PHD-THESIS SYNOPSIS

Studying and correcting the optical aberrations and reconstruction errors in localization based super-resolution microscopy

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1. Introduction

Optical microscope is an effective tool in the biological and biomedical research to investigate samples in non-invasive way. One main direction in the development of microscopes is to improve the resolution (to achieve larger magnification) and therefore resolve finer structures in the sample and acquire more detailed images. In the last few years, the evolvement of the so-called super-resolution fluorescent microscopy techniques was the most highlighted discovery in the microscopy. In 2014 the Nobel Prize in Chemistry was awarded for the development of super-resolved fluorescence microscopy. This kind of microscopes operates in the optical wavelength range and with resolution at few tens of nanometer. One of their advantages is that they do not need more complex sample preparation or optical imaging system than the conventional fluorescent microscopy technique. The main topic of my thesis is to study particularly one type of these new super-resolution microscopy techniques, the so called localization based super-resolution microscopy technique. The effects of optical aberrations and artifacts emerged during the image processing were analyzed. The sources of the errors appeared in the imaging were investigated and in all cases a correction or optimization method was suggested and realized.

2. Theoretical background

To quantify a microscope imaging ability, the spatial resolution is a great measure. Several concepts were emerged to define the resolution and determine the limit of that. Widely used ones are the Rayleigh and the Sparrow criterion for the resolution limit. These criterions define a Critical Distance (CD), where two point-like objects are placed at this distance and below that, these two points are not distinguishable after the imaging. Typically ~200 nm and ~ 500 nm resolutions can be achieved in lateral and axial direction, respectively, in the optical wavelength range and by using commercially available microscope objectives with high numerical aperture (~1.49). The resolution is limited by the diffraction of the light. However, the above mentioned limits can be exceeded using a trick during the imaging. The diffraction plays key rule in these super-resolution techniques too, but does not limit the

resolution. One type of these techniques is the localization based super-resolution microscopy technique. In the conventional fluorescence microscopy all of the fluorescent dye molecules placed in the excited area in the sample emit fluorescent photons in contrast to the localization based techniques where only a few molecules are in active state in the Region Of Interest (ROI) due to photochemical processes. These individual molecules are imaged by means of a high magnification imaging system and an ultrasensitive camera (EMCCD, Electron Multiplying CCD). The images of the molecules are fitted and the central positions (which are the positions of the molecules in the sample) are localized. Repeating this procedure by switching on new molecules and switching off the currently active ones, a map about the dye molecules can be achieved. This is a super-resolved image about the sample with a few tens of nanometer resolution. The localization based techniques are: PALM, PhotoActivated Localization Microscopy, STORM, Stochastic Optical Reconstruction Microscopy, FPALM, Fluorescence Photoactivation Localization Microscopy, dSTORM, directSTORM, GSDIM, ground state depletion microscopy followed by individual molecule return. These differ from each other in the way of photoswitching. I used dSTORM technique in the thesis.

Multi-color microscopy serves as an effective tool to observe separately several biological objects due to the specific fluorescent labeling. Two approaches to multi-color imaging can be distinguished. The sequential approach captures the two (or more) images at different times and uses the full size of the CCD chip. In the simultaneous approaches, the image is split into two spectral channels and then imaged onto different regions of the CCD chip. I favored the sequential (single channel) method in the thesis.

3. Objectives

The quality of the super-resolved image can be degraded by errors occurring during the imaging or in the image processing. In my thesis errors and artifacts were analyzed in localization based microscopy, which are caused by optical aberrations and the imperfectness of image reconstruction. These errors are rooted in the imperfectness of optical elements in the illumination or detection path or in the imperfect localizations as well. My aims were the following collected into points:

1. The epifluorescent illumination is a conventional illumination in the wide-field fluorescence microscopy. In this case the whole region of interest is illuminated and the out of focus signal is significant and can be disturbing for the imaging and degrade the quality of the final image. To reduce the out of focus signal, new illumination methods were invented, which illuminate the sample obliquely. These inclined illuminations have growing popularity but their practical implementation into the microscope systems is still challenging and that has yet to be studied in detail taking into consideration the effects of optical aberrations. **I modeled the various types of wide field illuminations in a realistic microscope system model by means of ray tracing method.**
2. Not only the optical elements placed in the illumination path can introduce significant aberrations but ones placed in the detector path too. One and the most significant of these optical aberrations is the chromatic aberration. This affects significantly the multicolor imaging which is an effective tool in the microscopy to detect separately different biological objects due to the multicolor labeling. In the multicolor detection an essential question is the co-registration of the images captured at different wavelengths. Because of the presence of the chromatic aberration a chromatic error emerges in the imaging: the image of a point-like object takes different place in the image plane depending on the emission wavelength. In the conventional microscopy techniques this error is corrected and eliminated well at that range of resolution (~200nm). But at the level of resolution of localization microscopy (~10-20nm) it is still significant and can lead misconception in the measurements.

Therefore a correction method is required to co-register the different color image channels at nanometers level. I provided both experimental and theoretical result to assess the effects of chromatic error and demonstrate a performance for co-registration.

3. Localization-based super-resolution microscopy image quality depends on further factors as well, such as dye choice and labeling strategy, microscope quality and user-defined parameters such as frame rate and number as well as the image processing algorithm. The imperfect imaging parameters can lead to appearance of artifacts on the final image. To eliminate or minimize these, an optimization is required for the parameters. Experimental optimization of these parameters can be time-consuming and expensive. **Therefore I created a software (TestSTORM) which models the whole imaging process in the localization based microscopy.**
4. The significance and quality of artifacts depends on the sample geometry and the imaging parameter such as labeling density, frame rate or pixel size. The overlapping molecule images cause mis-localizations and that is the main reason of the appearance of the artifacts. The aim is to reduce the probability of the overlapping. **Therefore I studied how possible artifacts can occur and how an optimization can perform to determine an optimal process window in the parameter space for imaging in case of four types of sample geometry.**

4. New scientific results

In the following I shortly present the new scientific results according to the pointed aims described above.

I demonstrated an analysis of optimal illuminations applied in wide-field microscopy, especially focusing on localization based microscopy issues arising because of the imperfect optical elements. I studied the possible illumination methods of region of interest (ROI) at several depths in the sample. I used a modeled microscope system in OSLO, optical design software. An essential question is to reduce the out of focus signal. This can be achieved with the inclined illumination techniques such as VAEM, Variable Angle Epifluorescence Microscopy, HILO, Highly Inclined and Laminated Optical sheet microscopy or with TIRF (Total Internal Reflection Fluorescence) illumination instead of conventional EPI illumination. I found that a correction was required after the EPI-TIRF transition for centered illumination in the TIRF mode. In case of deeper placed ROI illumination a calibration curve was added for the correction in case of HILO illumination. I investigated the effect of the positioning process of ROI in the sample by moving the objective. Due to focus dislocation, the ROI was not illuminated entirely, therefore additional correction was needed. A calibration curve was given for this additional correction too. TIRF, VAEM and HILO illumination can also be realized with movable lenses. By moving the lenses the required inclination and shift can be introduced according to the laws of geometrical optics. In the future I will study the issues mentioned above in such a system. I plan to carry out measurements in connection with the described issues using a standard layered sample. Another challenge in fluorescence microscopy is multicolor illumination. I made preliminary simulations with our modeled system at 405, 488 and 532 nm. It can be clearly seen that an illumination system optimized for a given wavelength is not appropriate for another one because of the high divergence of the beam caused by chromatic aberration.

I presented a method to assess the effect of chromatic error in multicolor localization based microscopy. Chromatic error causes image distortion on the sub-wavelength scale even for highly corrected microscope optics. I showed that in the single channel approach the most critical source of

this error is the lateral chromatic aberration introduced by the microscope objective. The distortion is a slowly varying function of optical aperture coordinates and is temporally invariant. I have presented a method for measuring chromatic offsets in practical microscope systems and compared the results with ray-tracing calculations. It showed good qualitative agreement. I presented a correction method also: co-ordinates from different color channels can be effectively mapped onto one another yielding co-localization precisions of the order of one super-resolved pixel or less. I demonstrated the effectiveness of the method in case of a measurement of real biological sample through observation of membrane protein clustering and vesicle formation.

Deformities can be caused by the imperfect localization procedure and non-optimized imaging parameters too. To study this question, I developed a program (TestSTORM) for modeling the whole imaging process in a localization based fluorescence microscope. Four types of sample are provided with different geometry: star, array, vesicles and lines. The algorithm creates the structure of the sample, generates a temporal trajectory of photoswitching fluorescent states to each dye molecule according to the three-state model (active, passive and bleached states) and simulates the image acquisition process. The output of the software is a frame stack which can be reconstructed and analyzed further with localization algorithms such as rainSTORM or rapidSTORM.

I demonstrated the effectiveness of the program for analyzing and exploring artifacts in localization microscopy. The z-dependent resolution, the effect of the labeling density on a structure, the high intensity ends and the bridge formations were analyzed with the star, array, lines and vesicles pattern, respectively. In the future I plan to develop TestSTORM further with some add-ons such as incorporated dye library with the parameters of widely used photoswitching dyes, 3D image acquisition with z-dependent PSFs and for the simulation of molecular diffusion imagery.

According to the above described new scientific results the following thesis statements can be made:

1. Several inclined illumination techniques such as TIRF, VAEM and HILO were analyzed and quantified in wide-field microscopy by means of OSLO ray-tracing optical design software. [T1]
2. The effect of chromatic offset was analyzed in multicolor localization based super-resolution microscopy and a calibration and correction method was invented and applied in measurements with biological samples. [T2]
3. A software (TestSTORM) was developed to generate test images for localization based microscopy, which incorporates the model of the whole imaging process. [T3]
4. Analysis of artifacts and imaging parameter optimization were carried out in case of four sample structures of which geometries are predefined in TestSTORM simulator program. [T4]

5. Publications

Related publications in peer reviewed journals:

[T1] József Sinkó, Gábor Szabó, and Miklós Erdélyi, "Ray tracing analysis of inclined illumination techniques," Opt. Express **22**, 18940-18948 (2014)
http://www.opticsinfobase.org/oe/abstract.cfm?URI=oe-22-16-18940

[T2] Miklos Erdelyi, Eric Rees, Daniel Metcalf, Gabriele S. Kaminski Schierle, Laszlo Dudas, Jozsef Sinko, Alex E. Knight, and Clemens F. Kaminski, "Correcting chromatic offset in multicolor super-resolution localization microscopy," Opt. Express **21**, 10978-10988 (2013)
http://www.opticsinfobase.org/oe/abstract.cfm?URI=oe-21-9-10978

[T3] József Sinkó, Róbert Kákonyi, Eric Rees, Daniel Metcalf, Alex E. Knight, Clemens F. Kaminski, Gábor Szabó, and Miklós Erdélyi, "TestSTORM: Simulator for optimizing sample labeling and image acquisition in localization based super-resolution microscopy," Biomed. Opt. Express **5**, 778-787 (2014)
http://www.opticsinfobase.org/boe/abstract.cfm?URI=boe-5-3-778

Other publications in peer-reviewed journals:

1. József Sinkó, László Dudás, Gábor Gajdátsy, Miklós Erdélyi, and Gábor Szabó, "Map-free line-scanning tomographic optical microscope," Opt. Lett. **36**, 4011-4013 (2011)
2. László Dudás, Gábor Gajdátsy, József Sinkó, Miklós Erdélyi, and Gábor Szabó, "Correction of error motion in a line-scanning tomographic optical microscope," Appl. Opt. **51**, 6319-6324 (2012)
3. László Dudás, József Sinkó, Miklós Erdélyi, and Gábor Szabó, "Confocal line-scanning microscope with modified illumination," Opt. Lett. **37**, 4293-4295 (2012)
4. Erdélyi Miklós, Sinkó József: Optikai pointillizmus: a lokalizációs optikai mikroszkópia, Fizikai Szemle, 2014. május, http://wwwold.kfki.hu/fszemle/archivum/fsz1405/ErdelyiM_SinkoJ.pdf

Conference posters, presentations

5. Laszlo Dudas, Miklos Erdelyi, Jozsef Sinko, Gabor Gajdatsy and Gabor Szabo, „Line-scanning optical tomographic microscope", 11th annual meeting of the European Light Microscopy Initiative (ELMI), Alexandroupolis, Greece, (2011. 06. 07-10.), Session VII.
6. L. Dudás, M. Erdélyi, J. Sinkó, G. Gajdátsy, G. Szabó: "Line-scanning tomographic optical microscope" poszter prezentáció, Focus on Microscopy (FOM2012) konferencia, 2012. április 1-4, Szingapúr

7. L. Dudás, J. Sinkó, M. Erdélyi, G. Szabó: "Line-scanning tomographic optical microscope" poszter prezentáció, 12th International ELMI meeting on Advanced Light Microscopy (ELMI2012), 2012. június 5-8 Leuven, Belgium
8. Jozsef Sinko, Eric Rees, Daniel Metcalf, Gabriele S Kaminski Schierle, Laszlo Dudas, Alex E Knight, Clemens F Kaminski and Miklos Erdelyi, „Correcting chromatic offset in multicolor super-resolution localization microscopy", Poster presentation, Frontiers in BioImaging, 3. July 2013- 4. July 2013., London, UK
9. József Sinkó, Róbert Kákonyi, Eric Rees, Daniel Metcalf, Alex E. Knight, Clemens F. Kaminski, Gábor Szabó, and Miklós Erdélyi, "TestSTORM: Simulator for optimizing sample labeling and image acquisition in localization based super-resolution microscopy,", Poster presentation, 14th International Advanced Light Microscopy meeting (ELMI 2014), 2014. május 20-23., Oslo, Norvégia