A versatile $2\pi$-tilting device for fluorescence microscopes

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Key words. Fluorescence light microscopy, confocal scanning laser microscopy, tilting device, point spread function, tilted view of cell nuclei.

Summary
A tilting device for biological specimens (rotation angle up to $2\pi$), especially fluorescence-labelled cell nuclei, was developed. It consists of a quartz glass capillary and a mounting adapter for the microscope stage. The applicability of the device was tested for several epifluorescence and confocal scanning laser fluorescence microscopes. The axis of rotation is perpendicular to the optical axis of the microscope. The capillary can be tilted around its axis at any desired angle or in equiangular steps. This can be done manually or by remote control using a stepping motor.

The three-dimensional (3-D), image-forming properties of the capillary system were experimentally examined using an inverse confocal scanning laser microscope. The results were compared with measurements obtained from the same microscope with the standard stage for plane slides with cover glasses. The measured point spread function suggested that in spite of aberration effects, the optical arrangement used allows a gain in the 3-D resolution by tilting the object.

Introduction
Light microscopy is one of the most powerful and widespread techniques in cell biology and cytogenetics. The recent advent of a variety of highly selective fluorescence probes (e.g. monoclonal antibodies, calcium- and pH-sensitive fluorochromes; Waggoner et al., 1989) requires a concomitant high spatial resolution in light microscopy to examine specific subcellular components. In cytogenetics, the rapid development of fluorescence in situ hybridization (for reviews see Lichter et al., 1991; Cremer & Cremer, 1992) during the last decade has resulted in specific painting of entire chromosomes, chromosomal subregions, individual genes and sequences of ribonucleic acids in cell nuclei. With this technique it became possible to study light microscopically the three-dimensional (3-D) organization of cell nuclei (Manuelidis & Borden, 1988; Trask et al., 1988; Popp et al., 1990; Geiger et al., 1991; Bischoff et al., 1993; Zirbel et al., 1993). Such investigations are relevant for the understanding of nuclear compartmentalization (Cremer et al., 1993).

With the increasing sensitivity of fluorescence in situ hybridization and the decreasing labelling size down to the level of individual genes (Ried et al., 1990), the demand for a 3-D microscopy with improved spatial resolution has increased. New methods such as confocal scanning laser fluorescence microscopy (Cremer & Cremer, 1978; Brakenhoff et al., 1985; Pawley, 1990) were introduced.

Light microscopes (epifluorescence as well as confocal systems), however, are far from ideal for 3-D imaging at high resolution. Normally, 3-D images are reconstructed from a series of optical sections obtained by subsequently incrementing the position of the focal plane. Due to the depth of field of the microscope objective (Sheppard, 1987) each optical section contains not only in-focus intensities from the region in the focal plane but also out-of-focus contributions. In addition, the spatial resolution in the direction of the optical axis is lower than perpendicularly in the focal plane using the conventional standard design with one objective (Richards & Wolf, 1959).

Those optical properties become a prominent image-forming component if the object dimensions of interest approach the diffraction limit. For example, in cell nuclei (typical diameter of the order of 10-20 µm) the territories of entire chromosomes cover regions of the order of about 2-5 µm in diameter.
(Bischoff et al., 1993; Rinke et al., 1994). This means that a detailed elucidation of the topology of targets within a territory (e.g. active genes vs. inactive genes; identified DNA sequences) is very difficult to obtain by present far-field optical procedures. A number of approaches are being made to overcome the present limitation of resolution in far-field light microscopy (Hell & Stelzer, 1992; Hell et al., 1994). They allow a considerable increase in optical resolution in one (axial) or two (lateral) directions (presently 75 nm; Hell, 1994: Hell et al., 1994a - a further reduction down to 35nm appears to be possible; Hell & Wichmann, 1994). A possible mean of improving the spatial resolution of the object in all three directions simultaneously is to rotate the object in an appropriate way and to register images at the different angles (Skaer & Whytock, 1975; Shaw et al., 1989; Bradl et al., 1992). Apart from a combination with these new approaches, rotation of the object would also improve 3-D imaging in conventional far-field fluorescence microscopy.

Here, we will present a versatile device based on developments described by Bradl et al. (1992) that can be adapted to nearly any far-field light microscope. This device allows full rotation (2π) of the specimen, so that the same resolution of a target distance within an object in all three object dimensions can be achieved.

Tilting device

As basically described by Bradl et al. (1992), a tilting device has been constructed with the option of 'general' applicability. By means of stage adapters the device can be mounted on many epifluorescence microscopes and confocal scanning laser microscopes with the capillary orientation parallel to the x- or y-direction. The device (Fig. 1) consists of an exchangeable quartz capillary (Glas & Technik, Berlin, Germany) with an average diameter of 0.2 mm and a length of 70-80 mm, in which the biological specimen in suspension can be sucked in. The thin end of the capillary is fixed in a brass bearing which is mounted into an aluminium block. The middle part of the capillary (i.e. the region of observation) is located in a long V-shaped groove in a teflon block. This prevents undefined lateral shifts of the capillary during rotation. The depth of the groove is determined in such a way that the capillary axis is kept above the teflon block.

Instead of the teflon blocks the object can be mounted in a plexiglas block, so that transmission light microscopy becomes possible. In this case, the capillary is located about 14mm above the stage of the microscope. Generally, with increasing magnification and numerical aperture (NA), the working distance of an objective decreases. For high-magnification objectives (63x, 100x) with high NA, typical values are between 50 and 100µm. Since the optical properties of those objectives for fluorescence microscopy were usually designed for use with cover glasses of about 170µm thickness, it is possible to focus through the entire capillary down to the bottom in the absence of a cover glass if sufficient matching of the refractive index can be achieved (see below).

The second end of the capillary (due to manufacturing reasons thicker) is located into a larger V-shaped groove in the aluminium frame and held by a copper feather. The entire tilting device has a size of about 100 x 50 x 27 mm. In order to adapt the device to confocal scanning microscopes with fast scan galvanometer stages, all components were constructed of light-weight materials.

The thin end bearing of the fixed capillary can be tilted manually or computer controlled by a stepping motor connected via a flexible shaft, so that motor-induced vibrations are minimized. The system has no restrictions on the tilting angle (0-360°/2π) or angle stepwidth.

The feasibility of object rotation by the described tilting device was tested by means of fluorescence microscopes or propidium-iodide-stained cell nuclei, which were sucked into the capillary, on the following epifluorescence and confocal scanning laser microscopes (CSLMs): Zeiss Standard 25, Zeiss Axioshot, Leitz Orthoplan, Leica TCS 4D, Zeiss LSM 10, Zeiss LSM 310, and a specially designed inverse CSLM (Stelzer et al., 1986; Stelzer, 1990). Figure 2 shows a typical example detected by the latter microscope. An inhomogeneously fluorescent microsphere of 8µm diameter in a buffer of glycerine and water (9:1) was excited by a 488-nm ion laser beam and detected via a filter combination suited for FITC emission. A Zeiss planapochromat, oil-immersion objective 100x/NA 1.4-336 (for λw 550nm). They allow a considerable increase in optical resolution in one or two directions (presently 75 nm; Hell, 1994: Hell et al., 1994a - a further reduction down to 35nm appears to be possible; Hell & Wichmann, 1994). A possible mean of improving the spatial resolution of the object in all three directions simultaneously is to rotate the object in an appropriate way and to register images at the different angles (Skaer & Whytock, 1975; Shaw et al., 1989; Bradl et al., 1992). Apart from a combination with these new approaches, rotation of the object would also improve 3-D imaging in conventional far-field fluorescence microscopy.

Refractive index matching

The optical properties of high-quality, multi-element objectives in fluorescence microscopes have normally been calculated for well-defined conditions (e.g. oil-immersion n = 1.482 at 293 K, thickness of the cover glass 170 µm). Ideally, the object should also fulfill these conditions. For biological specimens, however, the objects and the buffer systems differ from these ideals (e.g. for cytoplasm n = 1-35 8-1-3 74, or cell culture medium with n slightly greater than water n = 1-336; Lanni et al., 1986). In the case of the tilting device, the coverglass is omitted, and instead the quartz capillary provides two spherical surfaces in the optical pathway with n w 1-46 (for A w 550nm).

To reduce optical distortions caused by the capillary and...
by the different refractive indices, different combinations of immersion fluids were tested: (a) immersion oil (fluorescence free: $n = 1.482$) outside between the capillary and the objective; immersion oil inside the capillary for specimen suspension; (b) glycerine ($n = 1.4715$) inside and immersion oil outside; (c) glycerine inside and outside; (d) mixture of glycerine and specimen buffer (9:1) or glycerine and water (9:1), respectively. This combination inside and outside the capillary provided the best results as judged by visual observation.

**Influence on the point spread function**

The way in which the capillary in the microscope distorts and degrades the image of an object can be quantitatively described in terms of the point spread function (PSF). The PSF was determined by the method described by Hiraoka et al. (1990). For this purpose a set of optical sections of a 'point source' (microsphere 121 nm in diameter) at different positions along the optical axis was recorded.

The experiment was carried out using the inverse CSLM (Stelzer, 1990) with a Zeiss plan-apochromat oil-immersion objective 100x/1.3NA. Four different set-ups were compared to study effects caused by different immersion fluids and the combination with the quartz glass capillary.

In cases I-III, the beads registered were closest to the objective, next to the cover glass; in case IV, the microspheres were located in the capillary around the maximum distance from the objective lens due to their thermal movement. (I) Object slide with cover glass: immersion oil (fluorescence free) between the objective lens and the cover glass and between the cover glass and...
Fig. 2. Rotation of a fluorescent microsphere 5-8 μm in diameter registered with the capillary mounted on an inverse CSLM. (a-h) A sequence of central optical sections after tilting by increments of 45° (arrows). For refractive index matching a glycerine to water (9:1) mixture (inside and outside the capillary) was used with a planapochromat oil-immersion objective 100x/1.3NA. All images were computer aligned for visualization.

the slide as a suspension medium containing the beads (standard set-up oil/oil). (II) Object slide with cover glass: immersion oil (fluorescence free) between the objective lens and the cover glass and glycerine between the cover glass and the slide (standard set-up oil/glycerine). (III) Object slide with cover glass: immersion oil (fluorescence free) between the objective lens and the cover glass and water between the cover glass and the slide (standard set-up oil/water).

Fig 3. (a) Integrated intensity of images of a 'point source' (microsphere 121 nm in diameter) for a defocus series in pseudo 3-D representations (A) and in projections on the x- (B) and y- (C) axes for the standard set-up oil/oil (case I, see text). From a single bead different optical sections were registered, integrated and plotted as a function of x and y. (b) Integrated intensity distribution of the same microsphere in the xz-plane. (A) Pseudo 3-D representation; (B) projection on the x-axis; (C) projection on the z-axis. (c) Integrated intensity distribution of the same microsphere in the yz-plane. (A) Pseudo 3-D representation; (B) projection on the y-axis; (C) projection on the z-axis. Scale: μm as indicated in (A).
symmetry factor relative to the standard set-up (oil/oil)
the intensity distributions are shown. In addition, a
half maximum (FWHM) values of the projections of
normalized to 1. In Table 1, the mean of the full width
added pixel by pixel and the integrated maximum was
4). The image values of the optical sections were
axes are shown for case I (Fig. 3) and case IV (Fig.
-047µm) were registered. Image registration of each
sections were extracted to a size of 64 x 64
pixels around the centre of each bead slice.
The PSF and its projections on the three
axes are shown for case I (Fig. 3) and case IV (Fig.
4). The image values of the optical sections were
added pixel by pixel and the integrated maximum was
normalized to 1. In Table 1, the mean of the full width
half maximum (FWHM) values of the projections of
the intensity distributions are shown. In addition, a
symmetry factor relative to the standard set-up (oil/oil)

\[
\sigma = \frac{r_l}{r_r} = \frac{\rho}{\rho}\]

where \(r_l\) is the width of the intensity curve at 0.25
maximum (arbitrarily chosen value for a proper dis-
tinction) left of the maximum position and \(r_r\) is the
corresponding width right of the maximum position,
\(r_l\), \(r_r\), \(\rho\), \(\rho\) are the values for the standard set-up oil/oil.
\(\rho > 1\) means left and \(\rho < 1\) right asymmetry, \(\rho = 1\)
represents a symmetric distribution.
The results show that in the object slide
cases I-III the change of the immersion fluid had little
influence on the FWHM. For the capillary set-up the
resolution at FWHM was in the same range consider-
ing the x- and y-projections. Only the z-projections
showed a decrease by a factor of about 2.2 compared
with case I. This elongation effect is probably due to
aberrations induced by the capillary interfaces. The
asymmetry was compatible with that obtained by
standard set-ups, although the capillary effect may be
visible comparing x- and y-projections. Comparing the
lateral resolution of the capillary set-up with the axial-
resolution of the standard set-ups, an increase of
resolution can be obtained if the object is tilted by
appropriate angles. The relatively poor axial resolution
appears to be tolerable because target distances
within the object can be obtained from lateral image
slices after rotating the object into an optimal perspec-
tive. Thus, as a measure of 3-D resolution, the axial
FWHM of conditions I-III (conventional arrangement)
may be compared with the lateral FWHM obtained
with the tilting device (condition IV). If we compare the
smallest FWHM measured for each case [0.71 µm
(case II) axial; 0.38 µm (case IV) lateral] the tilting
device suggests a 3-D resolution better by a factor of
about 1.9; if we take the means (six in cases I-III; four
values in case IV), the improvement would be a factor of
2.1 times better.

Low-cost, automated set-up
The advantage of tilting biological specimen into an
optimal perspective for 3-D microscopy and thus in-
creasing the resolution may be a helpful tool in quanti-
tative analyses. For the determination of the absolute
distance between two image points, the object can be
tilted in such a way that both points are in the same
lateral plane (S. Dietzel, T. Cremer & C. Cremer,
unpubl. results). Distance measure ments can always
be made using the optical resolution in the x- and y-
directions.
The 2n-tilting device described here was
used in the construction of a low-cost, fully automated
3-D microscope with an expected 3-D distance resolu-
tion better than that of a conventional confocal fluo-
rescence microscope (J. Bradl et al., unpubl. results).
Apart from the capillary set-up, all components are
commercially available. The basic instrument for such
a system is a Zeiss Standard 25 epifluorescence
microscope. The tilting device is adapted to a step-
motor-controlled xy-stage (Fa. Märzhauser, Wetzlar,
Germany). For optical sectioning, the stage can be
moved in the axial direction by a step motor directly
coupled to the standard microscope focusing mechan-
ics. The xy-movement of the stage and the z-focus
are controlled by a transputer unit (MC 2000, Fa, ITK,
Lahnau. Germany) and can be operated by a joystick
or computer program. Each step motor has a resolu-
tion of 40.000 steps for 360°. An area of 75 x 25 mm
(xy-plane) can be scanned with a minimum theoretical
stepwidth of 0.01 µm and a

Fig 4. (a) Integrated intensity of images of a ‘point source’ (microsphere 121 nm in diameter) for a defocus series in pseudo 3-D
representations (A) and in projections on the x- (B) and y- (C) axes for the capillary set-up glycerine/glycerine (case IV, see text).
From a single bead different optical sections were registered, integrated and plotted as a function of x and y. (b) (integrated intensity
distribution of the same microsphere in the xz-plane. (A) Pseudo 3-D representation; (B) projection on the x-axis; (C) projection on
the 2-axis. (c) Integrated intensity distribution of the same microsphere in the yz-plane. (A) Pseudo 3-D representation; (B) projection
on the y-axis; (C) projection on the 2-axis. Scale: µm, as indicated in (A).
reproducibility for relocation of ±0.5 µm. The reproducibility of the focusing unit was measured by means of an inductive transducer (WL-1, Fa. Messotron, Seeheim, Germany). For a stepwidth of 200 nm the reproducibility was better than ±60 nm over an axial distance (z-direction) of 13 mm.

For image acquisition, a cooled black-and-white CCD camera (CF 8 RCC; Fa. Kappa, Gleichen, Germany) is used. The interline transfer CCD chip has a size of 8.8 x 6.6 mm, 739 x 575 pixels (x-y) and a pixel size of about 11 x 11 µm. For a 63x/1.4 oil-immersion objective, an additional magnification of 2 x is used, in order to satisfy the Nyquist criterion according to a theoretical resolution in the object plane of about 0.2 µm. The CCD camera is read out via an MFG-3M-AVS frame grabber board in single monitor display mode (Imaging Technologies Inc., MA, U.S.A.). It is possible to run the image acquisition software and the visualization of the image simultaneously on the same 20" monitor. The whole set-up is operated by a 50-MHz 80486 personal computer. For image acquisition, the software Optimas V4.00 (Bio-Scan Inc., WA, U.S.A.) running under MS-Windows 3.1 is used.

Preliminary results obtained with this system are presented in Fig. 5. The image sequence shows that objects can be tilted reproducibly.

Discussion

In this report, an improved tilting device is described that can be used with many epifluorescence and confocal scanning laser microscopes. The optical behaviour of the capillary set-up was tested in a high-resolution 3-D microscope and compared with standard set-ups of object slides with cover glasses for different conditions of refractive index matching.

A straightforward way to obtain a point spread function is to register the intensity distribution detected from a 'point-like' object. This condition was fulfilled by the beads used, which were about 2-7 times below the actual wavelength. Using smaller beads might result in a sharper PSF, but temperature-induced movements might result in broadening the detected PSF by averaging over several subsequently detected optical sections.

Using an inverse microscope with the fluorescent beads directly attached to the cover glass, the influence of different suspension media was tested. This was compared with the worst case for the capillary: i.e. the object at the maximum distance from the objective lens. To reduce optical distortions caused by the capillary and by the different refractive indices of the components in the imaging system (Hell et al., 1993), an appropriate index matching between immersion fluid outside the capillary, the quartz glass and the buffer fluid inside the capillary had to be achieved. However, the variation and index mismatch of the optical specimen cannot always be avoided in cases of biological material. In addition, the choice of the buffer fluid is restricted by its biochemical behaviour, which has to be appropriate for the specimen under investigation. The results obtained for the capillary suggest that the capillary did not have a major influence on the lateral resolution. Therefore, appropriate tilting of the object will allow an increased 3-D resolution and precision for distance measurements:

1 Two targets (A, B) located upon each other in the axial direction in the conventional confocal arrangement cannot be separated if their distance is ≤ 1 FWHM (axial). Since

![Image of Table 1](https://via.placeholder.com/150)

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Fig. 5. Rotation of a lymphocyte cell nucleus with FITC-labelled DNA material bound to the nuclear membrane. The images were registered with the capillary set-up mounted on the Zeiss Standard 25 microscope with a planapochromat oil-immersion objective 65x/1·4NA. For immersion fluid, a mixture of glycerine/hybridization buffer (9:1) was used. The images were registered with a cooled CCD-camera at an angle stepwidth of 10°. To overcome bleaching effects of the dye, the acquisition time was increased by 0·08 s for each subsequent image starting with 1 s.

The lateral PSF of the tilting device (using the same microscope) was observed to have a smaller FWHM, an improvement in the 3-D resolution of such targets is expected.

The precision of 3-D distance measurements depends on the resolution in both lateral and axial directions if arrangements without tilting are used. Using the tilting device, the object can be rotated in such a way, that the targets (A, B) are always located in the place with the smallest FWHM value.

The present results show the feasibility of the approach.

More detailed studies should include a statistical averaging of several measured point spread functions. From those data the average as well as the optimal conditions may be extracted. Furthermore, it should also be useful to register capillary point spread functions directly for other types of fluorescence microscopes. To optimize refractive index matching further for different types of biological specimens, other kinds of capillary material and immersion fluids should be tested.

A major advantage of a capillary device is that by means of object tilting, the same spatial resolution for all target
Distances within an object can be obtained. We expect that the tilting device described, in combination with conventional Abbe fluorescence microscopy, may allow a considerably improved spatial resolution and precision of 3-D distance measurements in the submicrometer range. If we assume an axial FWHM of 1-4 times the axial confocal FWHM (using the same objective lens and refraction index conditions), according to reasoning outlined above, an improvement in 3-D resolution of a factor of 2·1 x 1·4 ≈ 3 might be feasible. Furthermore, it is anticipated that the tilting device may also be used in combination with new techniques of superresolution far-field light microscopy. For instance the 4m and offset microscopy recently described (Hell & Stelzer. 1992: Hell et al., 1994a,b; Hell, 1994: Hell & Wichmann. 1994), or standing wave microscopes (Lanni et al., 1986, 1994) indicate that the optical resolution of far-field light microscopy is not restricted to the Abbé wavelength criteria. The capillary set-up described here may be mounted into such microscopes in order to obtain an improved 3-D distance resolution in all directions of the object.

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