Summary of the PhD thesis

Enhancement of the lateral resolution and the image quality in a line-scanning tomographic optical microscope

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1. Introduction

The purpose of microscopes is to provide an image about an object which is unresolvable for the human eye. Many types of optical microscopes with different parameters are used recently. One of the most important properties is the lateral resolution which sorely affects the details and the contrast of a microscope image at a given magnification. The basic microscope consists of an objective and an ocular lens, complemented by the proper illumination (Köhler, Epi) and detection (human eye, CCD). The lateral resolution of such a system is the distance at which two point-like objects can be distinguished on the image. It means that the lateral resolution depends on the size of the spot (PSF – point spread function) which is created by the optical system when a point-like object is used as a sample. The lateral resolution can be treated similarly in scanning microscopes, which are focusing a pattern (point, line) onto the sample and performing scanning by moving the structure. in this case the lateral resolution mostly depends on the dimensions of the scanning point (PSF) or line (LSF – line-spread function). In case of point scanning the focal spot can be modified in several ways (making the spot narrower in axial, lateral or both directions), by using interference (4pi, Theta microscopy), aperture manipulation (annular, coated aperture) or special polarization (radial polarization, vectorial aperture engineering). Multiphoton excitation or STED can also be applied. There are also methods for widefield microscopes that results resolution enhancement such as structured illumination (SIM) and localization based techniques (STED, PALM).

The fluorescent labeling of biological samples usually enhances the contrast of the images. However, if the sample is thicker than the depth of focus of the microscope, the out-of-focus light will decrease the contrast and sharpness of the image. An important advantage of the scanning microscopes is the possibility of confocal detection that filters the out-of-focus light, and thereby removes the image blur. That means at the same time, that the sample can be examined layer by layer. This so called optical sectioning property is also achievable in a widefield microscope, using SPIM, SIM or STORM.

Computed tomography (CT) is an essential tool in medical physics both for diagnostic and surgical purposes. The main advantage of the CT technology is its

ability to generate 3D images of different organs in vivo. This is performed by measuring the attenuation of the X-ray beams passing through the body. Repeating the measurement from several directions an image can be calculated from the collected data. OPT (optical projection tomography) practically applies the CT technology in the optical wavelength range, where laser sources are used instead of x-rays. This technique provides high-resolution three-dimensional images of fluorescent and non-fluorescent biological specimens in the micrometer to centimeter range.

Slit scanning procedure and tomographic data acquisition method is combined in LSTOM. Unlike the OPT, the LSTOM is a microscope technique, that makes the optical transfer function isotropic, and provides a lateral resolution enhancement of 18% compared to a traditional widefield microscope. In the tomographic data acquisition process projections are recorded from different directions using a diffraction-limited line. The scanning can be accomplished by galvo scanner, and the rotation can be performed by image rotation prism, mounted on a rotation stage. The rotation procedure is causing similar problems as the COR error (center of rotation) in the CT technology.

2. Objectives

My objective was to improve the lateral resolution of a line-scanning tomographic optical microscope (LSTOM) and to investigate and minimize the errors which can cause the degradation and quality loss of the recorded images.

1. The image quality and the lateral resolution of the LSTOM microscope are affected by the center of rotation error, which can be caused by the misalignment of the prism or by the rotating stage. My aim is to formulate an alignment criterion that can guarantee a sinogram recording process without image degradation or lateral resolution loss. I will study theoretically and experimentally what conditions and how precise alignment is necessary to satisfy this criterion.
2. The reconstruction errors are caused by the image rotation prism. Elimination of the introduced rotational error is possible by focusing through a birefringent plate and introducing astigmatism. My goal is to use the astigmatic line as the illumination pattern of the LSTOM microscope. The imaging performance of the system will be evaluated using the Richardson star pattern.
3. LSTOM microscope is modified to be capable of imaging fluorescent samples. Confocal detection will be added to achieve optical sectioning property. The performance of the new system will be checked by using test samples. The image quality and the lateral resolution will be investigated.
4. The lateral resolution of a line-scanning microscope was improved using phase manipulation of the illuminating beam and by applying confocal detection. I investigated the parameters which are influencing the line-spread function of the system. My aim is to demonstrate the resolution enhancement and investigate the applicability of the method in LSTOM microscope.

3. Materials and methods

Image quality of the reconstructed images had been studied numerically using Matlab environment. Projections were calculated using a point like object and the digital counterpart of the Richardson star pattern (Richardson test slide, US 2004/0227937A1), and the effect of the center of rotation error on the lateral resolution and image quality was investigated. The errors introduced by the misalignment of the prism were studied, and estimations were made for the alignment parameters using the ray-tracing software, OSLO.

Multiple LSTOM arrangements and a line scanner system were built. The lateral resolution was measured by means of single silver nanoparticles (with diameter 10–50 nm), single fluorescent bead (200nm Fluoresbrite Microspheres, Polysciences Inc.) and Richardson star pattern. In the case of single point like objects the line-spread function and the point-spread function was measured and fitted. The sample of multiple, randomly spaced nanodots and the Richardson star pattern was evaluated using Fourier-analysis, and the cut-off frequency of the modulation transfer functions was determined.

I have studied the deflection of the optical axis theoretically and experimentally in three LSTOM systems. The conjugate plane of the focal plane of the microscope objective was generated by splitting the optical path (Pellicle) and focusing the beam using a lens. A pinhole and a photo detector was placed into the focal plane and a sinogram was recorded. By evaluating the sinogram, the angle of the deflection and the extent of misalignment could be determined. The walk of the illumination beam on the entrance aperture of the focusing objective was also measured, using a CCD.

The astigmatic line was generated by focusing a beam using a Zeiss (M20x/0.4NA) and Olympus (M20x/0.4NA) objectives, through a calcite plate with a thickness of 0.5mm. The size of the astigmatic beam was reduced by a beam expander to match the beam size to the aperture of the focusing objective (Nikon M50x/0.55NA). The polarization of the incident beam and the calcite plate were rotated synchronously, and the scanning of the sample was performed by an X/Y/Z

piezo translator. The imaging performance of the astigmatic LSTOM system was evaluated using the Richardson star pattern.

LSTOM microscope that performs the rotation of the beam by a Pechan prism, and uses a galvo scanner, was modified to operate in fluorescent mode. The emitted light from the fluorescent object was detached by a dichroic beamsplitter and measured by photomultiplier (PMT). To achieve confocality, an objective and a slit was placed before PMT. The lateral resolution and the isotropy of the point-spread function were checked using a single fluorescent bead. The image quality and the optical sectioning property were demonstrated capturing the Convallaria Majalis test sample. Zeiss (M20x/0.4NA) objective was applied in both cases.

A line scanning microscope was built. The scanning line was created by a cylindrical lens and the scanning was performed by a piezo cube. Phase manipulation was carried out with an SLM. The aperture of the objective was divided into three sections and in the middle section a π phase shift was introduced, relative to the sidewise parts. As a result, structured scanning line with three peaks emerged in the focus of a high NA microscope objective (Nikon M100x/1.49NA Oil). Confocal detection - using a single line of the CCD camera with a pixel size of 0.1 AU – was used to measure only the central peak, excluding the effect of the sidewise peaks. The lateral resolution of the system was measured by means of silver nanoparticles.

All measurements were carried out with a diode pumped, frequency doubled Nd:YAG laser (Roithner DPSSL-532, λ=532nm,P_{max}=40mW). The output power of the laser source was modulated with a ~10kHz square wave and the lock-in detection technique was applied. Data acquisition was performed by a NI PXIe-6356 card.

Measurement control and data acquisition was implemented in LabView and the additional evaluation of the measured data was processed in Matlab.

4. New scientific results

1. ***I investigated the effect of center of rotation error caused by image rotating prism and rotator in line-scanning tomographic optical microscope (LSTOM) using numerical simulations and ray-tracing. I demonstrated that the lateral resolution degradation of the reconstructed image will be less than 2% when the amplitude of the COR error remains under 1/10 of the system's nominal lateral resolution.***

   A LSTOM microscope system requires precise rotation of the scanning line. In LSTOM, the COR error is caused by the misalignment of the Pechan prism or the imperfection of the rotation stage. Both sources of the COR error deflects the optical axis and makes the projections shifted relative to each other, that causes errors of the reconstructed image. I have studied the effects of the error using numerical simulations. The errors, introduced by the misalignment of the prism, were investigated using OSLO ray-tracer software, and estimations were made for the alignment parameters. My calculations showed that the lateral resolution degradation is expected to be less than 2%, if the amplitude of the COR error is less than 1⁄10 of the system's nominal lateral resolution. Alignment errors were investigated experimentally and theoretically in three different LSTOM setup. [T1]

2. ***I used an astigmatic line, produced by means of a birefringent plate, as a line illumination in LSTOM microscope. The method provides enhanced rotation stability compared to the precision of a rotation prism.***

   Astigmatic line was generated by focusing a linearly polarized laser beam through a p-type calcite plate. If the polarization of the incident beam and the calcite plate are synchronously rotated, the line will be rotated with the same angle. The rotation has large stability because both rotated optical elements are translation invariant and their slight lateral drift does not affect the axis of rotation. The alignment precision regarding the tilt of the plate is about 0.01°. I built a LSTOM system using astigmatic illumination and I investigated the imaging quality of the microscope. I found the captured images to be isotropic and free from reconstruction errors. This demonstrates that the rotation of the line was performed error-free. The lateral resolution was found 10% better compared to the resolution of a widefield microscope. [T2]

3. ***I constructed a confocal LSTOM microscope with fluorescent imaging capability. The captured images are error-free with a resolution improvement of 17% compared to a traditional widefield microscope.***

   I checked the imaging performance of the LSTOM system in confocal, fluorescent mode, using fluorescent test samples. The images captured from Convallaria Majalis test samples are free from reconstruction errors and aberrations, and they are demonstrating the optical sectioning capability of the system. The reconstructed image of 200nm fluorescent bead is showing that the point spread function of the system is isotropic; therefore the reconstruction is error-free. I found that the lateral resolution is 0.67μm (λ_{exc}=532nm, NA=0.4), which is in a good agreement with the theoretical value (0.665μm). Consequently, the resolution of LSTOM exceeds the resolution of a widefield microscope by 17%. [T1]

4. ***I improved the lateral resolution of a line scanning microscope by 37%, applying phase apodization on the illumination combined by confocal detection.***

   By modifying the phase structure of the incoming beam, I created an illumination LSF consisting of triple peaks in the focal plane. The central peak was narrower than the normal LSF, and only an area, illuminated by the central peak, was detected by means of confocality. I studied the effect of the obstruction ratio (ε) and the confocal slit width on the resulting LSF theoretically, using scalar diffraction theory. An optimum can be found at around ε=0.15 and the slit width should be kept below 0.5 Airy Units. The optical transfer functions were determined by means of Fourier analysis of captured images of silver nanoparticles (λ=532nm, 1.49 NA, Oil). The Rayleigh resolution limits were calculated from the determined cut-off frequencies. The values in the normal and modified illumination cases were 183±5nm and 115±5nm, respectively. That means an improvement of 37% in lateral resolution. Resolution enhancement is 36% and 48% compared to the theoretical resolution of line scanning (179nm) and widefield (218nm) microscopes. This method can be used in LSTOM microscope to achieve further resolution enhancement. [T3]

5. Publications

Publications in peer reviewed journals related to the thesis:

[T1] L. Dudás, G. Gajdátsy, J. Sinkó, M. Erdélyi, G. Szabó: "*Correction of error motion in a line-scanning tomographic optical microscope*" Appl. Opt. 51, 6319 (2012);
I: 1.689

[T2] J. Sinkó, L. Dudás, G. Gajdátsy, M. Erdélyi, G. Szabó: "*Map-free line-scanning tomographic optical microscope*", Opt. Let. 36, 4011-4013 (2011)
I: 3.385

[T3] L. Dudás, J. Sinkó, M. Erdélyi, G. Szabó: "*Confocal line-scanning microscope with modified illumination*" Opt. Let. 37, 4293 (2012)
I: 3.385

Other publications in peer-reviewed journals and a patent:

[E1] G. Gajdátsy, L. Dudás, M. Erdélyi, G. Szabó, "*Line-scanning tomographic optical microscope with isotropic transfer function"*, Journal of Optics 12 (11) 115505 (2010)
I: 1.99

[E2] G. Szabó, M. Erdélyi, G. Gajdátsy, L. Dudás, "*Optical microscope system and method carried out therewith for reconstructing an image of an object*", Patent Application WO/2009/030966 (2009)

[E3] M. Erdelyi, E. Rees, D. Metcalf, G. S. Kaminski Schierle, L. Dudas, J. Sinko, A. E. Knight, and C. F. Kaminski: „Correcting chromatic offset in multicolor super-resolution localization microscopy" Opt. Exp., Vol. 21, Issue 9, pp. 10978-10988 (2013)
I: 3.546

Conferences:

[E4] L. Dudás, "*Line-scanning tomographic optical microscope*", oral presentation, 11th international ELMI meeting, Alexandropoulis, Greece (2011)

[E5] L. Dudás, M. Erdélyi, J. Sinkó, G. Gajdátsy, G. Szabó: "*Line-scanning tomographic optical microscope*" poster presentation, Focus on Microscopy (FOM) conference, Singapore (2012)

[E6] L. Dudás, J. Sinkó, M. Erdélyi, G. Gajdátsy, G. Szabó: "*Line-scanning tomographic optical microscope*" poster presentation, 12th International ELMI meeting on Advanced Light Microscopy Leuven, Belgium (2012)