

## Application

Grant	Vernieuwingsimpuls Vici TTW 2018
Title	Ultra-resolution with visible light
Applicant	Prof. dr. B. Rieger
File number	17046
Your rebuttal on the referee reports is in progress	

## 1 - Assessment of the quality of the researcher

### Explanation

Criteria - the quality of the researcher: - in terms of profile fit in the target group; - in the top 10% of his/her international peer group; - academic excellence as demonstrated by numerous publications of international standing and/or other academic achievements; - inspiring enthusiasm for research and/or technology; - persuasiveness; - demonstrably capable of successfully developing own new innovative line of research; - has both a national and international prominent position; - demonstrable leadership and coaching skills. The Vici scheme aims at outstanding researchers only: the top 10% of his/her international peer group.

#### a.

What is your opinion on the past performance of the researcher (as demonstrated by publications and other relevant scientific achievements)?

#### comment referee 1

The past performance of the researcher is outstanding, particularly in the field of localization microscopy. By porting concepts from electron microscopy to localization microscopy he has truly advanced the field.

#### comment referee 2

The PI belongs to the few internationally well-recognized experts in the field of super-resolution microscopy with a special focus on data handling and analysis. He is especially well-known for his achievements and ideas in particle averaging methods, Fourier Ring Correlation Analysis to determine the spatial resolution of super-resolution images and his expert knowledge in cryogenic fluorescence imaging. He is definitely a very active leader in the field.

#### comment referee 3

Prof. Rieger is well known in the imaging field, particularly through his contributions to data analysis in super-resolution microscopy. Some of his seminal studies include the implementation of the FRC method in fluorescence nanoscopy, now a gold standard to evaluate resolution, and his work in single-particle analysis.

His impact in the research community can be seen by his regular invitation as a speaker in high-profile meetings.

#### comment referee 4

The applicant has excellent performance as a researcher. There is plenty of evidence in his research record of innovation and scientific advance in research that overlaps the computational and practical aspects of microscope imaging.

#### b.

Does the applicant belong to the top 10% of his/her international peer group? Which scientific achievements or talents of the applicant show that he/she belongs to this top?

#### comment referee 1

The applicant belongs to the top 10% of his peer group. As mentioned above, he has advanced the field of localization microscopy significantly. For example, his introduction of using FRC to measure resolution has become a standard tool in localization microscopy.

#### comment referee 2

Definitely, I would rather say he belongs to the top 1-3% of his international peer group. To be more precise, he is among the three worldwide leading scientists in the field. He has what it takes to push new methods to the next level by the development of new ideas and intelligent data analysis methods.

#### comment referee 3

Prof. Rieger is easily within the top 10% of his peer group. He has 6 >100 citation articles published in the last 10 years and regularly publishes in journals of high-standards. He also is known for actively participating in and organising internationally renowned meetings.

**comment referee 4**

I can confidently say that the applicant is one of the most highly talented researchers I know of in this field at this career stage. It is commonly accepted in the field that the interface between optical and computational imaging is the new frontier. Because of this, many researchers profess expertise in this area. However, very few of them really do much more than tweaking existing methods. The applicant's work, in contrast, has been truly innovative and has made significant advances by really bringing together methods of optics and computation.

**c.**

To what extent is there sufficient evidence that the applicant has the ability to lead and supervise a research group and support staff and to coach young researchers?

**comment referee 1**

The applicant has a long track record of successfully leading a research group and scientific projects as well as coaching young researchers.

**comment referee 2**

The applicant has a long-standing experience in supervision of students and postdocs. He is excellently networked in the community to coach and support also young researchers.

**comment referee 3**

The applicant already has an extensive track record of supervising students and postdocs. From his published work it is evident that the members of his team produce high-quality research.

**comment referee 4**

He has supervised ~30 bachelor and masters students. He was co-promoter of 7 PhD students and current promoter of 5 PhD students. He is or has supervised 5 postdocs. There is enough evidence here to convince me he can lead a group.

**2. - Assessment of the quality, innovative character and academic impact of the proposed research****Explanation**

Criteria - the quality, innovative character and academic impact of the proposed research - challenging content; - originality of the research question; - innovative scientific elements; - aimed at building up a new line of research; - potential to make important contributions to science; - effectiveness of proposed methodology; - international importance of the proposed research area.

**a.**

Please comment on the relevance of the problem and on the originality and challenging content of the proposal.

**comment referee 1**

Currently, functional imaging can be provided by fluorescence microscopy, but at a limited resolution, even with super-resolution methods. Cryo electron microscopy offers resolution in the Angstrom range, but cannot provide functional information. Therefore, pushing the resolution of FM to the single nm range would allow a direct combination with cryo-EM, and hence the combination of functional and structural imaging on the molecular scale. This is a big challenge in the field where currently no solution exists. Combining 4pi interferometric detection with STED and polarization controlled excitation is a truly novel concept for increasing sparsity of fluorescent signals.

**comment referee 2**

Super-resolution microscopy enabled us to study cells and structures with so far unmatched spatial resolution down to approximately 20 nm. So far higher resolutions cannot be achieved due to several problems including inefficient labeling of the target molecules and limited number of detected photons per emitter because of inefficient photon collection and fast photobleaching of the dyes. In order to enable ultrastructural investigations of e.g. molecular complexes supporting life the resolution of light microscopy has to be further increased. Here, the applicant suggests to bypass these limitations by developing cryo-light microscopy with 4pi detection geometry. This will enable him to increase the localization precision of fluorescent dyes by approximately an order of magnitude in all three dimensions and thus to achieve a spatial resolution in the few nanometer range. For temporal separation of individual emitters, the applicant proposes to use polarized irradiation to excite only a sparse subset of dyes at any time of the experiment. Finally, to bypass the problem associated with inefficient labeling the applicant proposes to use single particle averaging techniques developed by himself already very successfully (see recent publication in Nat Methods).

To summarize the proposed work is highly innovative and addresses several limitations of current super-resolution microscopy methods. It is highly challenging but given the experience of the applicant I am convinced that the proposed research will break new ground.

**comment referee 3**

The project proposed aims to develop a new method that combines the use of template-free single-particle analysis (SPA) recently developed by the applicants lab with a new optical super-resolution design. The new optical system will be based

on a STED-4Pi configuration and use a cryo-stage to image the sample at low temperature (akin to cryo-EM), effectively reaching a near-bleaching free regime. The resolution achieved in this optical design is still limited by the labelling density of the sample, to compensate for this factor the applicant proposes to use the computational analysis and modelling of hundreds of similar structures to generate supramolecular structural models with a precision as low as 1nm.

The proposed system is unique, and combines next-generation concepts in optical hardware, low-temperature imaging and computational analysis. It has a high-potential to impact the cell biology research field. Notwithstanding, it is important to note that the proposal has not identified a biological question that can be solved by the proposed system. The applicant does remark an interest in Nups and NPCs, as well as the potential application of this system to study protein and host-pathogen interactions. However, no discussion exists on what particular details in biology can be solved by the proposed design in comparison to other systems.

**comment referee 4**

Super-resolution is a very important area of microscopy research that is leading to important discoveries in the biomedical sciences. While it is very common now to perform imaging at the ~10nm scale, it is a huge challenge to move further down to the ~1nm scale. The proposal concerns the adaptation of microscope and image processing methods to enable this jump in resolution. This jump in resolution is a huge challenge that could not be achieved by optics alone. The approach presented here looks highly innovative.

**b.**

What are the innovative aspects of the proposal? Will the research break new ground by generating new concepts, a deeper understanding, new methods, etc.?

**comment referee 1**

The innovative aspect is the combination of 4pi, STED and polarization controlled excitation in cryo-FM to increase sparsity of fluorescent signals. For later correlation of cryo-FM and cryo-EM data, a novel particle picking strategy is presented. While I am very confident that the applicant and his group would be successful in implementing working package 1 (3D data fusion), I have serious doubts about the experimental realization of working package 2 (4pi cryo). From the optical point of view the presented projects are very well thought through. However, one of the biggest obstacles currently hindering to push the resolution further in cryo-FM is devitrification of the sample due to heating up the sample too much by the laser irradiation to achieve sufficient sparsity of the fluorescent signals for reaching high single molecule localization accuracies. Both publications (Chang et al., 2014, Nat. Methods; Liu et al., 2015, Sci. Rep.) which demonstrated successfully super-resolution cryo-FM in combination with cryo-EM highlighted this issue as one of the biggest problems. The applicant does not mention this anywhere in his proposal, nor does he propose a possible solution. The only hint that I could find was in one of his very recent publications (Hulleman et al., 2018, Small Methods), where a modified design for the holder of an EM grid in a setup for cryo-FM is proposed to improve heat transfer for the planned STED imaging. This is in my opinion a misconception, as the devitrification is a local problem (cf. Fig. S1 in Chang et al., 2014, Nat. Methods). The heat conductivity of vitrified water is very low (~400x lower than copper (Andersson & Suga, 2002, Phys. Rev. B)). Therefore, improved heat conductivity between EM grid and holder will probably not have a significant effect regarding the heat transfer in the sample where it is irradiated by the laser. Additionally, STED uses typically laser intensities that are 3-6 orders of magnitude higher than SMLM, which was used by Chang et al. and Liu et al. Because of that fact, a discussion of how the proposed method would deal with devitrification of the sample due to high laser intensities is crucial, but, unfortunately missing in the application. The overall goal of the proposal is to achieve 1 nm resolution in 3D. The whole project will not be successful if the laser intensities required for the polarized STED are too high to keep the sample in a vitrified state.

**comment referee 2**

There are several innovative aspects including the 4pi detection geometry combined cryo-light microscopy. The most innovative aspects are, however, the development of improved particle averaging methods and the polarized excitation to achieve temporal separation of emitters. The new method has the potential to revolutionize the field in the next years because it will enable for the first time near-field resolution (a few nanometers) by far-field imaging.

**comment referee 3**

The proposed system, and the underlying analytical methods to be applied are novel and may be groundbreaking by providing the capacity to image biological complexes at high-resolution in 3D. The system may enable new biological questions to be interrogated, but these are unfortunately not identified in the application.

**comment referee 4**

There are several ground breaking innovations in the proposal: Using combinations of photophysical and biological models, in addition to mathematical models of symmetry and statistics, enabled by efficient computation; Challenging optical systems (4pi, aberration and polarisation controlled STED) for imaging single molecules; The extraction of increased information about single molecules.

**c.**

What is your opinion on its potential to make a major contribution to the advancement of scholarship, science or technology (academic impact)?

**comment referee 1**

If the proposed project would be successful (cf. problematic issue in 2b), the impact would be huge. It would introduce a novel tool for structural biology that would open a wide range of new application possibilities. However, as mentioned above, a successful experimental implementation is very risky as one of the most crucial issues has not been discussed by the applicant.

**comment referee 2**

The potential of the proposed technique is very high for the advancement of scholarship, science and technology. The method can bridge the currently existing resolution gap between super-resolution microscopy and cryo-EM and x-ray crystallography and thus allow scientists for the first time to study the 3D molecular architecture of multiprotein complexes and molecular assemblies

**comment referee 3**

The proposed analytics and optical system will considerably advance the microcopy, optical physics and computational sciences field. The impact in the cell biology and biomedical field is unclear, particularly because the applicant doesn't intend to hire biologists or provides details of collaborations with cell biologists which will benefit from these developments.

**comment referee 4**

This is a different approach to most work in this field and it therefore brings something new. For this reason, it is likely to have a more significant impact than most technology development work taking place in this field. Its academic impact will therefore be high.

**d.**

To what extent is the proposed method effective? Please comment.

**comment referee 1**

As mentioned above, the working packages formulated in the proposal are very well thought through. However, working package 2 is missing one crucial topic that is currently one of the biggest problems for increasing resolution in cryo-FM. Without addressing this issue, a successful experimental implementation is very risky.

**comment referee 2**

The proposed method is highly effective, see details given above.

**comment referee 3**

The proposed system is perfectly design to achieve its main goal - the description of fluorescently labelled biological structures with isotropic resolution down to 1nm. It tackles most of the major challenges to achieve this bold aim. I believe this is a well designed proposal describing an effective method. Although highly risky, as there aren't that many groups having been able to formalise a 4pi-STED system, the track record of the applicant plus collaborations attest to formalise this project.

**comment referee 4**

From the descriptions provided, I have high confidence this will be effective. While the ambition is high, it is based upon sound groundings. Some of the methods will be challenging, but there is sufficient background in the applicant's own research and sufficient support from collaborators to make the advances feasible. The work is in two packages, which are related, but somehow independent. This means that delays or complications in one will not affect performance in the other.

### **3 - Assessment of the knowledge utilisation**

#### **Explanation**

Criteria - knowledge utilisation (KU) Potential - contribution to society and/or other academic areas; - disciplines and organisations that might benefit from the results. Implementation - action plan to allow the outcomes of the research project to benefit the potential knowledge users; - if and how the potential knowledge users will be involved; - (concrete) outcomes for society and/or other academic disciplines; - the period over which knowledge utilisation is expected to occur. The selection committee assesses: - whether the applicant has given a realistic description of the potential for knowledge utilisation - and to what extent the applicant has presented a concrete and convincing plan for the implementation of the available potential. - If a researcher is of the opinion that the proposed research is not appropriate for KU then he/she should explain why he/she thinks that KU is not applicable. The selection committee will assess the arguments given for this

**a.**

What is your opinion on the described potential for knowledge utilisation ?

**comment referee 1**

If a microscope could be constructed that would achieve 1 nm resolution in vitrified samples, the whole cryo-EM community would tremendously benefit from such a technology. There are many questions in structural biology that currently cannot be answered with cryo-EM or related methods alone. As the applicant has strong links to commercial partners, KU would seem very realistic.

**comment referee 2**

I am convinced that the proposed method can be used successfully to solve emerging problems already during the project period.

**comment referee 3**

The applicant has identified knowledge utilisation. Namely, the potential application of the proposed system to study biological systems, such as molecular complexes at the nanoscale, in health and disease. There is also a description on how both the hardware and software specifications will be provided to the community. The period for KU given is realistic.

**comment referee 4**

There is great potential for utilisation of the knowledge generated in this research. There is wide interest in the applications of these innovative microscopy methods. This will particularly benefit biological and medical sciences, with consequent benefits in health and wellbeing, in addition to the basic understanding of the basis of life.

**b.**

Please comment on the effectiveness and feasibility of the proposed approach for realising KU (implementation).

**comment referee 1**

The applicant has already established strong links to commercial partners as well as biological collaborators. I have no doubt that the applicant would be able to realize KU in the form of developing a commercial product that can be used for biological applications. The applicant also has a strong track record of making software and algorithms freely available for the community. The effectiveness and feasibility if of course also linked to whether the experimental realization is feasible at all (see 2b).

**comment referee 2**

The applicant will support KU by involving different companies who will profit from the developments.

**comment referee 3**

The applicant already has an outstanding track record of KU, particularly in the development of software widely up-taken by the community. I expect that the applicant will be able to fully realise the KU proposed.

**comment referee 4**

It is clear that the applicant has thought carefully about knowledge transfer. Some of the important developments will be software based and the applicant has already demonstrated the sharing of methods widely in the research community. The project will be in collaboration with the company Delmic, who could act as a channel for commercialisation of the systems. The applicant has connections to other industry partners too. The instruments themselves will be housed in a facility in Delft, to which others can have access, and there will be a user committee to gather feedback. This is a thorough plan for dissemination.

**c.**

Only answer this question in case the applicant argued that KU is not to be expected given the nature of the research proposal: Does the applicant convincingly explain why KU is not applicable for his/her research project (see also the information under criterion 3 listed above)?

**comment referee 1**

NA

**comment referee 2**

---

**comment referee 3**

NA

**comment referee 4**

Not applicable.

**4 - Final assessment**

**a.**

How do you assess the entire application? Please give your final scoring (A+/A/B/UF/U).

**comments referee 1**

02, A High quality, significance and recommendation for funding

**comments referee 2**

01, A+ Highest quality, significance and recommendation for funding

**comments referee 3**

02, A High quality, significance and recommendation for funding

**comments referee 4**

01, A+ Highest quality, significance and recommendation for funding

**b.**

Could you please summarize (point by point) the strengths and weaknesses of the grant application focussing on the candidate, proposal and knowledge utilisation?

**comment referee 1**

Strengths:

Candidate:

- substantial contributions to the field of SMLM
- high impact publications
- long track record for successfully leading a research group

Proposal:

- solid plan for data fusion, particle picking and averaging
- novel idea for generating sparsity of fluorescent signals under cryo conditions

KU:

- strong links with commercial partners
- remarkable examples such like DIPimage library

Weaknesses:

Candidate:

none

Proposal:

- One of the biggest problems in increasing the resolution in fluorescence microscopy under cryo conditions is devitrification due to high laser intensities. This issue is totally missing in the proposal. The applicant proposes STED for achieving high resolution, which particularly uses very high laser intensities. Without presenting a solution for this issue, a successful experimental realization is very risky.

KU:

none

**comment referee 2**

Excellent applicant with expert knowledge in all aspects required to perform the proposed research. Highly innovative and ground-breaking new concepts that have the potential to revolutionize the field. I am strongly supporting the proposal. There is no real weakness.

**comment referee 3**

I believe this is an outstanding project with only one major weakness - the lack of description of the biology studies the proposed system will enable, and how those studies cannot be addressed with other approaches.

**comment referee 4**

Strengths:

- The applicant has an excellent record of quality innovative research.
- The proposal is a genuine fusion of novel optical and computational methods, more so than most other work in this field.
- The methods are highly relevant to an expanding field of research in single molecule microscopy.
- There is likely to be uptake of the methods developed by users in the field, which will increase the benefit of this research.

Weaknesses:

- The two work packages are somewhat independent of each other, so could be run separately. But both are highly worthwhile and relevant to the overall theme, so this is only a minor issue.
- There is little explicitly presented in the proposal about the ultimate biomedical applications of the methods. However, there are close links with biological collaborators and past record in this area, so I am sure this will be covered. Hence, this is a minor issue.

## **Datamanagement**

### **Explanation**

Responsible data management is part of good research. For the collection/generation of data and the analysis of this data, timely measures need to be taken to ensure the storage and later reuse of the data. This means that prior to the start of the research project researchers must ascertain a) which data could be relevant and b) how these data could be stored. After a proposal has been awarded funding, the researcher will draw up a detailed data management plan in which the researcher explains how all relevant data will be made findable, accessible, interoperable and reusable (FAIR). The data management section( 2e) is not an assessment criterion for the research proposal. However for all the data management sections of these proposals, you can make suggestions and give advice that could be helpful for the researcher in drawing up the data management plan to be submitted after funding was awarded. See also [www.nwo.nl/datamanagement](http://www.nwo.nl/datamanagement)

-

If you have any suggestions or advice on the data management plan please state it here, otherwise type n/a.

#### **comment referee 1**

NA

#### **comment referee 2**

The applicant is an expert in data management. hence, I cannot recommend anything he does not already know....

#### **comment referee 3**

Data management seems to be fine.

#### **comment referee 4**

The plans are well thought out and adequate for this project.

## **Overall**

### **Overall rebuttal**

**Maximum number of words for the reaction on all referee reports**

1500