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Abstract. A device for selective plane illumination microscopy (SPIM) of three-dimensional multicellular spheroids, in culture medium under stationary or microfluidic conditions, is described. Cell spheroids are located in a micro-capillary and a light sheet, for illumination, is generated in an optical setup adapted to a conventional inverse microscope. Layers of the sample, of about 10 μm or less in diameter, are, thus, illuminated selectively and imaged by high resolution fluorescence microscopy. SPIM is operated at low light exposure even if a larger number of layers is imaged and is easily combined with laser scanning microscopy. Chinese hamster ovary cells expressing a membrane-associated green fluorescent protein are used for preliminary tests, and the uptake of the fluorescent marker, acridine orange via a microfluidic system, is visualized to demonstrate its potential in cancer research such as for the detection of cellular responses to anticancer drugs. © 2012 Society of Photo-Optical Instrumentation Engineers (SPIE). [DOI: 10.1117/1.JBO.17.10.101518]

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1 Introduction

Imaging of three-dimensional (3-D) samples, such as cell or tissue cultures, is a key problem in modern fluorescence microscopy. In particular, high axial resolution is required for obtaining information from individual focal planes and for possible reconstruction of a 3-D image. As an important step towards this goal, confocal^{1,2} and multi-photon^{3,4} laser scanning microscopy have been described more than 20 years ago. In addition, wide-field microscopy, with structured illumination, has been suggested to be a promising alternative method⁵ which has been improved considerably during the past few years.⁶ However, the disadvantage of all these methods is that for measuring each focal plane, the whole sample is exposed to light, and upon recording of numerous focal planes, the light dose sums up and may damage sensitive organisms like living cells.^{7,8} This problem may be overcome by single plane illumination microscopy (SPIM) where a light sheet is created perpendicular to the observation path either by a cylindrical lens or by scanning of a laser beam.^{9,10} In this case, only the plane under investigation is exposed to light, and successive measurements can be performed at low exposure of the whole sample when either the light sheet or the sample is shifted in axial direction.

Light sheet based microscopy, however, needs a considerable modification of existing microscopes with specimens being located either in special chambers^{11–13} or embedded in a matrix such as agarose.^{14,15} Often, completely new equipments are required. As an alternative to those systems, a comparably simple illumination device for SPIM is now adapted to a conventional inverse microscope. Multi-cellular spheroids, which in terms of cell morphology and function are more similar to tissue

than conventional two-dimensional cell cultures,^{16,17} are introduced and located in micro-capillaries of rectangular shape, where they are surrounded by culture medium or physiological solution under stationary or flowing condition. Since the light sheet is perpendicular to the surface of the capillary, the illuminated plane is well defined and, together with the microscope objective lens used for image detection, can be easily shifted in axial direction. This permits fluorescence microscopy of multiple planes at low light exposure.

2 Materials and Methods

Chinese hamster ovary cells, permanently transfected with a plasmid encoding for a membrane associated green fluorescent protein (CHO-pAcGFP1-Mem), are routinely grown in RPMI 1640 culture medium, supplemented with 10 percent fetal calf serum (FCS) and 1 percent Penicillin/Streptomycin at 37°C and 5% CO₂. After seeding 200 cells per well in a 96 well plate with cavities being coated by an agarose layer to prevent the cells from adhesion to the bottom, spheroids are grown for 5 days in the same medium up to a diameter of 200 to 300 μm .

For fluorescence microscopy, microcapillaries of borosilicate glass with rectangular shape and an inner cross section of 900 × 900 μm^2 (Hilgenberg, Malsfeld, Germany) are used. The thickness of their walls is 220 μm , thus, similar to a conventional cover glass used in high aperture microscopy. Culture medium or physiological buffer solution, such as Earl's Balanced Salt Solution, containing the cell spheroids is taken up by capillary forces. A final position of the spheroids is attained when the micro-capillaries are placed horizontally in the microscope (Axiovert 200, Carl Zeiss Jena, Germany). Spheroids in a micro-capillary do not move upon adjustment of the microscope table, therefore, no further fixation is necessary in stationary

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media. For applications in flowing media, capillaries coated with fetal calf serum (FCS) and containing spheroids are left for 4 h in the incubator (37°C, 5% CO₂) to assure appropriate adhesion. For visualizing the uptake of the fluorescent dye acridine orange (Molecular Probes, Eugene, OR), culture medium containing 5 μM acridine orange is pumped through the micro-capillary, at a temperature of 37°C and a velocity of 25 mm/min, using a dispensation pump (MS-1 REGLO, Ismatec Glattbrugg-Zürich, Switzerland), a water bath, and an appropriate tube (Tygon Standard R-3607, IDEX Health & Science, Wertheim, Germany) with a trap for potential bubbles. Solutions of the fluorescent dye, rhodamine 6G (2.5 μM in distilled water), are used for checking the beam waist of illumination.

For illumination of the samples, an argon ion laser, operated at 488 nm (Innova 90, Coherent, Palo Alto, CA), is used together with a single mode fiber-optic system (kineFlex-, Point Source, Southampton, UK). For measurements of acridine orange, this laser is replaced by a 470 nm laser diode directly coupled to the microscope (LDH 470 with driver PDL 800-B, PicoQuant, Berlin, Germany). The collimated laser beam, at the exit of this fiber, is expanded by a Galilei telescope (with lenses of $f'_1 = -10$ mm and $f'_2 = 80$ mm), and a resulting beam of 8 mm diameter is focused by a cylindrical lens (focal length: $f'_3 = 50$ mm) and deflected onto the spheroid by a 90 deg mirror, as illustrated in Fig. 1. With a numerical aperture, $A_N = 0.08$, the waist of the illumination beam can be limited to about 10 μm along the spheroid, as demonstrated in Fig. 2 for a test solution (2.5 μM rhodamine 6G). By an adjustable screw, the setup for beam deflection and focusing, deflection mirror and cylindrical lens, is coupled to the objective turret of the microscope, whereas, all other optical and mechanical components are fixed on the base plate of the microscope stage with a customized sample holder. Therefore, the light sheet and the objective lens can be moved, simultaneously, into vertical direction and all planes of the spheroid are imaged without re-adjustment of the microscope. A laser power of 0.042 mW, focused to a line sheet of 10 μm waist and 8 mm width, results in an irradiance of 52 mW/cm² for both lasers,

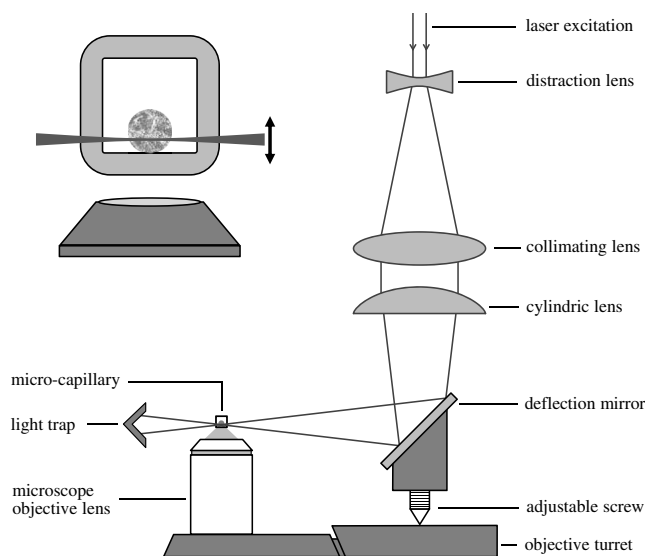


Fig. 1 Illumination device for light sheet based fluorescence microscopy coupled to an inverse microscope. Inlay: illumination of a spheroid within a micro-capillary. The cylindrical lens and the deflection mirror are fixed to the objective turret.

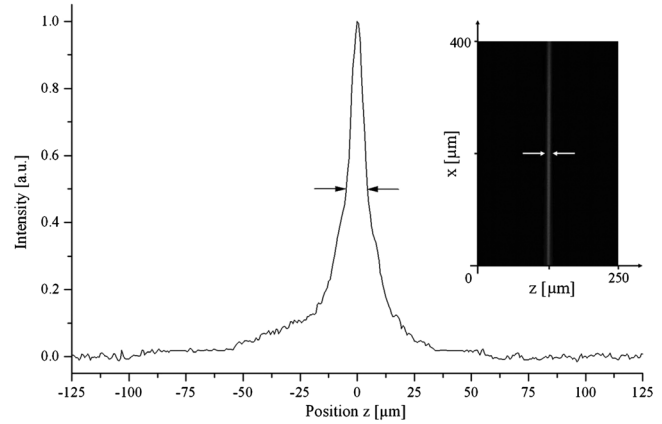


Fig. 2 Fluorescence profile of rhodamine 6G (2.5 μM) excited by a light sheet in the focus of the cylindrical lens ($f'_3 = 50$ mm). A width of 9.4 μm is measured at half maximum. The inlay shows the beam waist along a path length of 400 μm.

which corresponds to about half of the solar constant. An exposure time between 100 ms and 1 s is used for all images.

Fluorescence is commonly recorded by a 10×/0.30 or a 20×/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, Göttingen, Germany) with subsequent background discrimination from all images. Since the microscope is equipped with a Pascal 5 laser scanning head (Carl Zeiss Jena, Germany), light sheet based fluorescence microscopy is easily combined with confocal laser scanning microscopy (LSM) using the same excitation wavelength, a long pass filter for $\lambda \geq 505$ nm and an irradiance of 17 mW/cm². A recording time of 3 s, for each image, results in the same light exposure as for light sheet based fluorescence microscopy. With a pinhole adjusted to 80 percent of an Airy disk, layers of 10 μm diameter are again selected.

3 Results

SPIM images, of various layers of a CHO-pAcGFP1-Mem cell spheroid, recorded at distances of 20, 40, 60, and 80 μm from its edge are depicted in Fig. 3. While at distances of 20 and 40 μm, individual cells with brightly fluorescent membranes are well resolved, fluorescence patterns are becoming more diffuse along the path of excitation light through the spheroid, if distances of 60 or 80 μm from its edge are selected. This, most probably, results from increased scattering along the light paths of excitation from top to bottom, and emission, perpendicular to the image plane. Confocal LSM images, as seen in Fig. 4, again show well resolved cell layers at distances of 20 and 40 μm from the edge of the spheroid, whereas, some shading and decrease of resolution in the center of the spheroid occur at distances of 60 and 80 μm from this edge. Here, the paths of excitation and emission light, through the sample (perpendicular to the image plane), are comparably long, thus, causing pronounced scattering.

The same light dose of 52 mJ/cm² is used to record a single layer of the spheroid by SPIM or LSM. However, light exposure in SPIM experiments is restricted to individual layers, whereas, in LSM experiments, the whole sample is illuminated upon measurement of each layer. This implies that upon recording of multiple layers, the light dose sums up over the whole exposure time.

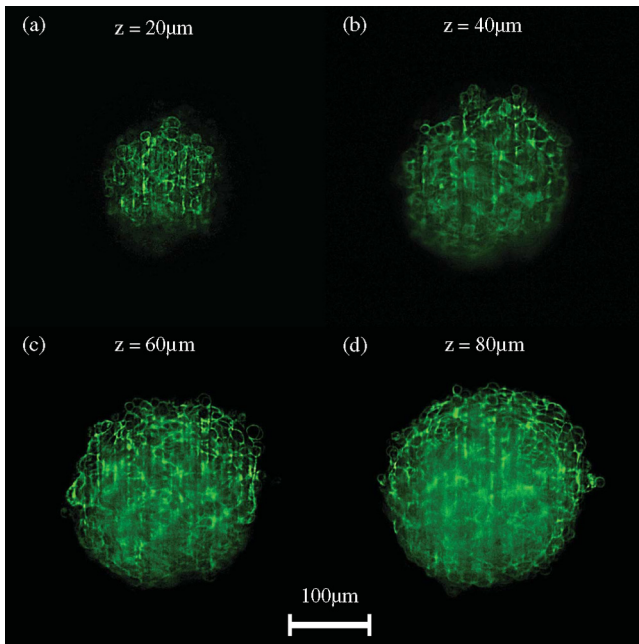


Fig. 3 SPIM images of various layers of 10 μm diameter of a CHO-pAcGFP1-Mem cell spheroid recorded at distances of 20, 40, 60, and 80 μm from its edge; light incidence from top to bottom (excitation wavelength: 488 nm; fluorescence detected at $\lambda \geq 515$ nm; light exposure: 52 mJ/cm^2 ; exposure time: 1 s each).

The uptake of acridine orange, pumped into the micro-capillary, by the multi-cellular spheroid after 5 and 30 min is depicted in Fig. 5(b) and 5(c), where its fluorescence is compared with the intrinsic fluorescence of the spheroids at 0 min, which is depicted in Fig. 5(a). The fluorescent marker, causing a pronounced increase of intensity—is localized in the outer cell

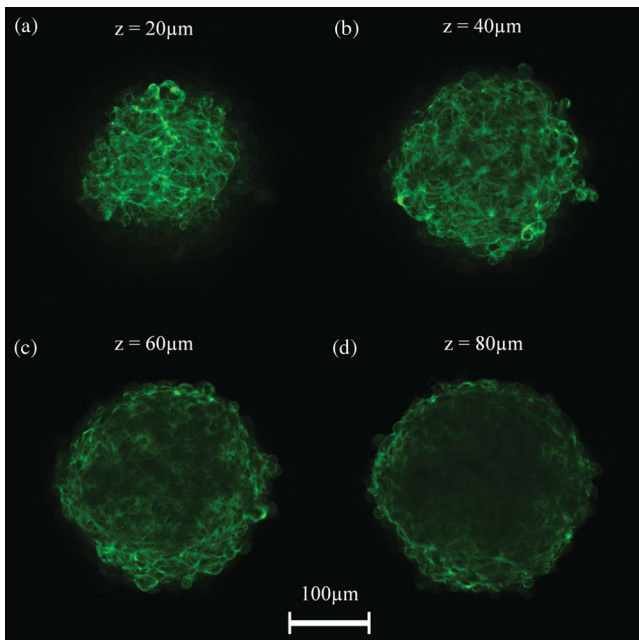


Fig. 4 LSM images of various layers of 10 μm diameter of a CHO-pAcGFP1-Mem cell spheroid recorded at distances of 20, 40, 60, and 80 μm from its edge; pinhole selected for 80% of an Airy disk (excitation wavelength: 488 nm; fluorescence detected at $\lambda \geq 505$ nm; light exposure: 52 mJ/cm^2 ; exposure time: 3 s each).

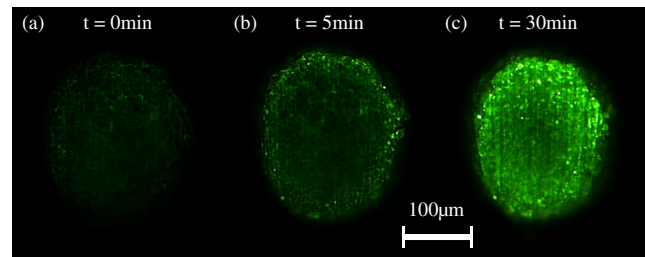


Fig. 5 Uptake of acridine orange (5 μM in culture medium) by a multi-cellular spheroid in a microfluidic system. Fluorescence intensity is recorded within a single layer of the spheroid at a distance of 40 μm from its edge after 0 min (a), 5 min (b), and 30 min (c) of application (flow rate: 9 $\mu\text{l}/\text{min}$; flow temperature: 37°C; detection range: $\lambda \geq 515$ nm; time is measured from the moment when the column of medium with acridine orange reaches the spheroid).

layers after 5 min and penetrates deeper into the spheroid after 30 min of incubation.

4 Discussion

An illumination device for light sheet based fluorescence measurements, with a conventional inverse microscope, is described and first experimental results are shown. The experimental setup appears well appropriate for selective measurements of individual layers of a 3-D sample, such as a cell spheroid, with a layer thickness of about 10 μm and a penetration depth of the light sheet into the sample of about 80 μm as demonstrated above. Smaller waists of the light sheet are attained when lenses of higher numeric aperture are used for illumination. However, a minimum axial resolution, $d = 1.22\lambda/A_N \leq 1$ μm , is only achieved within the focus of the laser beam while the waist is rapidly broadened outside this focus. A further problem arises when, upon imaging of smaller samples, the light sheet hits the bottom of the glass capillary or its curved edges. Novel micro-capillaries, of 600 \times 600 μm^2 inner diameter with a wall thickness of 120 μm and improved rectangular geometry (VitroCom, Mountain Lakes, NJ), reduced this problem and permitted distances between the light sheet and the glass bottom of even less than 20 μm . These micro-capillaries were used for recording the Figs. 5 and 6. Only at distances of about 10 μm or less, the micro-capillaries should be replaced by more appropriate micro-chambers¹¹ or by simple object slides, as used in oblique

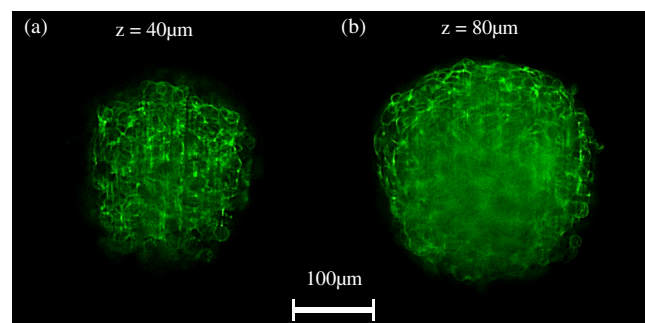


Fig. 6 SPIM images of two layers of 10 μM diameter of a CHO-pAcGFP1-Mem cell spheroid recorded with an additional 2.5 \times /0.075 objective lens at distances of 40 μm and 80 μm from its edge; light incidence from top to bottom (excitation wavelength: 488 nm; fluorescence detected at $\lambda \geq 515$ nm; light exposure: 52 mJ/cm^2 ; exposure time: 1 s each).

plane microscopy.¹⁸ Fluorescence can be measured with any microscope objective lens, even of high aperture and magnification, since the thickness of the micro-capillary is similar to a conventional cover slip, 170 μm , for which most microscope lenses are corrected. Therefore, moderate or even high axial resolution can be combined with high lateral resolution.

An inherent problem of light sheet illumination is that, due to scattering, the intensity of excitation light decreases, and fluorescence images become more diffuse and inhomogeneous (exhibiting some stripes) along the light path through the sample. Irradiation from different sides or rotation of the spheroids, under maintenance of the outer rectangular geometry of the capillary, could, therefore, increase the quality of fluorescence images. It should also be emphasized, that due to its rotational symmetry, it is often not necessary to image a whole spheroid to obtain relevant biological information, such as on penetration of metabolites or pharmaceutical agents. Geometric and chromatic aberrations might be reduced by insertion of an additional microscope lens with appropriate corrections, when the light sheet is focused into its aperture plane, as suggested in Ref. 19. However, when using a commercial objective lens (Carl Zeiss, plan-Neofluar $2.5 \times /0.075$ pol), some stripes in fluorescence images still exist, as shown in Fig. 6. Scanning of the laser beam, instead of using a cylindrical lens, could further improve image quality since undesired interferences, resulting from coherent illumination of the sample, might be excluded.

An essential advantage of the microcapillary system, utilized in this study, is that it can be easily combined with microfluidics, when nutrients, pharmaceutical agents or fluorescent dyes are inserted without moving the 3-D cell cultures. This has been demonstrated with the fluorescent marker acridine orange pumped with a flow rate of 9 μl per min, corresponding to a velocity of 25 mm/min, through the microcapillary. This opens up new perspectives of kinetic measurements in fluorescence diagnosis with low amounts of drugs and possibly short application times. Although a low flow rate, as used above, would be advantageous for cost-efficient drug screening, flow rates up to 1440 $\mu\text{l}/\text{min}$, corresponding to velocities up to 4000 mm/min, revealed to be possible under the present experimental conditions without detachment of the spheroids from the capillary. Pumped liquids can be either collected in a recipient, open system, or fed back to their source, closed loop. In the latter case, a total liquid volume of 200 to 300 μl is required, independently from pump velocity.

An attractive feature results from a combination of light sheet based fluorescence microscopy with other 3-D microscope techniques, such as structured illumination or confocal LSM. In the present setup, a laser scanning head is coupled to the inverse microscope so that SPIM and LSM can be used together. A possible application could include scanning of numerous planes of the sample by SPIM, preselection of its most interesting parts and high resolution LSM of these parts. This could keep the overall light exposure low and would, nevertheless, permit high resolution microscopy of the most interesting regions of the sample. In this context, it should be mentioned that by SPIM, as shown in Figs. 3 and 6, deeper layers in the spheroid, up to about 80 μm from its edge, than by LSM, less than 60 μm as illustrated in Fig. 4, can be excited without shading. This is probably due to a higher penetration depth of light when low aperture lenses are used and preferential forward scattering, such as Mie scattering, occurs.

Due to the very low light dose needed for SPIM, 52 mJ/cm² presently used for recording a single plane corresponds to 1/200 of a phototoxic light dose,⁸ this technique appears most promising for long exposure times or repetitive measurements. Applications may include imaging of 3-D cell cultures equipped with reporter molecules, such as tumor cell spheroids, transfected with genetically encoded biosensors.^{20–22} A spatially resolved analysis of drug response in 3-D models is of pivotal interest for anticancer compound research as gradients of hypoxia and nutrient distribution, which are observed in tumors and reproduced in tumor cell spheroids, influence the sensitivity towards anticancer drugs.²³ Since phototoxicity can interfere with anticancer drug effects, minimization of the light dose by SPIM again proves to be essential.

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