Micro axial tomography: A miniaturized, versatile stage device to overcome resolution anisotropy in fluorescence light microscopy

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With the development of novel fluorescence techniques, high resolution light microscopy has become a challenging technique for investigations of the three-dimensional (3D) micro-cosmos in cells and sub-cellular components. So far, all fluorescence microscopes applied for 3D imaging in biosciences show a spatially anisotropic point spread function resulting in an anisotropic optical resolution or point localization precision. To overcome this shortcoming, micro axial tomography was suggested which allows object tilting on the microscopic stage and leads to an improvement in localization precision and spatial resolution. Here, we present a miniaturized device which can be implemented in a motor driven microscope stage. The footprint of this device corresponds to a standard microscope slide. A special glass fiber can manually be adjusted in the object space of the microscope lens. A step-wise fiber rotation can be controlled by a miniaturized stepping motor incorporated into the device. By means of a special mounting device, test particles were fixed onto glass fibers, optically localized with high precision, and automatically rotated to obtain views from different perspective angles under which distances of corresponding pairs of objects were determined. From these angle dependent distance values, the real 3D distance was calculated with a precision in the ten nanometer range (corresponding here to an optical resolution of 10–30 nm) using standard microscopic equipment. As a proof of concept, the spindle apparatus of a mature mouse oocyte was imaged during metaphase II meiotic arrest under different perspectives. Only very few images registered under different rotation angles are sufficient for full 3D reconstruction. The results indicate the principal advantage of the micro axial tomography approach for many microscopic setups therein and also those of improved resolutions as obtained by high precision localization determination. © 2011 American Institute of Physics. [doi:10.1063/1.3632115]

I. INTRODUCTION

During the last decades, light microscopy has re-emerged as one of the fundamental methods in biomedical sciences and cellular biophysics. Typically, cellular and sub-cellular structures are analyzed by specific labeling with fluorophores which can be imaged using a fluorescence microscopy setup. A serious impediment to exploit the full potential of light microscopy to study cellular nanostructures, however, has been the conventional optical resolution of about 200 nm laterally and 600 nm axially, the Abbe-Rayleigh limit.^{1,2} This limit is still valid for all approaches using the basic conditions stated by Abbe and Rayleigh.

Thus, despite of all technical and optical improvements to overcome resolution limits in fluorescence microscopy, the determination of positions of cellular objects and the precision in distance measurements in three-dimensional (3D) microscopic imaging remains spatially anisotropic as a result of the Abbe-Rayleigh image diffraction conditions.³ This principal limitation has stimulated us to take up the idea of micro

axial tomography^{4–6} and to improve the setup in such a way that it can easily be mounted on any given type of microscope with a stage suitable for mounting of standard glass slides.

Micro axial tomography makes use of special glass capillaries^{4,7} or glass fibers^{6,8,9} as specimen carriers. This allows an automated multi-view 3D image acquisition^{9,10} and precise 3D image alignment of different perspectives of the same objects.¹¹ So far, micro axial tomography has been applied to 3D studies of cell nuclei after specific genome labeling¹² using a setup with an external stepping motor and a flexible shaft⁸ which due to mechanical insufficiencies appeared to be too laborious to be implemented in a routinely applied microscope. Nevertheless, it was also used to precisely measure focal depth dependent chromatic shifts.¹³

The aim of the design described and applied here was an improvement and a miniaturization of the micro axial tomography setup in such a way that it can be easily mounted on any given type of light microscopes with a stage suitable for standard glass slides (76 mm × 26 mm). The precision mechanics of a fully adjustable glass fiber carrier was constructed which allows for improved isotropic precision in 3D localization and distance measurements. In order to demonstrate the potential of this improved design, we show distance measurements

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FIG. 1. (Color online) Image of the miniaturized micro axial tomograph. The arrows indicate A: stepper motor; B: glass fiber; C: glass carrier for the specimen.

using a very simple standard microscope with low resolution optics. As a proof of concept, also an example of cell biology, a mouse oocyte during first cell division is shown and presented in 3D.

II. DESIGN AND CONSTRUCTION OF THE MINIATURIZED DEVICE

Several special critical design objectives had to be met in the development of the miniaturized instrument (Fig. 1) for precise measurements by fluorescence microscopy:

The radial play in the fiber bearings must not exceed a few nanometers. Therefore, the fiber bearings have been designed as V-grooves, engraved with a precision stylus into the left and the right fiber bearing block. Small bronze springs press the fiber down onto the two groove walls [Fig. 2(a)].

Being suspended on both sides between these precision bearings, the specially manufactured perfectly straight glass fiber (drawn at Physics Institute, University Heidelberg) represents a geometrically very well defined substrate for the attached objects, even when being rotated. However, when coupling the fiber to the drive motor, both the stepper motor shaft and the fiber axis have to be perfectly aligned with respect to each other to avoid warping of the fiber during rotation. Therefore, the two fiber bearing blocks are designed to be precisely adjustable laterally by slightly bending them sideways in the range of a few micrometers. This can be done by applying pressure from two opposite precision adjustment screws [Fig. 2(b)]. For the vertical alignment of the two critical axes (horizontal axes x and y), the motor mounting can be moved vertically by another precision adjustment screw. Since the whole tomograph body frame has been precision machined

from a single piece of brass, only a few micrometers of adjustment is necessary, and this has to be carried out only once after the manufacturing of the device and does not have to be touched again, e.g., after the replacement of a fiber.

To comply with the usual practice in biological microscopy, a standard microscope glass slide is used in the instrument to support the medium for the cells. Therefore, the tomograph has been designed to accept a standard microscope glass slide, cut to half of its length, to act as a support for either the immersion liquid or the cell nutrition medium. In the horizontal (x) direction (= direction of the fiber axis), the slide is self-adjusting by means of a bronze spring pressing it against two stops on one side. For the precise vertical leveling (z direction), three precision screws are provided which support the slide at three points. The slide is being firmly pressed down onto these three points by four bronze springs. These adjustments are rather critical because the available space between the fiber and the slide surface is kept to a minimum to allow the use of high-aperture immersion objectives with respect to short working distances. The design diameter of the fiber is $125\ \mu\text{m}$, and two standard $170\ \mu\text{m}$ cover slip pieces which are mounted onto the slide define the width and height of a channel in which the fiber rotates [Fig. 2(c)].

Next, the fiber axis must be aligned to be precisely parallel to the x movement of the microscope stage in order to keep the exact focus depth constant along the entire length of the fiber. This can be adjusted by use of three precision screws, two at the motor side and a single one at the other end of the tomograph. If the device is being used on an inverted microscope, accordingly longer screws allow for an upside-down positioning. Self-alignment of the device with regards to the microscope stage has been achieved by drilling a shallow

FIG. 2. (a) The rotatable fiber is held in position between two bearing V-grooves by forcing it down via single point contacts from the springs. (b) Layout of the micro axial tomograph (schematic top view = direction of the z axis). (c) Cross section of fiber in the immersion liquid chamber (schematic view along the fiber axis = x axis).

centering hole and milling a small V-groove into the stage. One of the tomograph beveled height adjustment screws rests with its apex in the bottom of the shallow hole, acting as a fixed pivotal point, and a distant screw rests in the opposite groove and thereby determines the lateral orientation of the instrument on the stage.

The coupling of the fiber to the motor has to be both user-friendly and rigid. Therefore, the fiber is simply attached by a small droplet of hot-melt adhesive applied to the end face of the motor shaft. To couple a fiber, the motor shaft is heated with the fine tip of a soldering iron set to about 125 °C, melting the glue droplet at the shaft end. Then, the fiber, firmly resting on its two bearings, is pushed towards the hot glue drop. It has been found to be of advantage to have the motor turning at a slow rate to equilibrate strains in the glue during the cooling phase. This simple method ensures an easy and robust handling of the fiber by the microscopist.

The diameter of the stepper motor used in this design has to be small enough to be fitted on the ground plane of a normal slide. Its diameter also determines the amount of free space left between the microscope stage and the objective. On the other hand, the torque should be sufficient to turn the fiber with a negligible backlash (hysteresis). Therefore, a model 0820-V-56-01 stepper motor from ARSAPE (Faulhaber, Schoeneich, Germany) was chosen. This motor has two separate field coils which are driven with switched direction direct current. The motor turns 18° per step resulting in 20 steps per one full rotation. An electronic stepper motor drive unit model AD-VL-01 (Faulhaber) is used to control the angular position of the motor.

III. DISTANCE MEASUREMENTS OF TEST OBJECTS

Test particles (green fluorescent microspheres of 200 nm diameter; Polysciences, Warrington, PA) were fixed on glass fibers (AR3 glass pulled to a homogenous diameter of 125 μm) using a specially designed capillary mounting device (Fig. 3). The device consists of a series of glass capillaries which can be filled by capillary forces with about 50 μL of cell or particle suspension and in which fibers can

FIG. 3. (Color online) Device for attaching objects, cells, and other bio-components to the glass fiber: small glass capillaries in the middle for the specimen suspension and grooves on the left and right to hold the fiber precisely in the center of the capillary.

be inserted for specimen preparation. After 30 min incubation time, the fibers with test particles were ready for mounting into the axial tomograph. To firmly bond particles, cells or other bio-components to fibers, the fibers can be coated with a polyvinyl-amine polymer exhibiting a multitude of positive surface charges.⁹

The micro axial tomograph was mounted on a motor driven, computer controlled microscope stage (Märzhäuser, Wetzlar, Germany) of a routine, upright fluorescence microscope (Zeiss Standard 25; Carl Zeiss Jena, Germany) which was equipped with a 40×/NA 0.75 (air) and a PlanAPO 63×/NA 1.40 (oil) objective lens and appropriate filter settings for the fluorescence dyes (here the FITC filter set was used: $\lambda_{ex.} = 450 - 490 \text{ nm}$; $\lambda_{em.} \geq 515 \text{ nm}$). For image acquisition, a cooled CCD b/w camera (Kappa CF 8 RCC; Kappa, Gleichen, Germany) was used.

Automated image acquisition was controlled by the software described in detail elsewhere.^{8,10,11} In short, from each angle of view, a stack of typically 80 images was taken from focal planes incremented by two micrometers each. To use all objects distributed around the fiber, a full turn of the fiber with 20 individual angular positions was recorded. The precision of the tilting angle is measured from the bead images using an alignment algorithm based on a weighted bipartite graph where the optimum transformation function is computed in a least squares manner based on the coordinates of the bary centers of the matched objects.¹¹ The lateral movement of the fiber was below 0.15% relative to the visible fiber length of 2000 μm between the two fiber bearings.⁹

Following the recording of the raw image stacks, the best focused image for each particle was included into a composite image constructed from each stack of 80 images, each representing a certain angular position. In all these 20 sum images, the x and y coordinates of each particle were automatically determined by fitting the intensity function to the point spread function (PSF) of the microscope objective with sub-pixel accuracy. To determine the precision of the distance measurements, two pairs of particles with approximately the same x position but differing at their y positions by a few micrometers were selected as test objects (Fig. 4).

To show the precision enhancement, the distance was calculated in two ways (Fig. 5; Table I): first the Euclidian distance was calculated “as usual” in microscopy from all three dimensions x, y, and z of the image stack:

$$d_{3d} = \sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2 + (z_1 - z_2)^2}. \quad (1)$$

Equation (1) includes the low resolution optical axis of the microscope (z). The tomographic approach is only to use the high resolution lateral axes (x,y) projection $d_{project}$ of the distance,

$$d_{project} = \sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2}. \quad (2)$$

Both measurements were done for each rotation angle. The first measurement results in 8–9 different distances depending on the number of acquired rotation angles (horizontal lines in Fig. 5) from which the median and standard deviation were calculated. The second measurement results in a sinus curve with the true distance d_{true} as the maximum and $\Delta\varphi$ as

FIG. 4. Rotation series of beads. Sum images acquired with the Zeiss standard 25 microscope, using a 40×/NA0.75 air objective at 0°, 36°, and −36° rotation angles. The arrows point at the two bead pairs (A, B) selected for the distance and localization measurements shown in Figs. 5 and 6 and Table I.

initial offset of the rotation angle:

$$d_{project} = d_{true} \cdot \sin(\varphi + \Delta\varphi) \quad (3)$$

Equation (3) was used for a Levenberg-Marquardt least squares fit with the maximum distance and offset as parameters, which gives the real distance and a standard deviation to this value.

Images of pairs of particles were acquired with both objective lenses. For the 63×/NA 1.40 (oil), the particles were embedded in 50% glycerol in water (refractive index $n = 1.40$) which was found to be the best compromise between viscosity (adapted to rotation conditions) and refractive index mismatch (oil/glass fiber). The measured tomographic and 3D distances for the two pairs of objects (A and B) are shown in Table I. Although a low resolution objective of NA = 0.75 (diffraction limited resolution laterally about 400 nm) was also used, the measurements applying the tilting projection image revealed a distance precision of less than $\pm 20 \text{ nm}$

FIG. 5. (Color online) Plot of the measured distance vs. rotation angle. The horizontal fit lines are averaged 3D distances [see Eq. (1)] measured from 3D image stacks acquired for each rotation angle. The sinus curves are fits of the 2D projections of the distance d_{project} [see Eq. (2)] in the lateral plane (xy-plane) with the true distance as maximum [see Eq. (3)]. Shown are the measurements with a $40\times/\text{NA}0.75$ (two right curves for A and B) and a $63\times/\text{NA}1.4$ (two left curves for A and B) objective for the particle pairs A and B (see Fig. 4).

which was considerably better than the values obtained from the averaged 3D measurements. Moreover, it is remarkable that the distance measurement precision was better for the results obtained with the low resolution $40\times$ lens than for the results obtained with the high resolution $63\times$ lens. This may be caused by reflection effects of the fiber due to insufficient adaptation of the refractive index. Despite the optical conditions that strongly differ from the optimum conditions, highly resolving objectives are calculated for; the distance precision measured in the particle arrangement of Fig. 4 revealed values of some ten nm only.

Figure 5 shows the result of averaged distance measurements obtained from series of 3D image stacks of several angular projections. With an increase of angular projections included into these mean values, the 3D distance accuracy can be improved. Under real biological specimen conditions, however, fluorescence photobleaching of the label has to be considered. Therefore, the axial tomographic distance determination using the maximum of the sinus curve (d_{true}) is more reliable and precise in routine practice.

Testing localization precision by simple line scans in x- and y-direction (Fig. 6) through one of the microbeads of pair

FIG. 6. Line scans in x- and y-direction through one of the microbead pair A in Fig. 4 at 0° rotation angle [see cross in Fig. 4]. The positions of the intensity maxima can be determined with the localization precision considerably below the nominal diffraction based resolution for the low resolution objective used ($\text{NA} = 0.75$).

A in Fig. 4 at 0° rotation angle revealed positions of intensity maxima with a localization precision considerably below the nominal diffraction based resolution ($\text{NA} = 0.75$).

IV. AXIAL TOMOGRAPHIC IMAGING OF A MOUSE OOCYTE

As a proof of concept, images of mature mouse oocytes' metaphase spindles were acquired with the axial tomography. For these experiments, the axial tomograph was mounted on the stage (Märzhäuser, Wetzlar, Germany) of a motorized Zeiss Axioplan2 imaging microscope equipped with a Zeiss Axiocam MR camera (Carl Zeiss Jena, Germany). The microscope provides a motorized filter wheel as well as a motorized objective mounting device equipped with filters for 4',6-DiAmidino-2-Phenylindole (DAPI), Fluorescein IsoThioCyanate (FITC), TetramethylRhodamine-IsoThioCyanate (TRITC), and CYanine 5 (CY5) and with $100\times/\text{NA}1.4$ (oil) and $63\times/\text{NA}1.4$ (oil) objective lenses. The setup is completed by a PC controlling stage, filter wheel, objectives, camera, and image acquisition by the Zeiss AXIOVISION 4 software.

The oocyte specimen was fixed on glass fibers and embedded in phosphate buffered saline (PBS) in order to find a compromise between refractive index adaptation and preservation of cellular morphology. The refractive index of PBS was 1.33. Special care had to be taken to prevent PBS from evaporation during the experimental time of about 30–60 min. In Fig. 7, sum images along optical axis of image stacks of a mouse oocyte fixed during the first cell division are shown, which were acquired at relative angles of 0° , 36° , 72° , and 90° using the $63\times/\text{NA}1.4$ (oil) objective lens. The spindle apparatus fluorescently stained with rabbit TACC3 protein antibodies is clearly visible from different perspectives. The 3D image region of the spindle apparatus was cut and deconvolved with the Huygens deconvolution software (Scientific Volume Imaging: www.svi.nl) (Fig. 7 insets). These

TABLE I. Results of distance measurements obtained from Fig. 5. For further details see text. Particle pairs A and B refer to Fig. 4.

Objective	Particle pair as shown in Fig.4	d_{true} (μm)	mean d_{3d} (μm)
$40\times/\text{NA}0.75$ (two right curves for A and B in Fig.5)	A	3.37 ± 0.02	3.41 ± 0.07
	B	5.66 ± 0.01	5.64 ± 0.08
$63\times/\text{NA}1.40$ (two left curves for A and B in Fig. 5)	A	3.03 ± 0.04	3.06 ± 0.09
	B	5.50 ± 0.03	5.87 ± 0.25

FIG. 7. Rotation series of a single mouse oocyte, sum images along optical axis of image stacks acquired at 0° , 36° , 72° , and 90° rotation angle with a $63\times/\text{NA } 1.4$ (oil) objective. The oocytes were fixed at the metaphase II stage and the TACC3 protein associated with spindle apparatus was visualized using rabbit anti-TACC3 and fluorophore-labeled goat anti-rabbit IgG antibodies. The orientation of the rotation axis follows the assignments in Fig. 4 (fiber axis = x-direction). Insets: Deconvolution images of the spindle apparatus, cuts from the rotation series at the respective angles were deconvolved using the Huygens software package.

deconvolved images were further processed with a raytrace volume rendering algorithm.¹⁴ Figure 8 shows comparisons of volume renderings of the deconvolved image stacks at 0° and 90° rotation angle. The improvement of image quality

and resolution by object rotation of 90° is clearly demonstrated. Further algorithms for 3D image processing and reconstruction are under development and will be published elsewhere.³²

FIG. 8. Volume rendering of the spindle apparatus of mouse oocytes (Fig. 7). Cuts from the 0° and 90° rotation angles in Fig. 7.

V. CONCLUSIONS

The idea of micro axial tomography was first realized about 20 years ago⁴⁻⁶ and successfully applied for automated multi-view 3D image acquisition.⁷⁻¹² Most of the early applications used a setup with an external stepping motor and a flexible shaft (e.g., Ref. 8) which suffered from mechanical insufficiencies in routine applications. Thus, the aim of the design described here was an improvement and a miniaturization of the setup in such a way that it can easily be handled without loss of precision as being necessary for modern high resolution microscopy techniques (e.g., Refs. 15 and 16).

Therefore, the precision mechanics of a fully adjustable glass fiber carrier was constructed which can allow for 3D precision localization and distance measurements considerably below the diffraction resolution limit.^{1,2} In order to demonstrate the potential of this improved design, we showed distance measurements using a very simple standard microscope with low resolution optics. Nevertheless, distance measurement precision of some ten nm only (corresponding here to an optimum optical resolution of 10–30 nm) was achieved without highly complex procedures of computer image sequence analysis and processing (see, e.g., Ref. 17). In addition, the improvement of image quality was shown by

3D imaging of a mouse oocyte and its spindle apparatus. Although a resolution improvement into the nanometer range was not obtained due to optically non-optimum preparation conditions in biological specimens, the 3D image indicates a structure resolution clearly beyond the diffraction limit of the given microscopic setup.

The number of angular projections taken was rather limited compared to similar axial tomographic imaging methods in electron microscopy, where usually large numbers of projections have to be acquired in order to improve the low signal-to-noise ratio after low energy electron radiation.¹⁸ In contrast to electron microscopy, the fluorescence techniques applied here provide a much better signal-to-noise ratio so that less image projections are necessary. Computer simulations of axial tomographic imaging in light microscopy and theoretical calculations of the resulting point spread function revealed three projections at least being necessary within a viewing angle of 120°. Merging these few projections resulted in an isotropic shape of the PSF.¹¹

Together with the specimen mounting device, the application is easy in laboratory handling and can be adapted to standard equipment, especially in such an environment where sophisticated near-isotropic 3D optical nanoscopy as, for instance, virtual volume super-resolution microscopy¹⁹ is not routinely available. Moreover, the micro axial tomograph may reach molecular localization and resolution conditions compatible with 3D nanoscopy which so far seems to require challenging interferometric optics.²⁰

VI. PERSPECTIVES

Novel approaches in light microscopy circumventing the Abbe-Rayleigh boundary conditions, enabled effective optical resolutions down to about 20 nm or even better (for review see Ref. 21). One of these methods using standard objective lenses is localization microscopy (for review see Ref. 22). It is based on the fundamental concept of labeling objects by different spectral signatures or using fluorophores that can be switched between two different spectral states to achieve a temporal isolation and, thus, a spatial separation of single signals. This allows the determination of object positions and distances, even if they are very close together (<Abbe-Rayleigh limit). All acquired positions of fluorescent molecules can then be merged into one image, in which the effective resolution is finally depending on the lateral and axial localization accuracy.

Early localization microscopy approaches (SPDM: spectral precision distance microscopy) have described a lateral localization precision of 50 nm or less for point like fluorescent objects, whereas the axial localization precision has been determined to about 100 nm.^{23–25} Other techniques [Photo Activation Localization Microscopy (PALM),²⁶ Fluorescence Photo Activation Localization Microscopy (FPALM),²⁷ and STochastic Optical Reconstruction Microscopy (STORM)²⁸] improved the localization accuracy using special fluorophores, which can be switched between two spectral states.

Recently, we have improved SPDM (SPDM: spatial position determination microscopy) localization microscopy to approach the precision range of less than 10 nm still using conventional fluorophores (e.g., fluo-

rescent proteins or Alexa dyes)^{15,16} which are switched to a “dark” state by a light-induced reversible photo bleaching.^{29,30}

So far, the application of all localization microscopy approaches depends on the precision to determine the position of a molecular object which at least in 3D mostly remains spatially anisotropic as a result of the Abbe-Rayleigh image diffraction conditions.^{3,31} Micro axial tomography in the device described here offers for the first time a technique that overcomes such restrictions in an easy to handle version. The localization data of particles presented here demonstrate the power of this approach. To elaborate further experiments and to test micro axial tomography for 3D localization nanoscopy (3D SPDM), especially for thick 3D conserved objects like entire cells, cell nuclei or cellular organelles will be the task of future investigations.

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