG kan istället ges medium, fosfatbuffrad saltlösning, isoton koksaltlösning eller liknande, alternativt lämnas obehandlade. Vi vill även få möjlighet att utföra motsvarande behandling med difteria-toxin eller kontrollösning på viltypmöss för att kunna utesluta att behandlingen i sig har en effekt på mössens immunsvar.

6.6 Behandling med sialinsyra eller sialinsyraliknande ämnen

Sialinsyra och liknande ämnen är i sig kända som ofarliga för däggdjur och har redan använts i försök publicerade av andra grupper. Vi vill behandla möss med ovan nämnda substanser som vi tror kan ha en terapeutisk effekt på infektionen. Djuren kommer att behandlas med substanserna intraperitonealt (maximalt 200 µl), intravenöst (maximalt 125 µl), intranasalt (maximalt 50 µl) eller intratrakealt (maximalt 100 µl). Substanserna kommer att vara lösta i medium, fosfatbuffrad saltlösning, isoton koksaltlösning eller liknande. Vissa möss kan komma att lämnas obehandlade, alternativt behandlas med lösningar utan sialinsyra eller liknande ämnen. Behandlingen kan komma att sker 1-2 gånger per individ, innan infektion, tillsammans med bakterier/virus eller i anslutning till infektion. Det finns inget skäl att tro att mössen blir mer påverkade av substanser eller substanser i kombination med bakterier/virus än vad de normalt blir vid en infektion med enbart bakterier eller virus.

6.7 Behandling med surfaktant eller surfaktantliknande ämnen

Lungsurfaktant och liknande ämnen är i sig kända ofarliga för däggdjur och har redan använts i försök (Borron *et al.*, 2000, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 278;L840–L847; Sender *et al.,* 2013, *PLoS One.* 8(3):e59896). Vi vill behandla möss med ovannämnda substanser som är beskrivet för att modifiera immunförsvar vid infektionen. Djuren kommer att behandlas med substanserna intranasalt (maximalt 50 µl), intratrakealt (maximalt 100 µl), intraperitonealt (maximalt 200 µl) eller intravenöst (maximalt 125 µl). Substanserna kommer att vara lösta i medium, fosfatbuffrad saltlösning, isoton koksaltlösning eller liknande. Vissa möss kan komma att lämnas obehandlade, alternativt behandlas med lösningar utan surfaktant eller liknande ämnen. Behandlingen kan komma att sker 1-2 gånger per individ; innan infektion, tillsammans med bakterier/virus eller i anslutning till infektion. Det finns inget skäl att tro att mössen blir mer påverkade av substanser eller substanser i kombination med bakterier/virus än vad de normalt blir vid en infektion med enbart bakterier eller virus.

6.8 Behandling med inhibitorer och silencing RNA

För att studera betydelsen av signaleringsvägar eller cytokiner i koinfektionsmodellen är det inte alltid möjligt att använda möss som saknar en eller flera gener viktiga för immunförsvaret, i vissa fall kan det krävas att inhibera en viss signaleringsväg eller cytokin. Därför vill vi få möjlighet att använda inhibitorer eller silencing RNA av signaleringsvägar eller cytokiner. Beroende på var det för försöket är mest relevant att reducera signaleringen eller cytokin-uttrycket kommer substanserna att administreras vid maximalt 2 gånger innan infektion samt maximalt 3 gånger i anslutning till eller efter infektion. Administrering sker intranasalt (maximalt 50 µl), intratrakealt (maximalt 100 µl), intraperitonealt (maximalt 200 µl) eller intravenöst (maximalt 125 µl). Substanserna kommer att vara lösta i medium, fosfatbuffrad saltlösning, isoton koksaltlösning eller liknande. På grund av vissa substansers begränsade löslighet kan dessa komma att lösas i små mängder DMSO eller liknande lösningsmedel. Kontrollgrupp kan lämnas obehandlad alternativt behandlas med motsvarande lösning utan aktiv substans. Denna lösning kan även innehålla en icke aktiv substans liknande den inhibitor som använts eller non-silencing RNA, dvs. RNA utan förmåga att tysta genuttryck. I samband med infektion kan infektionsförloppet komma att te sig annorlunda på grund av förändrad cytokinsignalering. De troligaste utfallet är ett snabbare infektionsförlopp och högre grad av disseminerad sjukdom. Djurens hälsa kommer därför att övervakas noga för att säkerställa att avbrytningspunkten inte överskrids.

6.9 IVIS-fotografering

I samband med vissa försök kan bilder komma att tas med en IVIS/Xenogen-kamera (eller likvärdig utrustning) under det att mössen är sövda. Fotografering tillåts vid ett tillfälle innan infektion/inmärkning av celler (referensbild) samt sker maximalt vid 2 tillfällen per dygn efter infektion/inmärkning av celler samt 1 gång i samband med avlivning.

Mössen kommer vid fotografering att ligga i en ändamålsenlig kammare med uppvärmt golv (cirka 37 grader) för att förhindra nedkylning till följd av sövning. Vi har använt denna teknik under många år och exponeringstiden är i regler ca 5 minuter, men vid applikationer som kräver högre känslighet kan exponeringstiden uppgå till maximalt 20 minuter. Vid exponeringstider som överstiger 5 minuter kommer ögonsalva att användas. Med hjälp av denna teknik kan man visualisera infektionsförloppet i en och samma mus över tid vilket gör att antalet möss kan hållas till ett minimum. Flera fotograferingar per dag är nödvändigt för att man ska kunna följa hur de märkta/fluorescerande/bioluminiscerande bakterierna/inmärkta cellerna sprids i djuret.

Då kamerans känslighet hämmas av päls kan mössen komma att klippas med ändamålsenlig trimmer. Lämplig hårborttagningskräm kan komma att appliceras efter att mössen klippts. Efter någon minut, då krämen verkat, tvättas området med vatten. Mössen kan komma att sövas under denna behandling. Behandling kan komma att upprepas vid behov.

6.10 Behandling med antibiotika

Vi vill få möjlighet att behandla mössen med antibiotika i avsikt att mildra sjukdomssymptom och undersöka behandlingens påverkan på bakteriens patogenicitet och värdens inflammatoriska svar. Kliniskt relevanta

antibiotika kan komma att administreras intravenöst (maximalt 125 µl), intraperitonealt (maximalt 200 µl), intratrakealt (maximalt 100 µl) eller intranasalt (maximalt 100 µl). Behandlingen kan komma att ske uptill en gång innan infektion samt upp till tre gånger efter infektion.

Behandlingsplanen där substans, dos, administreringssätt och frekvens samt volym anges måste godkännas av av veterinär tjänstgörande på Karolinska Institutet.

6.11 Provtagning

Utifall provtagningsvolymen överstiger 7 ml/kg/14 dagar skall vätskeersättning. Sammansättning, administrationssätt och volym avpassas i samråd med veterinär tjänstgörande vid Karolinska Institutet.

a. Beroende på försökets frågeställning kan förloppet av den aktuella behandlingen (med behandlingen avses här vad som står angivet under punkt 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8 och 6.10) komma att följas genom blodprovstagning (maximalt 20 µl) från svansen genom snitt eller borttagande av sårskorpa. Som mest kan 1 prov tas innan behandlingen (referensprov), maximalt 5 gånger under ett enskilt dygn efter behandlingen, maximalt 3 gånger under det därpå efterföljande dygnet, i övrigt får prov tas maximalt 1 gång per dygn efter behandlingen samt i samband med avlivning.

b. Vissa metoder för att exempelvis mäta inflammatoriskt eller metaboliskt svar eller identifiera bakteriekomponenter kräver större provvolymer än ovan angivna 20 µl. Vi vill då istället för vad som står angivet ovan använda kunna ta blod enligt följande; 120 µl blod får tas tre gånger från svansen efter en kort tids värmning med värmelampa eller dylik metod. Första provet tas omkring en vecka innan djuren genomgår behandlingen (referensprov), ett andra prov tas efter behandlingen och det tredje provet tas i samband med att djuret avlivas. En mus har en blodvolym på ca 72 ml per kilo kroppsvikt. För en mus med en vikt på 20 gram motsvara 120 µl knappt 9 % av blodvolymen.

Ovanstående provtagningsprotokoll (punkt 6.11a och 6.11b) får endast användas en gång per försök.

I samband med avlivning, under det att mössen är djupt sövda, vill vi ges möjlighet att ta blod genom hjärtpunktion alternativt från ögat (orbitalplexus). Omedelbart efter att provet har tagits kommer mössen att avlivas genom cervikal dislokation.

Upp till en timme innan avlivning vill vi ges möjlighet att administrera färglösning för att synliggöra lymfatiska kärl och lymfknutor. Färglösningen administreras genom injektion i trampdyna (maximalt 25 µl), subkutan injektion vid svansrot (ca 1-3 cm från rektum, maximalt 25 µl), subkutan injektion vid skulderblad nacke (maximalt 100 µl), intraperitonealt injektion (maximal 100 µl), eller administrering intranasalt (maximalt 50 µl) eller intratrakealt (maximalt 50 µl). I de fall injektion i trampdyna används hålls mössen sövda. Efter injektionen transporteras färgämnet från vävnaden via lymfatiska kärl till de dränerande lymfknutarna och färgar dessa, vilket gör att lymfknutarna lättare kan lokaliseras vid provtagningen. Då vi ofta provtar lymfknutor innan de hunnit förstoras som en följ av injektionen krävs infärgning för att provtagningen ska vara reproducerbar, dvs. att lymfknutorna alltid kan lokaliseras och att samtliga lymfknutor kan provtas. Metoden att infärga lymfknutor genom injektion av färglösning i svansrot alternativt trampdyna är beskriven sedan tidigare (Harell *et al.,* 2008, *J. Immunol. Methods* 20;170-4).

6.12 Behandling för isolering av peritoneala immunceller

För att isolera peritoneala immunceller kan mössen komma att injiceras intraperitonealt (maximalt 500 µl) koksaltlösning eller tioglycolate (max 250 µg) för att aktivera rekrytering av exempelvis makrofager till peritoneum. Efter 1-3 dagar avlivas mössen och celler isoleras.

När försöket avslutas kan vi komma att analysera blod, celler, vävnader och organ med avseende på förekomst av bakterier, virus och bakterierkomponenter. Vi studerar även bland annat histologiska och cellulära förändringar, inflammations- och immunologiska processer, signalmolekyler, blod- och vävnadskomponenter och liknande. Vi kan även komma att isolera organ, vävnader, benmärg, och celler samt bakterier för vidare *in vitro*-försök och cellkultur.

Översiktsschema försöksåtgärder etisk ansökan Koinfektioner

Förteckning över ingrepp där substanser tillförs försöksdjuren

Terminala ingrepp som sker under sövning är ej inkluderade.

Punkt	Beskrivning	Typ av ingrepp	Största tillåtna volym (µl): Intranasalt (inandning)	Intratrakealt (intubering)	Intraperitonealt (injektion)	Intravenöst (injektion)	Maximalt antal tillåtna behandlingar	Tätast tillåtna behandlingsintervall*	Kommentar
6.2a	Virus, bakterier och bakteriella substanser	Tillföra, virus, bakterier eller bakteriella komponenter	50	100	200	5ml/kg	4	3 dygn	
6.2b	Infärgning av lösning	Inmärkning/färgning	-	-	-	-	-	-	Ingreppet beskrivet under 6.2a ersätts helt eller delvis med färgad lösning.
6.2c	Fluorescerande inmärkning av lösning	Inmärkning/färgning	-	-	-	-	-	-	Ingreppet beskrivet under 6.2a ersätts helt eller delvis med lösning för inmärkning.
6.2d	Administrering av partiklar i lösning	Inmärkning/färgning	-	-	-	-	-	-	Ingreppet beskrivet under 6.2a ersätts helt eller delvis med lösning innehållande partiklar.
6.3a	Märkning in vivo	Inmärkning/färgning	100	100	200	5ml/kg	1	-	
6.3b	Cell-transfusion	Tillföra celler	50	100	200	5ml/kg	1	-	
6.4	Avlägsnande (depletion) av värdceller		100	100	200	200	5		Volymerna påverkas av att koncentrationen av substansen inte kan påverkas.
6.5	Avlägsnande av celler i DTR-möss	Utarma specifika cellpouplationer	50	-	100	-	1	-	Behandlingsplan för CD11c möss. Behandlingen har endast effekt på transgena möss med en human DTR-receptor.
6.5	Avlägsnande av celler i DOG-möss	Utarma specifika cellpouplationer	-	-	100	-	12	dagligen	Behandlingsplan för CD11c.DOG möss. Behandlingen har endast effekt på transgena möss med en human DTR-receptor.
6.6	Behandling med sialinsyra eller sialinsyraliknande ämnen		50	100	200	5ml/kg	5	-	
6.7	Behandling med surfaktant eller surfaktantliknande ämnen		50	100	200	5ml/kg	5	1 dygn	
6.8	Behandling med inhibitorer och silencing RNA		50	100	200	5ml/kg	5	1 dygn	
6.9	Märkning in vivo	Möjliggör IVIS-fotografering	100	100	200	5ml/kg	-	-	-
6.10	Behandling med antibiotika		100	100	200	5ml/kg	4	-	
6.12	Behandling för isolering av peritoneala immunceller		-	-	500	-	-	-	-

***Om inget annat behandlingsintervall angetts gäller följande begränsning, att inom en period av 6 timmar får maximalt följande volymer administreras;**

Intranasalt (inandning)	100 µl
Intratrakealt (intubering)	100 µl
Intraperitonealt (injektion)	500 µl
Intravenöst (injektion)	125 µl (undantag för punkt 6.4)

Förteckning över provtagningssheman

Terminala ingrepp som sker under sövning är ej inkluderade.

Punkt	Beskrivning	Typ av ingrepp	Maximal volym (µl)	Beskrivning
6.1	Identifiering och genotypning av möss	Biopsi/håltagning	20	Kan även utföras genom håltagning/biopsi i örat eller biopsi från svansspets.
6.10a	Blodtappning schema A	Blodprovstagning	20	Prov tas från svansspets. Maximalt 5 provtagningar under ett dygn, 3 under det efterföljnade dygnet, i övrigt 1 per dygn.
6.10b	Blodtappning schema B	Blodprovstagning	120	Prov tas från svans. Maximalt 3 provtaningar, med 1 veckas intervall varav 1 i samband med avlivning.

7. Vård och förvaring

Uppgifter ska lämnas dels om djurens vård och förvaring omedelbart före, under och omedelbart efter försöket, dels om vid vilken institution eller klinik förvaring sker och var försöket ska utföras

All skötsel och hantering av mössen sker av utbildad personal vid institutionen för Mikrobiologi, Tumör och Cellbiologis (MTCs) djurhus (Karolinska Institutet, Solna), Prekliniskt lab (Karolinska Institutet, Huddinge) eller Astrid Fagræus laboratoriet (Karolinska Institutet, Solna). Arbetet utförs enlighet med svensk lag om användning av försöksdjur.

Mössen förvaras i möjligaste mån gruppvis, undantag kan exempelvis ske för möss som genomgått skilda behandlingar, där övriga burmedlemmar avlivats på grund av sjukdom eller då mössen uppvisar aggressivt beteende mot varandra. Mössen förvaras i individuellt ventilerade burar då de inokulerats med bakterier. Mössen kommer att ha tillgång till spån/strö, bomaterial och klättermöjlighet; men även andra former av berikning kan förkomma. Mössen kommer att hållas i rum med kontrollerad dygnscykel, ca 12 timmar ljus/mörker. Mössen kommer att ha tillgång till föda och vatten *ad libitum*.

8. Djurens situation och försökets slutpunkt

Beskrivning ska göras av den påverkan på djuren som väntas, eventuella komplikationer, smärtupplevelser, beteendeförändringar m.m. Vidare motivering för klassificering av försökets svårhetsgrad lämnas (jämförs s. 1)

8.1 Djurens situation

Mössen kommer att uppleva ett visst obehag i samband med administrering av lösningar. Detta obehag är dock övergående och mössen förväntas inte uppleva en allmän sjukdomskänsla av behandlingarna i sig. De möss hos vilka immunceller har avlägsnats eller påverkats visar inget förändrat beteende i oinfekterat tillstånd, utan återhämtar sig lika snabbt och har samma aktivitetsnivå som obehandlade möss. Tidigare erfarenhet av metoderna visar att mössen snart efter behandling återgår till normalt beteende. Efter sövning med isofluran återhämtar sig mössen omgående, medan återhämtningen efter sövning med ketamin och xylazin tar längre tid. I det senare fallet kommer mössen därför att övervakas tills att de återfått grundläggande motorisk funktion, mössen kommer sedan att ha tillsyn tills de återhämtat sig. Den provtagning som utförs i form av upprepade små blodprov som tas från svansen verkar inte bekomma djuren.

8.2 Tillsyn av försöksdjur

a. Palpering vid upprepade intraperitoneala injektioner

I de fall lösning administreras intraperitonealt mer än 1 gång per dygn kommer mössen att bukpalperas innan varje injektion för att säkerställa att mössen inte ansamlar vätska. Om så är fallet kommer inte ytterligare injektioner genomföras intraperitonealt förrän mössen återhämtat sig, såtillvida att det inte är nödvändigt för avlivning av djuren. Om djuret visar symptom på peritonit kommer djuret att avlivas.

b. Tillsyn efter inmärkning av värdceller (punkt 6.3), avlägsnande av värdceller (punkt 6.4 eller 6.5), efter behandling med sialinsyra eller sialinsyraliknande ämnen (punkt 6.6), efter behandling med surfaktant eller surfaktantliknande ämnen (punkt 6.7), efter behandling med inhibitorer och silencing RNA (punkt 6.8), efter behandling med antibiotika (punkt 6.10) samt efter behandling för isolering av peritoneala immunceller (punkt 6.12)

Första dygnet efter behandlingen kommer mössens hälsostatus att bedömas 2 gånger, om det inte föreligger skäl för utökad tillsyn, mössen kommer därefter att bedömas 1 gång per dag. Behandlingarna i sig förväntas inte påverka djuren negativt utan påverkan ses först i samband med infektion. Det symptom som ibland kan uppträda är något nedsatt aktivitet.

c. Tillsyn efter infektion (gäller för punkt 6.2) samt vid IVIS-fotografering (punkt 6.9)

Infektionsdos som används vid virusinfektion är så låg att mössen som infekteras inte visar symptom på sjukdom. Baserat på tidigare erfarenhet förväntar vi oss att mössen kan då en viktminskning mellan dag 5-10 efter virusinfektionen. Kroppsvikt och hälsorstatus kommer därför att kontrolleras en gång om dagen efter virusinfektion.

1-3 dagar efter en intranasal infektion med bakterier utvecklar en del möss sjukdomssymptom, medan andra möss inte blir sjuka. Sjukdom observeras i undantagsfall under det första dygnet. Intratrakeal, intraperitoneal och intravenös administrering uppvisar i regel ett snabbare förlopp i de fall mössen blir sjuka, och en mer likartad progression mellan individer. Med dessa administrationssätt kan symptom uppkomma så tidigt som efter ca sex timmar men kan även ha ett mycket långsammare förlopp. Skillnaderna i förlopp beror i hög grad på vilken bakteriestam som använts och infektionsdos samt administrationssätt.

Djur som infekteras kan, i de fall de utvecklar sjukdom, komma att uppleva en allmän sjukdomskänsla. De vanligaste symptomen är att de blir trötta och mindre aktiva, de kan även få en något förändrad kroppshållning och piloerektion. Vid allvarligare sjukdom blir de allt mer inaktiva, får förvärrad kroppshållning och piloerektion, samt kan få dålig cirkulation i extremiteter. Påverkan på ögon (främst i form av porfyrin) är ovanlig vid infektion via luftvägarna men förekommer ibland vid intravenös infektion.

Baserat på tidigare erfarenhet förväntar vi oss inte att se en annorlunda symptombild i infektionsförsök för möss hos vilka immunceller avlägsnats jämfört med möss som enbart infekterats. Det förväntade utfallet av att immunceller avlägsnats är att sjukdomssymptom uppträder tidigare efter infektionen och att förloppet kan bli kortare. Vi kommer att avpassa våra kontroller av djurens hälsostatus med hänsyn till detta.

Djurens hälsostatus kommer att följas noggrant efter bakterieinokuleringen och bedömas enligt Karolinska Institutets bedömningsmall. Dygn 1-3 kommer mössens hälsostatus att bedömas 3-5 gånger per dygn. Dygn 4 och fram till försökets avslutande kommer mössens hälsostatus att bedömas 1-3 gånger per dygn. Om det bedöms som nödvändigt under försökets gång kommer mössen att bedömas med tätare intervall.

8.3 Bedömningsmall

Vid bedömning av djurens hälsostatus kommer Karolinska Institutets bedömningsmall ("Bedömning av djurhälsa för smågnagare och kanin vid misstänkt ohälsa") att användas.

8.4 Avbrytningspunkter

a. Möss som genomgått infektion enligt punkt 6.2

Möss som uppvisar en totalscore som överstiger 0.6 kommer att avlivas. Den förhållandevis höga avbrytningspunkten krävs då vi studerar en infektion som dels kan ge systemisk påverkan men där vissa möss även kan tillfriskna spontant och därför kan vi inte använda en lägre avbrytningspunkt. Om mössen uppvisar en totalscore som överstiger 0.2 vid sista tillsyn före nattuppehåll kommer de att avlivas. Denna lägre avbrytningspunkt gäller dock inte vid försök där mössen kan ges tillbörlig tillsyn även nattetid.

b. Möss som genomgått annan behandling

Avbrytningspunkten är en totalscore som uppgår till eller överstiger 0.4 för möss som inte är infekterade (punkt 6.2) men som genomgått någon av följande behandlingar;

- Inmärkning av immunceller – märkning in vivo (punkt 6.3)
- Avlägsnande (depletion) av värdceller (punkt 6.4)
- Avlägsnande av celler i DTR-möss (punkt 6.5)
- Behandling med sialinsyra eller sialinsyraliknande ämnen (punkt 6.6)
- Behandling med surfaktant eller surfaktantliknande ämnen (punkt 6.7)
- Behandling med inhibitorer och silencing RNA (punkt 6.8)
- IVIS-fotografering – märkning in vivo (punkt 6.9)
- Behandling med antibiotika (punkt 6.10)
- Behandlingen för isolering av peritoneala immunceller (punkt 6.12)

9 Anestesi- och avlivningsmetoder

Användning av narkosmedel, bedövningsmedel, smärtlindrande medel och lugnande medel ska anges liksom avlivningsmetod som ska användas

9.1 Anestesi

a. Anestesi - Inhalation

Vi använder företrädelsevis isofluran som sövningsmedel eftersom sövningen snabbt kan induceras och mössen återhämtar sig omgående och inte visar tecken på att vara påverkade i någon större utsträckning. Med undantag för IVIS-fotografering (punkt 6.9) hålls mössen i regel bara sövda under några minuter.

Mössen kommer att sövas med cirka 4 % isofluran. Vid längre sövningar exempelvis vid IVIS-fotograferingen kan narkosen induceras med en hög koncentration, cirka 4 %, av isofluran för att sedan vidmakthållas med en lägre koncentration dvs. ned till cirka 2.5 % isofluran.

b. Anestesi - Injektion

För vissa applikationer behöver vi hålla mössen sövda under en längre tid, detta gäller främst vid intranasal och intratrakeala behandlingar. Samtidigt behöver vi då få åtkomst till mössens luftvägar vilket gör det omöjligt att kontinuerligt kunna tillföra isofluran. I vissa fall påverkar inhalationsanestesi kvalitén på de prov som tas och vi måste då ha möjlighet att kunna använda injektionsanestesi. Mössen kan därför även komma att sövas genom intraperitoneal injektion (maximalt 250 µl) bestående av en kombination av ketamin (80-100mg/kg kroppsvikt) och xylazin (5-10mg/kg kroppsvikt). Mössen kommer i dessa fall att tillföras värme under sövningen samt behandlas med ögonsalva.

Eventuellt kan annan narkos, smärtlindring, lugnande medel användas efter godkännande av veterinär tjänstgörande på

Karolinska Institutet.

c. Eutanasi

Mössen avlivas genom cervikal dislokation efter nedsövning med isofluran alternativt ketamin och xylazin.

Mössen kan även avlivas genom intraperitoneal injektion med en letal dos (80-100mg/kg) av pentobarbital (Allfatal® eller motsvarande) följt av avblodning via bukaorta (exsanguinering via *aorta abdominalis*) eller cervikal dislokation.

Annan avlivningsmetod kan användas efter godkännande av veterinär tjänstgörande på Karolinska Institutet.

10 Undantag

Jag ansöker om följande undantag, markerade med kryss i nedanstående tabell	
Undantag från djurskyddsförordningen	
☐	9 § Höns för äggproduktion får inte hållas i andra inhysningssystem än sådana som uppfyller hönsens behov av rede, sittpinne och sandbad. Inhysningen skall ske på ett sådant sätt att dödlighet och beteendestörningar hos hönsen hålls på en låg nivå.
☐	10 § Nötkreatur som hålls för mjölkproduktion och som är äldre än sex månader skall sommartid hållas på bete.
☐	11 § Andra nötkreatur än sådana som hålls för mjölkproduktion skall sommartid hållas på bete eller på annat sätt ges tillfälle att vistas ute.
☐	14 § Svin skall hållas lösgående.
☐	15 § första stycket Fixeringsanordningar för svin får inte användas annat än tillfälligtvis.
☐	16 § Boxar för svin och för kalvar upp till en månads ålder skall vara försedda med strö av halm eller annat jämförbart material.
Undantag från Jordbruksverkets föreskrifter och allmänna råd om försöksdjur	
☐ *	10 kap. 2 §, att icke-destinationsuppfödda djur av arter som ska vara destinationsuppfödda inte får användas i försök.
☐	10 kap. 4 §, att herrelösa och förvildade tamdjur inte får användas i försök.
☐	10 kap. 5 §, att djur som har fångats i naturen inte får användas i försök.
☐	10 kap. 8 §, att hotade arter inte får användas i försök.
☐	10 kap. 8 §, att primater inte får användas i försök.
☐	11 kap. 8 §, att ett djur som kan uppleva smärta när bedövningen har avklingat, inte behöver behandlas med smärtstillande metoder eller avlivas.
☐	12 kap. 10 §, att använda andra avlivningsmetoder än de som anges i dessa föreskrifter.
☐	14 kap. 2 §, att immunisering inte får ske genom injektion i tass eller trampdyna.
☐	14 kap. 3 §, att immunisering inte får ske genom injektion i lymfknuta.
☐ **	14 kap. 4 §, att immunisering inte får ske genom injektion i huden på djur av familjerna råttdjur (Muridae) och hamsterartade gnagare (Cricetidae).
☐	14 kap. 5 §, att immunisering inte får ske genom injektion i muskulaturen på mus och andra djur av jämförbar eller mindre kroppsstorlek.
☐	14 kap. 9 §, att det inte är tillåtet att odla monoklonala antikroppar från hybridom i bukhålan på ett djur, så kallad ascitesmetod.
☐ **	14 kap. 7 §, att boosterinjektioner för att förstärka antikroppssvaret inte får göras med ett kortare tidsintervall än fyra veckor när långtidsverkande (depågivande) adjuvans används vid framkallande av ledsjukdomar.
☐	14 kap. 19 §, att djurförsök inte får genomföras i syfte att ta fram en dödlig dos eller koncentration av ett ämne eller en blandning av ämnen.
☐	15-26 kap., att djur ska hållas och skötas enligt vad som framgår av dessa föreskrifter.

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Diarienummer

* Ansökan ska skickas till Uppsala djurförsöksetiska nämnd
** Ansökan ska skickas till Stockholms norra djurförsöksetiska nämnd

Motivering och beskrivning av sökta undantag

11 Populärvetenskaplig sammanfattning

Beskrivning ska göras om försökets syfte och nytta, vilket lidande djuren kommer att utsättas för samt information om antal och typ av djur som ska användas och hur kraven på ersättning, begränsning och förfining i 19 § djurskyddslagen (1988:534) uppfylls. Denna sammanfattning ska skickas med som bilaga till ansökan, se bilaga 1.

Underskrift sökande

Datum	Underskrift
2015/10-20	Birgitta Henriques Nilsson
	Namnförtydligande B. HENRIQUES NORLÉN

Underskrift ansvarig föreståndare Institutionen för Mikrobiologi- Tumör och Cellbiologi (MTC)

Datum	Underskrift
2015/10-20	
	Namnförtydligande MARTIN ROTTENBERG

Underskrift ansvarig föreståndare Astrid Fagræus Laboratorium (AFL)

Datum	Underskrift
2015/10/20	Helene Fredlund
	Namnförtydligande HELENE FREDLUND

Underskrift ansvarig föreståndare Pre-Kliniskt Laboratorium (PKL)

Datum	Underskrift
22/10/2015	
	Namnförtydligande

Kompletterande uppgifter till ansökan

OBS! Om nya eller kompletterande uppgifter muntligen tillförs under ärendets beredning ska dessa antecknas nedan genom beredningsgruppens försorg eller av den djurförsöksetiska nämnden.

se bil 2

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Diarienummer

Beredningsgruppens förslag till nämndens ställningstagande

Se bil. 3

2015.12.15		BIBORKA BERECZKY-VERESS
Datum	Underskrift	Namnförtydligande
2015-12-15	Dagmar Galtei	DAGMAR GALTEIR
Datum	Underskrift	Namnförtydligande
Datum	Underskrift	Namnförtydligande

Nämndens fastställande av försökets svårhetsgrad

☐ Ringa svårhet ☒ Måttlig svårhet ☐ Avsevärd svårhet ☐ Terminal ☐ Terminal/organ

Utvärdering i efterhand

☐ Försöket ska utvärderas i efterhand enligt nedanstående förutsättningar, i vilka delar och vilken aspekt

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Diarienummer

Den djurförsöksetiska nämndens beslut (avd. 1)

Om inte annat sägs i beslutet gäller detta under fem år från dagen för beslutet.

☐ Godkänns ☐ Avslås

☒ Godkänns med följande villkor: D.4 på ansökan ska vara avbrytningspunkt.

För motivering, se bil. 3

Nämnden fastställer avgiftsbeloppet till

6 000 kr.

Datum	Ordförandens underskrift
17.12.2015	Namnförtydligande Thomas Arvefors

~~För motivering och eventuell avvikande mening se bifogat protokoll.~~ Information om hur du överklagar bifogas i ~~förekommande~~ fall.

Fördelning av ansökningar och placering av de regionala djurförsöksetiska nämnderna

Ansökningarna om planerade djurförsök ska fördelas mellan de regionala djurförsöksetiska nämnderna enligt förteckningen nedan. Om en ansökan gäller planerade djurförsök som berör flera nämnders verksamhetsområden ska ansökan fördelas till den nämnd inom vars verksamhetsområde huvuddelen av försöken ska genomföras.

Nämnd	**Verksamhetsområde**
Stockholm norra avdelning 1 och 2	Den del av landskapet Uppland som ingår i Stockholms län
Stockholm södra	Den del av landskapet Södermanland som ingår i Stockholms län samt Gotlands län
Uppsala	Uppsala, Västmanlands, Dalarnas och Gävleborgs län
Linköping	Södermanlands, Östergötlands, Jönköpings, Kronobergs, Kalmar och Örebro län
Malmö/Lund	Blekinge, Skåne och Hallands län
Göteborg avdelning 1 och 2	Västra Götalands och Värmlands län
Umeå	Västernorrlands, Jämtlands, Västerbottens och Norrbottens län

Trots det som står ovan ska följande gälla:
Stockholm norra djurförsöksetiska nämnd ska få alla ansökningar som avser planerade djurförsök som innebär ett frångående av bestämmelserna i 14 kap 4 § Statens jordbruksverks föreskrifter och allmänna råd om försöksdjur (SJVFS 2012:26) eller är i enlighet med bestämmelserna i 14 kap 12 § samma föreskrifter.

Uppsala djurförsöksetiska nämnd ska få alla ansökningar som avser planerade djurförsök om klinisk prövning av veterinärmedicinska läkemedel.

Uppsala djurförsöksetiska nämnd ska även få alla ansökningar som avser planerade djurförsök där man begär undantag från kravet på destinationsuppfödning.

Umeå djurförsöksetiska nämnd ska få alla ansökningar som avser planerade djurförsök vid myndighet tillhörande försvarsdepartementets ansvarsområde

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Bilaga 1 – Populärvetenskaplig sammanfattning

Den populärvetenskapliga sammanfattningen ska publiceras på Jordbruksverket webbplats. Språket ska därför vara lättförståeligt även för de som inte arbetar med försöksdjur samtidigt.

Titel

Studie av immuncellers betydelse för immunsvaret vid infektioner orsakade av luftvägspatogen

Försökets varaktighet

Försöken kommer att pågår i maximalt 4 veckor efter bakterieinfektioner och upp till 3 månader efter virusinfektioner för att observera influensa-inducerade förändringar av immunförsvaret, men kan komma att avslutas tidigare.

Sökord (nyckelord, max 5)

Luftvägspatogen, infektion, lunginflammation, immunsvar, sepsis

Försökets syfte (enligt 3 kap 1 § SJVFS 2012:26) – markera med kryss

Grundforskning	x
Vilka effekter sjukdomar, ohälsa eller annat avvikande tillstånd har på människor, djur eller växter samt hur de ska undvikas, förebyggas, diagnosticeras eller behandlas	
Utvärdering, påvisande, reglering eller modifiering av fysiologiska tillstånd hos människor, djur eller växter	
Forskning som syftar till förbättring av djurens välfärd	
Utveckling, tillverkning eller testning av kvalitet, effekt och säkerhet av läkemedel, livsmedel, foder och andra ämnen eller produkter	
Artskydd	
Skydd av den naturliga miljön för att bevara människors eller djurs hälsa eller välfärd	
Rättsmedicinska undersökningar	
Användning i högskoleutbildning eller i utbildning som syftar till att förvärva, upprätthålla eller utveckla yrkesfärdigheter	
Upprätthållande av kolonier av genetiskt förändrade djur som inte används i andra försök	

Beskriv försökets mål (de vetenskapliga frågorna, eller de vetenskapliga, kliniska behoven som ska mötas).

Studiens syfte är att studera bakteriella virulensfaktorers funktion i infektions- och koloniseringsmodeller samt hur värdens immunsvar och inflammation påverkar sjukdomsförloppet.

Vilka nyttor förväntas komma ur försöket (vilka vetenskapliga framsteg kommer att nås, hur kan människor eller djur dra nytta av försöket)?

Genom en ökad förståelse för infektionsprocessen och växelspelet mellan bakterien och immunsvaret får vi bättre möjligheter att förhindra infektion genom nya vacciner och behandlingsmöjligheter. Bättre förebyggande åtgärder och behandlingsalternativ skulle drastiskt kunna minska dödligheten till följd av bakteriella luftvägsinfektioner. Att kunna förhindra infektion i större utsträckning skulle också kunna minska det ofta livslånga lidandet och funktionsnedsättning som är associerat med exempelvis hjärnhinneinflammation.

Vilka arter ska användas och antalet individer?

Mus musculus, 5000 st

Vilka är de förväntade negativa effekterna på djuren och vilken är den förväntade svårhetsgraden?

Den förväntade svårighetsgraden är måttlig. Vi kommer att administrera lösningar vilket enligt vår tidigare erfarenhet innebär ett snabbt övergående obehag. Den provtagning som utförs verkar inte bekomma djuren. Djur som infekteras kan, i de fall de utvecklar sjukdom, komma att uppleva en allmän sjukdomskänsla. De vanligaste symptomen är att de blir trötta och mindre aktiva, de kan även få en något förändrad kroppshållning och ruggig päls. Vid allvarligare sjukdom blir de allt mer inaktiva, får förvärrad kroppshållning och piloerektion, samt kan få dålig cirkulation i extremiteter. Den avbrytningspunkt som används och den tillsyn som ges syftar till att försöksdjuren inte ska bli svårt sjuka.

3R-aspekter

1. Ersätta (Replace)

Förklara varför djur måste användas och varför djurfria alternativ inte kan användas.

Vi använder försöksdjur som ett komplement till andra experimentella metoder, däribland olika former av cellkulturer. Djurförsök står sällan eller aldrig ensamma utan är en av flera metoder som vi använder, alla med sina olika styrkor och svagheter, som tillsammans hjälper oss att besvara en frågeställning. Även om man måste vara försiktig i att överföra resultat från djurförsök till människa har djurmodeller ett oersättligt värde för oss. Det fungerande biologiska system som försöksdjuret utgör med olika celltyper, organ och vävnad med specialiserad funktion, barriärer som separerar olika delar djuret, olika typer av immunceller

etc. medför en komplexitet som inte går att återskapa på konstgjord väg.

I våra försök vill vi bland annat studera hur bakterier kan spridas från en plats till en annan, exempelvis kan möss vara koloniserade i de övre luftvägarna utan att uppvisa symptom. Bakterierna kan sedan sprida sig ner till lungorna och där utveckla lunginflammation för att sedan sprida sig vidare i djuret. Den forskning vi bedriver syftar till att förstå vilka faktorer hos bakterien som krävs för att kolonisera och sedan orsaka olika former av sjukdom. För att förstå detta måste vi även studera hur bakteriens virulensfaktorer interagerar med ett komplext biologiskt system, något som vi bara kan göra med försöksdjur. De virulensfaktorer vi studerar kan vara sådana som möjliggör för bakterien att fästa vid de olika celltyperna som finns i exempelvis luftvägarna. Det kan också röra sig om faktorer som gör att bakterien kan undvika att upptäckas eller bekämpas av värdorganismens immunceller och faktorer som gör att värdorganismen svarar med en mycket stark inflammation, något som kan vara mycket skadligt för värdorganismen själv.

2. Begränsa (Reduce)

Förklara hur ni har försäkrat er om att använda så få djur som möjligt.

Efter varje försök utvärderas resultatet av försöket. Dels utvärderas den data som genererades, men det är också viktigt att utvärdera hur försöket utfördes rent tekniskt. Baserat på resultatet av det enskilda försöket och sammantaget med tidigare försök, fattas beslut om ytterligare försök kommer att utföras. Det kan också vara nödvändigt att beräkna om den är möjligt att kunna uppnå statistisk signifikans för en eventuell skillnad som uppmättes i initiala försök med avseende på antalet djur som i så fall skulle behövas (så kallad power analysis).

3. Förfina (Refine)

Förklara valet av art och varför den valda djurmodellen är den mest förfinade. Beskriv de insatser som görs för att minimera djurens eventuella lidande.

Försöksmodeller baserade på möss är mycket vanligt förekommande inom infektionsforskning i allmänhet och så även inom forskning på luftvägsinfektioner. För att kunna jämföra våra resultat och data med andra forskargrupper runt om i världen måste vi kunna använda liknande forskningsmetoder. Under decennier har forskare världen över upprättat ett bibliotek av genetiskt modifierade musstammar. Vissa av dessa musstammar har genetiska förändringar som gör att de saknar faktorer som är centrala i försvaret mot bakteriella infektioner. Denna typ av genetiskt modifierade möss är oumbärliga för att kunna förstå hur bakterier interagerar med värdorganismen. För att kunna studera hur olika bakteriestammar och isolat som hos patienter gett upphov till olika typer av sjukdom kan vi karaktärisera bakteriernas förmåga att orsaka olika former av sjukdom.

Vi har strävat mot att använda nya tekniska lösningar när det gäller hur vi administrerar lösningar som vi tror på sikt kommer att ge mer robusta resultat och därför leda till att färre försöksdjur behöver användas totalt sett.

Vi har även använt oss av metoder med märkta bakterier som gör att vi kan följa infektionsprocessen i en och samma individ under en längre tid vilket gör att antalet djur som behövs för försöken kan minskas.

Vid potentiellt stressande moment kommer försöksdjuren att sövas.

Under försöket använder vi lägsta möjliga avbrytningspunkt, baserat på klinisk bedömning, som är möjlig för att försöket ska vara informativt. Vi har även en mycket hög tillsynsfrekvens för att säkerställa att försöksdjuren inte drabbas av för allvarlig sjukdom. Mössen har fri tillgång till mat och vatten samt en berikad miljö under hela försöket.

Följande ska fyllas i av nämnden

Försökets svårhetsgrad

☐ Ringa svårhet ☒ Måttlig svårhet ☐ Avsevärd svårhet ☐ Terminal ☐ Terminal/organ

Utvärdering i efterhand

Försöket ska utvärderas i efterhand ☐ Ja ☒ Nej

Om ja, ange i vilka delar och ur vilken aspekt.

Diarienummer

Tillägg eller ändringar som påverkar den populärvetenskapliga sammanfattningen

Observera att nämnden här endast ska notera ändringar eller tillägg som behövs för att den populärvetenskapliga sammanfattningen ska bli korrekt.

N 235/15
Bil. 2

Hej Dana. N 235/15

Nedan har vi svarat på de frågor som ställts från kommittén. Hoppas ni tycker att detta är tillfylles. Återkom annars om ni behöver mer information.

1. *Vävnadsprov som används för genotypning skall vara så liten som möjligt (max 1-2mm)*

I möjligaste mån kommer vi att använda de vävnadsprov som tas i samband med öronmärkning. Mängden vävnad som tas kommer att hållas till ett minimum och väntas inte överstiga 2 millimeter.

2. *Du skall inte hänvisa till referenser i ansökan. Alla viktiga uppgifter från referenserna skall beskrivas i ansökan*

Vi är medvetna om att all relevant information ska framgå i ansökan. Vi har därför försökt att förklara och redogöra för ingrepp och behandlingar på ett fullgott sätt. Referenserna som anges är i första hand till för att undvika tvetydighet i vad vi åberopar i vår ansökan. Det djuretiska tillståndet är giltigt i flera år och det är troligt att det under denna tid kommer att användas av fler gruppmedlemmar.

3. *6:2 Beskriv processer*

Mössen kommer att infekteras intranasalt (maximalt 50 µl), intratrakealt (maximalt 100 µl), intraperitonealt (maximalt 200 µl) eller intravenöst (maximalt 5 ml/kg) med virus. Beroende på hur substansen administreras kan mössen kommer att sövas under infektionen som beskrivet under punkt 9, Anestesi- och avlivningsmetoder. I regel kommer mössen att infekteras med bakterier en vecka efter virusinfektion.
För att kunna utvärdera hur lösningen eller bakterierna fördelas kan infektionslösningen innehålla eller enbart bestå av en färglösning, fluorescerande substans eller märkta partiklar. Administreringen av dessa substanser kommer i dessa fall således inte utgöra ett ytterligare ingrepp utan ingår som beståndsdel i infektionslösningen.

4. *6:3 hur många injektioner i.v; i.p får djuren?*

Vi vill naturligtvis inte att djuren ska genomgå fler behandlingar än nödvändigt och försöker därför minimera antalet. I möjligaste mån kommer behandlingslösningar att kombineras för att minska antalet ingrepp. Antalet ingrepp varierar mellan de olika behandlingarna, men vi kommer att göra maximalt 2 förberedande ingrepp. Detta innebär som exempel att mössen infekteras med virus för att sedan ges i regel en vecka för återhämtning. Om vi använder CD11c.DTR möss kommer de att behandlas med difteri-toxin, för att utarma CD11c-celler (en injektion i.p) och med exempelvis klodronat (en injektion i.v.) för att utarma makrofager. Dessa två behandlingar har utförts flera gånger tidigare och har ingen synbar negativ inverkan på djuren. Däremot påverkas infektionsförloppet, som går snabbare, varför mössen kommer att få frekvent tillsyn efter infektion med bakterier som kan ges genom en injektion i.v. eller i.p.

5. *Angående hur djuren påverkas av fluorescerande lösning alternativ inmärkta partiklar.*

Vid användandet av fluorescerande lösning kommer vi exempelvis att använda FITC vilket är en väl beskriven metod för inmärkning som vi använt tidigare utan problem. För andra typer av inmärkning av bakterier, värdceller eller administrering av partiklar kommer vi att använda produkter såsom pHrodo, rediject etc. som är kommersiella produkter framtagna för att märka bakterier eller vädceller in vivo. På samma sätt är de partiklar, Qdot eller liknande, som vi avser att använda kommersiellt tillgängliga och framtagna för detta ändamål. Dessa metoder ska därför inte påverka djuren menligt och djuren kommer att få frekvent tillsyn.

6. *6:4 Påverkan på djuren? och 6:5 Påverkan på djuren?*

I de fall vi tidigare utarmat värdceller, exempelvis alveolära makrofager, makrofager i mjälte, CD11c-celler och neutrofiler, har vi inte sett att djuren påverkats negativt av dessa behandlingar i sig. Däremot har infektionsförloppet efter infektion med bakterier i vissa fall visat ett snabbare förlopp och vi är därför mycket noga med tillsynen under denna typ av försök. Symptombilden är dock oförändrad jämfört med försök med obehandlade möss.

7. *6:10 hur påverkas djuren av antibiotika behandling. Sövs djuren när de behandlas intranasalt?*

Vid all intranasal behandling (oavsett syftet med ingreppet) kommer djuren att sövas. Antibiotikabehandlingen väntas inte påverka djuren negativt, syftet är att se hur effektivt olika typer av bakteriella infektioner kan behandlas. I gynnsamma fall kommer vi att uppnå en terapeutisk effekt, dvs. reducerad infektion, i andra fall kommer infektionen att vara opåverkad och skiljer sig då inte från våra vanliga infektionsförsök. Behandlingsschemat kommer att utarbetas tillsammans med en veterinär.

8. *6:11 b. Injektioner i trampdyna, lymfknuta, skall inte ges. Vi förstår att syftet inte är att immunisera, men önskar att du motiverar varför du måste injicera i trampdyna, lymfknutor. Hur omhändertas djuren efter att ha vaknat upp efter injicering i trampdyna. Har vi förstått rätt att djuren avlivas max 1 timme efter injicering i trampdyna, lymfknuta*

Administrering av färglösningen genom injektion i trampdyna med maximalt 25 µl sker bara i samband med avlivning och ger oss möjlighet att synliggöra lymfatiska kärl och lymfknutor. Avsikten är således att djuren inte kommer att vakna upp mellan behandling och avlivning, vi förväntar oss därför inte att behandlingen kommer att påverka djuret negativt.

9. *Förtydliga hur olika moment kommer att kombineras. Hur många försöksmoment kan ett djur gå igenom? Kommer vissa försöksmoment att upprepas? Hur många gånger kommer ett djur att sovas?*

I regel kommer vi att göra sammanlagt 2 förberedande ingrepp/experimentella behandlingar samt märkning av djuren. I undantagsfall kan vi behöva göra 3 behandlingar det gäller exempelvis när vi t.ex. använder konditionella mutantmöss som kräver detta för att få den önskade fenotypen. Det kan t.ex. även bli aktuellt i samband med genotypning av möss vi sedan vill använda i försök. Vi vill naturligtvis inte att djuren ska lida mer än nödvändigt och försöker minimera antalet behandlingar. Vi vill kunna kombinera ihop en viss "förberedande ingrepp/experimentella behandlingar" med en annan behandling. Detta beror på hur bakterierna påverkas av en infektion. Ansökningsmässigt kompliceras detta ytterligare av att vi har många olika musstammar att arbeta med.

10. *Hur länge kan djuren gå med symptom som motsvarar avbrytningspunkt 0.6p*

Om djuren uppnår en score som överstiger 0.6 kommer de att avlivas omedelbart. I realiteten gäller detsamma för ett djur med en score av 0.6 då detta djur inte kan lämnas utan nästintill konstant tillsyn. Denna avbrytningspunkt föreslogs av den djuretiska nämnden i samband med en tidigare ansökan med motiveringen att djuren förväntades bli systemiskt påverkade av infektionen.

11. *Hur många injektioner kan ett djur få?*

Antalet injektioner är beroende på vilken typ av förberedande ingrepp som används. Vi vill också understryka att det stora flertalet av mössen inte kommer att genomgå någon som helst förberedande behandling innan infektion. Ett mindre antal möss kommer att få en förberedande behandling och för ett fåtal kommer två förberedande behandlingar att kombineras.

N235/15

Vid utarmning av makrofager administreras en substans i lunga eller intravenöst vid ett tillfälle. Även vid utarmning av CD11c-celler ges substansen vid ett tillfälle, intraperitonealt eller till lunga. Den förberedande behandling som kräver flest injektioner är antikroppsmedierad utarmning av neutrofiler där vi vid tidigare försök funnit att tre injektioner krävs för att förhindra att neutrofiler frisätts från benmärgen efter infektion med bakterier. Tidigare har dessa injektioner getts 24 och 16 timmar innan samt i samband med infektion.

N235/15
B:1 3

Beredningsgruppens förslag till nämndens ställningstagande N235/15 Birgitta Henriques Normark

Beredningsgruppen föreslår att ansökan godkänns efter kompletterande uppgifter.

Syftet med studien är att studera förändringar i immunförsvaret som orsakas pneumokocker. Dessa infektioner kan hos svaga personer (som t.ex. lider av annan sjukdom) eller som är mera mottagliga för infektionen leda till döden. Det behövs mera kunskap om varför detta uppkommer så att nya behandlingar kan utvecklas.

Jordbruks verket

De regionala djurförsöksetiska nämnderna

BESLUT

2019-09-12

Dnr 11367-2019
Delg.

Stockholms djurförsöksetiska nämnd

Birgitta M Henriques Normark

Din ansökan om etiskt godkännande av djurförsök, se bil. A

Nämndens beslut

Stockholms djurförsöksetiska nämnd bifaller din ansökan och godkänner ditt försök ur etisk synpunkt och du kan därmed utföra ditt försök enligt nedanstående villkor.

Nämnden bedömer försökets svårhetsgrad som måttlig.

Nämnden beslutar att försöket inte ska utvärderas i efterhand.

Nämnden bestämmer avgiften för prövningen till 15 000 kr.

Detta godkännande gäller till och med den 12 september 2024.

Villkor för beslutet

Beslutet gäller under förutsättning att:

1. Försöksledare för försöket är Birgitta M Henriques Normark.
2. Föreståndare är Elisabet Andersson.
3. Försöket genomförs på i bil. A angivna försöksdjursanläggningar med där angivna dnr för anläggningarna.
4. Försöket genomförs i enlighet med bil. A och med i bil. A:1 redovisade kompletteringar.

Beskrivning av ärendet

Du som försöksledare har ansökt om etiskt godkännande av djurförsök enligt 21 § djurskyddslagen (1988:534) för försöket "Studie av patogen och värdsvar vid infektioner orsakade av Streptococcus pneumonia, Staphylococcus aureus och Klebsiella".

Du har lämnat in en ansökan där du beskriver hur försöket ska genomföras och vilket lidande som kan förväntas eller som djuren maximalt får utsättas för. Du har också

angett vilken nytta du förväntar dig att detta försök kommer att ha för människan, djur eller miljön.

Kompletterande information till ansökningen redovisas sålunda i bil. A:1.

Nämndens beredningsgrupp har föreslagit att godkänna ansökningen på de skäl som framgår av bil. A:2.

Motivering

Gällande regler

Av 21 § djurskyddslagen framgår bland annat följande.

- För att få använda sig av djur i ett djurförsök krävs ett godkännande från etisk synpunkt av en regional djurförsöksetisk nämnd innan användningen får påbörjas.
- Vid prövningen av ett sådant ärende ska försökets betydelse vägas mot lidandet för djuret. Försöket ska utifrån graden av lidande hos djuret klassificeras i någon av kategorierna terminal, ringa svårhet, måttlig svårhet eller avsevärd svårhet.
- Vid prövningen av ärendet ska det även beslutas om försöket ska utvärderas i efterhand.
- En djurförsöksetisk nämnd får återkalla ett godkännande om djurförsöket inte utförs enligt godkännandet.

Av 2 a § Statens jordbruksverks föreskrifter (SJVFS 2008:19) om avgifter i vissa ärenden enligt 67 § djurskyddsförordningen (1988:539) framgår att den som ansöker om etiskt godkännande av djurförsök ska betala en avgift enligt följande:

1. Ansökan om ändring av ett befintligt etiskt godkännande som riskerar att inverka negativt på djurs välfärd.....6 000 kr
2. Ansökan som rör pilotstudie och ärende som endast innebär användning av djur som inte hålls i en försöksdjursanläggning, dvs. privatägda djur, djur i anläggning som är godkänd för offentlig förevisning enligt 37 § djurskydds-förordningen (1988:539) eller fritt levande vilda djur.....8 000 kr
3. Ansökan som inte omfattas av punkt 1, 2 eller 3.....15 000 kr

Nämndens bedömning

Se bil. A:2.

Svårhetsgraden för försöket fastställs till måttlig.

Nämnden bedömer att ansökningen faller under 2 a § 3 Statens jordbruksverks föreskrifter (SJVFS 2008:19) om avgifter i vissa ärenden och att du därför ska betala 15 000 kr.

Hur du överklagar

Du kan överklaga detta beslut till Centrala djurförsöksetiska nämnden. Överklagandet ska vara skriftligt. När du överklagar ska du skriva

1. vilket beslut du överklagar,
2. hur du vill att beslutet ska ändras, och
3. varför du tycker att det ska ändras.

Du ska skriva till Centrala djurförsöksetiska nämnden men skicka eller lämna överklagandet till Stockholms djurförsöksetiska nämnd, Stockholms tingsrätt, box 8307, 104 20 Stockholm

Ditt överklagande måste ha kommit in till Stockholms djurförsöksetiska nämnd inom tre veckor från den dag som du tagit del av beslutet. För offentlig part räknas dock tiden för överklagande från beslutsdagen.

Övriga upplysningar

Om du har betalat en annan avgift än den som nämnden har beslutat kommer du att få en tilläggsfaktura eller kreditfaktura från Jordbruksverket. Om du överklagar avgiftsbeslutet kommer en kopia på överklagandet att skickas till Jordbruksverket så att verket tillfälligt kan stoppa fakturan i vårt ekonomisystem till dess att överklagandet har avgjorts. Beroende på utgången ska fakturan därefter antingen betalas omgående eller makuleras.

Om du vill göra ändringar i detta beslut behöver du skicka en ansökan om ändring av ett befintligt etiskt godkännande till den regionala djurförsöksetiska nämnden. Nämnden bedömer i så fall om ändringen riskerar inverka negativt på djurs välfärd eller inte och om en ny etisk prövning behöver göras. Det krävs en avgift för ändringar i ett befintligt etiskt godkännande.

Om föreståndare eller försöksledare ska bytas ut under detta godkännandes giltighetstid är detta också en sådan ändring av beslutet.

Jonas Härkönen

Vice ordförande i Stockholms djurförsöksetiska nämnd

Nämnden skickar kopia för kännedom till

Länsstyrelsen i Stockholms län
Tillståndshavare Christer Säfholm
Föreståndare Elisabet Andersson
Försöksdjursveterinär Christer Säfholm

bil. A

11367-2019
2019-09-02

Jordbruks verket

FÖRSÄTTSBLAD
2019-09-02 09:59

Sökande

Ingrid Birgitta M Henriques Normark
Karolinska Institutet
Institutionen för Mikrobiologi, Tumör och Cellbiologi
Nobelsväg 16, 17177 Stockholm
birgitta.henriques@ki.se
Mobil: +46706780317

Stockholms djurförsöksetiska nämnd
Stockholms tingsrätt Box 8307
104 20 STOCKHOLM
stockholm@rdn.jordbruksverket.se

Ansökan om etiskt godkännande av djurförsök

Elektroniska underskrifter för denna handling:

I egenskap av föreståndare har jag utan invändningar tagit del av det planerade djurförsöket.
I egenskap av försöksledare medger jag att ansökan skickas till den regionala djurförsöksetiska nämnden.

För att kunna verifiera underskrifterna kan du behöva öppna detta dokument i exempelvis Adobe Reader.

Underskrivet av: Ingrid Birgitta M Henriques Normark Karolinska Institutet Försöksledare

Filer som ingår

2019-09-02_0959-02 Ansökan om etiskt godkännande av djurförsök.pdf
Kontrollsumma (SHA-256): 2440D2BA93848A2FF60E72A9E3C0E9C4633D196531E0F1F1389EF8AE7C8C8332

2019-09-02_0959-03 Populärvetenskaplig sammanfattning.pdf
Kontrollsumma (SHA-256): 6DB32A8BEAE2AC05CC4D8D2A982C43DC40EDE3B7FA06D87F4DF25C4835EDAA34

2019-07-09_1220-04 Flödschema etiskt tillstånd.pdf
Kontrollsumma (SHA-256): 39591C9B41ACBBAF25EBC9B54E7605B0B24B95ADA0549E85F308DE0453A80A73

2019-09-02_0959-05 Förändringar i Ansökan om etiskt godkännande av djurförsök.pdf
Kontrollsumma (SHA-256): C24C129BC0AAEEE889593D7441B1F281095E417BFF4F0AAA5A394535C15C459B

Jordbruks verket

Ansökan om etiskt godkännande av djurförsök

Tidigare inskickade versioner av ansökan

Ansökans id 1808 2019-07-09 12:21

Innehållsförteckning

1 Grunduppgifter

1.1 Försöksledare (Sökande)

Namn:	Ingrid Birgitta M Henriques Normark
Organisation:	Karolinska Institutet
Institution/avdelning:	Institutionen för Mikrobiologi, Tumör och Cellbiologi
c/o:	
Adress:	Nobelsväg 16, 17177 Stockholm
E-postadress:	birgitta.henriques@ki.se
Telefonnummer:	
Mobilnummer:	+46706780317
Faxnummer:	

1.2 Ansvarig veterinär

Namn:	Christer Säfholm
c/o:	
Adress:	Komparativ medicin (KM), Karolinska Institutet, 17177 Stockholm
E-postadress:	christer.safholm@ki.se
Telefonnummer:	
Mobilnummer:	

1.3 Tillstånd att använda försöksdjur

Nedan visas uppgifter från tillståndet som det såg ut när denna sammanställning gjordes – eventuella ändringar som har gjorts i tillståndet efter det visas inte här.

Diarienummer:	5.2.18-3236/17
Organisation:	KAROLINSKA INSTITUTET, DEPARTMENT
Institution:	Avdelningen för komparativ medicin
Slutdatum:	2022-04-18
Tillståndshavare:	CHRISTER SÄFHOLM
Föreståndare:	ELISABET ANDERSSON
Veterinär/expert:	Christer Säfholm
Ändamål:	Biomedicinsk forskning i syfte att förstå grundläggande livsfunktioner, sjukdomsmekanismer, utveckla nya/förbättra behandlingar av människans sjukdomar, samt utbildning och träning i LAS

Djurarter:	Gerbil (Gerbillinae), Hamstrar (Cricetinae), Kanin (Oryctolagus cuniculus), Marsvin (Cavia porcellus), Mus (Mus musculus), Råtta (Rattus norvegicus), Signalkräftor (Pacifastacus leniusculus), Zebrafisk (Danio rerio), Spansk revbensalamander (Pleurodeles waltl), Afrikansk klogroda (Xenopus laevis), Mexikansk axolotl (Ambystoma mexicanum), Vattensalamander (Notophthalmus viridescens), Västafrikansk klogroda (Xenopus tropicalis), Nejonöga (Lampetra fluviatilis), Mindre skogsmus (Apodemus sylvaticus), Gräsand (Anas platyrhynchos), Iller (Mustela putorius), Krabbmakak (Macaca fascicularis), Nordamerikansk näbbmus (Cryptotis parva), Rhesusapa (Macaca mulatta), Större skogsmus (Apodemus flavicollis), Sorkar (Arvicolinae), Tamsvin (Sus scrofa domestica)	
Specifika villkor:		
Hållandesätt:	Försöksdjursanläggning	
Försöksdjursanläggningar:	32-2748/91	Institutionen för farmakologi
	34-5480/94	Gärtuna, byggnad 681R
	35-6352/01	Karolinska Institutet, Fysiologiska Institutionen Hus 95:21
	34-3590/94	95:3, södra delen, Karolinska institutionen
	35-998/99	Astrid Fagreus laboratorium (hus 95:56)
	35-765/01	Astrid Fagreus laboratorium (hus 95:56)
	31-2487/10	Astrid Fagreus Lab
	31-5159/10	Astrid Fagreus lab
	31-3029/11	Berzelius väg 35, rum B-121
	31-495/12	rum F224 och F230 och 231 i byggnad 95:33
	5.2.18-2239/13	Wallenberg, Hus 95:17 med adress Von Eulers väg 5 och Berzelius väg 17, Stockholm.
	35-1079/02	Byggnad 95:33, rum F 224 och F 230.
	5.2.18-11066/13	Retziuslaboratoriet, byggnad 95:55, Retzius väg 8, Stockholm
	5.2.18-8359/15	rum A1:01011/12 och A3:01038/39 i byggnad A1 och A3 samt rum R300B005 i MR center
	5.2.18-11233/15	KM Wallenberg, hus 95:17 och 95:48
	5.2.18-11254/15	rum 224 samt rum 231 i byggnad 95:33
	5.2.18-12375/15	Berzelius väg 35, rum B-117a

1.4 Etisk nämnd

Stockholms djurförsöksetiska nämnd
Stockholms tingsrätt Box 8307
104 20 STOCKHOLM
Telefon: 08 561 650 00
Fax: 08-653 34 44
stockholm@rdn.jordbruksverket.se

1.5 Försökets titel

Studie av patogen och värdsvar vid infektioner orsakade av Streptococcus pneumoniae, Staphylococcus aureus och Klebsiella.

1.6 Övriga upplysningar

Tidigare försök

Försöket bygger helt eller delvis på ett tidigare försök med diarienummer N93/13

Motivering
Vi kommer fortsatt att studera mekanismer för vad som gör att dessa mikrober orsakar sjukdom och ämnar att leta efter nya kandidater för behandling och vaccin. Dessa frågor är ännu inte lösta. Vi kommer att använda beprövade metoder.
Vi vill därför, om denna ansökan godkänns, kunna föra över alla djur som står på N93/13 till detta tillstånd. Överföring av eventuella djur som är i försök vid aktuell tidpunkt sker efter veterinärtillsyn.

Godkännandets giltighetstid

Försöket beräknas pågå till 2024-06-03

Samråd

☑ Samråd har skett enligt 3 kap. 4 § L 150.

Personalens kompetens

☑ De personer som är involverade i försöket har tillräcklig kompetens enligt 6 kap. L 150.

Sekretess

☑ Önskar sekretess för vissa uppgifter i ansökan.

*Motivering:*Då vi avser använda tillståndet i projekt som kan leda till kommersialiserbara resultat inom området behandlingsstrategier/immunaktivering vid pneumokockinfektion, önskar vi av patenträttsliga skäl att ansökan omfattas av sekretess enligt nedan. Detta för att både de funktionella mutanter och signaleringsvägar som står angivna i val av djurart och försökets tids- och genomförandeplan ger tydliga uppgifter om vad vi avser att studera. Sålunda begär vi sekretess för Försöksgrupp 2 och Fösöksgrupp 3 under avsnitt 4.

Betalning

Betalningsmetod:	Faktura
Ordernummer:	79925
Belopp:	15 000 kr

2 Syfte m.m

Syftet med försöket enligt Jordbruksverkets föreskrifter och allmänna råd om försöksdjur

- ☑ 1 Grundforskning
- ☑ 2 Forskning om vilka effekter sjukdomar, ohälsa eller annat avvikande tillstånd har på människor, djur eller växter samt hur de ska undvikas, förebyggas, diagnosticeras eller behandlas
- ☐ 3 Forskning som innebär utvärdering, påvisande, reglering eller modifiering av fysiologiska tillstånd hos människor, djur eller växter
- ☐ 4 Forskning som syftar till förbättring av djurens välfärd
- ☐ 5 Utveckling, tillverkning eller testning av kvalitet, effekt och säkerhet av läkemedel, livsmedel, foder och andra ämnen eller produkter. Detta gäller endast i de syften som avses i 2-4
- ☐ 6 Forskning som syftar till artskydd
- ☐ 7 Skydd av den naturliga miljön för att bevara människors hälsa eller välfärd
- ☐ 8 Skydd av den naturliga miljön för att bevara djurs hälsa eller välfärd
- ☐ 9 Rättsmedicinska undersökningar
- ☐ 10 Användning i högskoleutbildning eller i utbildning som syftar till att förvärva, upprätthålla eller utveckla yrkesfärdigheter under förutsättning att användningen framgår av utbildningens kursplaner, och är nödvändig med hänsyn till syftet med utbildningen.
- ☐ 11 Framställning och upprätthållande av en genetiskt modifierad djurstam
- ☐ 12 Annat - gäller endast för försök som sannolikt inte orsakar lidande i lika stor eller större utsträckning än ett nålstick som utförts enligt god veterinärmedicinsk praxis

Beskrivning av syftet

Beskrivning av vad försöket syftar till att uppnå, ta reda på, fastställa eller framställa genom att utföra detta försök. Syftet bör vara specifikt för detta försök samt entydigt, realistiskt och genomförbart. Syftet ska inte förväxlas med nyttan med försöket, som angetts nedan.

Streptococcus pneumoniae är en Gram-positiv bakterie och infektion med pneumokocker är en av de vanligaste orsakerna till sjuklighet och dödlighet i världen. Pneumokocker orsakar allt från milda luftvägsinfektioner, öroninflammation och bihåleinflammation till svårare infektioner såsom lunginflammation, blodförgiftning och hjärnhinneinflammation. Vi har fortfarande för lite kunskap om de faktorer som påverkar pneumokockernas förmåga att orsaka sjukdom. Eftersom de vacciner som finns har begränsad effekt i de stora riskgrupperna, små barn och äldre, och antibiotikaresistens har blivit ett allt större problem i världen, finns det ett stort behov av att hitta nya sätt att förebygga och behandla pneumokockinfektioner, t.ex. genom att försöka utveckla nya vacciner.

I likhet med S. pneumoniae är Staphylococcus aureus en Gram-positive bakterie som asymptomatiskt koloniserar människor, men som även kan ge upphov till lunginfektioner, och allvarlig invasiv sjukdom. Pneumokocker och Stafylokocker inducerar båda en kraftig inflammation, dvs. en aktivering av det medfödda immunförsvaret. Det är således väsentligt att studera immunförsvarets roll vid infektionerna. Det kan bland annat göras genom att använda möss som är defekta i receptorer och andra faktorer som är väsentliga för det medfödda immunförsvaret och jämföra dem med vildtypmöss.

Klebsiella pneumoniae är en Gram negativ bakterie som är en vanlig orsak till urinvägsinfektioner, men orsakar också lunginflammation och andra allvarliga infektioner särskilt hos äldre och multisjuka patienter. Den ökande andelen antibiotikaresistenta bakterier gör att behandlingsalternativen blir allt färre för dessa vanliga infektioner. WHO har satt upp Klebsiella på sin högsta prioritetslista för bakterier där vi omgående behöver nya antibiotika. Därför är det av största vikt att vi kan studera dessa infektioner in vivo och försöka hitta nya mekanismer för hur dessa bakterier verkar, vilket kan leda till nya sätt att behandla dessa infektioner.

Sammanfattningsvis vill vi med denna ansökan fortsätta detaljstudier av infektioner orsakade av tre luftvägspatogener för att:

1)Testa nya biologiska behandlingsmetoder/substanser.
2)Försöka utveckla nya vacciner.
3)Studera hur bakterierna orsakar sjukdom och immunförsvarets roll vid infektionerna.
4)Förstå olika cellpopulationers roll genom analysering, selektiv eliminering eller cellöverföring.
5)Göra imaging studier av infektionerna

Svårhetsgrad

Måttlig

Beskrivning av försökets slutpunkt

Slutpunkten är det tillfälle då försöksledaren planerar att avsluta försöket och inte göra några fler observationer. Slutpunkten är den tidpunkt där försöksledaren utgår ifrån att ha uppnått syftet med försöket. Den kan anges som en viss tidsperiod (t.ex. djuren går i försöket över en viss tid) eller när något specifikt händer (t.ex. djuren utvecklar vissa symtom eller visar ett visst beteende). Slutpunkten ska inte förväxlas med avbrytningspunkten som är den i förväg satta gränsen för ett djurs lidande då djuret av djurskyddsskäl ska tas ur ett djurförsök oavsett om försökets slutpunkt har uppnåtts.

För kolonidjur ej i försök: Kolonidjur som inte används för försöket kan användas för organuttag och avlivas upp till 24 månaders ålder.

För avelsdjur: honor som går i avel avlivas efter 6 kullar; hanar och honor kan efter avslutad avel användas för organuttag och avlivas upp till 24 månaders ålder.

För djur i försök: Mössen kommer att vara minst 4 veckor vid försökets start. Försöken kommer att pågå i maximalt 4 veckor efter infektioner, men kan komma att avslutas tidigare om till exempel djur visar sjukdomssymptom. För mer detaljer vänligen se individuella försöksgrupper.

Dokumentationskrav

Om det finns internationella eller nationella krav på att djurförsök måste göras eller krav om att vissa bestämda riktlinjer måste följas, ska uppgifter som styrker behovet av försöket anges.

Inga dokumentationskrav.

Beskrivning av nyttan för människa, djur eller miljön

Beskrivning av nyttan för människa, djur eller miljö. Det bör även angetts på vilket sätt resultaten väntas få betydelse för den medicinska eller biologiska utvecklingen. Om försöket innebär grundforskning bör det vara beskrivet vilka framsteg eller nya rön som kan förväntas på längre sikt. Om försöket innebär en fortsättning på eller en upprepning av ett tidigare försök bör det här vara beskrivet vilka tidigare resultat forskaren har kommit fram till samt motiverad varför det är nödvändigt att fortsätta eller upprepa försöket.

Genom en ökad förståelse för infektionsprocessen och växelspelet mellan bakterien och immunsvaret får vi bättre möjligheter att förhindra infektion genom nya vacciner och behandlingsmöjligheter. Bättre förebyggande åtgärder och behandlingsalternativ skulle drastiskt kunna minska dödligheten till följd av bakteriella luftvägsinfektioner. Att kunna förhindra infektion i större utsträckning skulle också kunna minska det ofta livslånga lidandet och funktionsnedsättning som är associerat med exempelvis hjärnhinneinflammation.

Egen etisk avvägning

Egen beskrivning hur sökande har resonerat när de har kommit fram till att nyttan med försöket överväger lidandet för djuren.

Studiens syfte är att studera bakteriella virulensfaktorers funktion i infektions- och koloniseringsmodeller samt hur värdens immunsvar och inflammation påverkar sjukdomsförloppet. Genom en ökad förståelse för infektionsprocessen och växelspelet mellan bakterien och immunsvaret får vi bättre möjligheter att förhindra infektioner genom nya vacciner och behandlingsmöjligheter. Detta är ytterst viktigt särskilt i ett läge då vi ser en ökning av antalet bakterier som är resistenta mot antibiotika och det finns en risk för att vi inte kommer att kunna förebygga och behandla vanliga infektioner. Bättre förebyggande åtgärder och behandlingsalternativ skulle också drastiskt kunna minska dödligheten till följd av bakteriella luftvägsinfektioner. Således är det av största nytta för mänskligheten att vi kan utföra dessa experiment även om de kan betyda visst lidande för djuren. Alternativa likvärdiga metoder saknas.

Beskrivning av alternativa metoder

Beskrivning av varför djur måste användas för att uppnå syftet med försöket, vilka eventuella alternativa metoder som kommer att användas, vilka metoder som har övervägts men som inte är möjliga att använda samt vilka databaser som har använts vid sökande efter alternativa metoder.

Andra likvärdiga metoder saknas.
In vivo försöken kompletteras med cellbaserade (in vitro) försök för att minska antal djurförsök maximalt. Med dagens tekniker finns tyvärr ingen metod som t ex cellkulturer eller datorsimuleringar som kan ersätta ett intakt helkroppssystem. Vi finner att de nedan nämnda metoderna utgör olika sätt att optimalt ge svar på våra frågeställningar. Andra, alternativa, metoder till de nedan beskrivna har givetvis tagits i beaktande vid valet av vetenskapliga metoder.

Beräkning av antalet djur

Förklaring av principerna hur forskaren har kommit fram till det antalet djur som ska användas, t.ex. statistiska beräkningar samt hur det är säkerställt att så få djur som möjligt kommer att användas.

Vi kommer att använda ett antal olika bakteriestammar och musstammar och vissa musstammar kommer från egen avel (på Karolinska).
Ofta används fler hanar än honor för experimenten och felaktiga genotyper kan förekomma under avel. För att få tillförlitliga data och statistisk signifikans behöver experimenten ofta upprepas flera gånger. Mössen kommer även att avlivas vid olika tidpunkter. Med utgångspunkt från detta har vi beräknat antalet möss som krävs.

3 Djurarter m.m

3.1 Mus (Mus musculus)

Antal: 10000

Motivering av valet av djurart

Experimenten kan endast göras på däggdjur. Möss erbjuder stora möjligheter för genetiska studier och studier av patogenes. Därför är det mycket värdefullt att använda möss i våra infektionsstudier. Vi använder oss av etablerade metoder för att studera bakterieinfektioner i möss.

Djuren kommer från egen avel, ackrediterade säljare såsom JAX, Charles river, Janvier eller framställas av oss genom (och under) KCTT, core facilitet vid KI.

Hållandesätt

- Försöksdjursanläggning
  - 5.2.18-11233/15 KM Wallenberg, hus 95:17 och 95:48 - Djuren förvaras i IVC standardburar enligt lagstiftning. Samtliga djuravdelningar följer Karolinska Institutets miljöberikningsplan. Djuren grupphålls i möjligaste mån, om experiment eller veterinärmedicinska skäl inte föreligger eller om hane med unik genotyp föds i en kull och inte kan poolas. Eventuella problem handläggs i samråd med veterinärenheten vid Karolinska Institutet. Möss kan komma att föras över på andra godkända etiska tillstånd och kan i samband med detta byta djurhus.
  - 5.2.18-2239/13 Wallenberg, Hus 95:17 med adress Von Eulers väg 5 och Berzelius väg 17, Stockholm. - Djuren förvaras i IVC standardburar enligt lagstiftning. Samtliga djuravdelningar följer Karolinska Institutets miljöberikningsplan. Djuren grupphålls i möjligaste mån, om experiment eller veterinärmedicinska skäl inte föreligger eller om hane med unik genotyp föds i en kull och inte kan poolas. Eventuella problem handläggs i samråd med veterinärenheten vid Karolinska Institutet. Möss kan komma att föras över på andra godkända etiska tillstånd och kan i samband med detta byta djurhus.
  - 35-998/99 Astrid Fagreus laboratorium (hus 95:56) - Djuren förvaras i IVC standardburar enligt lagstiftning. Samtliga djuravdelningar följer Karolinska Institutets miljöberikningsplan. Djuren grupphålls i möjligaste mån, om experiment eller veterinärmedicinska skäl inte föreligger eller om hane med unik genotyp föds i en kull och inte kan poolas. Eventuella problem handläggs i samråd med veterinärenheten vid Karolinska Institutet. Möss kan komma att föras över på andra godkända etiska tillstånd och kan i samband med detta byta djurhus.
  - KM Biomedicum 5.2.18-19251/17 och KMA
    Djuren förvaras i IVC standardburar enligt lagstiftning. Samtliga djuravdelningar följer Karolinska Institutets miljöberikningsplan. Djuren grupphålls i möjligaste mån, om experiment eller veterinärmedicinska skäl inte föreligger eller om hane med unik genotyp föds i en kull och inte kan poolas. Eventuella problem handläggs i samråd med veterinärenheten vid Karolinska Institutet. Möss kan komma att föras över på andra godkända etiska tillstånd och kan i samband med detta byta djurhus.

4 Försökets genomförande

Sammanställning av försöksgrupper och åtgärder inom dessa:

Försöksgrupp: 1

Undergrupp: Avel och genotypning

Djurart: Mus (Mus musculus)

Åtgärd 1: Avel och genotypning

Åtgärd 2: Induktion av genmodifiering

Åtgärd 3: Blodprov

Försöksgrupp: 2

Undergrupp: Avlägsnande (depletion) av värdceller

Djurart: Mus (Mus musculus)

Åtgärd 1: Behandling med liposom/vesiklar eller med antikroppar, receptorligander eller liknande

Åtgärd 2: Difteri-toxinbehandling

Åtgärd 3: Blodprov

Åtgärd 4: Anestesi

Försöksgrupp: 3

[redacted]

4.1 Försöksgrupp: 1

-Avel och genotypning för att upprätthålla stammar.
-Induktion av genmodifiering genom att injicera difteri-toxin (som ej orsakar sjukdom hos möss), vilket krävs för att tillfälligt slå ut dendritiska celler och makrofager (två typer av vita blodkroppar).
-Vävnadsinsamling från djur

Djur från denna försöksgrupp kan ingå i alla övriga försöksgrupper.

4.1.1 Undergrupp: Avel och genotypning

Avel och genotypning för upprätthållande av olika musstammar.
Detta inkludera men begränsar oss inte till stammar som är nämnda under 3.1 Motivering av valet av djurart.

För stammar som bär gener som tillåter att man tillfälligt kan slå ut dendritiska celler och makrofager (två typer av vita blodkroppar) behövs en induktion via difteri-toxin för borttag av gener eller för att slå på gener.
För CD11c.DTR resulterar behandlingen i att majoriteten av de dendritiska cellerna i mjälten är borta under den första och andra dagen efter injektionen. Under dag 3-4 återkommer ungefär 50% av de dendritiska cellerna. För CD11c.DOG mössen är 80% av de dendritiska cellerna i mjälten borta under första och andra dagen efter behandlingen.
Vi vill kunna använda dessa möss för att studera de dendritiska cellernas och makrofagers betydelse vid infektion med mikroorganismer.

Genom blodprov och/eller vävnadsprov vill vi kunna följa eventuella effekter en substans kan ha på blodsystemet eller andra organen. Det vill också ge oss möjlighet att kontrollera hur effektiv behandling / utarmning / aktivering är.

Antalet djur är en uppskattning och kan komma att öka eller minska något men ingår i det totala antalet sökta djur.

Mus (Mus musculus), 2000 st, Försöksdjursanläggning

Beskrivning av ras, stam och egenskaper som kan medföra lidande för djuren samt motivering av valet av djur med dessa egenskaper

Experimenten kan endast göras på däggdjur. De stora möjligheter för genetiska studier som möss erbjuder gör detta djur mycket värdefullt for dessa infektionsstudier.

Nedan beskrivs de stammar som kommer att används i våra studier. Generellt gäller för alla våra musstammar att vi inte förväntar oss någon fenotyp som innebär lidande för djuren (med undantag för beskrivna stammar med fenotyp enligt nedan). Avbrytningspunkten är 0.4p på KI-mallen (med undantag för de stammar som vi beskriver fenotypen för nedan), och skulle fenotyp uppkomma som når 0.4p avslutas aveln omedelbart och vi inkommer då med ett tillägg till ansökan.

Musstammar som kommer att användas är:

• Vildtypsmöss eller möss med spontana mutationer: t.ex. C57BL/6, MF1, C3H/He, BALB/c, SVJ129, CD1, SWISS eller liknande vildtypstammar.

Utöver vildtypmöss kommer vi att använda genetiskt modifierade möss som saknar en eller flera gener eller på andra sätt förändrade gener viktiga för immunförsvaret. Då nya musstammar hela tiden utvecklas vill vi ha möjlighet att använda stammar som har förändringar i det medfödda och/eller det adaptiva immunsvaret alternativt förändringar av fysiologisk betydelse för det medfödda och/eller det adaptiva immunsvaret eller bakteriens förmåga att orsaka sjukdom. Förutsättningen för att mössen ska kunna användas är att de vid normaltillstånd inte har klinisk score som överskrider 0.4p på KI-mallen.

Nedan finns några exempel av olika metoder som kommer att användas för att generera genetiskt modifierade möss:

• Cre/Flip-möss: Diverse musstammar som bär på Cre/CreER-enzymet (används för att ta bort genetiska regioner som flankeras med loxP sites) eller Flip/FlipER enzymet (används för att ta bort

genetiska regioner som flankeras med Frt sites). Cre/Flip-genen ger ingen märkbar fenotyp och innebär inget lidande för musen. Uttrycket av (Cre, CreER eller Flip) sker med hjälp av regulatoriska element som kan specificera uttrycket till valda typer av celler, vilket gör att modifikationen blir mer begränsad jämfört med traditionella möss. Dessa stammar finns in house eller kan rekvireras från t.ex. andra forskningsgrupper, Jackson lab, EMMA konsortiet och uppvisar ingen fenotyp. De kommer att korsas med musstammar som bär på genetiska regioner flankerade med loxP respektive Frt sites för att ta bort genetiska regioner (d.v.s. man tar bort genetiskt material i specifika cellpopulationer och därmed minskas inverkan på djuret) och/eller sätta igång uttryck av markörgener (t.ex. fluorescerande proteiner, luciferas, b-galactosidase m.fl.), vilket gör att vi kan följa celldelningar och organbildning (så kallad "cell-tracing").

• Reportermöss : Diverse indikatorstammar som bär på olika markörgener (t.ex. fluorescenta proteiner, luciferase, b-galactosidase, RNA-barcode) där en stoppkassett flankerad av loxP eller Frt sites hindrar markörgenens uttryck men vars uttryck kan aktiveras genom att indikator-stammen korsas med Cre/CreER eller Flip/FlipER uttryckande musstammar. Markörgener ger inget lidande för musen. Celler som uttrycker markörgenen får en distinkt färg när vävnadssektioner eller dissekerade organ läggs under mikroskop, vilket gör att de infärgade cellerna kan studeras. Indikator-stammarna finns in house eller kan rekvireras från t.ex. andra forskningsgrupper, Jackson lab, EMMA konsortiet och uppvisar ingen fenotyp.

• Konditionella loxP/Frt-möss: Diverse konditionella musstammar där en viss genetisk region har flankerats med loxP eller Frt sekvenser. Dessa stammar finns in house eller kan rekvireras från t.ex. andra forskningsgrupper, Jackson lab, EMMA konsortiet och uppvisar ingen fenotyp. De konditionella stammarna kommer att korsas med Cre/CreER- eller Flip/FlipER-uttryckande musstammar (som oftast redan korsats med en indikatorstam, se ovan). När loxP/Frt-möss korsats inaktiveras den del av arvsmassan som flankerats med loxP/Frt sekvenser, beroende på Cre eller Flip stam kan detta kräva induktion via tamoxifen.

• En musstam med inducerbar expression av Difteritoxinreceptorn möjliggör en lokalt avgränsad och specifik eliminering av relevanta celltyper genom administrering av difteritoxin (se respektive beskrivning av experiment nedan). Dessa konditionella stammar förväntas inte ha en fenotyp före aktivering.

• Musstammar som bär på CAS9 som tillåter in vivo CRISPR genmodifiering, där en virusvektor som bär på Cre och sgRNA injiceras. Såsom klassiska CRISPR/Cas9 genererade transgena musstammar som hjälper att minska tid och antal möss som behövs för att korsa tillbaka genetiska förändringar till rätt bakgrund med traditionella metoder.

• Knockoutmöss: Olika musstammar där en viss gen har tagits bort, s.k. knockout möss, kommer att användas. Dessa stammar finns in house eller kan rekvireras från t.ex. andra forskningsgrupper, Jackson lab, EMMA consortiet. Dessa stammar kommer att hållas som heterozygota linjer i de allra flesta fallen och uppvisar ingen fenotyp som innebär lidande för musen. En del av dessa stammar kan gå som homozygoter utan att uppvisa någon fenotyp. De kan komma att korsas med varandra; med reporters; med olika Cre/CreER eller Flip/FlipER uttryckande stammar (som redan korsats med indikatorstam eller konditionell stam) för att kunna studera hur olika gener samverkar under utveckling. Vi förväntar oss ingen fenotyp som orsakar lidande. Dessa knockout linjer är t.ex.: Nkx2.2, Foxa2, Alk5, TgfB inklusive stammar med defekter som påverkar immunförsvaret. Detta inkluderar bland annat men begränsar oss inte till möss som är defekta med avseende på t.ex.:
• Presentation av antigen: såsom MHC klass I eller/och II receptor
• Igenkänning av patogen eller stress-signaler samt signaleringsvägar: såsom NOD och Toll-liknande receptorer, MyDD88, TIRAP, Mal, RIG-I, IPS.1, TRIF
• Aktivera och styra immunsvaret och bildning av immunceller: såsom Interferonreceptorer såsom IFNR och IRF3, interleukiner/cytokiner och dess receptorer såsom interleukinreceptorer, IL-1, IL-6, IL-8, IL-17, IL-18, ICE; TNF receptorer, och G-CSF.
• Receptorer som är av vikt för igenkänning av mikrobiella komponenter: såsom PGRP; Scavenger receptorer, lektiner, såsom SIGN-R1, SIGLEC, och integriner, såsom CR3, och MRC-1 eller andra receptorer som är av vikt för:

• Gener som påverkar inflammasomen: såsom tex Caspase-1, ASC, NLRC4, NAIP5.
• Inhibitorer av signaleringsvägar: såsom tex A20, IRAK-M, Tollip, IB-;
• Collektiner: såsom SP-A eller möss med förändringar i Glykanstruktur såsom CMAH.
• Gener som påverkar bakteriens förmåga att binda till värdceller: såsom Platelet Endothelial Adhesion Molecule (PECAM-1) och Poly Immunglobulin Receptor (pIgR).

• Transgena musstammar: olika musstammar där en viss gen överuttrycks under specifika regulatoriska element. Denna typ av stammar är viktiga för att förstå hur för mycket av ett protein påverkar normal utveckling och vid sjukdom. Dessa stammar kommer inte att uppvisa en fenotyp som innebär lidande för musen. Dessa stammar kan korsas med tex Cre/CreER och reporters.

• Olika reporter-musstammar eller knockout- och/eller konditionella knockoutstammar eller transgener kan komma att genereras via core-faciliteten KCTT, Karolinska Institutet och under deras etiska tillstånd eller av annan core facilitet internationellt (under deras etiska tillstånd). Reporterstammar kommer att bära på olika genregulatoriska segment och en markörgen (t.ex. fluorescerande proteiner, luciferase, b-galactosidase). Nya knockouter och/eller konditionella musstammar eller möss med överuttryckta gener som identifieras i vår forskning och där det inte ännu finns befintliga genmodifierade möss kan behöva genereras. Vi förväntar oss ingen fenotyp som innebär lidande hos dessa möss.

Stammar med fenotyp:

• Stat3 knockoutstammar eller inducerade konditionella Stat3 stammar:
o LysM-Cre x Rosa26-stopflox-DTA: dessa möss har ett reducerat antal neutrofiler
o LysM-Cre x Stat3flox: dessa möss uttrycker inte transkriptionsfaktorn Stat3 i myeloida celler
o LcK-Cre x Stat3flox: dessa möss uttrycker inte transkriptionsfaktorn Stat3 i T-celler
o Spc-Cre x Stat3flox: dessa möss uttrycker inte transkriptionsfaktorn Stat3 i epitelceller

Fenotypen av musstammarna som är angivna ovan avviker inte från den hos vildtypmöss, men det kan hända de uppvisar ett annorlunda infektionsförlopp än möss med intakt immunförsvar och detta vill vi studera.
Undantaget är:
• LysM-Cre x Stat3flox som i vissa fall utvecklar kolit vid 12-20 veckors ålder. Vid hälsobedömningar kommer särskild uppmärksamhet ges för tecken på kolit. Vanligtvis kommer dock mössen vara yngre än 12 veckor då försöken utförs, och bör därför vara opåverkade.
• LysM-Cre x Rosa26-stopflox-DTA kan en mild svullnad på svans/leder uppstå spontant, svullnaden kan vara i några dagar och sedan försvinna. Mössen kan dock röra sig fritt oförhindrat. Detta påverkar inte djurets välbefinnande negativt och vi önskar därför undantag för detta gällande avbrytningspunkt. 0.4p på KI mallen gäller som avbrytningspunkt men med undantag för svullnad på svans/led under max 5 dagar.

Anledningen till att vi vill använda möss som saknar en eller flera gener eller en särskild typ av cell är för att få kunskap om dessa geners betydelse för immunförsvaret. Vi kommer även att studera bakteriestammar med olika egenskaper samt delar av bakterier och ligander eller inhibitorer till olika receptorer för att lättare kunna förstå vilka faktorer som påverkar sjukdomsutvecklingen. På så sätt kan vi få kunskap som leder till utveckling av effektivare strategier för behandling och prevention av dessa bakteriella infektioner.

Kön: båda könen kommer att användas, men för vissa experimenten kan det vara fler hanar än honor.

Kön: Kön: båda könen kommer att användas, men för vissa experimenten kan det vara fler hanar än honor.

Genetiskt modifierade djur används: Ja

Diarienummer på tillstånd samt beskrivning och motivering av metoder.

Se ovan beskrivning av djurens egenskaper.
Vi kommer inte att generera helt nya GMO-stammar under detta tillstånd. Skulle ny stam tas fram sker detta under kommersiellt företag eller core facilitets etiska tillstånd tex KCTT vid KI.
Vi använder befintliga GMO stammar för avel samt korsningar mellan dessa. Dnr för GMO är KMW:5.5.18-14683/18. KMB: 5.5.18-12320/17. KMA:5.5.18-123221/17.
KMF (tidigare AFL): 5.5.18-13103/18.

4.1.1.1 Beskrivning av de åtgärder/ingrepp djuren kommer att utsättas för

Åtgärd 1: Avel och genotypning

Ingående beskrivning av åtgärden och hur djuret kommer att påverkas

Vad djuret kommer att utsättas för inklusive försöksperiodens längd för det enskilda djuret.
Beskrivning av hur djuret kommer att påverkas – eventuellt fysiskt och psykiskt lidande.

Hona och hane sätts samman för avel. 1-2 honor per hane. Om två honor i samma bur blir dräktiga ungefär samtidigt, separeras honorna från varandra ca 1 vecka före beräknad födsel.

Flera av våra musstammar avlas som heterozygota linjer och måste genotypas. För detta kommer öronbitar som blir över från ID-märkningen att användas i möjligaste mån och detta görs vid avvänjningen, dvs ca 3 veckors ålder, för att undvika att störa djuren mer än nödvändigt. Skulle inte genotypningen fungera tas en ny öronbiopsi i första hand och i sista hand en yttersta milimetern av svanstippen.

Beskrivning av hur djuret kommer att påverkas av åtgärden – eventuellt fysiskt och psykiskt lidande:

Biopsiering kan orsaka snabbt övergående smärta och liten stress på djuret.
Vi förväntar oss inte att avel av våra stammar eller korsningar mellan stammar kommer att orsaka ett ökat lidande hos djuren.
Undantagna är djur i Stat3-aveln där vissa fenotyper kan utveckla kolit vid 12-20 veckors ålder och/eller en mild svullnad på svans/leder kan uppstå spontant. Mössen kan dock röra sig obehindrat. Detta påverkar inte djurets välbefinnande negativt. (se under beskrivning av Stat3-stammar). Denna risk ökar med stigande ålder.

Motivering av åtgärden – varför den behöver göras och varför beskrivet sätt är det bästa sättet att utföra åtgärden på

Åtgärden behövs för att upprätthålla genmodifierade stammar, då krävs avel och genotypning.

Beskrivning av vad som ska göras för att minska djurets lidande – både farmakologisk lindring och övriga insatser liksom ökad tillsyn

Öronbiten som blir över från id-märkningen hos djuret används i största möjligaste mån.
Vi håller stammar som heterzygoter för att undvika utveckling av fenotyp som kan inverka negativt på djuret.
För de stammar som eventuellt utvecklar fenotyp, har vi samma avbrytningspunkt som för avelsdjuren.

Åtgärd 2: Induktion av genmodifiering

Ingående beskrivning av åtgärden och hur djuret kommer att påverkas

Vad djuret kommer att utsättas för inklusive försöksperiodens längd för det enskilda djuret.
Beskrivning av hur djuret kommer att påverkas – eventuellt fysiskt och psykiskt lidande.

I vissa fall där CreER eller FlipER ingår kommer vi att administera tamoxifen (eller mifeprestone eller annan östrogenreceptoriducerare). Alternativt kan genuttrycket ändras med hjälp av tetracykliner om mössen eller cellerna bär på tetO-systemet.

Tamoxifen administreras genom oral gavage, intraperitoneal (ip) eller subcutan (s.c.) injektion på dräktiga honor eller andra möss. I vissa fall kan även musens diet eller vatten komma att kompletteras med ämnen som doxycyklin, tetracyklin, RU486 eller tamoxifen.

Tamoxifen som administreras oralt kommer att lösas upp i majsolja (10-20 mg/ml). Administrationen av tamoxifen kommer att ske 1-5 ggr/djur (1 ggr/dag) och dosen är väletablerad (0.1ml/10g mus). Både hård och mjuk sond kommer att användas beroende på stam och vad den som utför administrationen är upplärd på.

Tamoxifen som administreras via ip kommer att lösas upp i majsolja (10-20 mg/ml) och 0.1 ml/10g mus administreras i buken 1 gång per dag maximalt 5 ggr/djur.

Tamoxifen som administreras subcutant kommer att lösas upp i majsolja (10-20 mg/ml) och administrationen kommer att ske en gång per dag maximalt 5 ggr/djur och dosen är väletablerad (0.1ml/10g mus). Den maximala volym som administreras subkutan kommer inte att överstiga 10 ml/kg vilket är enligt riktlinjerna från veterinärerna.

Beskrivning av hur djuret kommer att påverkas av åtgärden – eventuellt fysiskt och psykiskt lidande:

Tamoxifen kan leda till penisprolaps eller ljumskbråck hos hanar.
Tamoxifenbehandling av dräktiga honor kan leda till komplikationer vid födsel. Vi har inte observerat detta hos de stammar som vi använder.

Intraperitonal injektion kan leda till peritonit.

I övrigt förväntar vi inte några effekter på djuret dels då vi styr CreER/FlipER uttrycket till specifika cellpopulationer alternativt samlar upp embryon före de föds.

Motivering av åtgärden – varför den behöver göras och varför beskrivet sätt är det bästa sättet att utföra åtgärden på

Konditionella genmodifikationer måste induceras, dvs genborttag eller ingångsättande, vilket görs med tamoxifen då dessa stammar bär på CreER eller FlipER eller tetracykliner för tetO-systmet.

Beskrivning av vad som ska göras för att minska djurets lidande – både farmakologisk lindring och övriga insatser liksom ökad tillsyn

Adminstrationsmetod väljs i möjligaste mån utifrån tidigare publikationer samt kollegors erfarenheter så att vi inte behöver testa själva.
Vi använder i första hand gavage för administration och i sista hand subcutan injektion.
Om vi behöver använda upprepade intraperitonal injektioner palperas buken mellan administrationerna.

Vid behandling med tetracykliner i vattnet kontrollerar vi att djuren dricker och vid behov tillsätts 0,5-5% sucrose i vattnet för att ta bort smaken av tetracyklinerna.

Åtgärd 3: Blodprov

Ingående beskrivning av åtgärden och hur djuret kommer att påverkas

Vad djuret kommer att utsättas för inklusive försöksperiodens längd för det enskilda djuret.
Beskrivning av hur djuret kommer att påverkas – eventuellt fysiskt och psykiskt lidande.

Blodprover kan komma att tas för genotypning och/eller immunologisk undersökning. Volymen överstiger aldrig 10% av den totala blodvolymen under 14 dagar (enligt rekommendation i SJVFS 2017:40, Saknr L150). Om provtagning sker upprepande gånger (max 10 prover/mus) oftast 5ul och maximalt ca 20 mikroliter. Som mest kan 1 prov tas innan behandlingen (referensprov), maximalt 5 gånger under ett enskilt dygn efter behandlingen, maximalt 3 gånger under det därpå efterföljande dygnet, i övrigt får prov tas maximalt 1 gång per dygn efter behandlingen samt i samband med avlivning, se flödesshemat.

Vissa metoder kan kräva större provvolymer än ovan angivna max 20 µl. Vi vill då istället för vad som står angivet ovan och i flödesschemat kunna ta blodprov enligt följande: första provet tas cirka en vecka innan behandlingen (referensprov), ett andra prov tas under/efter behandlingen, och det tredje provet tas i samband med avlivningen. Max 120ul tas totalt under 14 dagarsperioden, dvs ofta ca 60ul kommer att tas vid provtillfälle 1 och ca 60 ul vid provtagning 2, vilket totalt inte kommer att överstiga 10% av kroppsvikten. Prov 3 tas i samband med avlivning och räknas inte in i de 10%. Beroende på hur mycket som tas vid en enskild provtagning kommer vi att se till att mössen återhämtar sig mellan provtagningarna. Minsta vila mellan prov 1 och 2 är en dag. Se provtagningsschemat nedan:

Blodprov 1: tas 0-7 dagar dvs innan behandling
Blodprov 2: tas 7-14 dagar dvs under/efter behandling
Blodprov 3: tas i samband med avlivning

En mus har en blodvolym på ca 72 ml per kilo kroppsvikt. För en mus med en vikt på 20 gram motsvarar 120 µl knappt 9 % av blodvolymen.

Övervakning av eventuella blodförändringar i djuren görs genom blodprovstagning från svansen genom snitt eller borttagande av sårskorpa. Blodproven kan också tas från svansven eller vena saphena (hasven) på vakna djur. Sista blodprovet kan komma att tas via punktion av hjärtat eller orbitalt under narkos (isoflurane). Omedelbart efter att provet har tagits kommer mössen att avlivas genom cervikal dislokation. I vissa fall kan mössen komma att sövas i samband med provtagning. Detta är för att underlätta provtagning samt för att minska infektionsrisken för personalen.

Beskrivning av hur djuret kommer att påverkas av åtgärden – eventuellt fysiskt och psykiskt lidande:

Lidandet av blodprovstagning från svansen förväntas vara lindrig. Den provtagning som utförs i form av upprepade små blodprov som tas från svansen verkar inte bekomma djuren.

Motivering av åtgärden – varför den behöver göras och varför beskrivet sätt är det bästa sättet att utföra åtgärden på

Proven är nödvändiga för att kunna få information om hematologiska, kemiska och immunologiska förändringar under behandling med substanser för att kunna följa substansens nedbrytning/upptag.

Beskrivning av vad som ska göras för att minska djurets lidande – både farmakologisk lindring och övriga insatser liksom ökad tillsyn

Om möjligt sker blodprovstagningen via kanyl eller prickning av ven med skalpell för att minimera påverkan hos djuret. Men även den provtagning som utförs i form av upprepade små blodprov som tas från svansen genom borttagningen av sårskorpa verkar inte bekomma djuren.

4.1.1.2 Svårhetsgrad och avbrytningspunkt

Svårhetsgrad: Måttlig

Beskrivning av avbrytningspunkten

Avbrytningspunkten för djur i avel och kolonidjur: 0.4p på KI-mallen avbrytningspunkten för övriga åtgärder i denna försöksgrupp är: 0.4p på KI-mallen.
Om penisprolaps eller ljumskbråck utvecklas avlivas djuret om det påverkas av prolapsen eller ljumskbråcket (0.4p) annars får djuret vara kvar till experimentell slutpunkt är uppnådd. Stammar som utvecklat fenotyp i form av svullen svans/leder begär vi undantag från detta i upp till 5 dagar då eventuell svullnad ska ha lagt sig.

4.1.1.3 Efter försöket

Avlivningsmetod: Halsdislokation

Om metoden inte gäller alla djur ska de djur anges som ska avlivas på detta sätt.

Avlivning genom hastig vridning-sträckning-tänjning av halskotpelaren på ett sådant sätt att ryggmärgen slits av.

Bedövningsmetod

Annan metod

Beskrivning av bedövningsmetoden

Avlivning genom hastig vridning-sträckning-tänjning av halskotpelaren på ett sådant sätt att ryggmärgen slits av.

Kontrollmetod för att säkerställa att djuret är dött

- Halsdislokation

Beskrivning av kontrollmetoden

Avlivning genom hastig vridning-sträckning-tänjning av halskotpelaren på ett sådant sätt att ryggmärgen slits av.

Avlivningsmetod: Koldioxid

Om metoden inte gäller alla djur ska de djur anges som ska avlivas på detta sätt.

Djur placeras i en bur där koldioxid koncentrationen sakta höjs tills djuren är bedövad, sedan djuren blivit medvetslösa kan koncentrationen ökas snabbt tills andningen upphör.
Alla möss genomgår halsdislokation efter behandling med koldioxid för att verifiera att död inträffat.
Hastig vridning-sträckning-tänjning av halskotpelaren på ett sådant sätt att ryggmärgen slits av.

Bedövningsmetod

Annan metod

Beskrivning av bedövningsmetoden

Koldioxid enligt ovan.

Kontrollmetod för att säkerställa att djuret är dött

- Kontroll att cirkulationen har upphört
- Halsdislokation

Beskrivning av kontrollmetoden

Alla möss genomgår halsdislokation efter behandling med koldioxid för att verifiera att död inträffat. Hastig vridning-sträckning-tänjning av halskotpelaren på ett sådant sätt att ryggmärgen slits av.

Avlivningsmetod: Överdos av narkosmedel

Om metoden inte gäller alla djur ska de djur anges som ska avlivas på detta sätt.

Överdos av narkosmedel t.ex. avertin ip (0.015ml, 2.5% averting/g kroppsvikt) eller pentobarbital-Allfatal® (80.100 mg/kg) följt av avblodning via bukaorta (exsanguinering via aorta abdominalis) eller cervical dislokation.

Alternativt överdos med isofluran via inhalation

Djur som ska perfunderas sövs djupt med narkosmedel varefter de perfunderas med paraformaldehyd (4% lösning) eller PBS

Bedövningsmetod

Narkosmedel

Beskrivning av bedövningsmetoden

Överdos av narkosmedel, se ovan.

Kontrollmetod för att säkerställa att djuret är dött

- Avblodning
- Halsdislokation

Beskrivning av kontrollmetoden

Vid perfusion avblodas djuret.
Vid halsdislokation görs en hastig vridning-sträckning-tänjning av halskotpelaren som gör att ryggmärgen slits av.

Djuren kommer att återanvändas i andra försök som inte omfattas av denna ansökan

Beskrivning av vad djuren kommer att användas till efter försöket har avslutats.

Vi vill kunna föra över djur från avelskolonin, dvs de har endast ingått i åtgärd 1 (avel och genotypning), till andra etiska tillstånd. Detta ligger i linje med 3R (reducera) då andra grupper kan ta del av djur som vi annars skulle ha avlivat tex pga fel genotyp. All överföring sker i samråd med veterinär.

Djuren ingår i andra grupper i denna ansökan

Beskrivning av vilken/vilka övriga grupper djuren kommer att ingå i.

Djur från denna grupp kan använda i en av de andra försöksgrupperna (2 och/eller 3).

4.2 Försöksgrupp: 2

Avlägsnande av värdceller genom:
- Behandling med liposom/vesiklar eller med antikroppar, affibodies, receptorligander eller liknande
- Difteri-toxinbehandling

Vävnadsinsamling från djur
Anestesi av djur vid behandling

Djur från denna försöksgrupp kan ingå i övriga försöksgrupper.

4.2.1 Undergrupp: Avlägsnande (depletion) av värdceller

För att studera immuncellers roll vid pneumokockinfektion kan immunceller, eller andra celler av betydelse vid infektion, komma att avlägsnas, hämmas i sin funktion eller i sin interaktion med bakterien. Effekten uppnås genom behandling med liposom/vesiklar eller med antikroppar, affibodies, receptorligander eller liknande. En musstam med inducerbar expression av Difteriatoxinreceptorn möjliggör en lokalt avgränsad och specifik eliminering av relevanta celltyper genom administrering av difteriatoxin (se respektive beskrivning av experiment nedan). Behandlingen påbörjas innan, i samband med eller efter att mössen infekteras och pågår sedan under infektionsförsöket.

Antalet djur är en uppskattad siffra och kan komma att öka eller minska något men är en del av det totala antalet djur som vi söker för.
Djur från denna försöksgrupp kan ingå i övriga försöksgrupper

Mus (Mus musculus), 1000 st, Försöksdjursanläggning

Beskrivning av ras, stam och egenskaper som kan medföra lidande för djuren samt motivering av valet av djur med dessa egenskaper

Vänligen se Försöksgrupp 4.1

Kön: Vänligen se Försöksgrupp 4.1

Genetiskt modifierade djur används: Ja

Diarienummer på tillstånd samt beskrivning och motivering av metoder.

Vänligen se Försöksgrupp 4.1

4.2.1.1 Beskrivning av de åtgärder/ingrepp djuren kommer att utsättas för

Åtgärd 1: Behandling med liposom/vesiklar eller med antikroppar, receptorligander eller liknande

Ingående beskrivning av åtgärden och hur djuret kommer att påverkas

Vad djuret kommer att utsättas för inklusive försöksperiodens längd för det enskilda djuret.
Beskrivning av hur djuret kommer att påverkas – eventuellt fysiskt och psykiskt lidande.

1. Liposom/vesikelbehandling: populationer av fagocyterande celler kommer att avlägsnas genom att liposomer innehållande den verksamma substansen klodronat administreras. Kontrollgrupp kan istället ges isoton koksaltlösning, fosfatbuffrad lösning alternativt liposomer som ej innehåller den verksamma substansen klodronat eller motsvarande, alternativt lämnas obehandlad. Uppemot 90% av alla makrofager i en population avlägsnas genom liposombehandlingen.

2. Behandling med antikroppar, affibodies, receptorer eller receptorligander: specifika populationer av immunceller avlägsnas eller hämmas i sin funktion genom att en lösning innehållande antikroppar eller receptorligander specifika för en eller flera cellpopulationer administreras. Kontrollgrupp kan istället ges isoton koksaltlösning, fosfatbuffrad lösning alternativt antikroppar eller affibodies med annan specificitet eller liknande, alternativt lämnas obehandlad. Metoden möjliggör en mer precis utarmning av specifika immuncellspopulationer än vad som är möjligt genom liposombehandling.

Liposom-/antikropps-/affibody/-receptorer-ligand- eller kontrollösning kommer antingen att injiceras intravenöst (maximalt 5ml/kg), intraperitonealt (maximalt 20 ml/kg), intratrakealt (maximalt 100 µl, se beskrivning nedan) eller intranasalt (maximalt 100 µl). De volymer som administreras vid respektive behandlingsmetod är nödvändiga för att effektivt kunna avlägsna immunceller, särskild Kupferceller i lever och makrofager i mjälte kräver en stor volym lösning för att utarmas. I samband med intratrakeal administrering av lösning intuberas mössen. För att utvärdera hur lösningen fördelas i luftvägarna kan färglösning administreras i likhet med vad som är beskrivet för intranasal och intratrakeal administrering nedan. De volymer som används vid intratrakeal och intranasal administrering är nödvändiga då lösningen måste nå lungorna där den har effekt.

Då immunceller nybildas kan mössen komma att behandlas med liposomer, antikroppar, affibodies, ligander etc alternativt motsvarande kontrollösning vid upprepade tillfällen för att säkerställa att cellpopulationen är utarmad. Behandlingen kan därför komma att ske upp till tre gånger innan infektion (t.ex. 72, 48, 24, 12 och/eller 6 timmar) samt upp till tre gånger efter infektion.

Behandling/injektion kan innebära en viss stress hos djuret och ger kortvarig, snabbt övergående smärta. Själva administreringen ger ingen effekt på djuret. De möss hos vilka immunceller har avlägsnats eller påverkats visar inget förändrat beteende i oinfekterat tillstånd, utan återhämtar sig lika snabbt och har samma aktivitetsnivå som obehandlade möss. Tidigare erfarenhet av metoderna visar att mössen snart efter behandling återgår till normalt beteende.

Vid intratrakeal (intubering) administration använder vi bedövning. Mössen bedövas genom i.p. injektion av en kombination av ketamin (80-100 mg/kg kroppsvikt) och xylazin (5-10 mg/kg kroppsvikt). Mössen kommer i dessa fall tillföras värme under sövningen samt behandlas med ögonsalva. Mössen observeras noggrant för att bekräfta att de är helt bedövade. Detta sker genom att kontrollera för långsammare andningsfrekvens, ingen reaktion av extremiteterna när de lyfts i nacken, och ingen reaktion när bakbenen stimuleras. Under sövningen behandlas mössen med ögonsalva och värme tillförs. Under den intratrakeala administrationen, vilken bara tar några sekunder, är mössen placerade på en intubationsplattform. Munnen öppnas försiktigt med hjälp av ett laryngoskop för att kunna se öppningen av trakea. En trubbig nål på en spruta som innehåller suspension sätts precis ovanför luftröret och lösningen administreras. Nålen dras ut snabbt och musen tas från plattformen och placeras på en värmedyna. När mössen är helt återhämtade (tar cirka 30 minuter) läggs de tillbaka i sina förvärmda burar.

Motivering av åtgärden – varför den behöver göras och varför beskrivet sätt är det bästa sättet att utföra åtgärden på

Behandling med liposom/vesiklar eller med antikroppar, receptorligander eller liknande är en väl-etablerad metod för att ta bort värdceller. Det är en metod med minimalt lidande hos djuren som gör att vi kan studera värdcellers roll vid bakterieinfektion. Genom denna teknik kringgår vi till stor del användandet av transgena möss vilket drastiskt reducerar antalet djur som behövs för att genom avel få fram rätt genotyp för experimenten.

Metoden att utarma populationer av fagocytiska celler med klodronat är etablerad sedan länge. Effektiviteten av utarmningen kommer att kontrolleras och vid behov justeras antalet behandlingar (sker i samråd med veterinär). Vi kommer att behålla behandlingen (antal och volym) så låg som möjligt.

Beskrivning av vad som ska göras för att minska djurets lidande – både farmakologisk lindring och övriga insatser liksom ökad tillsyn

Administrationsmetod väljs i möjligaste mån utifrån tidigare publikationer samt kollegors erfarenheter så att vi inte behöver testa själva om möjligt.
Om vi behöver använda upprepade intraperitonal injektioner palperas buken mellan administrationerna. Vid nedsövning av djur (se beskrivning av anestesi) använder vi värmedyna och ögonsalva. Den uppvaknande musen får vakna upp i sin bur med extra värme. Första dygnet efter behandlingen kommer mössens hälsostatus att bedömas 2 gånger, om det inte föreligger skäl för utökad tillsyn, mössen kommer därefter att bedömas 1 gång per dag. Behandlingarna i sig förväntas inte påverka djuren negativt utan påverkan ses först i samband med infektion. Det symptom som ibland kan uppträda är något nedsatt aktivitet.

Åtgärd 2: Difteri-toxinbehandling

Ingående beskrivning av åtgärden och hur djuret kommer att påverkas

Vad djuret kommer att utsättas för inklusive försöksperiodens längd för det enskilda djuret.
Beskrivning av hur djuret kommer att påverkas – eventuellt fysiskt och psykiskt lidande.

1. CD11c.DTR-mössen ges en dos difteri-toxin (maximalt 4 ng/g kropssvikt) intraperitonealt (maximalt 100 µl) eller intranasalt (maximalt 50 µl), ca 6-24 timmar innan infektion. Metoden är beskriven sedan tidigare och ger en övergående utarmning av immunceller.

2. CD11c.DOG möss behandlas cirka 24 timmar innan infektion med intraperitoneal injektion (maximalt 100 µl, 8 ng/g kroppsvikt) av difteri-toxin. Mössen kan därefter komma att injiceras vid upprepade tillfällen med 1-2 dagars mellanrum. Detta kan pågå i upp till 12 dagar och är inte associerat med viktnedgång eller minskad överlevnad hos mössen, dvs max antal injektioner är 12. Vid intranasal administrering av lösning kommer volymen att uppgå till maximalt 50 µl och administreras vid ett tillfälle, den maximala dosen av toxiner är 8 ng/g kroppsvikt. Metoden är beskriven sedan tidigare.

3. Kontrollgrupp till CD11c.DTR och CD11c.DOG kan istället ges medium, fosfatbuffrad saltlösning, isoton koksaltlösning eller liknande, alternativt lämnas obehandlade. Vi vill även få möjlighet att utföra motsvarande behandling med difteri-toxin eller kontrollösning på vildtypmöss för att kunna utesluta att behandlingen i sig har en effekt på mössens immunsvar.

Vi förväntar oss i regel ingen påverkan på djuret av difteri-toxinbehandlingen.

Motivering av åtgärden – varför den behöver göras och varför beskrivet sätt är det bästa sättet att utföra åtgärden på

Det är en metod med minimalt lidande hos djuren som gör att vi kan studera värdcellers roll vid bakterieinfektion. Genom denna teknik kringgår vi till stor del användandet av transgena möss vilket drastiskt reducerar antalet djur som behövs för att genom avel få fram rätt genotyp för experiment.

Beskrivning av vad som ska göras för att minska djurets lidande – både farmakologisk lindring och övriga insatser liksom ökad tillsyn

Vänligen se åtgärd 1: Behandling med liposom/vesiklar eller med antikroppar, affibodies, receptorligander eller liknande.

Åtgärd 3: Blodprov

Ingående beskrivning av åtgärden och hur djuret kommer att påverkas

Vad djuret kommer att utsättas för inklusive försöksperiodens längd för det enskilda djuret.
Beskrivning av hur djuret kommer att påverkas – eventuellt fysiskt och psykiskt lidande.

Vänligen se beskrivning i försökgrupp 1 (Åtgärd 3: Blodprov)

Motivering av åtgärden – varför den behöver göras och varför beskrivet sätt är det bästa sättet att utföra åtgärden på

Proven är nödvändiga för att kunna få information om hematologiska och kemiska förändringar under behandling med substanser för att kunna följa substansens nedbrytning/upptag samt för att säkerställa att cellpopulationen är utarmad/cellerna är färgad.

Beskrivning av vad som ska göras för att minska djurets lidande – både farmakologisk lindring och övriga insatser liksom ökad tillsyn

Vänligen se beskrivning i försökgrupp 1 (Åtgärd 3: Blodprov)

Åtgärd 4: Anestesi

Ingående beskrivning av åtgärden och hur djuret kommer att påverkas

Vad djuret kommer att utsättas för inklusive försöksperiodens längd för det enskilda djuret.
Beskrivning av hur djuret kommer att påverkas – eventuellt fysiskt och psykiskt lidande.

1. Anestesi - Inhalation
Vi använder företrädelsevis isofluran som sövningsmedel eftersom sövningen snabbt kan induceras och mössen återhämtar sig omgående och inte visar tecken på att vara påverkade i någon större utsträckning. Med undantag för IVIS-fotografering hålls mössen i regel bara sövda under några minuter.
Mössen kommer att sövas med cirka 4% isofluran. Vid längre sövningar exempelvis vid IVIS-fotograferingen kan narkosen induceras med cirka 4% av isofluran för att sedan vidmakthållas med en lägre koncentration dvs. ned till cirka 2.5% isofluran.

2. Anestesi - Injektion
För vissa applikationer behöver vi hålla mössen sövda under en längre tid, detta gäller främst vid intratrakeala och intranasala behandlingar. Samtidigt behöver vi då få åtkomst till mössens luftvägar vilket gör det omöjligt att kontinuerligt kunna tillföra isofluran. I vissa fall påverkar inhalationsanestesi kvalitén på de prov som tas och vi måste då ha möjlighet att kunna använda injektionsanestesi. Mössen kan därför även komma att sövas genom intraperitoneal injektion bestående av en kombination av ketamin (70-100 mg/kg kroppsvikt) och xylazin (2-10 mg/kg kroppsvikt). Mössen kommer i dessa fall att tillföras värme under sövningen samt behandlas med ögonsalva. Alternativ anestesi kan användas vid behov efter rekommendation av veterinär.

Nedsövning med isofluran kan orsaka stress innan djuret är helt nedsövt. Efter sövning med isofluran återhämtar sig mössen omgående, medan återhämtningen efter sövning med ketamin och xylazin tar längre tid. I det senare fallet kommer mössen därför att övervakas tills att de återfått grundläggande motorisk funktion, mössen kommer sedan att ha tillsyn tills de återhämtat sig.

Eventuellt kan annan narkos, smärtlindring, lugnande medel användas efter samråd med veterinär.

Motivering av åtgärden – varför den behöver göras och varför beskrivet sätt är det bästa sättet att utföra åtgärden på

För vissa applikationer behöver vi hålla mössen sövda för kort eller under en längre tid, detta gäller främst vid intranasal och intratrakeala behandlingar där vi måste säkerställa att lösningen når lungorna. För att undvika att kvalitén på prov som tas blir påverkade av inhalationsanestesin måste vi ha möjlighet att kunna använda injektionsanestesi.

Beskrivning av vad som ska göras för att minska djurets lidande – både farmakologisk lindring och övriga insatser liksom ökad tillsyn

Om vi behöver använda upprepade intraperitonal injektioner palperas buken mellan administrationerna. Vid nedsövning av djur (se beskrivning av anestesi) använder vi värmedyna och ögonsalva. Den uppvaknande musen får vakna upp i sin bur med extra värme. Övervakning efter behandling sker minst 1 gång per dag i 48 timmar.

4.2.1.2 Svårhetsgrad och avbrytningspunkt

Svårhetsgrad: Måttlig

Beskrivning av avbrytningspunkten

Avbrytningspunkten i denna försöksgrupp: 0.4p på KI-mallen.
Stammar som utvecklat fenotyp i form av svullen svans/leder begär vi undantag från detta i upp till 5 dagar då eventuell svullnad ska ha lagt sig.

4.2.1.3 Efter försöket

Avlivningsmetod: Överdos av narkosmedel

Om metoden inte gäller alla djur ska de djur anges som ska avlivas på detta sätt.

Överdos av narkosmedel t.ex. avertin ip (0.015ml, 2.5% averting/g kroppsvikt) eller pentobarbital-Allfatal® (80.100 mg/kg) följt av avblodning via bukaorta (exsanguinering via aorta abdominalis) eller cervical dislokation.

Alternativt överdos med isofluran via inhalation

Djur som ska perfunderas sövs djupt med narkosmedel varefter de perfunderas med paraformaldehyd (4% lösning) eller PBS

Bedövningsmetod

Narkosmedel

Beskrivning av bedövningsmetoden

Överdos av narkosmedel, se ovan.

Kontrollmetod för att säkerställa att djuret är dött

- Avblodning
- Halsdislokation

Beskrivning av kontrollmetoden

Vid perfusion avblodas djuret.
Vid halsdislokation görs en hastig vridning-sträckning-tänjning av halskotpelaren som gör att ryggmärgen slits av.

Avlivningsmetod: Halsdislokation

Om metoden inte gäller alla djur ska de djur anges som ska avlivas på detta sätt.

Avlivning genom hastig vridning-sträckning-tänjning av halskotpelaren på ett sådant sätt att ryggmärgen slits av.

Bedövningsmetod

Annan metod

Beskrivning av bedövningsmetoden

Avlivning genom hastig vridning-sträckning-tänjning av halskotpelaren på ett sådant sätt att ryggmärgen slits av.

Kontrollmetod för att säkerställa att djuret är dött

- Kontroll att cirkulationen har upphört

Beskrivning av kontrollmetoden

Avlivning genom hastig vridning-sträckning-tänjning av halskotpelaren på ett sådant sätt att ryggmärgen slits av.

Avlivningsmetod: Koldioxid

Om metoden inte gäller alla djur ska de djur anges som ska avlivas på detta sätt.

Djur placeras i en bur där koldioxid koncentrationen sakta höjs tills djuren är bedövad, sedan djuren blivit medvetslösa kan koncentrationen ökas snabbt tills andningen upphör.
Alla möss genomgår halsdislokation efter behandling med koldioxid för att verifiera att död inträffat.
Hastig vridning-sträckning-tänjning av halskotpelaren på ett sådant sätt att ryggmärgen slits av.

Bedövningsmetod

Annan metod

Beskrivning av bedövningsmetoden

Koldioxid enligt ovan.

Kontrollmetod för att säkerställa att djuret är dött

- Kontroll att cirkulationen har upphört
- Halsdislokation

Beskrivning av kontrollmetoden

Alla möss genomgår halsdislokation efter behandling med koldioxid för att verifiera att död inträffat.
Hastig vridning-sträckning-tänjning av halskotpelaren på ett sådant sätt att ryggmärgen slits av.

Djuren kommer att återanvändas i andra försök som inte omfattas av denna ansökan

Beskrivning av vad djuren kommer att användas till efter försöket har avslutats.

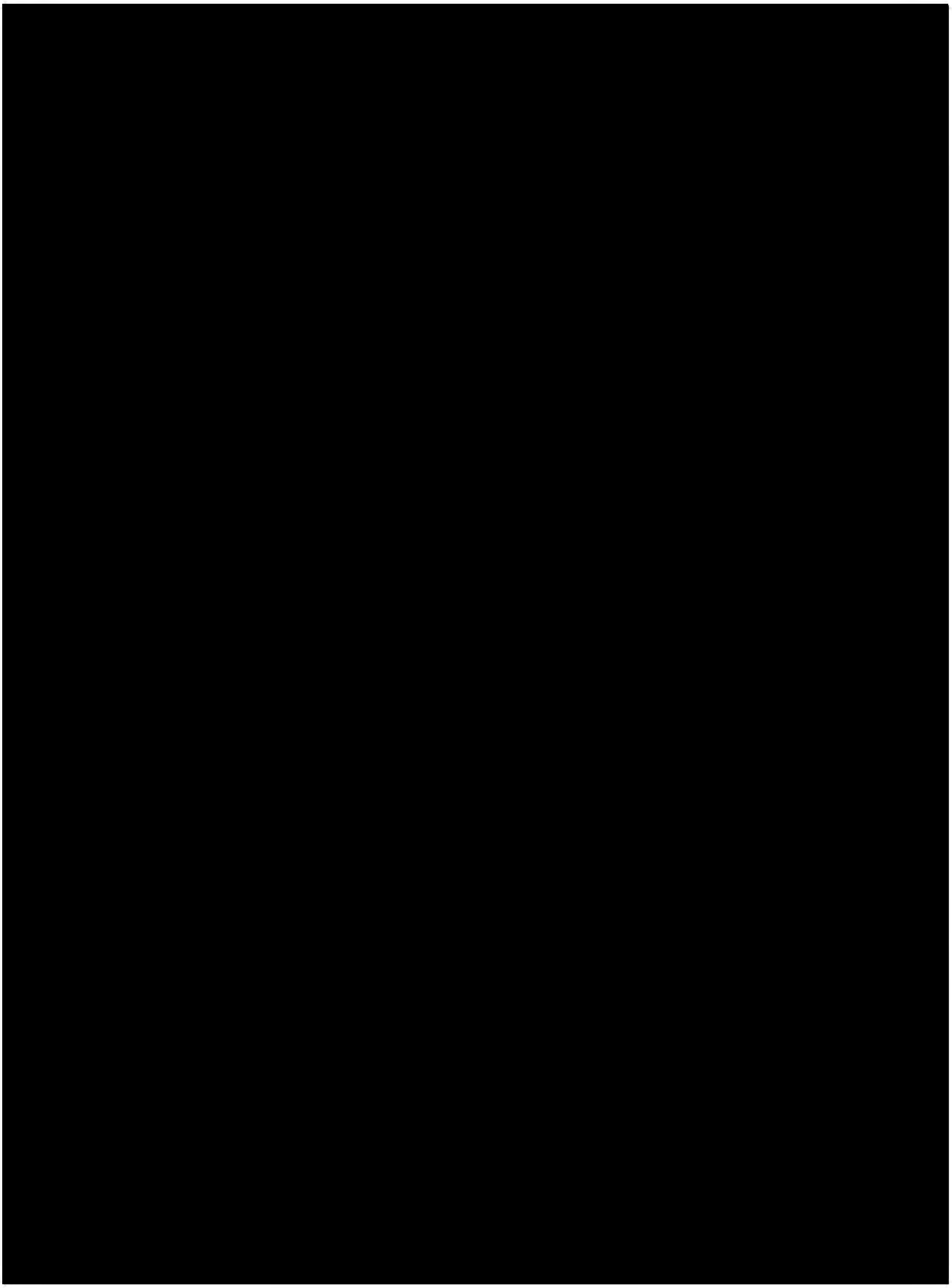
Vi vill kunna föra över djur till andra etiska tillstånd. Detta ligger i linje med 3R (reducera) då andra grupper kan ta del av djur som vi annars skulle ha avlivat tex pga fel genotyp. All överföring sker i samråd med veterinär.

Djuren ingår i andra grupper i denna ansökan

Beskrivning av vilken/vilka övriga grupper djuren kommer att ingå i.

Djur från denna grupp kan användas i försöksgrupp 3.

4.3 Försöksgrupp: 3



Beskrivning av ras, stam och egenskaper som kan medföra lidande för djuren samt motivering av valet av djur med dessa egenskaper

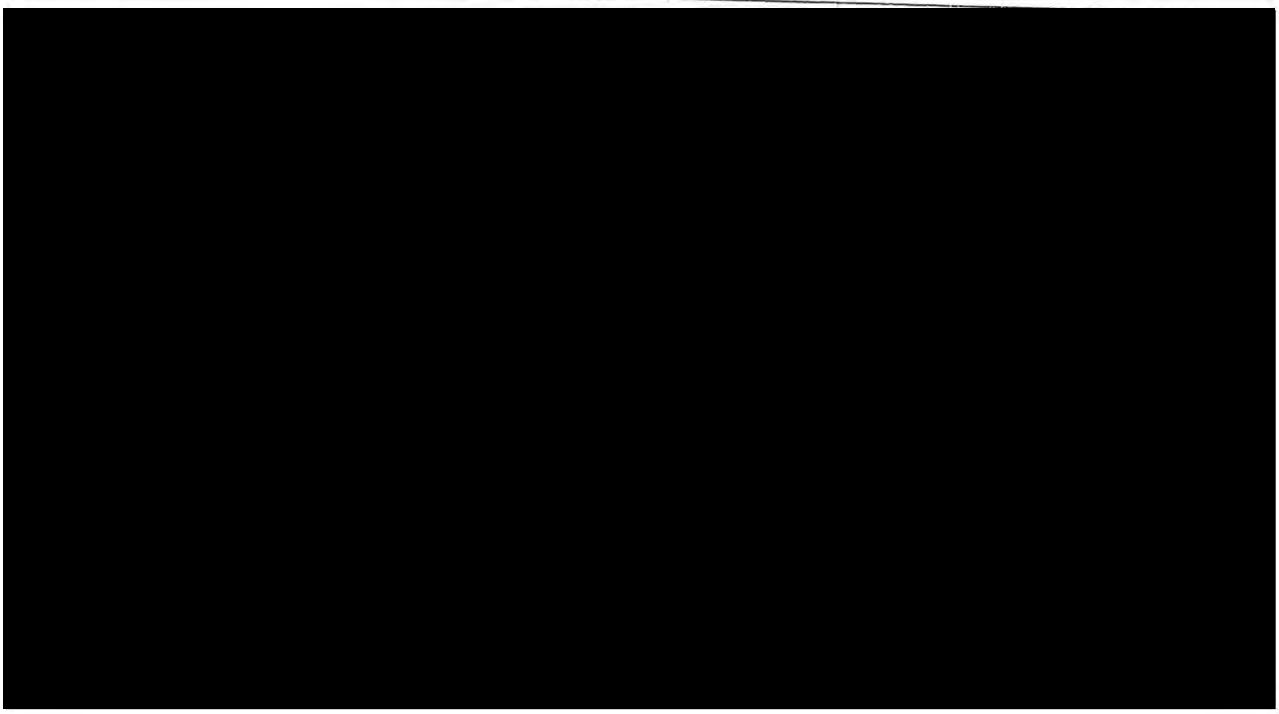
Vänligen se Försöksgrupp 1.

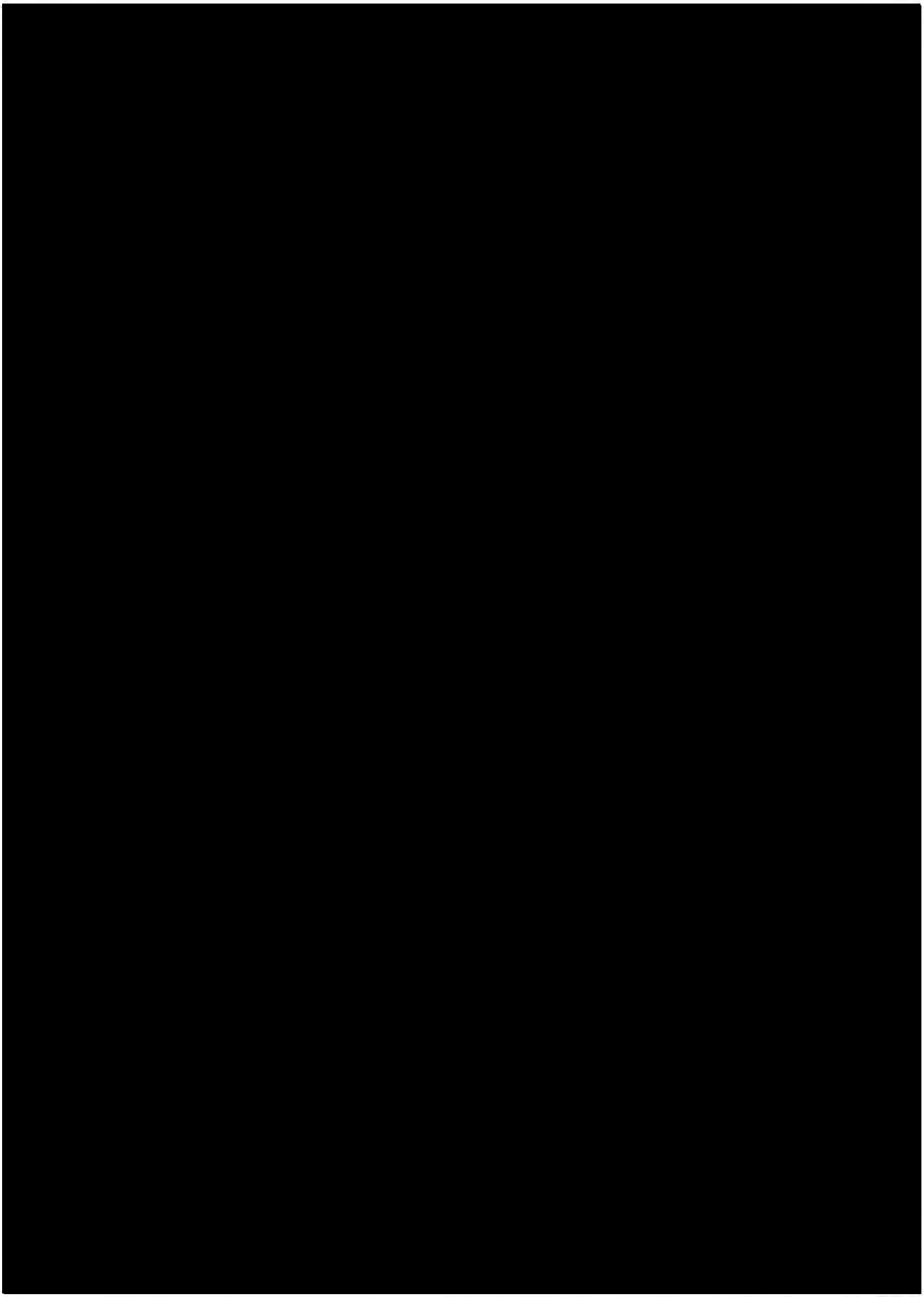
4.3.1.1 Beskrivning av de åtgärder/ingrepp djuren kommer att utsättas för

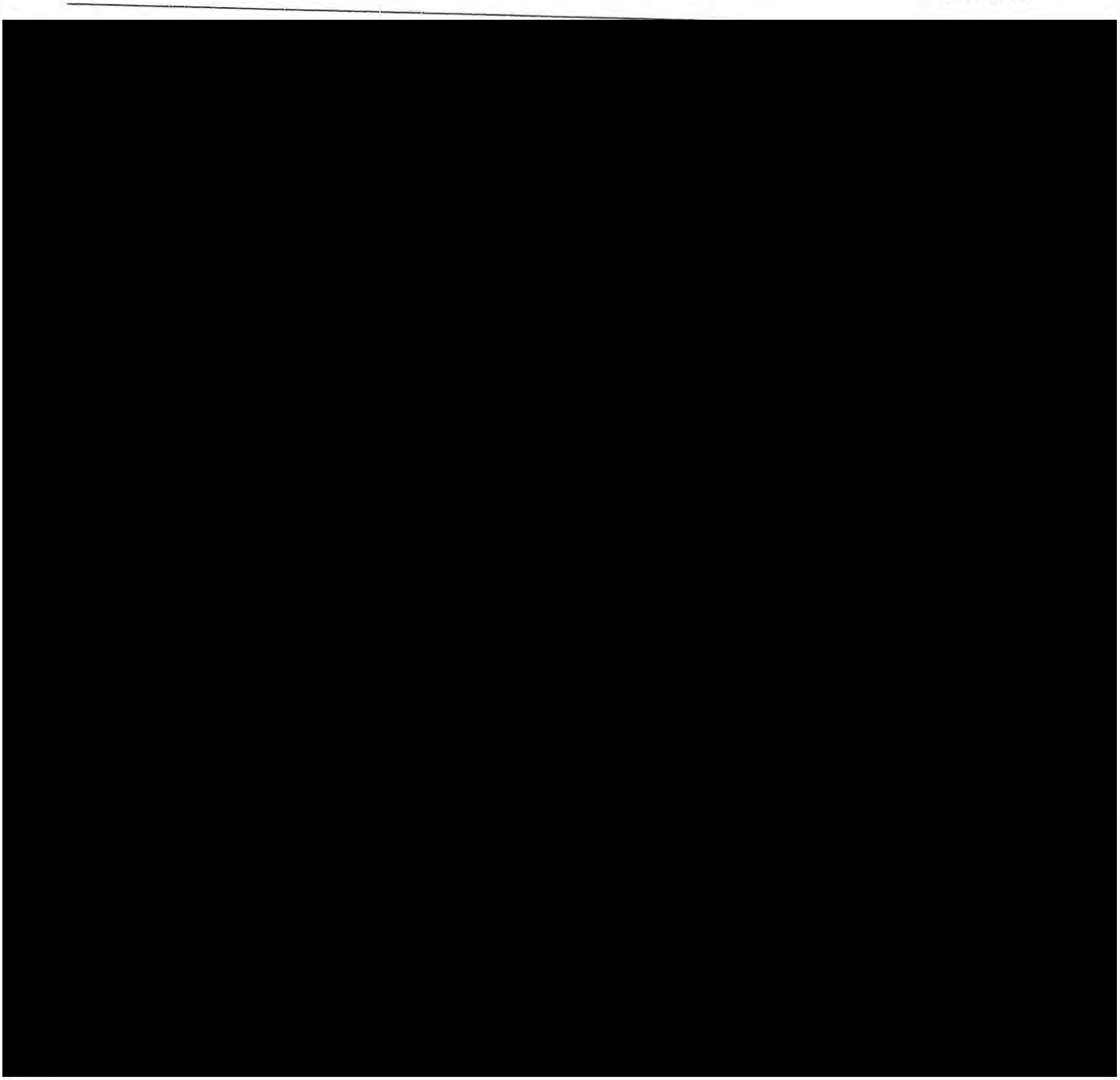
Åtgärd 1: Infektion

[redacted]

[redacted]







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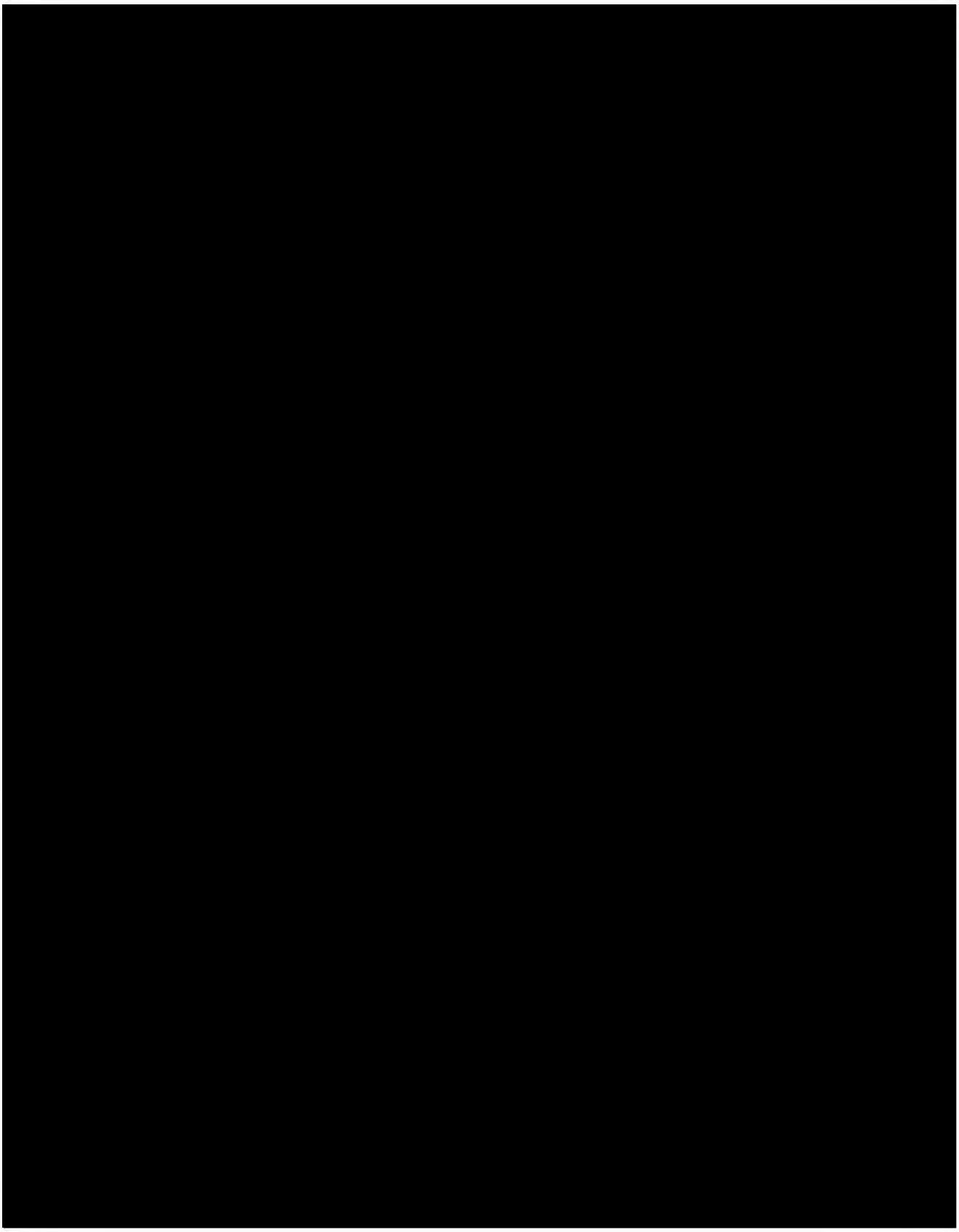

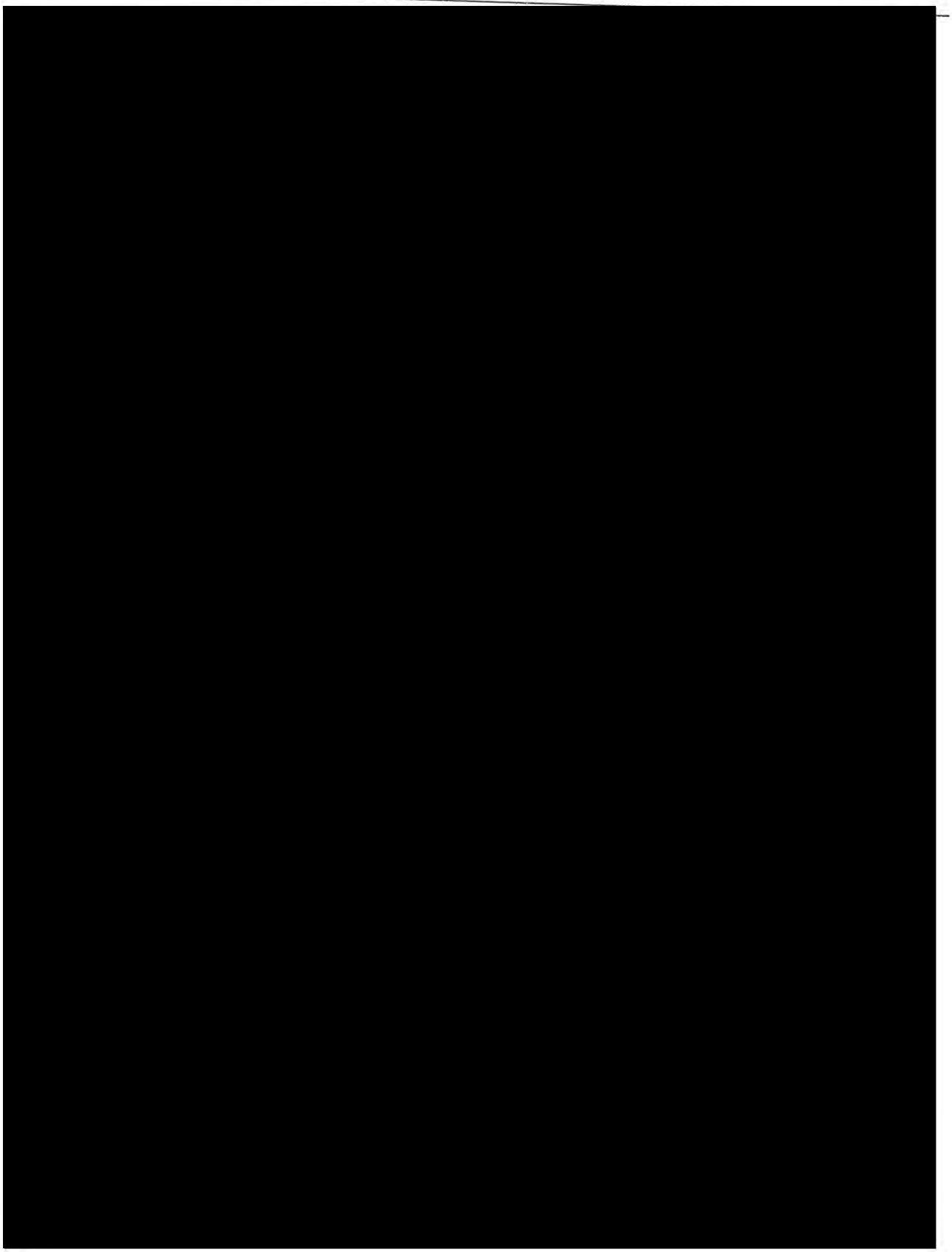

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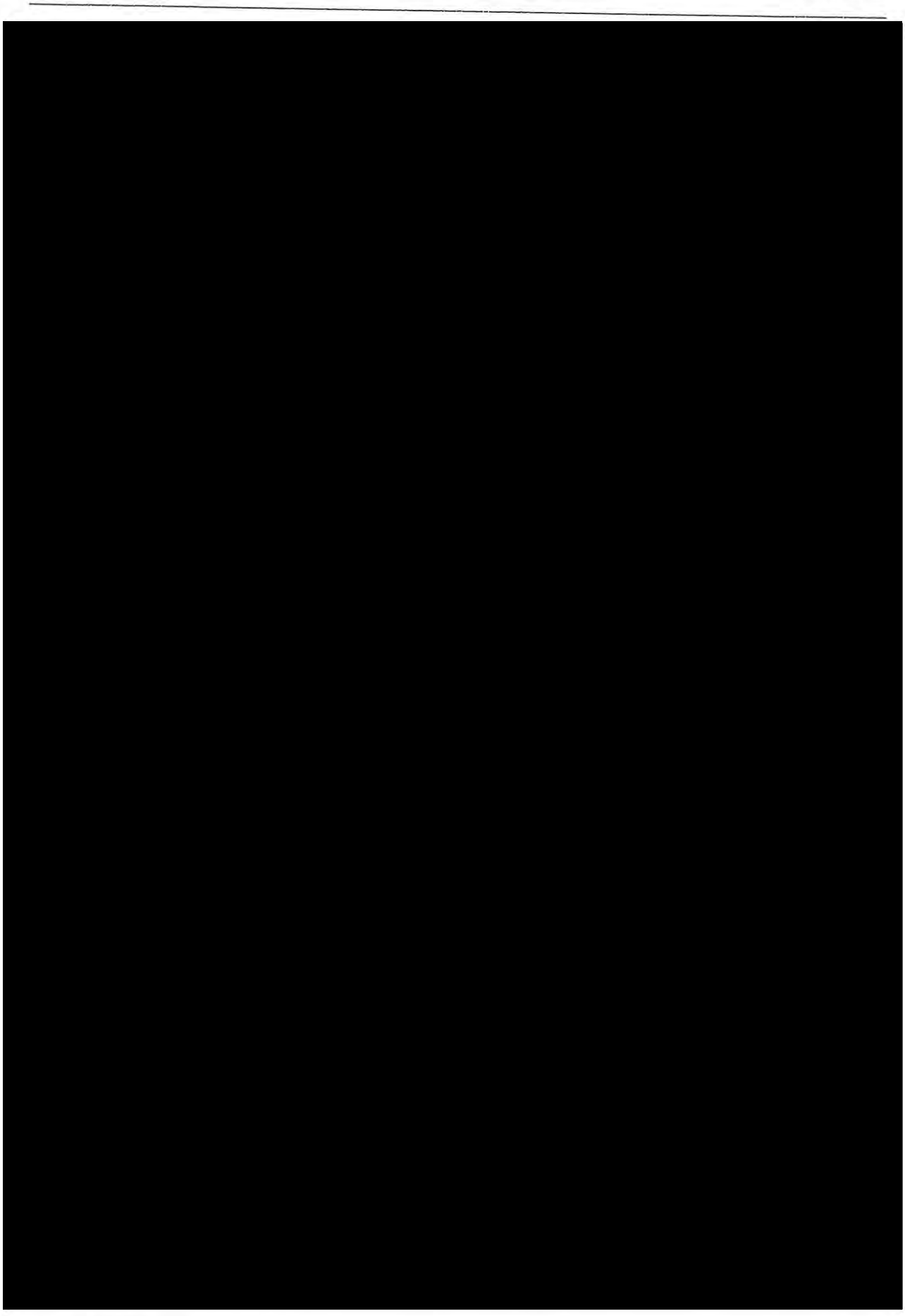


[redacted]

[redacted]

5 Undantag, sammanfattning

Populärvetenskaplig sammanfattning

Titel

Studie av bakterier och värdsvar vid luftvägsinfektioner

Sökord

[redacted]

Varaktighet

[redacted]

Använda djurarter

[redacted]

Syfte med försöket

- ☑ 1 Grundforskning
- ☑ 2 Forskning om vilka effekter sjukdomar, ohälsa eller annat avvikande tillstånd har på människor, djur eller växter samt hur de ska undvikas, förebyggas, diagnosticeras eller behandlas
- ☐ 3 Forskning som innebär utvärdering, påvisande, reglering eller modifiering av fysiologiska tillstånd hos människor, djur eller växter
- ☐ 4 Forskning som syftar till förbättring av djurens välfärd
- ☐ 5 Utveckling, tillverkning eller testning av kvalitet, effekt och säkerhet av läkemedel, livsmedel, foder och andra ämnen eller produkter. Detta gäller endast i de syften som avses i 2-4
- ☐ 6 Forskning som syftar till artskydd
- ☐ 7 Skydd av den naturliga miljön för att bevara människors hälsa eller välfärd
- ☐ 8 Skydd av den naturliga miljön för att bevara djurs hälsa eller välfärd
- ☐ 9 Rättsmedicinska undersökningar
- ☐ 10 Användning i högskoleutbildning eller i utbildning som syftar till att förvärva, upprätthålla eller utveckla yrkesfärdigheter under förutsättning att användningen framgår av utbildningens kursplaner, och är nödvändig med hänsyn till syftet med utbildningen.
- ☐ 11 Framställning och upprätthållande av en genetiskt modifierad djurstam
- ☐ 12 Annat - gäller endast för försök som sannolikt inte orsakar lidande i lika stor eller större utsträckning än ett nålstick som utförts enligt god veterinärmedicinsk praxis

Beskrivning av syftet med försöket

Beskrivning av vad forskaren syftar till att uppnå, ta reda på, fastställa eller framställa genom att utföra detta försök.

Studiens syfte är att studera bakteriella virulensfaktorers funktion i infektions- och koloniseringsmodeller samt hur värdens immunsvar och inflammation påverkar sjukdomsförloppet.

Beskrivning av nyttan av försöket

Beskrivning av nyttan för människa, djur eller miljö. Hur resultaten väntas få betydelse för den medicinska eller biologiska utvecklingen och när det gäller grundforskning vilka framsteg eller nya rön som kan förväntas på längre sikt.

Genom en ökad förståelse för infektionsprocessen och växelspelet mellan bakterien och immunsvaret får vi bättre möjligheter att förhindra infektion genom nya vacciner och behandlingsmöjligheter. Bättre förebyggande åtgärder och behandlingsalternativ skulle drastiskt kunna minska dödligheten till följd av bakteriella luftvägsinfektioner. Att kunna förhindra infektion i större utsträckning skulle också kunna minska det ofta livslånga lidandet och funktionsnedsättning som är associerat med exempelvis hjärnhinneinflammation.

Beskrivning av vilken påverkan försöket förväntas ha på djuren

Beskrivning av vilka negativa effekter försöket förväntas ha på djuren och vad som ska hända med djuren efter försöket.

[redacted]

3R - Ersätta (Replace)

[redacted]

3R - Begränsa (Reduce)

Vad forskaren har gjort för att försäkra sig om att de använder det minsta möjliga antalet djur.

[redacted]

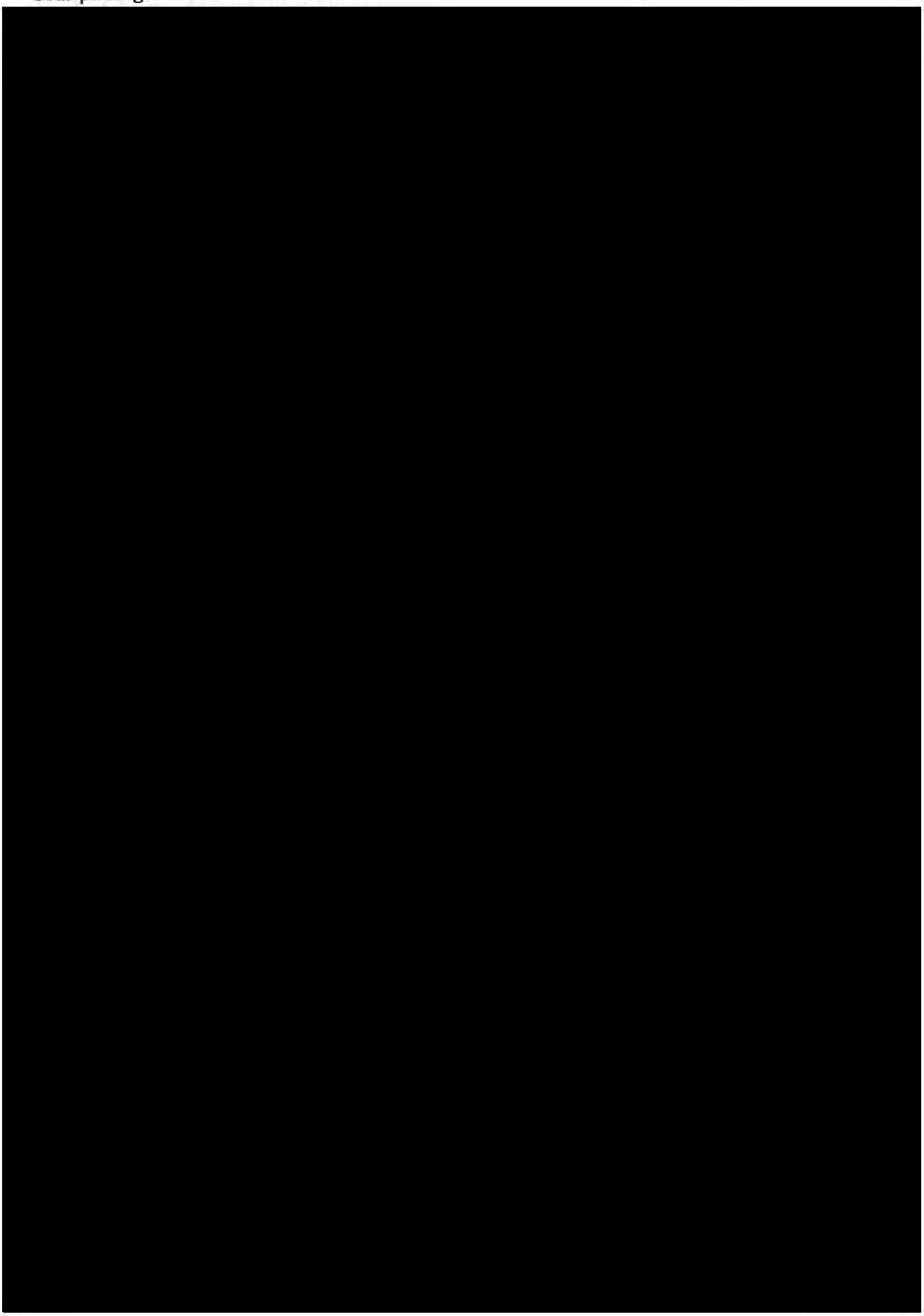
3R - Förfina (Refine)

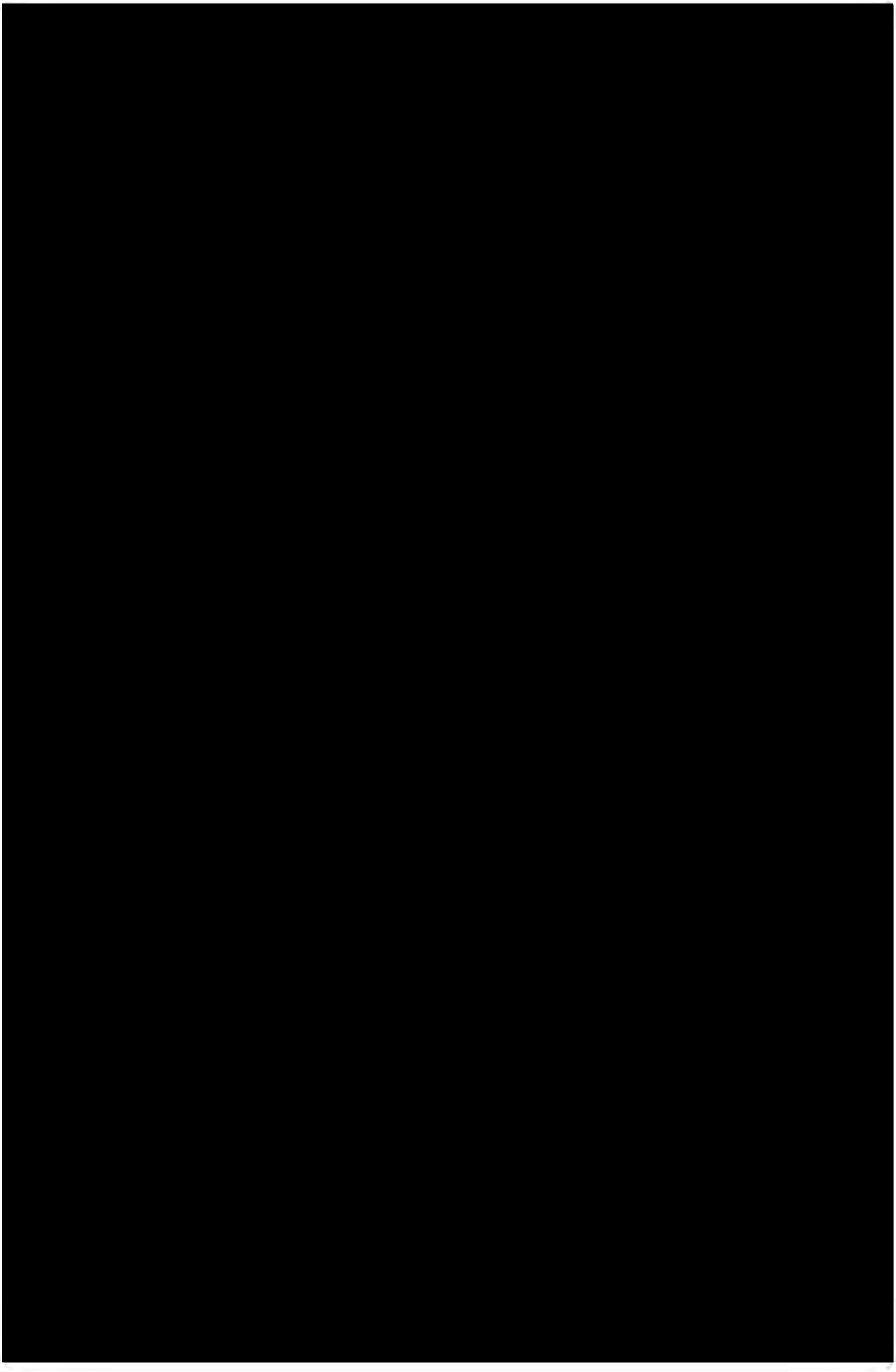
Förklaring till valet av djurart och varför de metoder som används är de mest skonsamma med hänsyn till att uppnå syftet med försöket. Beskrivning vad forskaren gör för att minimera djurens eventuella lidande.

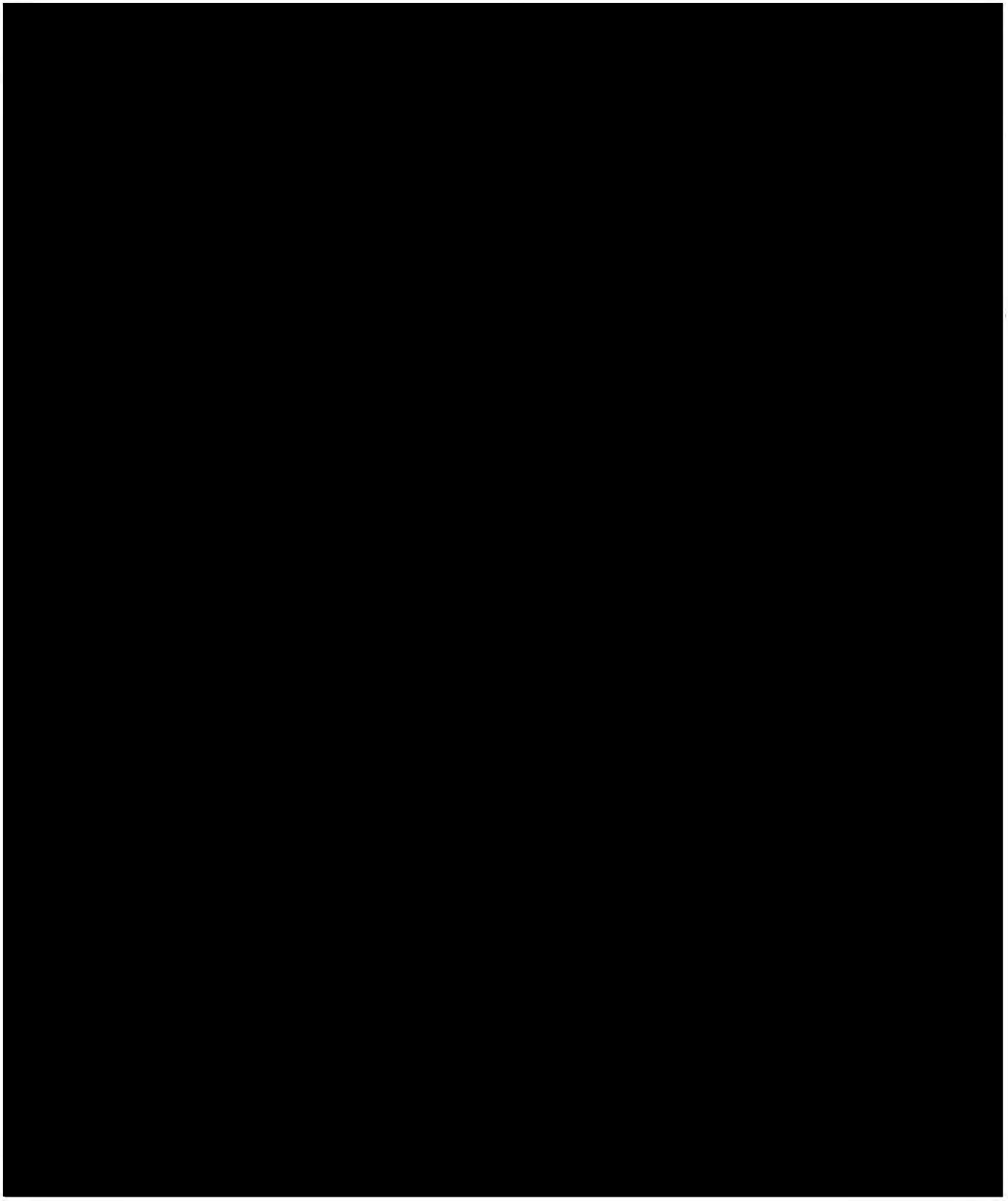
[redacted]

bil. A:1

Svar på frågorna ställda till ansökan om tillstånd 11367-2019: Birgitta Henriques Normark







11367-2019

VB: 10376-2019 Svar på frågor BG 2 (ansökan nr 3)

[redacted]

bil. A:2

Stockholms djurförsöksetiska nämnd

Ansökan 11367-2019

Syftet med försöket är att studera bakteriella virulensfaktorer (metoder som bakterier använder sig av för att orsaka skada eller sjukdom hos värden) funktion i infektions- och koloniseringsmodeller samt hur värdens immunsvar och inflammation påverkar sjukdomsförloppet.
Det finns fortfarande för lite kunskap om de faktorer som påverkar vissa bakteriers förmåga att orsaka sjukdom. Dessutom bidrar antibiotikaresistens till att behandlingsalternativ för vanliga infektioner, som ger svåra besvär för äldre och multisjuka patienter, minskar.

Beredningsgrupps 2 förslag till nämndens ställningstagande
Beredningsgrupp anser att nyttan överväger lidandet och föreslår att ansökan bör
- Godkännas

Giltighetstid bör vara:
- 5 år från beslutsdatum

Försökets svårighetsgrad bedömer beredningsgruppen som:
- Måttlig

Utvärdering i efterhand
- Nej

Avgift
Beredningsgrupp bedömer avgiften som kategori
- 4. 15 000 kr

Handläggningstid
- Handläggningstiden kommer såvitt beredningsgruppen nu kan bedöma inte bli längre än 40 arbetsdagar

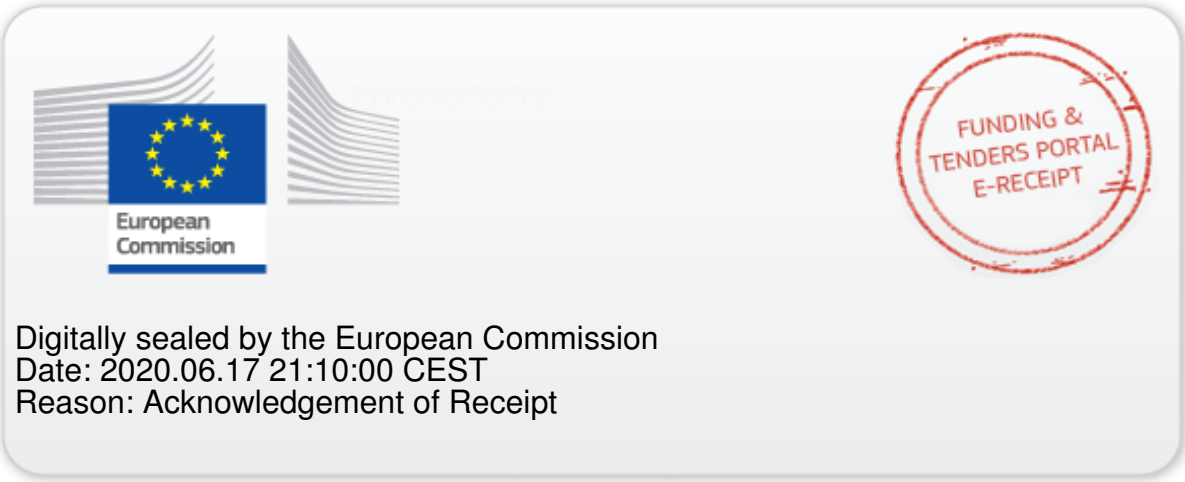
Ändringar som påverkar den populärvetenskapliga sammanfattningen
- Nej

Komplettering (bifogas som bilagor)
Ansökan har kompletterats:
- Frågor och svar via mail 2019-07-31

Undantag
- Nej

Deltagare i beredningsgruppen samt datum för sammanträde
Anna Kers Hagberg, Annica Wohlin Wottrich, Irja Eggertsen, Lars Larsson

Reservation
- Ingen



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