Theoretical and experimental study of line-scanning Tomographic Optical Microscopes

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1. Introduction

Optical microscopy is an important technique for a vast number of applications in science and engineering. It is particularly important in life sciences as it allows one to investigate intact samples including living cells in the range from a few microns to several hundred nanometres. Its non-invasive imaging property provides advantages that are not found in other methods such as electron microscopy. To efficiently exploit these advantages, optical imaging systems and techniques with resolution beyond the wave optical limit (superresolution) are in the focus of a very intense research in modern microscopy.

One of the main parameters of an imaging system is its resolution, which is limited by the diffraction of light waves and the aberration of the applied optical elements. The aberration can be reduced by increasing the quality of the applied optical elements, and in ideal case one arrives at the resolution limit set by the diffraction. This limit can be described by the Rayleigh or Sparrow criteria. In case of optimal illumination in the visible spectral range, the minimum resolvable distance (critical distance) of an optical system is approximately the half of the applied wavelength, corresponding to about 200nm lateral and 400nm axial resolution. Improving this limit has been a source of continuing research of different methods with major successes such as confocal microscope, 4Pi microscope, multiphoton microscope, structured illumination, stimulated emission depletion (STED) microscope, localization microscopes (PALM, STORM), near field imaging and so on. Some of the abovementioned methods can reduce the critical distance with several percents whilst others provide around ten times higher resolution. However, most of these methods require fluorescent indicators or special samples with limited geometry and material properties. In case of non-linear techniques intensive illumination is needed, which can damage the biological samples (photobleaching).

In contrast to the conventional microscopes, several techniques aim to obtain detailed information from the inner structure of a sample. Techniques like confocal microscopy, total internal reflection fluorescent (TIRF) microscopy and optical projection tomography (OPT) acquire this information working in the optical spectral range. Confocal microscopy is one of the earliest methods developed for improved resolution along the optical axis. Filtering out the irrelevant intensity information coming outside from the investigated region of the sample, it provides $\sqrt{2}$ times larger axial resolution than conventional optical microscope. Confocal imaging is achieved by point illumination and raster scanning of the entire image, which is a rather time consuming procedure. OPT practically applies the computer tomography (CT)

technology in the optical wavelength range, where laser sources are used instead of x-rays. Both OPT and CT performs indirect imaging, where the absorption property of the sample is measured. In the simplest case the sample is illuminated through by collimated beams and the transmitted intensity is measured, meanwhile the light source and the detector are moved together along a line. The recorded transmission data as a function of the detector's position is called projection. Depending on the actual technical design of the system, the sample or the beam can be rotated. Projections – captured at different angular positions – compose the sinogram and the cross section image is reconstructed with a computer algorithm such as filtered back projection (FBP). In OPT the detected intensity by a CCD pixel is proportional to the integrated absorption of all the voxels through which the actual laser beam travels. There is a significant difference between CT and OPT. In the optical regime diffraction cannot be neglected and the laser beam cannot be considered as a straight ray, when longitudinal projections are acquired. Thus, OPT is limited by the depth of focus (DOF) of the optical system, which scales inversely with the square of the numerical aperture (NA). To achieve a sufficiently high DOF, the numerical aperture has to be kept at a relatively low value, which also decreases the spatial resolution. This fact indicates that the OPT is not suited to perform superresolution imaging. Moreover, the thickness of the applied sample is limited and the optical path difference depends on the angle of scanning in case of non-cylindrically symmetric sample.

In present dissertation a novel, indirect imaging technique that combines the principle of tomographic reconstruction with the arrangement of a line-scanning confocal microscope is studied comprehensively.

2. Objectives

My main aim was to build and study a novel, non-interferometric, microscopic device referred to as **T**omographic **O**ptical **M**icroscope (TOM), which successfully combine the scanning arrangement of a line-scanning confocal microscope and the indirect imaging principle of tomographic reconstruction, providing enhanced lateral resolution. As a first step, I performed numerical simulations to investigate the accuracy of the applied reconstruction algorithm and determine the optimal value of the reconstruction parameters such as the number of projections and the step size of scanning. To demonstrate the resolution enhancement of TOM method, I designed and built two optical systems. The first arrangement measured the projections of the given sample in transmission mode, while the second acquired the reflected intensity data applying slit scanning confocal technique. The variation of the scanning direction was achieved by sample and beam rotation. Based on the recorded images of the arrangements, the resolution limit and imaging properties of TOM method were experimentally investigated.

A numerical code was developed to simulate the applied projection recording and reconstruction processes and make the adjustment of the second device easier. Both the preliminary measurements and the results of simulations showed that the quality of the reconstructed image strongly depends on the axial runout of the beam or sample rotating mechanism. To reduce the effect of this runout, an *in-situ* axial error correction technique was developed and tested.

Elimination of the introduced rotational error is possible by using an optical element, which can generate and rotate the illumination structure on the sample's surface and insensitive for the lateral misalignment of the rotation axis. The necessary optical element can be created applying a birefringent plane plate. Such a birefringent plate is able to change the polarization distribution of the illumination beam. In ideal case the properly polarized beam can be focused into a tighter spot or line, which can further improve the resolution of the imaging system. The feasibility of an optical element with the mentioned advantages was also investigated theoretically and experimentally.

3. Materials and methods

First the imaging capabilities of the proposed TOM arrangements had been studied numerically. I implemented the FBP reconstruction algorithm using MATLAB software environment, to optimize the parameters of the recorded sinograms. The sinogram recording process of TOM – both in transmission and reflection modes – were also modeled by an individually developed code. Distortions in the focused intensity distribution, the image degrading effect of the rotation error and the proposed correction procedure were also taken into account.

Two TOM arrangements were built to determine the available resolution enhancement. In transmission mode, a non-transparent edge was imaged on the surface of the sample. The position of the edge was shifted and the transmitted total intensity was measured behind the sample. The sinogram could be obtained by rotating the sample and repeating the measurement. The non-transparent edge was generated by a spatial light modulator (SLM). A reflective grid with different periods on a silica substrate was used as a test sample. To collect the transmitted intensity, a microscope objective (M = 4, NA = 0.1) was applied. With this objective the arrangement could be used as a coherently illuminated conventional microscope as well. The resolution limit of TOM and this conventional microscope was compared based on the recorded images.

In reflection mode, the illumination and scanning scheme of a line-scanning confocal microscope was applied. An adjustable slit was illuminated by a diode-pumped, frequency-doubled Nd:YAG laser. The produced cylindrical beam was focused on the sample by a cover-slide-corrected *Zeiss LD 'Plan-Neofluar'* microscope objective (M = 20, NA = 0.4). Since the back aperture of the objective was illuminated only by the zero order of the diffraction pattern of the slit, the intensity distribution in the focal plane was diffraction-limited. A galvo scanner was used for beam scanning and the scanning direction was changed by the rotation of a Pechan prism. An *in situ* correction method was developed to realign the deviated rotational axis – due to the rotational error – with the galvo scanner. The direct imaging performance of TOM arrangement was measured using a standard test pattern (*Richardson Test Slide, Bio-Microtech, US 2004/0227937 A1*). The recorded images were compared with the images of two commercially available point-scanning confocal devices (*Zeiss Axiovert 135M, Olympus Fluoview FV1000*) using the same microscope objective and sample.

To design a birefringent optical system, which is able to create the diffraction limited illumination and eliminate the effect of the rotational error in TOM, the commercially available ray tracing software, OSLO was used. Using OSLO, I was able to calculate the polarization maintaining efficiency of microscope objectives. The generated intensity and polarization conditions by focusing through a birefringent plane parallel plate were also studied with the software. To verify these calculations, an optical arrangement was built and the intensity distribution in the focus was measured.

4. New scientific results

1. I investigated the influence of the reconstruction parameters of FBP on the quality of the TOM image. The results of my calculations showed that decreasing the step size of scanning (Δn) the finer details of the object can be resolved: however, a smaller step size increases the computational time proportionally to the square of the number of steps. Decreasing Δn beyond a certain limit, the resolution of the reconstructed image can not be increased further. With increasing the angular resolution of the sinogram ($\Delta \phi$) the contrast of the image improves. The values of Δn and $\Delta \phi$ determine the frequency resolution of the image in the Fourier or reciprocal space. Assuming that the object does not contain information above a certain frequency limit in the reciprocal space, Δn has to be at least as small as it is required by the Shannon sampling criteria. To obtain a reconstructed image completely free from artefacts, an infinite number of projections is necessary, which means that $\Delta \phi$ must converge to zero. [T1, T2]

2. I designed and built a TOM arrangement in transmission mode with sample rotation. I experimentally showed that its resolution exceeds the resolution of a conventional optical microscope with coherent illumination, using the same objective. Images of a reflecting equidistant grid with 4μm period were recorded by TOM and the optical microscope. The critical distance (CD), which is half of the minimum observable period, proved to be larger than 2μm in case of the optical microscope. In contrast, the resolution of TOM was better than this value. The NA of the applied objective was 0.1 and the wavelength of illumination was 660nm. The intensity modulation of the grid could not be observed in the CCD image of the optical microscope, but it was resolved in the reconstructed image of TOM. The Fourier spectrum of the recorded images was also compared and the characteristic frequency component belonging to the 2μm CD was resolved only by the TOM. [T1]

3. To reduce the scanning time when a high number of projections are recorded, the arrangement was redesigned and rebuilt using line-scanning and reflection method. I applied two different methods to change the scanning direction, namely sample and beam rotation. In case of both solutions, the axial runout of the rotating mechanism seriously degraded the reconstructed image quality. The motion error is always present in a

mechanical rotation system and can be divided into to parts. The synchronous error, which is repeated round to round, can be measured separately from the image recording and corrected subsequently. To correct the randomly changing asynchronous error, an *in-situ* runout measurement and correction process is necessary. I developed such a method for sample rotation as well as for beam rotation. In case of sample rotation a lens was placed behind the sample and its focal point was aligned in the plane of the sample's front surface and next to the sample. As the scanning beam passed over the focal point the transmitted light was collected by the lens and an intensity peak was measured. This peak indicated a virtual axis of rotation in each scanning position. During the reconstruction, the recorded projections were realigned relative to this axis. The solution was similar as applying beam rotation. A part of the illumination beam was coupled out and focused through a pinhole, which was declared the virtual axis. As the beam was rotated, the coupled out part of the beam missed to the pinhole due to the motion error. The deflected beam and the deflected axis of rotation were realigned with the line-scanning mechanism. Applying the abovementioned methods, I could reduce the effect of the synchronous and asynchronous motion error beyond the resolution limit of TOM. [T2]

4. I demonstrated that resolution of the line-scanning TOM arrangement in reflection mode exceeds the resolution of a commercially available point-scanning confocal microscope, using the same microscope objective. First the intensity distribution of the illumination pupil was measured. Its full width at half maximum (FWHM) proved to be 0.664 ± 0.014μm. This value is very close to the theoretical data ($0.5\lambda/NA = 0.665$μm where NA = 0.4, λ = 532nm). In the case of a point-scanning confocal microscope, the FWHM of the illumination pupil's PSF is $0.61\lambda/NA$, according to the theory. The PSF of a confocal system is the product of the PSF of the illumination and the PSF of the detection pupils. If we assume the same NA in the illumination and detection paths, the measured FWHM result indicates that the lateral resolution of TOM exceeds the resolution limit of a point-scanning confocal microscope. The resolution difference was demonstrated by recording images of the Richardson star sample with TOM and a point-scanning confocal device (*Zeiss Axiovert 135M*). According to the images, the CD of TOM was 0.443μm (λ = 532nm) while the CD of the confocal microscope was 0.517μm (λ = 543nm). Thus the resolution of TOM, even corrected for the wavelength, is 15% better than that of a confocal microscope. [T2]

5. My aim was to design an optical element, containing a birefringent plane plate, which is able to create the diffraction limited illumination line and eliminate the effect of the rotational error. As a first step I investigated the possibility of independent generation of ordinary and extraordinary foci by linearly polarized incoming beams using uniaxial crystals whose optical axis is parallel to their surface. The results of my calculation of the polarization conditions and the overlap integral of a refractive and a catadioptric system showed an intensity loss of almost 10% when a high quality refractive imaging system was used. I showed that this intensity loss can be reduced to 2% by means of an appropriate catadioptric objective. The difference between the two systems is not only the sum of the intensity degradation, but also the level of degradation, which is greater at the edge of the four quarters of the beam behind the objective. At high numerical apertures the inhomogeneous illumination distorts the focus shape and the impaired cylindrical symmetry introduces optical aberrations. I have shown that the shape of the generated focus suffers serious distortion at high numerical apertures even if a catadioptric imaging system is used. [T3]

6. It is known that a cylindrically polarized illumination beam can increase the resolution of an imaging system. To utilize this property I proposed and studied a special polarizer, which contains a birefringent plane parallel plate and transform the polarization of the incoming beam into radially and azimuthally polarized beams. This radial and azimuthal polarizer (RAP) was built and experimentally studied. The simulation results showed that by focusing through a birefringent plane parallel plate – the optical axis of which is perpendicular to its surface – two foci are generated. The ordinary and extraordinary foci are azimuthally and radially symmetric, respectively. I showed that the generated focus shape has an impaired cylindrical symmetry if a linearly polarized incident beam is applied. The introduced focus separation was demonstrated and studied experimentally. [T4]

5. Publications

Publications in peer reviewed journals and a patent related to the thesis:

[T1]   Szabó G., Erdélyi M., Gajdátsy G., Dudás L., Optical microscope system and method carried out therewith for reconstructing an image of an object, *Patent Application* **WO/2009/030966** (2009).

[T2]   Gajdátsy G., Dudás L., Erdélyi M., Szabó G., Line-scanning tomographic optical microscope with isotropic transfer function, *Journal of Optics* **12**(11) 115505 (2010).

[T3]   Gajdátsy G., Erdélyi M., Analysis of focus distortion based on birefringence, *Journal of Optics A* **9** pp.: 982–987 (2007).

[T4]   Erdélyi M., Gajdátsy G., Radial and azimuthal polarizer by means of a birefringent plate, *Journal of Optics A* **10** (2008).

Other publications in peer-reviewed journals:

[1]   Erdélyi M., Bereznai M., Gajdátsy G., Bor Zs., Three-dimensonal focus manipulation by means of a birefringent plate, *Optics Communications* **281** pp.: 4807-4811 (2008).

[2]   Gajdátsy G., Benedek F., Kokavecz J., Szabó G., Kornis J., Improved fiber optic device for in situ determination of electrolyte stratification in lead-acid batteries, *Review of Scientific Instruments* **80** 125108 (2009).