

Ultra-resolution with visible light

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I want to thank all reviewers for their valuable time reading and commenting my proposal. In particular I was delighted that all reviewers see me as an outstanding researcher in the field and rank me easily within the top 10% of my peer group.

Please find below my response to the individual reviewers.

Reviewer 1 (A):

I especially thank the reviewer for his/her compliments on my knowledge utilization for “strong links with commercial partners” and “remarkable examples such like DIPimage library” and my “substantial contribution to the field of single molecule localization microscopy”. This Vici project “would introduce a novel tool for structural biology that would open a wide range of new application possibilities.”

The reviewer voices only one concern, returning at several points, namely “devitrification of the sample”. I.e. laser heating could locally raise the temperature above the glass temperature changing amorphous ice to crystalline ice above 135 K. Indeed the reviewer is right that I should (and could) have discussed this in the proposal. More so as I had considered this issue to such an extent that we monitored heating of the sample in our current single objective lens cryo-setup and performed finite-element simulations (COMSOL) of the heat transfer under laser illumination for the proposed setup. In short, the reviewers concerns appear to be no problem and devitrification will not happen in our setup as the temperature only rises to max. 94 K.

The long version: **1)** If we only consider fluorescence light microscopy any ice crystals occurring above the glass temperature are invisible! This will therefore not affect the imaging *with light* of macromolecular complexes at 1 nm resolution at all. If we consider correlative imaging with electrons after imaging with light, ice crystals would render the sample opaque to electrons, but this is highly unlikely as detailed in the following. **2)** The two literature references given by the reviewer state devitrification as a big problem, but they both use a very different setup (and different fluorescent labels) than in my proposal. They use irradiation with 1.5 kW/cm² @405 nm as upper bound, but we irradiate at 800 nm, which has a ~2000x lower cross section in tissue [1], implying that CW STED intensities with 1-10 MW/cm² are acceptable. In addition, we will not use highly absorbing carbon film as support that heats the sample. COMSOL simulations for our proposed setup with absorption in ice, a realistic illumination scheme, sample holder geometry and cooling showed ~14 K temperature increase from 80 K to 94 K, which is well below the critical temperature of 135 K. Even stronger, of this 14 K increase, only ~2 K stem from the laser and the rest from environmental room temperature irradiation. This suggests that heat shields between the objectives and sample could be added to reduce the temperature increase to a mere 2 K. Preliminary experiments on our current single objective system show only about 10 K temperature increase (steady state) when using 1 W of laser power (resulting in about 10 MW/cm² on the sample). **3)** The different strategies that the two papers suggest for reducing the heating effect are fully compatible with our setup, so we could adopt both if needed.

Reviewer 2 (A+):

I thank the reviewer for all his/her compliments, in particular on the “highly innovative and ground-breaking” nature of the proposal.

Reviewer 3 (A):

I thank the reviewer for his/her compliments that “the proposed system is perfectly designed to achieve its main goal - the description of fluorescently labelled biological structures with isotropic

resolution down to 1nm". The reviewer raised as only minor weakness of the proposal "the lack of description of the biology studies" except the Nuclear Pore Complex (NPC) and host-pathogen interactions and that I "do not intend to hire biologists".

My project has the primary objective to develop the setup and algorithmic methodology. Biological studies will be explored in the end phase. Infrastructure for this is already available at the faculty and I have an extensive network of biological collaborators to do so. Examples of explorative biological studies are next to the mentioned identification of nucleoporins in the NPC, identification and structuring of the Guanylate binding proteins involved in the binding to the parasitophorous vacuole in intracellular pathogen defense [2] and identification and structuring of clathrin-coated endocytic vesicles [3]. The latter two in close collaboration with Arjen Jacobi (TU Delft), mentioned in the proposal.

Reviewer 4 (A+):

I thank the reviewer for his/her compliments on the project being a "genuine fusion of novel optical and computational methods". The reviewer raised as only minor point that "ultimate biomedical applications of the methods" are not explicitly mentioned but incorporated via "close links with biological collaborators and past record in this area". Indeed, my view on biological applications is that they can only be meaningfully addressed in collaboration with biologists. Please also see the comment for Reviewer 3 on this point.

- [1] S.L. Jacques. *Optical properties of biological tissues: a review*. Physics in Medicine and Biology, 58(11):R37, 2013.
- [2] E. Kravets, D. Degrandi, Q. Ma, T.-O. Peulen, V. Klümpers, S. Gelekyna, R. Kühnemuth, S. Weidtkamp-Peters, C.A.M. Seidel, K. Pfeffer. *Guanylate binding proteins directly attack Toxoplasma gondii via supramolecular complexes*. eLIFE, 5:e11479, 2016.
- [3] M. Kaksonen and A. Roux. *Mechanisms of clathrin-mediated endocytosis*. Nature Reviews Molecular Cell Biology, 19:313-326, 2018.

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