

# Sample holder for axial rotation of specimens in 3D microscopy

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**Key words.** 3D imaging, axial tomography, confocal laser scanning microscopy, light sheet fluorescence microscopy, microcapillary, SPIM.

## Summary

In common light microscopy, observation of samples is only possible from one perspective. However, especially for larger three-dimensional specimens observation from different views is desirable. Therefore, we are presenting a sample holder permitting rotation of the specimen around an axis perpendicular to the light path of the microscope. Thus, images can be put into a defined multidimensional context, enabling reliable three-dimensional reconstructions. The device can be easily adapted to a great variety of common light microscopes and is suitable for various applications in science, education and industry, where the observation of three-dimensional specimens is essential. Fluorescence z-projection images of copepods and ixodidae ticks at different rotation angles obtained by confocal laser scanning microscopy and light sheet fluorescence microscopy are reported as representative results.

## Summary for nonexperts

In common light microscopy samples are often located on glass slides or specific dishes, and observation is only possible from one side, namely perpendicular to the slide. However, for larger three-dimensional specimens observation from different views is desirable. Therefore, we developed a sample holder permitting rotation of the specimen perpendicular to the light path of the microscope. The specimen is placed within a glass capillary of cylindrical shape which is optically coupled to a surrounding capillary of rectangular shape fixed under the microscope. By rotating the inner tube, the specimen can thus be observed from any desired perspective perpendicular to its longitudinal axis. Using established techniques of three-dimensional microscopy, e.g. confocal laser scanning or light sheet fluorescence microscopy, image stacks are recorded under different angles and used for reconstructing the three-dimensional shape of the object. Fluorescence z-projection images of copepods and ixodidae ticks at different rotation angles are reported as representative results. In addition, the device can be easily

adapted to a great variety of common microscopes and is suitable for various applications in science, education and industry, where the observation of three-dimensional specimens is essential.

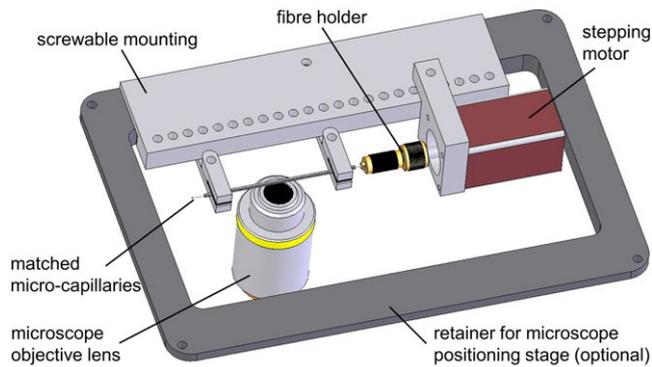
## Introduction

Light microscopy has become a well-established method in scientific research, medical diagnosis and industrial quality management. Samples are usually embedded between glass slides or within a specific dish such that observation is only possible from one perspective, namely perpendicular to the glass slide or the bottom of the dish. This allows for imaging and analysis of the specimen's surface facing the microscope objective lens including adjacent parts of the sample.

Especially three-dimensional (3D) objects require microscopic methods with high axial resolution, e.g. confocal laser scanning microscopy (CLSM; Pawley, 1990; Webb, 1996) or structured illumination microscopy (Neil *et al.*, 1997; Gustafsson *et al.*, 2008). Here, individual planes of a sample can be measured selectively upon illumination of the whole specimen permitting a single-sided 3D reproduction. In addition, in larger specimens, image quality often decreases with increasing distance from the sample surface facing the objective lens, since the penetration depth of excitation light is limited. These limitations can be overcome by rotating the sample within an appropriate holder. Another benefit of rotation is an increase of resolution by moving the vertical axis of a sample into the horizontal plane.

In contrast to CLSM and structured illumination microscopy, sample rotation has already been implemented in light sheet fluorescence microscopy (LSFM). This method, also known as selective plane illumination microscopy (SPIM), is becoming more and more popular (Huisken & Stainier, 2009; Santi, 2011; Bruns *et al.*, 2012) since planes under investigation are illuminated selectively, thus keeping the total light exposure very low (Schneckenburger *et al.*, 2012). However, most light sheet microscopes (e.g. Huisken *et al.*, 2004; Fahrbach & Rohrbach, 2010; Ritter *et al.*, 2010) are stand-alone devices with implemented illumination and specialized

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**Fig. 1.** Device for rotation of three-dimensional samples applicable to the x, y-stage of microscopes either directly by a screwable mounting or by a retainer compatible to common positioning stages.

sample rotation setups that cannot be combined with commercial microscopes.

For small samples in the range of a few micrometres (e.g. single cells, cell nuclei) the problem was faced by Bradl *et al.* (1994) and Staier *et al.* (2011) presenting a tilting device which allows specimens to be rotated up to  $\pm 90^\circ$ . By contrast, a sample holder for axial rotation of the specimen up to  $360^\circ$  and independent of the sample size is described in this manuscript. It provides good optical quality and can be combined with further add-on modules (e.g. microfluidics, tempering). Furthermore, the sample holder can be adapted to a variety of commercially available microscopes and is highly independent from illumination or detection techniques. Applications of the sample holder for axial specimen rotation are shown for CLSM and LSM. Fluorescence z-projection images of copepods and ixodidae ticks at different rotation angles are reported as representative results.

## Material and methods

### Sample holder

The module for rotation of 3D samples is depicted in Figure 1. The mounting can be screwed directly onto the x, y-stage of the microscope or adapted to a retainer compatible to common positioning stages used in modern microscopes.

For fluorescence microscopy two matched microcapillaries of borosilicate glass are used. The central part of the device consists of a fixed outer glass capillary of rectangular shape with a length of 50 mm, an inner cross-section of  $600 \mu\text{m} \times 600 \mu\text{m}$  and a wall thickness of  $120 \mu\text{m}$  (VitroTubes, VitroCom Inc., Mountain Lakes, New Jersey, USA). Within this outer rectangular capillary another capillary of cylindrical shape is placed (length: 75 mm, inner diameter:  $400 \mu\text{m}$ , outer diameter:  $550 \mu\text{m}$ ). The spacing between inner and outer capillary as well as the inner capillary containing the sample are filled with an appropriate immersion fluid. The outer rectangular capil-

lary is clamped at both sides to the mounting permitting optical access for detection from below by a microscope objective lens (Fig. 1). Furthermore, clamping prevents displacement during the measurement and supports rotation of the inner capillary along the longitudinal axis without tension.

For rotation of the inner capillary a computerized stepping motor with microstep positioning control (Nema 8 – ST2018S0604-A, SMC11, Nanotec Electronic GmbH & Co.KG, Germany) with an angular resolution of  $0.45^\circ$  (800 increments in 1/4-step mode) is used. This resolution can be improved to  $0.1125^\circ$  without further gear modifications (resulting in 3200 increments in 1/16-step mode). A bare fibre holder with delrin jaws (EPH-SR, Newport Corporation, Irvine, California, USA) for strain relief is adapted to the shaft of the stepping motor holding the inner round capillary with radial symmetry to the motor shaft. Here, the presented module is attached to the stage of the inverted microscope Axiovert 200M (Carl Zeiss Jena, Germany).

The stepping motor is driven by a small and flexible stand-alone control unit providing different running modes. It allows for manual rotation, for continuous rotation with selectable speed and for stepwise rotation with adjustable increments. Each mode is working in clockwise as well as in counter clockwise direction.

### Sample preparation and mounting

The sample is located within the inner round capillary. The appropriate method for sample preparation depends on the sample itself and its desired surrounding medium. There are different ways of inserting the sample, mainly dependent on its dimension: Small samples – compared to the inner diameter of the round capillary – can be soaked in by capillary forces or inserted using a pipette, larger samples – similar to the size of the inner diameter of the capillary – can be soaked in by connecting the capillary via a tube either to a pump or a syringe.

It is important to assure that the sample in the capillary does not move upon rotation. Therefore, it has to be either attached to the inner surface, or embedded in a gel or its size has to match the size of the inner diameter of the capillary so that it supports itself against the wall. The inner capillary containing the specimen is then inserted into the rectangular capillary and clamped to the shaft of the stepping motor. Now the rectangular capillary is fixed by the clamps, and the spacing between the two capillaries is filled with an immersion fluid by pipetting it next to one opening of the rectangular capillary so that it is soaked in by capillary forces.

Exemplary experiments presented in this paper were carried out using glycerol as sample environment and as immersion fluid. The advantage of glycerol is that its refractive index  $n_{589\text{nm}} = 1.473$  is very similar to the refractive index of the capillaries (borosilicate glass:  $n_{589\text{nm}} = 1.474$ ) so that aberrations are minimized. However, experiments carried out on

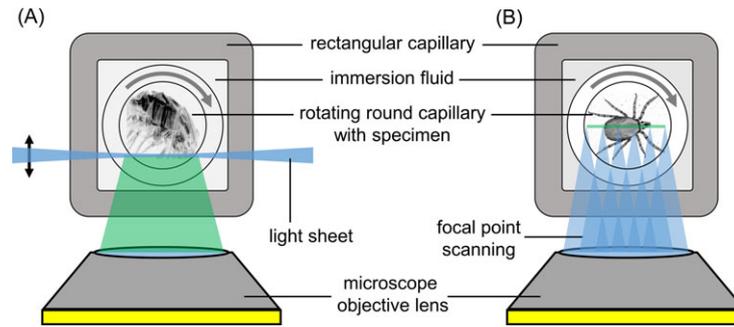


Fig. 2. Setup for fluorescence excitation and detection using (A) light sheet fluorescence microscopy (LSFM) or (B) confocal laser scanning microscopy (CLSM).

living specimens, e.g. living cells, require a physiological medium (typical refractive index: 1.33–1.37) as sample environment, and, therefore, lower refraction indices of the sample holding capillary and the immersion fluid are preferred to minimize aberrations.

As the sample sizes in the experiments reported below were similar to the size of the inner diameter of the capillary they were soaked in by a pump and supported themselves against the wall, so that further embedding or attachment was not necessary.

#### Microscopy techniques

The device for rotation of 3D samples can be used in combination with numerous microscopy techniques including epillumination and transillumination microscopy (e.g. bright field illumination, dark field illumination, phase contrast). Two techniques for detection of specific layers within the sample are described in detail.

**Light sheet fluorescence microscopy.** In LSFM a light sheet is created in perpendicular direction to the observation path either by a cylindrical lens (Bruns *et al.*, 2012; Fahrbach *et al.*, 2013; Chardès *et al.*, 2014) or by scanning of a laser beam (for a review see Huisken & Stainier, 2009; Santi, 2011). Thus, only the plane under investigation is exposed to light, and successive measurements can be performed at low light exposure of the whole sample when either the light sheet or the sample is moved in axial direction, as depicted in Figure 2(A).

For illumination of the sample a collimated 470 nm laser diode (LDH 470 with driver PDL 800-B, PicoQuant, Germany) is directly coupled to a LSFM module (Bruns *et al.*, 2014). The collimated laser beam is expanded by a telescope, focused by a cylindrical lens and deflected onto the specimen by a 90° mirror. At a numerical aperture  $A_N = 0.08$  the waist of the illumination light sheet is 6–10  $\mu\text{m}$  along the whole specimen. The light sheet and the objective lens can be moved simultaneously in vertical direction, and – using a correction of optical path lengths inside and outside the sample (Bruns

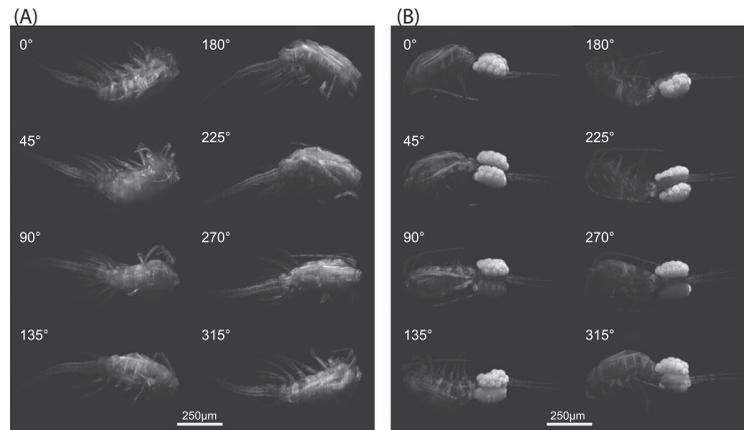
*et al.*, 2014) – all planes of the specimen are imaged without readjustment of the microscope. The sample holder can therefore be used with microscope objective lenses corrected for air. This permits easy exchange of lenses with different numerical apertures and magnifications even during a measurement cycle. In addition, objective lenses designed for an immersion medium (e.g. oil, water, glycerol) were tested successfully.

In the present experiments, fluorescence is detected by a 10x/0.30 or a 20x/0.50 microscope objective lens, a long pass filter for  $\lambda \geq 515$  nm and an integrating CCD camera (AxioCam MRc with software AxioVision 4.8.2, Carl Zeiss MicroImaging GmbH).

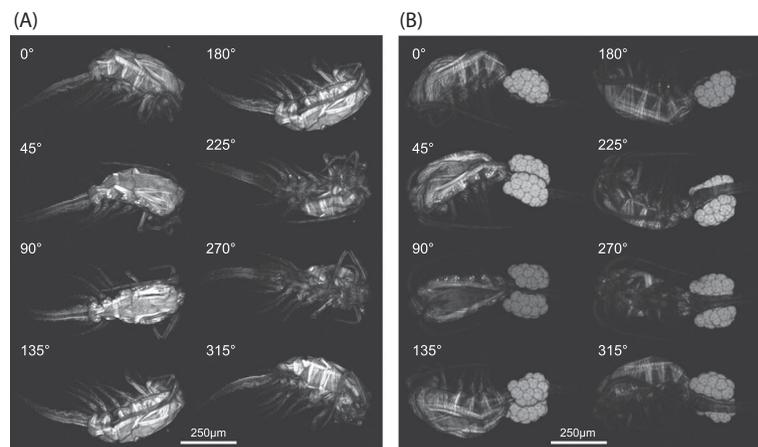
**Confocal laser scanning microscopy.** For illumination of the samples a Pascal 5 laser scanning head (Carl Zeiss Jena) equipped with an argon ion laser operated at 488 nm is used. The laser beam is focused by the microscope objective lens into the image plane and shifted by the scanning head to generate a two-dimensional image (Fig. 2B). By moving the objective lens in vertical direction one can generate a 3D data set (optical sectioning).

Fluorescence is detected by a 10x/0.30 or a 20x/0.50 microscope objective lens, a long pass filter for  $\lambda \geq 505$  nm and a photomultiplier tube. With a pinhole adjusted to 80% of an Airy disk, layers of 10  $\mu\text{m}$  thickness or less are again selected.

Data acquisition is equal for both microscopy methods. At a given rotational position two-dimensional images of different layers are recorded by shifting the image plane in axial direction with fixed increments (usually 5–10  $\mu\text{m}$ ), thus resulting in a z-stack of images. Then the inner capillary holding the specimen is rotated by a well-defined angle (e.g. 45° or 22.5° resulting in eight or 16 increments of a full circle), and another z-stack is recorded. Therefore, 3D stacks recorded under various angles are collected. The individual stacks can be either linked to each other (multiview registration) and fused (content-based multiview fusion) to one 3D rendered output image (Pitrone *et al.*, 2013) or projected separately for each angle into one image (z-projection). Here, z-projections of the individual data sets are presented.



**Fig. 3.** Fluorescence z-projection images of a copepod (A) without and (B) with egg sac incubated with acridine orange ( $10 \mu\text{M}$ , 30 min) recorded by light sheet fluorescence microscopy (LSFM) at eight single rotation steps. Light incidence from top to bottom (excitation wavelength: 470 nm; fluorescence detected at  $\lambda \geq 515 \text{ nm}$ ; sequence of stacks:  $\Delta z = 5 \mu\text{m}$ ; 80 images each; lateral resolution:  $\Delta x = 1.0 \mu\text{m}$  obtained by 10x/0.30 objective lens).



**Fig. 4.** Fluorescence z-projection images of a copepod (A) without and (B) with egg sac incubated with acridine orange ( $10 \mu\text{M}$ , 30 min) recorded by confocal laser scanning microscopy (CLSM) at eight single rotation steps (excitation wavelength: 488 nm; fluorescence detected at  $\lambda \geq 505 \text{ nm}$ ; (A) sequence of stacks:  $\Delta z = 3.75 \mu\text{m}$ , 100 images; (B) each z-stack recorded with intervals  $\Delta z = 6 \mu\text{m}$ ; 90 images; lateral resolution:  $\Delta x = 1.0 \mu\text{m}$  obtained by 10x/0.30 objective lens).

### Specimens

Experiments for demonstration of the sample holder for axial rotation were carried out on copepods and ticks.

*Copepods* belong to the group of small crustaceans resident in sea water as well as in fresh water. They represent the main disposal of marine zooplankton and, therefore, play an important role within the marine food chain. Copepods are typically 0.2 to 2 mm long and have a compact-shaped body with an armoured exoskeleton and large antennae. They possess a single median compound eye and a segmented thorax. The narrow abdomen contains five segments. Copepods do not have any heart or circulatory system (Barnes, 1980).

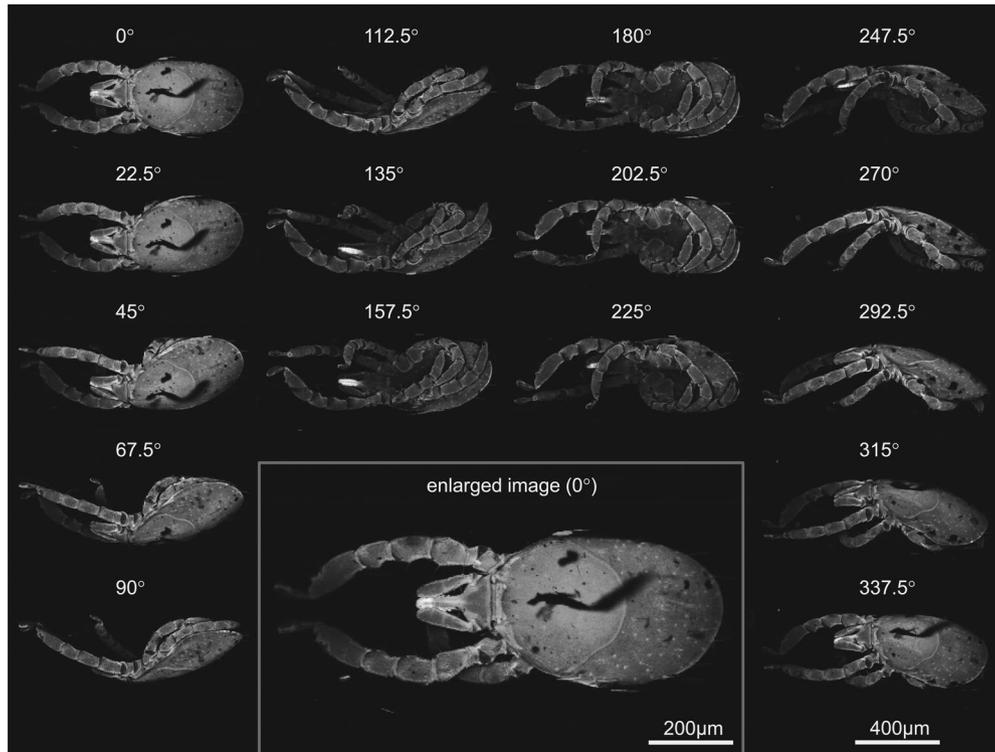
*Ticks* are small arachnids in the order of parasitiformes (sub-order: ixodidae). Ticks are nonpermanent ectoparasites, living by hematophagy on the blood of mammals, birds, and reptiles [Anderson, (2002)]. They are vectors of a number of diseases.

Ixodidae ticks undergo three primary stages of development: larval, nymphal and adult. Within the larval state they have a size of  $500 \mu\text{m} \times 400 \mu\text{m}$  and possess six legs (compared to eight legs in further states).

Copepods were stained in a 70% ethanol solution with the fluorescent dye acridine orange ( $10 \mu\text{M}$ ) for 30 min. Ixodidae ticks were prepared in a 70% ethanol solution without staining. The stained copepods and the unstained ixodidae ticks were washed afterwards in glycerol and soaked into the round capillary as described in Section above.

### Results

Fluorescence images of stained copepods were acquired by LSFM (Fig. 3) and CLSM (Fig. 4). In addition, CLSM was used for autofluorescence imaging of ixodidae ticks (Fig. 5).



**Fig. 5.** Autofluorescence z-projection images of an ixodidae tick in the larval state recorded by confocal laser scanning microscopy at 16 single rotation steps (excitation wavelength: 488 nm; fluorescence detected at  $\lambda \geq 505$  nm; sequence of stacks:  $\Delta z = 8.5 \mu\text{m}$ ; 50 images each; lateral resolution:  $\Delta x = 1.0 \mu\text{m}$  obtained by 10x/0.30 zobjective lens).

#### *Light sheet fluorescence microscopy*

In addition to the low light dose needed for light sheet based experiments, this method is characterized by a comparably high penetration depth of light into the specimen, as reported earlier (Bruns *et al.*, 2012) and proven by the images depicted in Figure 3. This Figure shows fluorescence z-projections of stained copepods without (A) and with egg sac (B) at light incidence from top to bottom. Fluorescence z-projection images of eight angular positions are depicted in each case with increments of 45°. Due to orthogonal detection (perpendicular to the image plane) solid parts like the egg sac may generate shadows (e.g. Fig. 3B at 270°).

#### *Confocal laser scanning microscopy*

In CLSM excitation and detection occur from the same side. This prevents shadowed areas and leads to a highly detailed visualization of the specimen, in particular of its superficial parts, as depicted in Figure 4. This Figure shows again fluorescence z-projection images of eight angular positions of stained copepods without (A) and with egg sac (B) and proves the high contrast of CLSM images.

As a further example z-projections of autofluorescence images (CLSM) of ixodidae ticks are depicted in Figure 5 for 16 rotation angles. This figure gives again detailed information

with high contrast, in particular from the superficial parts of the sample.

#### **Discussion**

This manuscript describes rotation of 3D samples located in a micro-capillary. The sample holder for axial rotation is easily adapted to the positioning stage of a conventional microscope. A stepping motor permits adjustment of any rotational angle. Any microscopy technique – including transmission, scattering or fluorescence microscopy – can be used, as exemplarily demonstrated for CLSM and LSFM.

In the present experiments, the geometry of the inner capillary matches the size of the specimen in such a way that it supports itself against the capillary wall. Therefore, no further fixation is necessary. For other applications and other specimens immobilization might be a prerequisite and can be achieved by embedding gels (e.g. agarose, phytigel) or by appropriate cellular or biochemical adhesion (for a protocol see Bruns *et al.*, 2014). In the latter case with liquid sample environment the module for rotation can be combined with a microfluidic system. For measurements in flowing media, where a dynamic incubation and, therefore, a dynamic environment is desirable, it is possible to plug the inner round capillary to a silicone tubing. Using a peristaltic pump one can measure the

uptake kinetics of fluorescent dyes, pharmaceutical agents or drugs. For a closed-loop microfluidic setup the required liquid volume can be reduced to about 300  $\mu\text{L}$ . A combination of a microfluidic setup and LSFM was recently described (Bruns *et al.*, 2014).

In the present experiments, glycerol is used for embedding the sample. With a refractive index  $n = 1.473$  glycerol matches the refractive index of the rotating borosilicate glass capillary. Due to this homogeneous refractive index, image quality is not impaired by the way of sample holding. However, in general index matching of the inner round capillary with the medium surrounding the sample and the immersion medium filling the space between the two capillaries is crucial. Experiments with samples in aqueous solution using the round glass capillary for sample holding would result in aberrations due to missing compensation of the curved interface. To avoid defocusing effects of the illumination and the detection light for samples embedded in water, culture medium or low concentrations of agarose gel an index matched tubing material for the round capillary is favourable. In this case, fluorinated ethylene propylene with a refractive index  $n = 1.338$  would be an appropriate nonfluorescent capillary material. Its use was demonstrated in long-term light sheet microscopy of zebrafish by Kaufmann *et al.* (2012) and Weber *et al.* (2014). Water should be used as immersion fluid filling the space between the two capillaries then. Index matching would also permit the use of high aperture objective lenses without or with very low aberration, if ultimate resolution is desired.

Aberrations due to the thickness of the glass capillaries can be excluded since the additional thickness of 220  $\mu\text{m}$  (for both capillaries) is close to a conventional cover slip (approximately 170  $\mu\text{m}$ ).

When using air objective lenses with samples surrounded by media others than air (e.g. glycerol, water) care has to be taken upon recording of  $z$ -stacks. Different refractive indices result in different optical pathways. The factor by which the shift of the objective lens differs from the resulting shift of the focal plane is calculated from the refractive indices and the numerical aperture of the objective lens. This factor has to be taken into account when choosing the increment for  $z$ -stack recording to avoid distortion of the 3D reconstruction.

A main advantage of the present module is that a specimen can be observed from any axial direction. In addition, in view of increasing resolution in microscopy, sample rotation may be helpful, since most commonly lateral resolution is higher than axial resolution. The technique of 3D optical microscopy using tilted views to circumvent the fundamental problem of low axial resolution was introduced by Shaw *et al.* in 1989 and Shaw in 1990. Exemplarily, in wide-field fluorescence microscopy  $\Delta z = n \lambda / A_N^2$  is about 7.5 times larger than  $\Delta x = 0.61 \lambda / A_N$ , if a fluorescence wavelength  $\lambda = 500 \text{ nm}$ , a numerical aperture  $A_N = 0.3$  and a refractive index  $n = 1.35$  are assumed. Thus, by rotating the sample one can improve

the axial resolution to a high and isotropic resolution in all three dimensions (Bradl *et al.*, 1996; Heintzmann & Cremer, 2002).

A comparison between LSFM and CLSM is given in the Figures 3 and 4, where in both cases sequential layers of 5–10  $\mu\text{m}$  thickness are selected and used for  $z$ -projection images. Due to the low numerical aperture used for LSFM, preferential forward scattering causes a higher penetration depth of excitation light in comparison with CLSM, where higher apertures are commonly used. Therefore, LSFM may give more information about deeper layers within a specimen, but may also produce some artefacts, e.g. stripes or shadows. CLSM on the other hand gives preferential information on more superficial layers of the specimen with a high image contrast which is typical for a scanning technique.

To conclude, in comparison to one-view standard imaging the sample holder presented in this paper allows for multiview imaging using the microscopy method of choice. The user is able to choose exactly the view and plane of interest which is of great importance, especially when examining larger complex samples, e.g. in developmental biology.

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