Referee's report on PhD Thesis of Ing. Martin Ovesný titled "Computational methods in single localization microscopy".

Martin Ovesný has chosen a topical field for his study. The subject of his thesis is aimed at the development of new software to analyze SMLM data and to obtain super-resolution images.

The thesis is organized into six chapters. 1) Introduction that provides a brief list of the methods of super-resolution optical microscopy, with a subchapter describing the scope of the thesis. 2) Single molecule localization microscopy in which a brief description of SMLM principles and related technical problems is presented. 3) ThunderSTORM in which the structure of this software package is introduced together with a brief explanation of the design of particular steps in data processing. 4) Dual-objective microscope describing the construction of microscope, its calibration and performance. 5) Improving temporal resolution of 3D SMLM which seems to be the most important part of the thesis with a detailed description of achievements in the field of high-density 3D-SMLM. 6) Conclusion. In total this thesis has 119 pages of text including 40 Figures, followed by an extensive list of references.

While the majority of thesis text is clear and easy to read, there are also some parts that are not well written. For example, it is difficult, if not impossible to capture the design of ThunderSTORM cross-correlation procedure just by reading the text presented in p. 50. Similarly, the subchapter on the use of wavelets is quite vague. Obviously, the development of ThunderSTORM has been an enormously big project. Therefore it is clear that the ThunderSTORM structure, its underlying technical solutions and reasons for their choice cannot be outlined in detail within the framework of a single thesis 120 pages long. However, an alternative to the rather sketchy Chapter 3 could be just a brief introduction to the software structure at the beginning of a chapter based on the selection of a particular ThunderSTORM part with the highest personal contribution of the student, presented in eligible detailed way. Such an option could be considered for oral presentation of the thesis.

The quality of thesis layout is very high. Unfortunately, the use of hard glossy paper led to practical problems with keeping this book open at a desktop. Number of misprints is negligible. The author used appropriate language and style. However, I have one critical comment regarding the formal aspects of the thesis. Many figure legends do not present sufficient information about the figure because they are just a title without an eligible description of presented items. For example, the legend to Fig. 3.2 does not provide any clue to understand why the 1st and the 3rd molecule were merged, but not the 2nd molecule. Similarly, it is not presented in the legends to Figs. 3.9 and 3.10 how the input data were acquired.

A few specific comments on the text and list of trivial errors are presented below:

p.2 - Note that the terms chromophore and fluorophore are not interchangeable.

p.2, row 6 from bottom - It is better to say that "molecule can absorb" instead of "single electron can absorb". Not photon, but a valence electron is excited to a higher quantum state.

p.4 - Eq.1.1 does not represent Abbe sine condition, but just the Abbe's formula describing objective resolution.

p.6, row 7 - Not the excitation wavelength is used in the Rayleigh formula, but the emission wavelength. Not the magnification of the objective is a primary factor to ensure that images comply with the Nyquist criterion, but the size of pixels of the detector.

p.7- TIRF is not a tool to achieve true optical sectioning since it makes it possible to visualize only the surface layer of an examined sample that is in contact with microscope slide.

p.9 - A fuzzy description of SNOM that does not discriminate between the excitation light and fluorescence detector.

p.11 - In addition to 4Pi, there are also other possibilities to improve axial resolution of STED microscopy. In particular, high axial resolution can be achieved using annular phase mask to control the intensity distribution in the focus of depletion beam.

p.35 - Symbols used in Eq.3.7 are not properly explained. Moreover, the related reference (Šonka et al. 2007) cannot be found in the reference list.

p.47 - Fig.3.2 – insufficient figure legend.

p. 104 - It is not clear from the presented text how the adequacy of gaussian approximation of PSF can be improved by using better corrected lenses.

A clear proof of the quality of achieved results is the result of evaluation of existing software packages for SMLM data processing performed by Sage et al. (Nature Methods, 2015). ThunderSTORM was indicated as the best software in the category long-sequence (LS) data processing. As the processing of high-density (HD) is concerned, the position of ThunderSTORM was in about the mid of the list of compared software packages. However, this feature of ThunderSTORM was dramatically improved in the new 3D-version described in the Chapter 5. In the last chapter of the thesis, titled Conclusions, there is a summary of achievements of Guy Hagen's team without a clear specification of personal contribution of Martin Ovesný. This important information must be present by the student during his thesis presentation on December 5, 2016.

Conclusions:

Despite some criticism raised above, the main results presented in this thesis can be referred as new and valuable. The thesis of ing. Martin Ovesný meets the requirements of Ph.D. program, thus **the author can be given the desired degree**.

Prague, November 4, 2016

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