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Review of the PhD-Thesis in Biophysics, presented by Mgr. Tomáš NÁHLÍK, Faculty of Science, University of South Bohemia

with the title: "Microscopy – Point Spread Function, Focus, Resolution"

Vienna, 04.01.2016

The thesis of Tomáš Náhlík deals with a very important topic of Biophysics, as it examines and discusses **the application of physical instruments as the microscopes to biological objects and samples** and studies their interaction in a very thorough and advanced way. Namely, the question in this respect is always, what do we see by the microscope and what is the true image, which we are supposed to see. Just now, in this age of great technical advancements, it is a chance to optimize and improve the instruments themselves as well as their results by optimizing their interpretation. This is just what Tomáš Náhlík is attempting to do in his presented thesis.

The thesis is solidly based on 4 scientific articles already published, 2 in journals with Impact Factor, and 2 without.

The thesis first describes in a well understandable and comprehensive way the state of the art in its introduction, divided into three parts, the matter of light emission and light diffraction, the problems of correct focus of an image, and the differences between resolution, by which microscopes are usually characterized, and the distinguishability versus the discriminability of individual points.

With modern digital imaging additional problems occur and ask for optimal software application. Tomáš Náhlík's personal approach to a better solution of the problem of interpreting microscopic images is the **point spread function;** he also compares his approach with others used by some of his colleagues.

The special aim of this thesis is to introduce methods for **better discrimination of the objects in the microscopic image.** To fathom the outer limits of this discrimination nanoparticles were studies and simulated. Only by a solid understanding of the principles of microscopy, and here especially of light microscopy, it is possible to go further into improvements of results. Therefore simulations of created images by the light microscope were made and compared to the

real achieved images from the microscope in use. From this, new algorithms for advanced image analysis were developed. For better understanding, different models of the Point Spread Function (PSF) were created.

As equipment for his studies, Tomáš Náhlík used a specific microscope developed in his institute, the Nanoscope, especially versatile and adapted for the experiment needs and with a camera of down to 20x20 nm pixel size. Experiments were also done with Nanoparticles at the CLSM (Confocal-Laser-Scan Microscope). The analysis was based on Z-scan of the latex particles in bright field imaging. The core of PSF is searched for by PDG-model (Point Divergence Gain).

Some questions I would like to pose to the candidate:

1) What basic principles must be considered for optimal setting of the microscope used?
2) On what does the size of the PSF and the PSF core depend?
3) What are the specific properties and adjustments of the nanoscope?

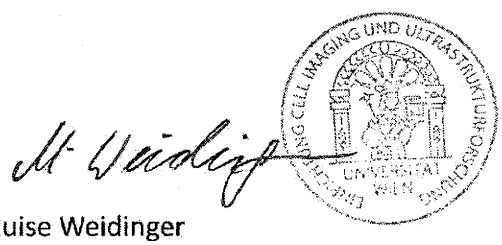
The results of the thesis consist of developed algorithms used for software improval of image analysis. These mathematical calculations are not quite my field of competence, since I do not develop software. I do see that the goal and the approach to a solution are correct, since as electron microscopist I deal a lot with discrimination of individual points in practice. Interesting I find the topic of focus as we vary the focus for different purposes in electron microscopy and the contrast plays an important role. I am looking forward to the discussion in this respect.

In conclusion, I appreciate the intensive effort to improve the interpretation of microscopic images as near to truth as possible by optimizing the best possible microscope technique with the best possible camera technique and the best possible software to achieve ever better imaging in future.

Some minor suggestions to the thesis of Tomáš Náhlík would be:

1) Biological images should be better described by mentioning the name of the sample and the type of cells as well as adding magnification bars.
2) In view of the comprehensiveness of this work, I recommend that it be published as a monograph, but that a native speaker of English language should review and correct it first.

Finally, I wish to say that I was very happy to read and review this excellent thesis and greatly recommend it for PhD- defensio. Consequently, I recommend that after successful defensio, the candidate Mgr. Tomáš Náhlík may take his doctorate degree and be awarded the academic title of PhD.



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Review: phd thesis Tomas NAHLIK
Microscopy-Point Spread Function, Focus, Resolution

Mr. Nahlik reports in his thesis the development of a novel computer algorithm, which allows to re-construct a three dimensional image of a microscopic object from a sequence of images obtained by standard optical bright-field microscopy with a resolution below Abbe´s diffraction limit. Resolution below the diffraction limit is obtained due to the small pixel size of modern CCD cameras which correlates to a high spatial resolution. The possibility to obtain such "super-resolution microscopy" is an important and fundamental issue of modern microscopic technologies, was successfully realized up to now only for fluorescence microscopy. In that case fluorescence labeling is needed to obtain images and temporally controlled emission from individual molecular labels allow to resolve the intensity distribution within the Airy discs of individual fluorophores. The novel technology would, however, give superresolution 3-dimensional images under label free conditions.

The technology described by Mr. Nahlik is thus a fundamental and novel extension of modern microscopy.

In his thesis Mr. Nahlik gave a clear and broad introduction into the theory of the fundamentals of microscopic image reconstruction under the limits of the variety of lens aberrations and limits of the CCD cameras. Especially simulations based on the extended Nijboer-Zernike diffraction theory were used to generate full three-

dimensional images off the limits of individual focal planes. These extended simulations were applied to a detailed study of the dependence of the discriminability of two real points in the image in dependence of their microscopic distance. Such simulations resulted also in the definition of "resolution" after application of the novel algorithms and allowed to understand the influence of aberration of the system and electric noise of the CCD array. Also the term "focal point" needed a redefinition for a full three-dimensional image. Image processing resulting from these considerations were than based on information theoretical approaches using information entropy as a discriminator to optimize the information content extracted from the series of images. It could be demonstrated that the optimal resolution available is limited by the actual pixel size used. Higher integrated CCD array would thus yield a resolution of probably 10 times larger when compared to normal diffraction limited microscopes.

Experimental verification of the theoretical and computational approach was presented using samples of latex particles of different size and density. He could show that the experimental results are in line with the simulations and in accord with the theoretical predictions.

First applications to living cells showed possible future applications. The collection of publications in peer reviewed journals demonstrates also the quality of he work.

In summary, Mr. Nahlik presented an excellent overview on these novel computational algorithms, their theoretical foundation and their applicability to generate full three-dimensional images from standard bright-field microscopes with a resolution far below the refraction limit and without using any chemical labelling techniques. It is clear that such a novel technology must be further evaluated in different fields and that this exceeds the limits of a single phd-thesis.

I recommend, therefore, this consistent and detailed work to be defended as a self-contained and completed thesis.

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Brno, January 7, 2016

Review of doctoral thesis submitted by Tomáš Náhlík

The thesis deals with an important topic of resolution capabilities of optical microscopes and degradations caused by the optics. This is an old topic but the author tackles it from a slightly different perspective than many other authors, namely from the point view of information contained in the acquired digital image rather than concentrating purely on optical phenomena. I appreciate this approach and find it interesting also for further research.

The thesis consists of a theoretical part (about one third) and a practical one (about two thirds) followed by several appendices. The theoretical part reviews the relevant terms and phenomena of optics as well as basic image processing aspects. The experimental part contains methods, obtained results and discussion. Because mostly experimental research was carried out, the thesis contains numerous figures and tables illustrating the results of the experiments (both simulated and real data).

In my opinion, the main contribution of the thesis is the development of a simulation framework capable of producing theoretical 3D PSF images including the effect of selected optical aberrations and comparison of theoretical PSF images with experimental ones obtained using beads of various diameters. Such comparisons were performed for different conditions and dependence on selected parameters was studied (especially on illumination intensity and wavelength). Additionally, the thesis presents several image processing methods suitable for images containing signal from point sources or small objects. Mainly, an approach to determine the core of a blurred image of a point-like source is studied.

The experimental part contains also some sections that I do not find much innovative or persuading. For example, Least Information Loss (LIL) conversion of 12bit image to 8bit image seems to be just a simple linear stretch operation applied to 12bit image before its conversion to 8bit. Such operation is known and used in many software packages for many years for visualization purposes (for analysis purposes no conversion should be performed). Also it is not explained what advantage it brings to introduce the terms discriminability, distinguishability and resolution in a new sense. The idea of having such "digital world terms" is interesting but the terms are not properly introduced (e.g., there is no distingushability example in 5.3, example of not discriminable particles in fig. 5.22 contains valey between maxima, also in fig.5.23 it is unclear why the peaks are not discriminable) and no application is shown. Moreover the definitions depend on bit depth, which is very unfortunate.

Concerning the major weaknesses of the thesis I have to mention the following:

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1) The comparison between simulated and real images is primarily done visually, not quantitatively; also tuning of the simulator parameters (e.g., optical aberration strengths) to resemble real data is done by hand, not using some optimization procedure.

2) Discussion of the obtained results is rather limited; some sections contain plenty of figures but very little text.

3) Comparisons with other available methods is often missing, especially there is no comparison of suggested entropy-based focusing criterion with other available autofocusing functions.

4) Many definitions are imprecise or vague, e.g. "phenomenon of Point Spread Function" (p.1, function is not a phenomenon), PSF is "transfer function" (p.1, p.3, not true, transfer function is a Fourier transform of PSF), "transfer function is understood as a process" (p.3, function is not a process), "focus is a special distance" (p.14, focus is not a distance), Eq. 2.8. (p.17, symbols are wrongly defined after the equation), references to Fig. 2.9 (p.20, exchanged A and B), "proper color channel is only selected" (p.29, channel selection is not segmentation), "g is convolution function" (p.34, g is not function, it is kernel), "deconvolution kernel" (p.35, should be convolution kernel), unexplained where half of green pixels disappear in fig. 5.2 (p.49), "it can be proven" (p.56, where is the proof or reference to it?), "contour corresponds to the center" (p.56, contour is not a point), values in the last row of table 5.4 incorrect (copied from previous row), "automatic procedures to suppress the PSF" (p.73, PSF cannot be suppressed!), "bright field microscopy better than CLSM" (p.73, better in what respect?), "agarose means comparable" (p.73, I doubt, agarose can cause change of refractive index and completely different properties), "phase contrast" (p.76, non-systematic, why sudden change to phase contrast?), "red lines" and "positions 184, 150" in fig. 5.35 (p.81, not present in the figure), "position" is "part of" (p. 82, point is not region), "I was not able" (p.92, you could put optical filters in front of camera), "deconvolution kernel in blind deconvolution" (p.93, then blind would become non-blind), "cannot be processed" (p.94, why, processing at higher bit depth is even advantageous), "3D model of the cell" (p.94, p.98, very distant future, with bright-field impossible to reach), fig. 7.2. (p.97) – suddenly morphological processing of non-point sources without any previous introduction of morphology operations, not related to previous text, Appendix A – not clear for which experiment these settings hold (also too long listings including needless information like zeros).

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5) Some parts of state-of-the-art are obsolete, e.g. Trainable segmentation (p.33-34) with references from 1990s or VEM method from 1980s (p.92).

6) Some conclusions are questionable or incorrect, especially the claim that "in these days are limits for detecting of small objects given by resolution (number of pixels) of used camera" (p.91), I disagree, limit is given by Shannon-Nyquist sampling theorem, it does not make sense to sample microscope image using very high-resolution cameras because the image is band-limited. Also how can knowledge of PSF lead us to definition of optimal focus" (p.91)? Why do we have "minimal intensity of the electrical field in focus" (p.91)?

7) Typesetting is very poor, there are many pages with very large free space left and also fonts in figures are often extremely small to read.

8) English language needed improvement, there are basic mistakes like missing "it" before "was" (e.g., twice in annotation), wrong word order like "are presented limits" (p.1) or "is discussed the problem" (p.2), wrong "'s" usage like "samples's" (p.3) or "lens' aperture" (p.18), wrong passive voice like "can be consider" (p.5, p.33, p.34) or "can be transform" (p.18), missing "s" like "light interact" (p.5, p.6), "on figure" (everywhere) or "on institute" (p.40), wrong modal verb usage like "it can works" (p.40), "be" missing in passive voice like "can easily selected" (p.40), "in according to" (p.44), etc.

9) Many references are incomplete (e.g., missing source, just authors and title, examples: 16, 34, 41-44, 48, 62, 67-69, 72, 78).

Although the thesis deserved a major revision based on the comments above, in spite of these weaknesses, it is clear that the author did a lot of work (mainly experimental), is capable of publishing the results (especially publication IV is relevant to the topic of the thesis) and deserves therefore the PhD title.

Michal Kozubek