RESEARCH ARTICLE

Side Views in 3D Live Cell Microscopy – Innovative Concepts of Illumination for Novel Applications

Authors
Verena Richter and Herbert Schneckenburger

Affiliation
Institute of Applied Research, Aalen University, Beethovenstr. 1, 73430 Aalen, Germany

Corresponding author
Herbert Schneckenburger
Email: herbert.schneckenburger@hs-aalen.de

Abstract

Commonly trans-illumination or epi-illumination techniques are used in optical microscopy, e.g. phase microscopy, fluorescence microscopy or inelastic light scattering (Raman microscopy). However, an increasing number of applications requires optical excitation perpendicular or under a certain angle to the optical axis. Methods include Mie Scattering Microscopy with angular resolution for label-free studies of cell or tissue morphology as well as Total Internal Reflection Fluorescence (TIRF) Microscopy with an evanescent electromagnetic field penetrating only about 100 nm into a sample and permitting selective studies of cell surfaces, e.g. membranes. Presently, conventional wide field or confocal microscopy are often replaced by Light Sheet Fluorescence Microscopy (LSFM) where individual planes of a specimen are illuminated from the side without any light exposure or phototoxic damage of adjacent parts of the sample. Finally, Axial Tomography permits samples to be observed from various sides with a high and isotropic resolution in all three dimensions. All these methods require experimental setups which are not integrated in conventional microscopes. Therefore, we report on some technical solutions implemented in specific modules or add-ons for these microscopes.

Keywords: Optical microscopy, living cells, Mie scattering, fluorescence, TIRF, light sheet, axial tomography

1. Introduction

In optical microscopy trans-illumination (including phase and interference contrast) as well as epi-illumination are well established methods with the latter one having gained considerable importance in fluorescence or light scattering (e.g. Raman) microscopy. However, in some cases illumination from the side or under well-defined angles is required, and specific experimental setups are needed. Techniques include Mie Scattering Microscopy with angular resolution, Variable-angle Total Internal Reflection Fluorescence (VA-TIRF) microscopy, Light Sheet Fluorescence Microscopy (LSFM) or Axial Tomography. In the present manuscript these techniques of "side view illumination" are described and experimental solutions how they can be integrated in a conventional microscope are presented. In all cases some exemplary applications are given.

2. Methods and Applications

2.1 Mie Scattering

Scattering microscopy may give valuable information on cell or tissue morphology. In particular, Mie scattering shows some characteristic angular behaviour depending on the size, shape and internal structure of cells and particles in the micrometer range. This scattering behaviour has been utilized for more than 30 years for characterization of various types of cells [1–3] or for measurement of morphological changes of cells undergoing necrosis or apoptosis [4, 5]. Figure 1a shows the angular distribution of light scattering from a small object field of 4–6 individual cells prior to (control) and subsequent to apoptosis (induced by 2 µM staurosporine after 4 h). While the cells show pronounced changes of their morphology after initiation of apoptosis (characterized by shrinking and almost spherical shape, see inset of the Figure), the scattering intensity increases and exhibits pronounced oscillations, as described in the literature for Mie scattering [6].

Considering Mie theory, backward scattering is less pronounced, but seems to contain more detailed information on nuclear morphology than forward scattering [4, 7]. To assess high-angle scattering, various goniometers [2] have been described in the literature, but also microscopes with angular resolution [8–10] are of increasing interest, since in addition to light scattering they permit visual control of single cells and their sub-structures.

For light scattering microscopy with angular resolution, we previously used illumination with a focus in the aperture plane of the microscope objective lens [5]. For this purpose, the collimated beam of a laser diode was deflected by a mirror under a variable angle and guided as a parallel beam to the image plane at the entrance of the inverted microscope. The further optical path within the microscope resulted in a focus within the aperture plane of the objective lens and – according to the position of the focus – a parallel beam under variable inclination in the plane of the sample (Fig. 1b). Backscattered light collected by the same objective lens was focused by a so-called Bertrand lens to an exit pupil with a pinhole located in its centre, by which an angle of 180 ± 1° was selected prior to light detection by a photomultiplier. The angles of incidence ("scattering angles") were limited to 190 - 220° in order to avoid a strong overlap by specular reflection on the cover slip (around 180°) and to exclude comparably weak signals above 220° from detection.

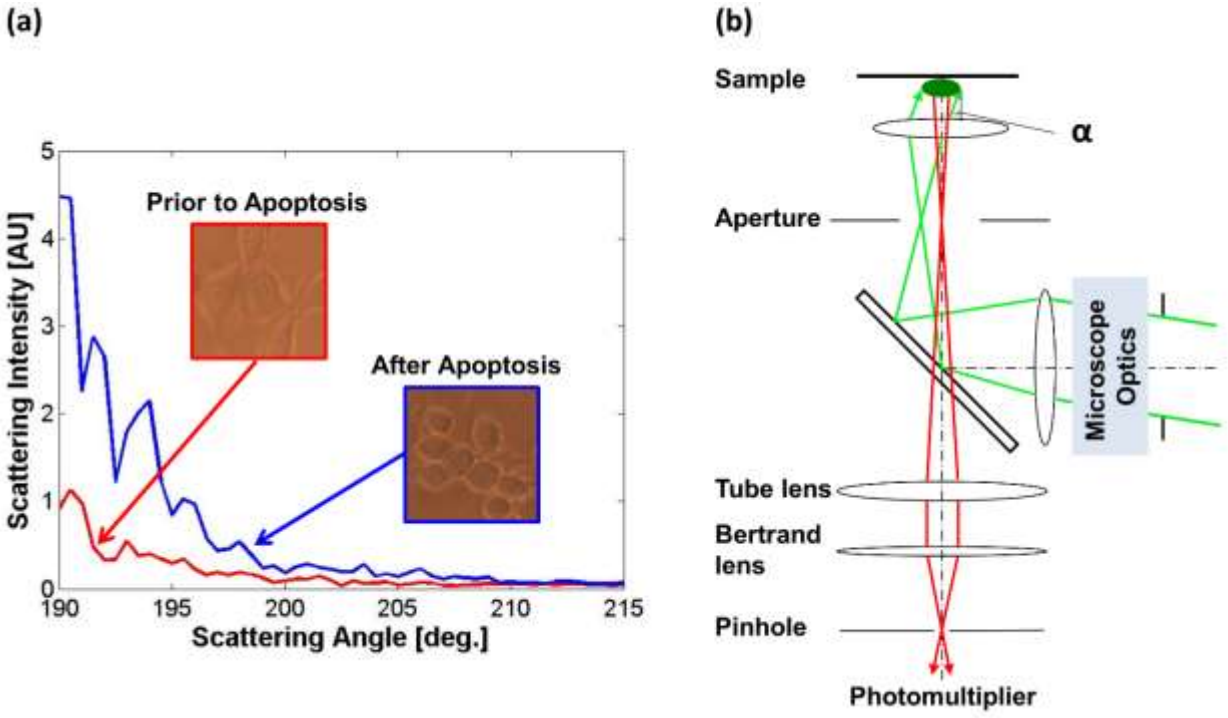

Figure 1 (a) Angular behaviour of Mie scattering of 3T3 fibroblasts prior to and subsequent to apoptosis (induced by 2 µM staurosporine after 4h); (b) modified inverted microscope (Axiovert 200M, Carl Zeiss Jena, Germany) for Mie scattering experiments with angular resolution.

2.2 Total Internal Reflection Fluorescence (TIRF) Microscopy

When the angle of incidence is below about 120° (i.e. more than 60° inclination towards the optical axis), light may be totally reflected on a cell-substrate surface, while its evanescent electromagnetic field penetrates a small distance (typically about 100 nm) into the specimen. Absorption by cell membrane associated molecules and subsequent fluorescence may thus permit selective membrane imaging. Therefore, objective type Total Internal Reflection Fluorescence (TIRF) Microscopy with numerical apertures around 1.45 is now a well-established technique [11], which meanwhile has been commercialized by several industrial companies (e.g. Zeiss, Olympus, Nikon). Disadvantages of this technique, however, are a very limited range of angles of incidence and a small number of available objective lenses with high numerical aperture and magnification. These problems, however, are overcome by prism type TIRF microscopy, where a laser beam hits the specimen under variable angles, e.g. in a special microscope condenser, as depicted in **Figure 2**. Based on the angular dependence of penetration depth of the evanescent wave an algorithm has been developed for fluorescent membrane markers as well as for markers homogeneously distributed in the cytoplasm to calculate cell-substrate distances [12].

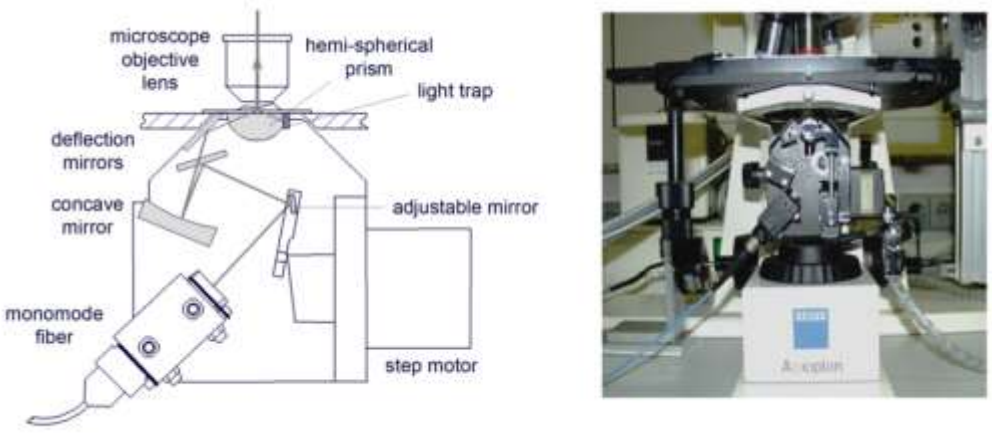

Figure 2 Condenser for TIR illumination under variable angle; schematic (left) and laboratory equipment with an additional light path for transillumination and phase contrast microscopy (right).

So far, TIRF microscopy has been applied for measurements of focal adhesions, cell-substrate contacts [13], protein dynamics [14], as well as endocytosis or exocytosis [15, 16]. **Figure 3** gives an example for HeLa cervical carcinoma cells, where membrane associated paxilline has been fused with Enhanced Yellow Fluorescent Protein (EYFP). While conventional epi-illumination excites EYFP all over the cells, TIR illumination excites selectively EYFP bound to paxilline in focal adhesions of the cell towards the substrate.

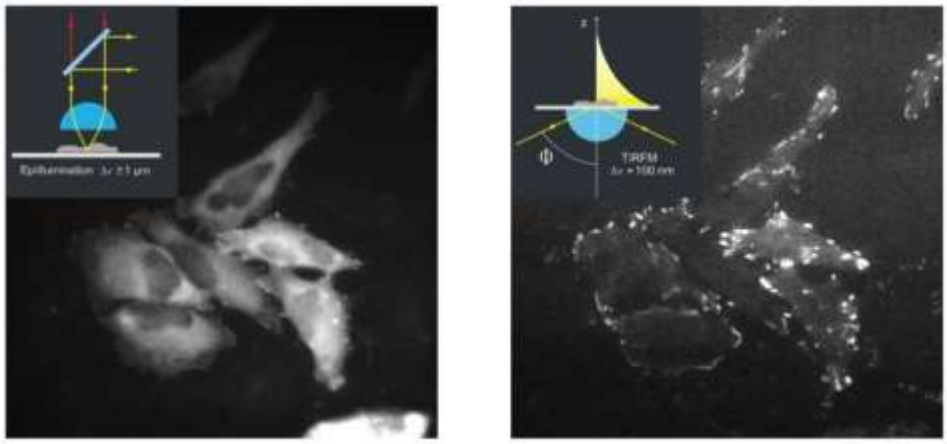

Figure 3 Comparison of epi-illumination (left) and TIR illumination (right) for excitation of membrane associated Paxilline – EYFP in HeLa cervical carcinoma cells. Insets: Beam alignment for epi-illumination and TIR illumination.

Variable-angle TIRF microscopy has so far been applied to studies of cell-substrate topology, e.g. after depletion of cholesterol or application of photosensitizing agents [17] as well as after cell transfection with tumor suppressor genes [18]. While glioblastoma cells with a tumour suppressor gene showed a "folded" surface topology with cell-substrate distances varying between about 100 and 300 nanometres, the wild type tumour cells were characterized by almost constant distances of about 100 nm from the substrate. This might offer a possibility to distinguish between cells of different malignancy.

2.3 Light Sheet Fluorescence Microscopy (LSFM)

In recent years light sheet microscopy has proven to be a valuable method for 3D imaging of biological specimens [19, 20]. In contrast to established methods, e.g. confocal

laser scanning microscopy (LSM) [21, 22] or structured illumination microscopy [23], samples are illuminated perpendicular to the detection path by a light sheet which is commonly created by a cylindrical lens or by scanning of a laser beam. If either the light sheet or the sample is moved in axial direction, individual planes can be recorded successively with each plane being exposed only once to laser irradiation. This minimizes light exposure of the whole sample and reduces photobleaching and phototoxic damage to living cells or tissues [24, 25], in particular for long observation times.

Light sheet microscopes can be stand-alone instruments (e.g. Lightsheet Z1, Carl Zeiss Jena, Germany) or add-ons for existing microscopes, e.g. the miniaturized light sheet module for commercially available inverted microscopes described by Bruns et al. [26]. As shown in Figure 4a, the divergent beam of a fibre is collimated, passes an aperture and is focused by an achromatic cylindrical lens with a numerical aperture of $A_N = 0.08$, thus creating a beam waist around 6 µm and a depth of focus around 100 µm. Figure 4b shows that this module can be integrated into the positioning stage of a commercial inverted microscope. This permits versatile light sheet imaging including 3D, spectral and fluorescence lifetime imaging as well as a combination of light sheet microscopy with other imaging techniques, e.g. Structured Illumination Microscopy (SIM) [27]. While conventional microscope object slides can be used for TIRF microscopy, LSFM requires completely different sample holders. Micro-capillaries, e.g. in combination with micro-fluidics, permit experiments to be performed with very low amounts of culture media or reagents, e.g. fluorescent dyes or cytostatic drugs [28]. In addition, sample rotation as reported by Bruns et al. [29] is easy to perform as described in Section 2.4. Figure 5 gives an example of 3D imaging of Chinese hamster ovary (CHO) cell spheroids by Light Sheet Fluorescence Microscopy (LSFM) including the illumination and detection scheme, exemplary images of individual planes and 3D reconstruction.

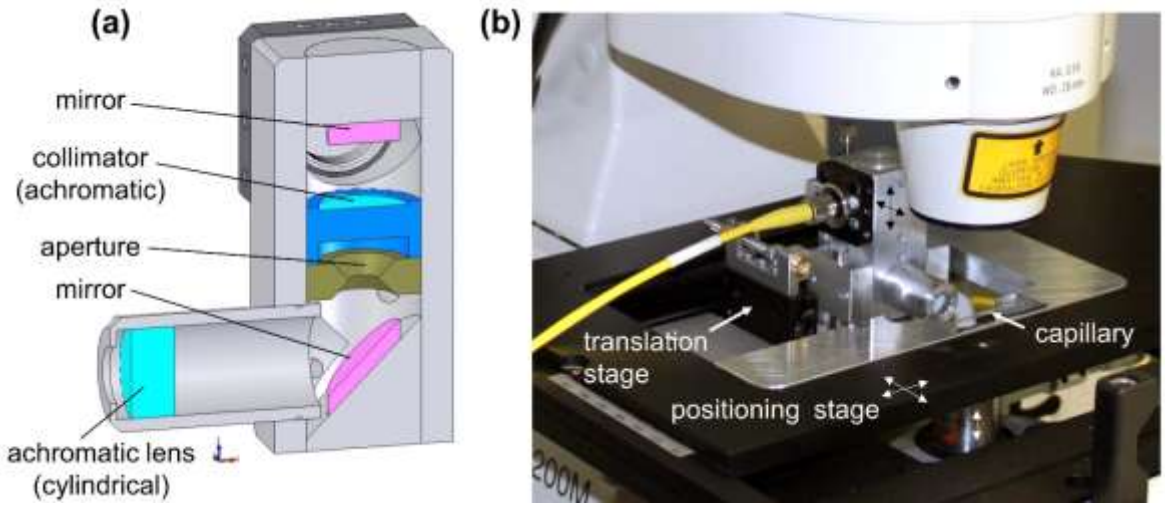

Figure 4 Miniaturized Light Sheet Module: (a) technical setup of the illumination unit; (b) adaptation to an inverted microscope (reproduced from Ref. 26 with modifications).

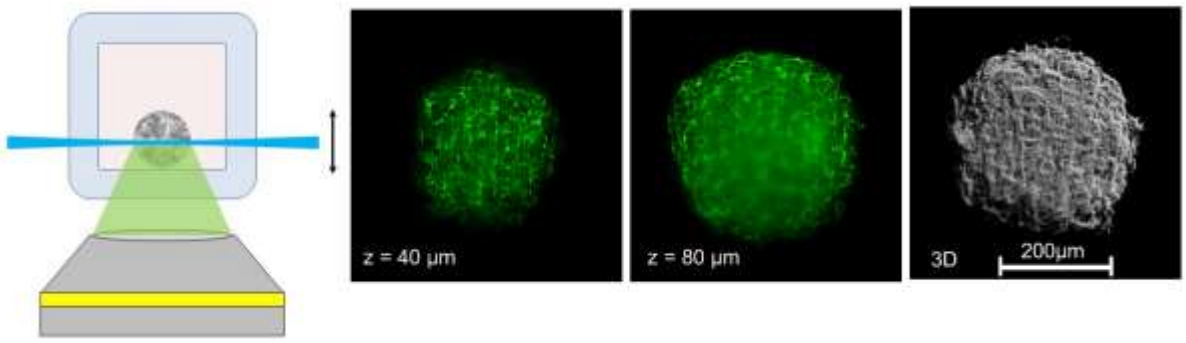

Figure 5 3D imaging of CHO cell spheroids with membrane associated Green Fluorescent Protein (GFP). Light Sheet Fluorescence Microscopy (LSFM) using rectangular micro-capillaries (scheme, 2 single planes and 3D reconstruction); beam waist: Δz = 5–10 µm, focal depth: Δy = 100–200 µm (reproduced from Ref. 33 with modifications).

2.4 Axial tomography

In microscopy it is often beneficial to observe a sample from different sides. This may give additional information, since cells or organelles, which appear superimposed in one direction, may be well separated in another one. In addition, sample rotation permits to overcome the anisotropy of optical resolution, which is always lower in axial than in lateral direction. Therefore, devices for tilting small samples (e.g. single cells) [30, 31] or for rotation of samples of variable size up to 360° [29] were developed.

Figure 6 shows a device for rotation of three-dimensional samples applicable to the x, y-stage of a conventional microscope, which

allows Axial Tomography to be combined with various microscopy methods such as Light Sheet Microscopy or Laser Scanning Microscopy [28]. A microcapillary system with a rotating inner capillary containing the specimen and an outer rectangular capillary coupled by immersion fluid permits multiscale measurements from single cells in agarose gel [32] and cell spheroids [33] to small organisms [29]. In addition to glass capillaries, cylindrical fluoroethylene propylene capillaries (FEP), whose refractive index of 1.33 to 1.35 fits that of the sample, can be used with a water immersion lens to ensure optimum imaging independent of the sample geometry. In this case, no second (outer) capillary is needed. Rotation of the cylindrical capillary is performed by a computerized stepping motor with micro-step positioning control and an angular resolution of 0.45°. The stepping motor is driven by a small and flexible stand-alone control unit providing different running modes. It allows for manual rotation, continuous rotation with selectable speed or stepwise rotation with adjustable increments. In axial tomography, z stacks can thus be recorded and combined in a 3-D image or a z projection for each angle. This greatly improves the potential of visualizing the specimen and its microenvironment.

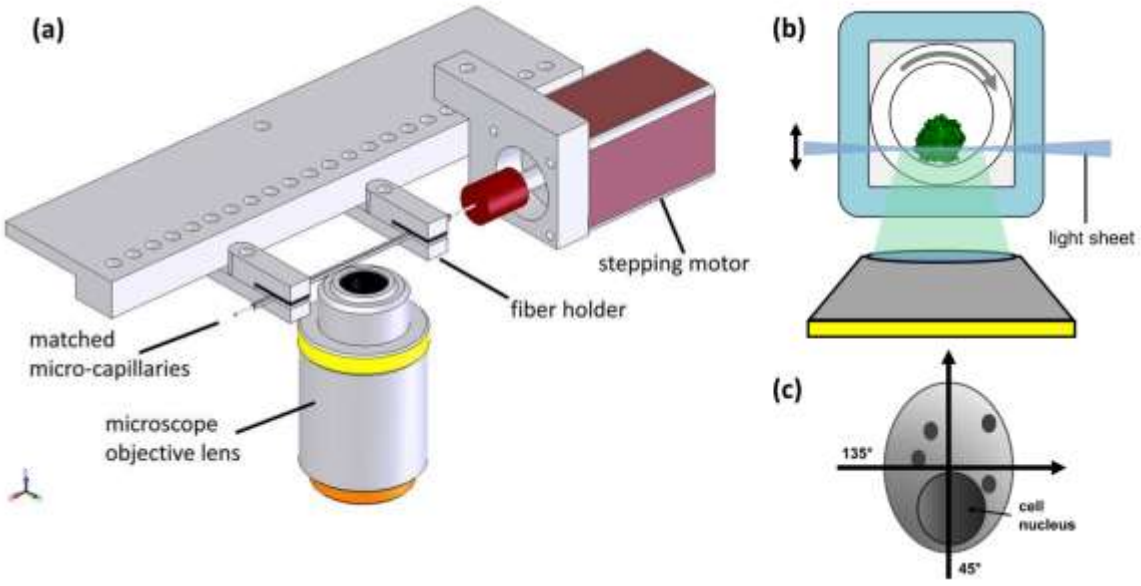

Figure 6 Device for rotation of 3-D samples applicable to the x, y-stage of a microscope (a), combination with Light Sheet Microscopy (b), and application to microscopy of single cells with the light sheet passing either through or aside the cell nucleus (c).

In Figure 7 a series of applications are depicted: Single CHO cells with membrane associated GFP are either observed under one angle (0°), while images for all other angles are calculated (upper part of Fig. 7a), or measured under several angles upon sample rotation (lower part of Fig. 7a). Obviously, image quality is increased considerably in the second case. Figure 7b shows an MCF-7 cell spheroid incubated with fluorescent Quantum Dots including fluorescence views under 90°, either calculated from 0° or measured after sample rotation with increased resolution (see e.g. the two Quantum Dots within the marked circle). As an example for micro-organisms fluorescence images of a copepod are depicted in Figure 7c for various angles of observation.

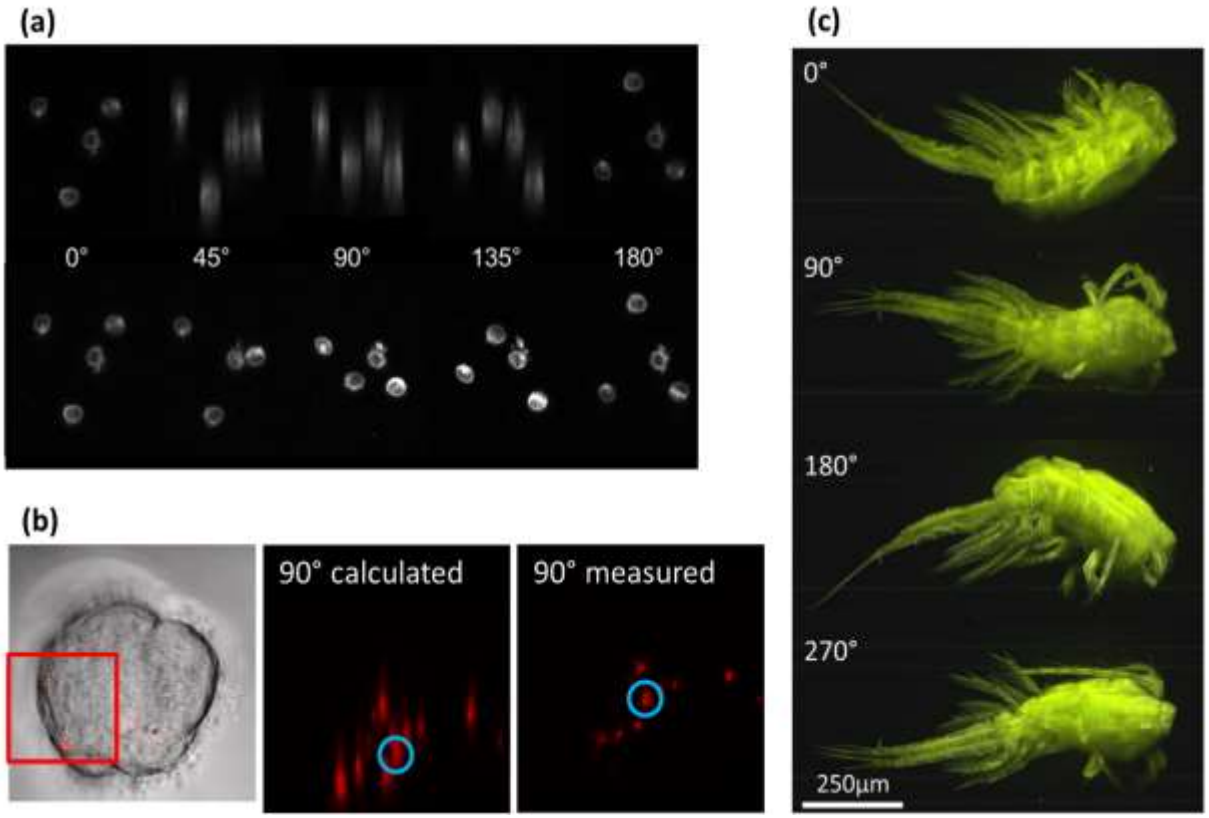

Figure 7 Axial Tomography for multiscale measurements: (a) single CHO-pAcGFP1-Mem cells under variable angles, either calculated from 0° direction (upper part) or measured upon sample rotation (lower part); (b) MCF-7 cell spheroid incubated with QTracker including calculated or measured side views at 90°, and (c) copepod incubated with acridine orange; excitation wavelength: λ_{ex} = 488 nm; detection range: $\lambda_d \geq 505$ nm.

3. Conclusion

In the present manuscript four methods are described, where the direction of incident light is perpendicular or under a certain angle to the optical axis of a microscope. These methods may give additional information, e.g. on cell morphology (Mie scattering) or on cell-substrate topology (variable-angle TIRF microscopy). They may be useful to reduce the light dose in 3D microscopy by LSFM and increase resolution in diverse image planes after rotation of the specimen. However, application of these methods requires either novel instruments or the use of various add-ons (e.g. miniaturized modules) for customary microscopes. Many of these add-ons, e.g. those described in the present manuscript, are commercially not available, so that the user may be caused to construct his own technical equipment. His effort may be compensated by numerous novel and original applications.

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References

(1) Brunsting, A, Mullaney, PF. Differential Light-Scattering from Spherical Mammalian-Cells, Biophys. J., 1974, 14: 439–453, doi: 10.1016/S0006-3495(74)85925-4.

(2) Mourant, JR, Johnson, TM, Doddi, V, Freyer, JP. Angular dependent light scattering from multicellular spheroids, J. Biomed. Opt., 2002, 7: 93–99, doi: 10.1117/1.1427053.

(3) Mourant, JR, Johnson, Carpenter, S, Guerra, A, Freyer, JP. Polarized angular dependent spectroscopy of epithelial cells and epithelial cell nuclei to determine the size scale of scattering structures, J. Biomed. Opt., 2002, 7: 378–387, doi: 10.1117/1.1483317.

(4) Mulvey, CS, Curtis, AL, Singh, SK, Bigio, IJ. Elastic Scattering Spectroscopy as a Diagnostic Toolfor Apoptosis in Cell Cultures, IEEE Journal of Selected Topics in Quantum Electronics, 2007, 13(6): 1663–1670, doi: 10.1109/JSTQE.2007.910115.

(5) Richter, V, Voit, F, Kienle, A, Schneckenburger, H. Light scattering microscopy with angular resolution and its possible application to apoptosis, J. Microsc., 2015, 257(1):1–7, doi: 10.1111/jmi.12180.

(6) Bohren, CF, Huffman, DR. Absorption and Scattering of Light by Small Particles, Wiley-Interscience, New York, 1998, doi: 10.1002/9783527618156.

(7) Drezek, R, Dunn, A, Richards-Kortum, R. Light scattering from cervical cells throughout neoplastic progression: Influence of nuclear morphology, DNA content, and chromatin texture, J. Biomed. Opt., 2003, 8: 7–16, doi: 10.1117/1.1528950.

(8) Cottrell, WJ, Wilson, JD, Foster, TH. Microscope enabling multimodality imaging, angle-resolved scattering, and scattering spectroscopy, Opt. Lett., 2007, 32: 2348–2350, doi: 10.1364/OL.32.002348.

(9) Rothe, T, Schmitz, M, Kienle, A. Angular and spectrally resolved investigation of single particles by darkfield scattering microscopy, J. Biomed. Opt., 2012, 17: 117006, doi: 10.1117/1.JBO.17.11.117006.

(10) Schmitz, M, Rothe, T, Kienle, A. Evaluation of a spectrally resolved scattering microscope, Biomed. Opt. Express, 2011, 2: 2665–2678, doi: 10.1364/BOE.2.002665.

(11) Axelrod, D. Selective imaging of surface fluorescence with very high aperture microscope objectives. J. Biomed. Opt., 2001, 6–13, doi: 10.1117/1.1335689.

(12) Stock, K, Sailer, R, Strauss, WSL, Lyttek, M, Steiner, R, Schneckenburger, H. Variable-angle total internal reflection fluorescence microscopy (VA-TIRFM): realization and application of a compact illumination device, J. Microsc., 2003, 211: 19–29, doi: 10.1046/j.1365-2818.2003.01200.x.

(13) Axelrod, D. Cell-substrate contacts illuminated by total internal reflection fluorescence, J. Cell. Biol., 1981, 89: 141–145, doi: 10.1083/jcb.89.1.141.

(14) Sund, SE, D. Axelrod, D. Actin dynamics at the living cell submembrane imaged by total internal reflection fluorescence photobleaching, Biophys. J., 2000, 79: 1655–1669, doi: 10.1016/S0006-3495(00)76415-0.

(15) Betz, WJ, Mao, F, Smith, CB. Imaging exocytosis and endocytosis, Curr. Opin. Neurobiol., 1996, 6: 365–371, doi: 10.1016/s0959-4388(96)80121-8.

(16) Oheim, M, Loerke, D, Stühmer, W, Chow, RH. The last few milliseconds in the life of a secretory granule, Eur. J. Biophys., 1998, 27: 83–98, doi: 0.1007/s002490050114.

(17) Wagner, M, Weber, P, Strauss, WSL, Lassalle, HP, Schneckenburger, H. Nanotomography of cell surfaces with evanescent fields, Advances in Optical Technologies, 2008, Vol. 2008, Article ID 254317, doi: 10.1155/2008/254317.

(18) Wagner, M, Weber, P, Baumann, H, Schneckenburger, H. Nanotopology of cell adhesion upon variable-angle total internal reflection fluorescence microscopy (VA-TIRFM), J. Vis. Exp., 2012, 68: e4133, doi: 10.3791/4133.

(19) Huisken, J, Swoger, J, del Bene, F, Wittbrodt, J, Stelzer, EHK. Optical sectioning deep inside live embryos by SPIM, Science, 2004, 305(5686): 1007–1009, doi: 10.1126/science.1100035.

(20) Santi, PA. Light sheet fluorescence microscopy: a review, J. Histochem. Cytochem. 2011, 59(2): 129–138, doi: 10.1369/0022155410394857.

(21) Pawley, J. Handbook of biological confocal microscopy, Plenum Press, New York, 1990.

(22) Webb, RH. Confocal optical microscopy, Rep. Prog. Phys., 1996, 59: 427–471.

(23) Neil, MA, Juskaitis, R, Wilson, T. Method of obtaining optical sectioning by using structured light in a conventional microscope, Opt. Lett., 1997, 22(24): 1905–1907, doi: 10.1364/OL.22.001905.

(24) Schneckenburger, H, Weber, P, Wagner, M, Schickinger, S, Richter, V, Bruns, T, Strauss, WSL, Wittig, R. Light exposure and cell viability in fluorescence microscopy, J. Microsc., 2012, 245: 311–318, doi: 10.1111/j.1365-2818.2011.03576.x.

(25) Pampaloni, F, Chang, BJ, Stelzer, EHK. Light sheet-based fluorescence microscopy (LSFM) for the quantitative imaging of cells and tissues, Cell Tissue Res., 2015, 362(1): 129–141, doi: 10.1007/s00441-015-2144-5.

(26) Bruns, T, Bauer, M, Bruns, S, Meyer, H, Kubin, D, Schneckenburger, H. Miniaturized modules for light sheet microscopy with low chromatic aberration, J. Microsc., 2016, 264(3): 261–267, doi: 10.1111/jmi.12439.

(27) Gustafsson, MGL, Shao, L, Carlton, PM, Wang, CJR, Golubovskaya, IN, Cande, WZ, Agard, DA, Sedat, JW. Three-dimensional resolution doubling in wide-field fluorescence microscopy by structured illumination, Biophys. J., 2008,

94(12): 4957–4970, doi: 10.1529/biophysj.107.120345.

(28) Bruns, T, Schickinger, S, Schneckenburger, H. Single plane illumination module and micro-capillary approach for a wide-field microscope, J. Vis. Exp., 2014, 15(90):e51993-1–e51993-10, doi: 10.3791/51993.

(29) Bruns, T, Schickinger, S, Schneckenburger, H. Sample holder for axial rotation of specimens in 3D microscopy, J. Microsc., 2015, 260(1): 30–36, doi: 10.1111/jmi.12263.

(30) Bradl, J, Rinke, B, Schneider, B, Edelmann, P, Krieger, H, Hausmann, M, Cremer C. Resolution improvement in 3D fluorescence microscopy by object tilting, Microsc. Anal., 1996, 11: 9–11, doi: 10.1002/1361-6374(199712)5:4<171:AID-BIO1>3.0.CO;2-K.

(31) Staier, F, Eipel, H, Matula, P, Evsikov, AV, Kozubek, M, Cremer, C, Hausmann, M. Micro-axial tomography: a miniaturized, versatile stage device to overcome resolution anisotropy in fluorescence light microscopy, Rev. Sci Instrum., 2011, 82(9): 093701, doi: 10.1063/1.3632115.

(32) Richter, V, Bruns, S, Bruns, T, Weber, P, Wagner, M, Cremer, C, Schneckenburger, H. Axial tomography in live cell laser microscopy, J. Biomed. Opt., 2017, 22(9):91505, doi: 10.1117/1.JBO.22.9.091505

(33) Bruns, T, Schickinger, S, Wittig, R, Schneckenburger, H. Preparation strategy and illumination of 3D cell cultures in light-sheet based fluorescence microscopy, J. Biomed. Opt., 2012, 17(10): 101518, doi: 10.3390/ijms16035375.

