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Life sciences require the third dimension

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Novel technologies are required for three-dimensional cell biology and biophysics. By three-dimensional we refer to experimental conditions that essentially try to avoid hard and flat surfaces and favour unconstrained sample dynamics. We believe that light-sheet-based microscopes are particularly well suited to studies of sensitive three-dimensional biological systems. The application of such instruments can be illustrated with examples from the biophysics of microtubule dynamics and three-dimensional cell cultures. Our experience leads us to suggest that three-dimensional approaches reveal new aspects of a system and enable experiments to be performed in a more physiological and hence clinically more relevant context.

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Introduction

Advances in the life sciences are strongly related to the ability to observe dynamic processes in live systems and to mimic relevant *in vivo* conditions.

For instance, cells usually grow and differentiate in soft, jelly-like, three-dimensional environments provided by, for example, the extracellular matrix (ECM). Consequently, the relevance of any experiment that reduces the number of dimensions or constrains the temporal resolution should be carefully evaluated. In particular, the introduction of hard surfaces (e.g. cover slips) adds elements that are usually not present in living systems. In the resulting flat and essentially two-dimensional situation, the dramatic change in the surface-over-volume ratio and the hard surface itself induce the cell to adapt by changing its metabolic function and in general its protein expression. An alternative interpretation is that such a system selects for cells that can adapt to such an environment. This most likely pushes any biological system's response into a realm that is at least less physiologically relevant.

On a different level, in biophysical studies microtubules are often observed close to a hard surface, which could account for why their behaviour in this setting differs from what is observed in a more physiological situation. For instance, the microtubule growth rates and catastrophe frequencies are force-dependent [1,2] and in *S. pombe* microtubule bundles seem to bend [3] rather than depolymerise spontaneously when they touch the yeast's cell surface.

Although the scientific community is now starting to realize the importance of ''introducing the third dimension'' in biology [4••], the main drive does not stem from basic research in cell biology but rather from the clinicians [5], in other words from those who would like to take advantage of the results of modern molecular biology [6].

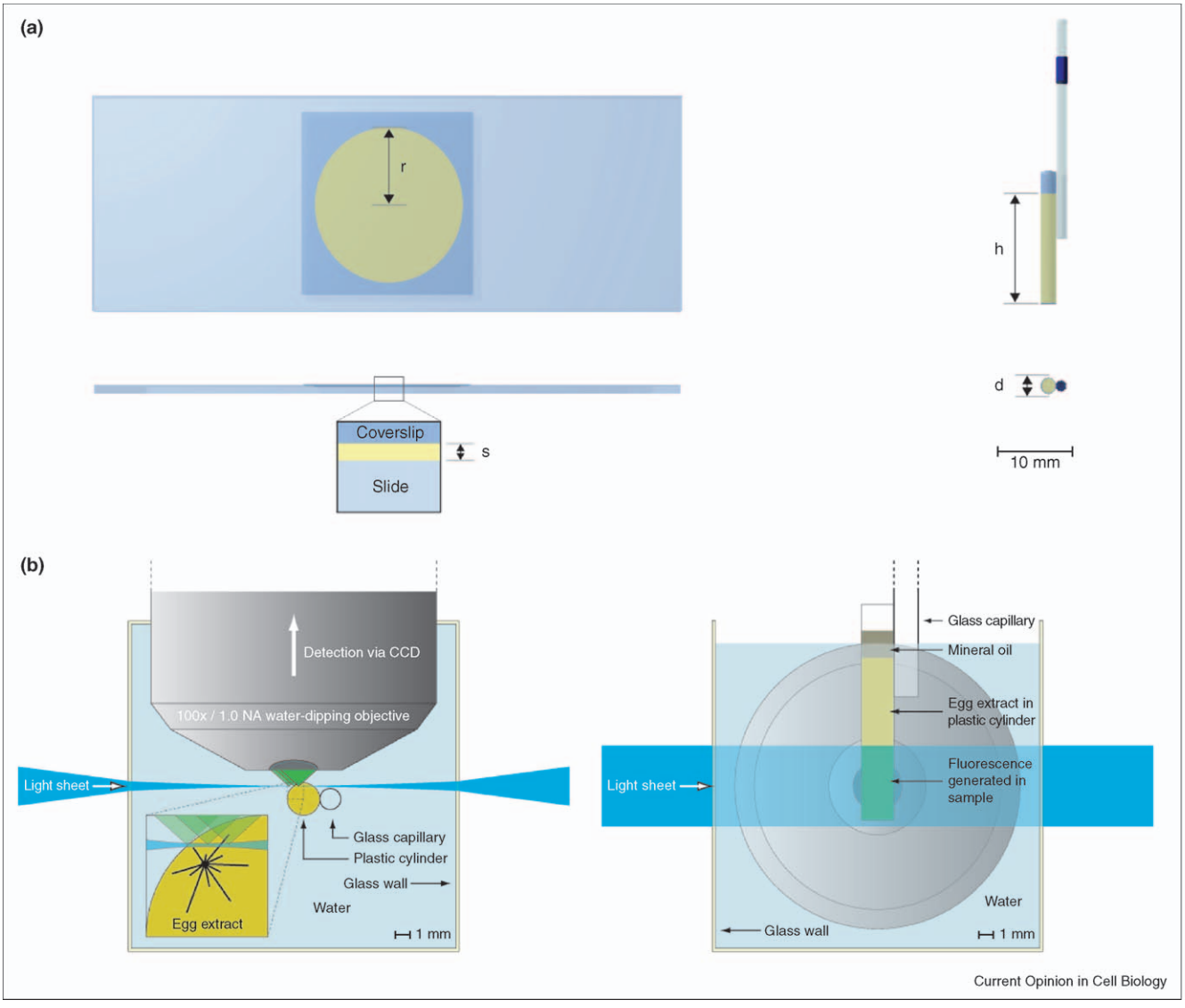
In the following we shortly outline current approaches to three-dimensional imaging with an emphasis on light-sheet-based technology. The applicability is illustrated with an example from microtubule biophysics and a novel approach to cell biology.

Modern three-dimensional microscopy

Quantitative analysis of live three-dimensional structures requires fast optical sectioning. Confocal fluorescence microscopy works well in relatively thin samples; in large objects, however, the signal is scattered and rejected by the pinhole. An excellent resolution is retained by physically sectioning the sample [7], but this destroys the sample irretrievably and is not applicable to live preparations. Most methods that have been developed to enhance the resolution — e.g. 4Pi-confocal [8], I^5M [9] and STED microscopy [10] — require an excellent control over the phase of the wavefront of the light and hence cannot address challenges encountered in multicellular objects. Nevertheless, some of the recent developments, for example with I^5M by Gustafsson [11••], indicate an impressive potential for improvement in resolution. Other imaging techniques for large samples, such as optical projection tomography (OPT) [12] and micro magnetic resonance imaging (μMRI) [13], cannot take advantage of fluorescent proteins and hence lack specificity. Two-photon microscopy, which many [14] regard as the best technique for use with sensitive biological material, suffers from a moderate resolution [15]. It is currently not clear how much damage two-photon microscopy actually creates with its relatively high average intensities (several mW).

Our suggestion for an optically sectioning instrument is based on light-sheet technology and is termed single plane illumination microscopy (SPIM) [16••]. It operates on four principles: illumination with a light sheet, observation along at least one direction perpendicular to the illumination plane, rotation of the sample about an axis parallel to gravity, and a stationary chamber with the immersion medium (Figure 1c,d). It owes much to the 'Ultramikroskop', an orthogonal, darkfield illuminator invented by Siedentopf and Zsigmondy in 1903 [17] to

Figure 1



Two- and three-dimensional sample preparation for the study of dynamic microtubules. **(a)** Comparison of sample preparation for conventional two-dimensional and novel three-dimensional microscopy. Left: drawing of the chamber geometry using coverslips and slides for conventional sample preparation. The chamber dimensions are defined by the radial extent of the sample r and the distance between coverslip and slide s. Right: drawing of the hollow plastic polymer cylinders used to measure microtubule dynamics in a SPIM. As an example for *in vitro* studies, the location of the *Xenopus laevis* egg extract is indicated in yellow. The length of the plastic polymer cylinder is h and the inner diameter of this cylinder is d. Typical values are r = 1 cm, s = 20 μm, h = 1 cm, and d = 2 mm. With these assumptions, surface-over-volume ratios of $2/s = 0.1\ \mu m^{-1}$ for the conventional sample preparation and $4/d + 1/h = 0.0021\ \mu m^{-1}$ for the semi-open plastic cylinder result. Thus, in a SPIM experiment the surface-over-volume ratio is reduced by factor of about 50 as compared to conventional sample preparation. **(b)** Top-view (left) and side-view (right) drawings of the SPIM imaging chamber. The detection objective lens is immersed into the water-filled chamber. The plastic polymer cylinder with the sample is placed in front of the objective. The microtubule asters developing from *Xenopus laevis* egg extract are polymerized inside the cylinder. With this approach, microtubule asters without surface contact can be observed. A drop of mineral oil protects the extract from contact with air. The illuminating light sheet enters from the side, while the fluorescence light is detected perpendicular to the illumination plane. For an optimal imaging quality the path of the light in the egg extract must be minimized by appropriately choosing the position of the recorded aster. All drawings are to scale.

visualize nm-sized gold particles. The concept has been used in ophthalmic instruments [18] and in a macroscope used by Voie to observe the cochlea [19]. Fuchs described such a device [20] to observe microbes while Huber [21] reconstructed mm-sized samples using scattered light. The orthogonal arrangement of point illumination and point detection was also used in confocal theta fluorescence microscopy [22] with lenses of high numerical aperture.

In SPIM a three-dimensional data set is recorded by scanning the sample through the stationary light sheet while recording the fluorescence light with a camera. The sample can be as small as a few micrometers (e.g. microtubule asters or yeast cells), in the 100s of micrometers range (e.g. Madin-Darby canine kidney [MDCK] cysts or endothelial spheroids) or even as large as several millimetres (e.g. zebrafish or medaka embryos). The properties of the detection lens depend on the necessary working distance and on the material required for the embedding procedure (agar, liquid, gas). Since the sample is attached to a stage it can be rotated as well as translated, meaning that three-dimensional image stacks can be recorded along different directions [23]. These independently recorded data sets can be combined into a single three-dimensional data set with a spatial resolution that is dominated by the lateral resolution of the detection system. However, from a practical point of view, the most important advantage of SPIM is that only those parts in the sample that are observed are in fact illuminated. Out-of-focus light is not generated. With single-photon excitation and laser powers in the μW range, a SPIM ensures dramatically reduced photobleaching, is less phototoxic and is particularly well suited to the observation of live and dynamic processes.

Case study: microtubule asters

In our studies of microtubule asters, we transfer the two-dimensional experiments of microtubule dynamic instability [24] performed between two closely spaced glass flats (Figure 1a) to a three-dimensional environment (Figure 1b) and use the SPIM for imaging (Figure 1c,d). Experiments are performed *in vitro* using *Xenopus laevis* egg extracts, providing us with a physiological yet biochemically easily modifiable system. Three-dimensional sample preparation ensures a minimal area of artificial surfaces and unconstrained development of the asters in three dimensions (Figure 1b). Apart from addressing the fundamental questions of microtubule dynamics, this approach allows us to phrase questions that specifically focus on three-dimensional aspects of microtubule structural dynamics.

Among these issues are the centrosome's three-dimensional movement and rotation during aster polymerization and spindle formation. Analysis of the structural homogeneity of the aster allows us to relate the angular microtubule distributions to the centrosome's internal structure. Structural configurations (e.g. for the centrosomes' centrioles) are well-known from electron microscopy [25,26]. This raises the question of whether these configurations have effects on the spatial dependency of microtubule nucleation that are observable in live systems. Surfaces obviously impair microtubule growth [1,2] and significantly influence aster structure. Hence, it is crucial to either avoid or precisely characterize the asters' surface contact in these studies. In our SPIM data sets, the evaluation of three-dimensional microtubule length distributions over time effectively takes all of the asters' microtubules into account. This provides a very good statistical basis to test and improve theoretical models of dynamic instability [27–29]. Finally, the elastic properties of the microtubules can be determined from the thermal fluctuations of the filaments' three-dimensional position and geometry.

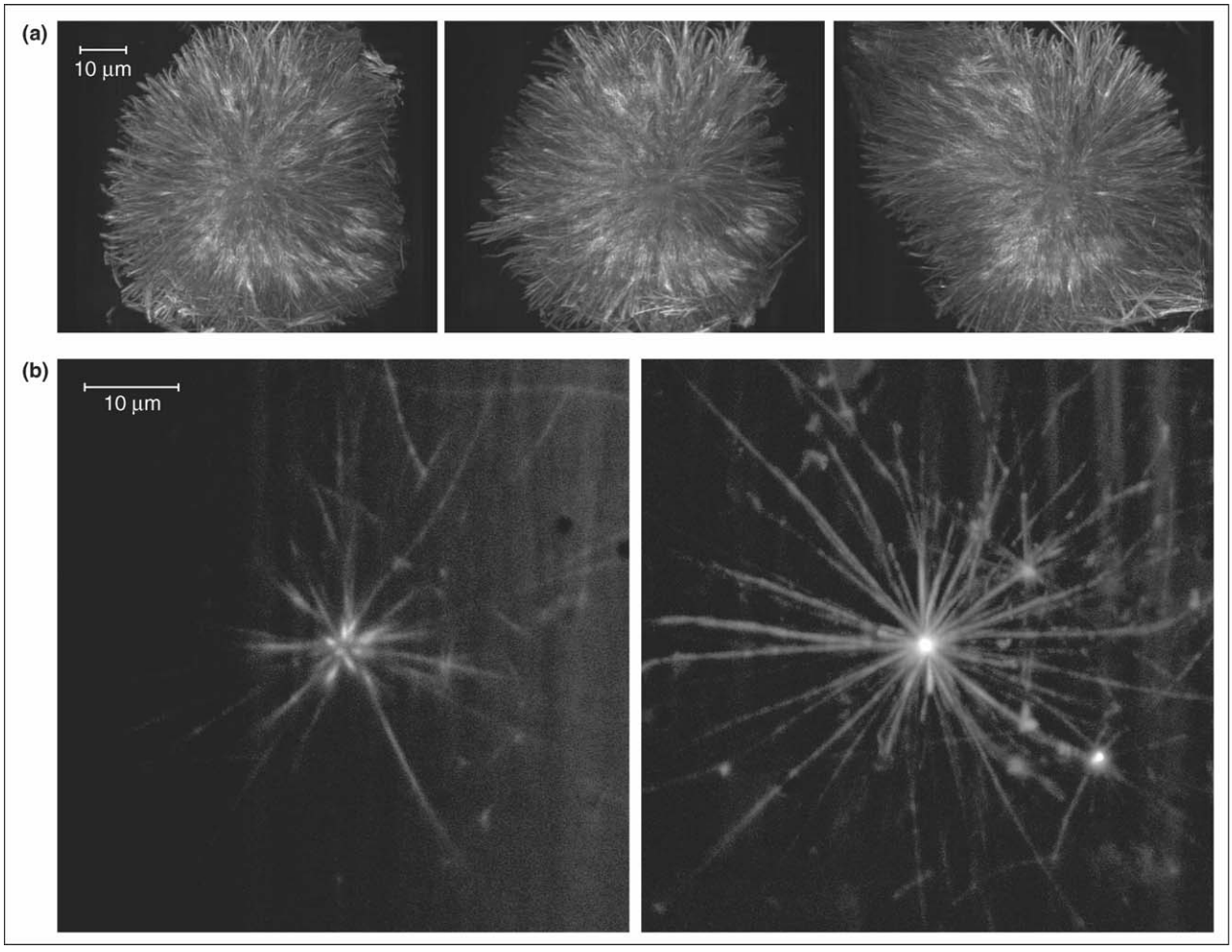
Unluckily, neither at EMBL nor as far as we know in other laboratories have fast three-dimensional aster dynamics been successfully investigated with confocal fluorescence microscopes, mainly because of fluorophore photo bleaching. With single-view SPIM, however, we achieve a time resolution of three seconds for the entire three-dimensional volume of a typical interphasic aster without a significant effect of photo bleaching even after 15 minutes of continuous observation. Multi-view SPIM (mvSPIM) records the entire three-dimensional volume of a stabilized microtubule aster with an isotropic resolution. The aster data sets are recorded along several directions and fused by image processing. The resulting isotropy (Figure 2a,b,c) is a crucial feature in the quantitative investigation of three-dimensional structures.

The three-dimensional imaging and sample preparation in SPIM provide several advantages for the investigation of cytoskeletal filament dynamics. The imaging yields three-dimensional structural information instead of two-dimensional projections of fluorescent structures. Unconstrained filament growth along all dimensions eliminates uncharacterized interactions of the sample with artificial surfaces. Additionally, the strongly reduced surface-over-volume ratio in SPIM sample preparation (Figure 1a,b) minimizes possible surface effects, for example the unspecific adsorption of proteins. While the surface area is minimized in SPIM experiments, the visibility of these surfaces in the three dimensional data sets still allows us to clearly assess whether phenomena of microtubule dynamics are associated with proximity to or contact with a surface.

SPIM technology for three-dimensional cell culture

So far relatively few data are available on how cells interact and communicate with each other in the

Figure 2

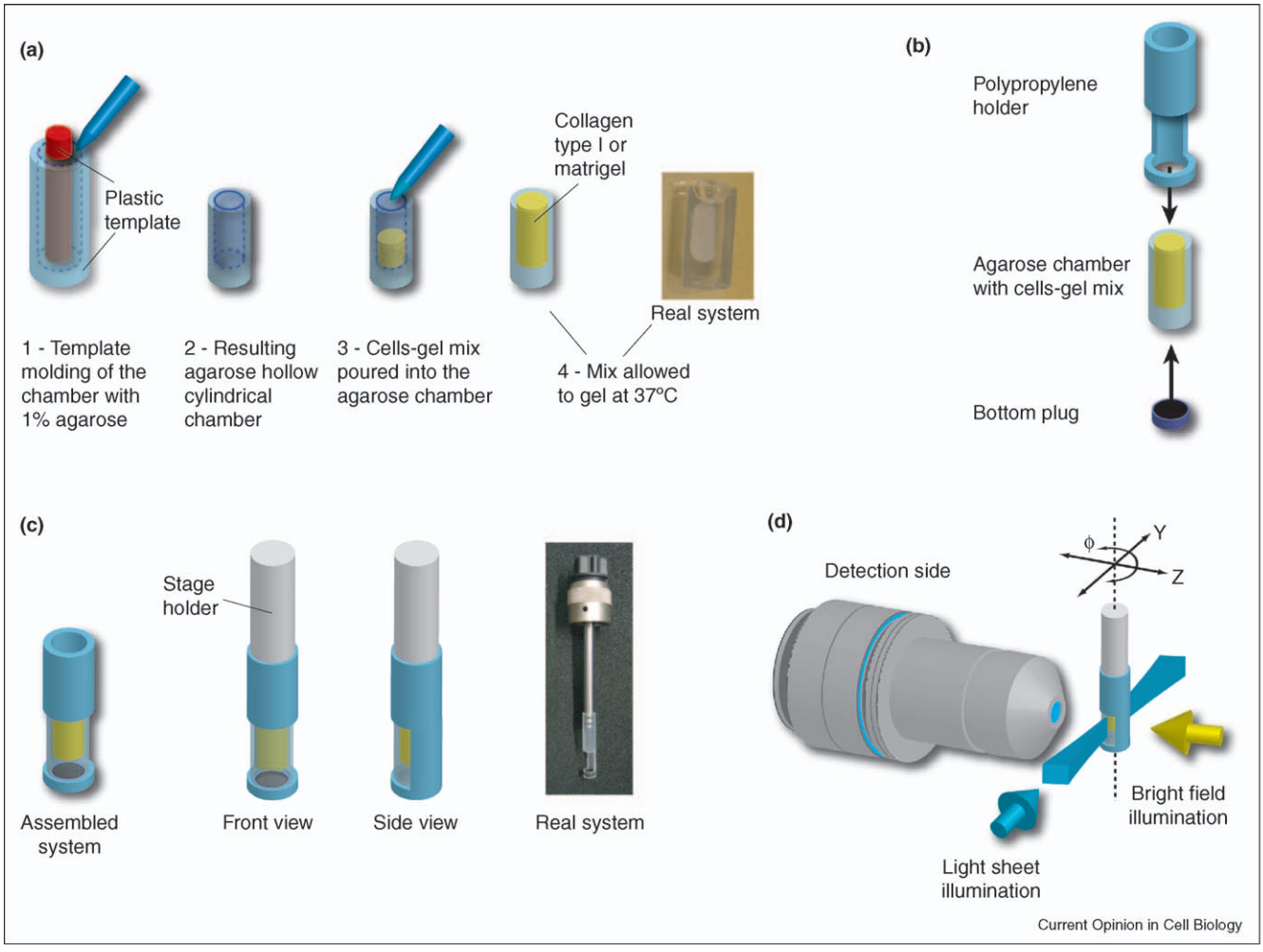


SPIM images of fixed and unfixed microtubule asters. **(a)** Maximum intensity projections of a taxol-stabilized aster polymerized in agarose/BRB80. The fused and deconvolved SPIM data set contains 309 planes at 300 nm spacing and is based on seven recorded angles (−45°, −30°, −15°, 0°, 15°, 30°, 45°). In order to illustrate the isotropy of deconvolved multi-view SPIM images, projections are shown along different angles (20°, 60° and 130°). The images were recorded using a CCD chip with a size of 1344 × 1024 pixels and a dynamic range of 10 bits. Due to a very high concentration of taxol, tubulin-taxol crystals are visible in the central region of the aster. The tubulin was labelled with Alexa-488. **(b)** SPIM images showing an aster in a high-speed interphasic *Xenopus laevis* egg extract. Left: single frame of the data set. Right: maximum intensity projection of the data set, which contains 68 planes at 300 nm spacing. Microtubules that are oriented in parallel to the plane of observation appear dimmer than perpendicular microtubules. This effect is avoided in multi-view SPIM imaging. The tubulin was labelled with TAMRA. Carl Zeiss W 100×/1.0.

three-dimensional context of a tissue. Cell fate in living organisms — for example polarization, growth, migration or apoptosis [5,30,31,32•,33,34] — is determined by three-dimensional and temporal information exchange between neighboring cells as well as by cues from the microenvironment (e.g. ECM proteins or growth factors). Ultimately, the whole physiology of healthy or pathologic organisms depends on information flow and processing that is based on both biochemical and mechanical cues [35•,36]. We believe that the life sciences are currently undergoing a paradigm shift towards the investigation of cells maintained in an environment closely mimicking the mechanical, chemical and cytological properties of real tissues [4••]. An improved understanding of how cells react to physiological stimulations and constraints is leading to substantial scientific and technological advancements in cancer research [37•,38], immunology [39,40•] and tissue engineering [41,42]. Indeed, in three-dimensional cell cultures, the boundaries between *in vivo* and *in vitro* experiments tend to disappear, which has important implications, particularly for drug discovery [30].

We are currently developing a basic 'SPIM-compatible' technology to investigate three-dimensional cell cultures.

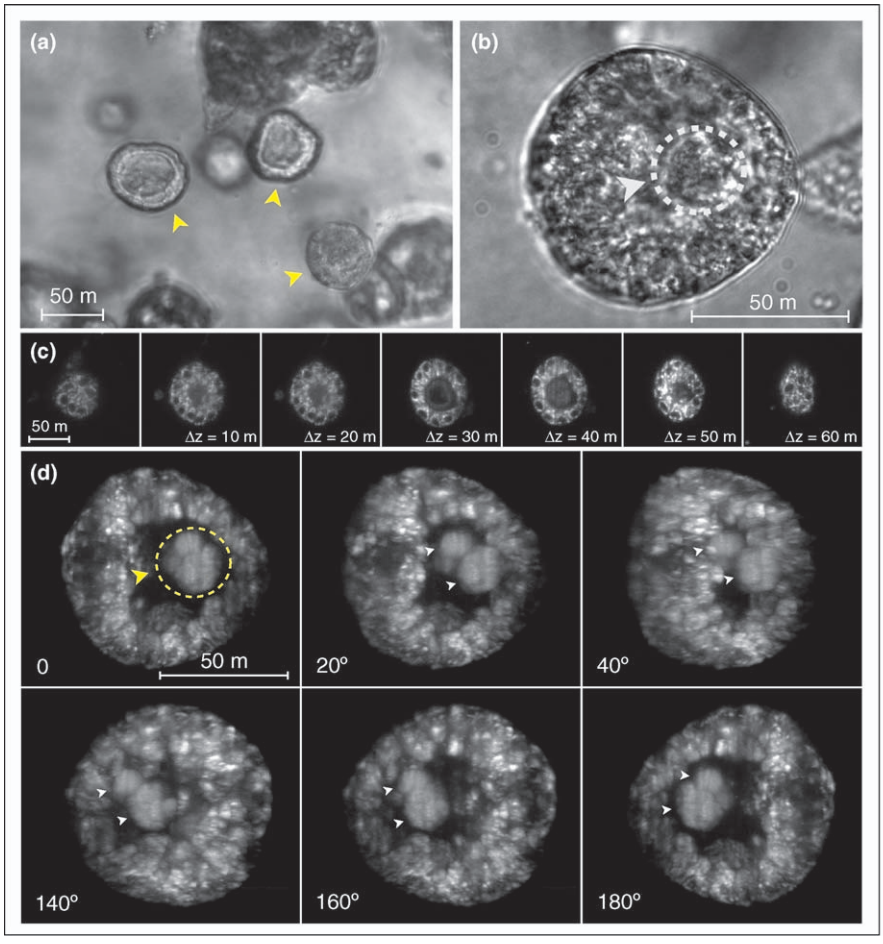
Figure 3



Sample preparation and mounting procedures for the observation of three-dimensional cell cultures with SPIM. **(a)** Low-melting-point agarose is used to form a container that can be filled with different three-dimensional cell culturing materials. 1. Molding of the hollow agarose cylindrical chamber: liquid low-melting-point 1% agarose is poured into a template and allowed to gel by cooling to room temperature. 2. Resulting agarose cylinder after removal from the template. The wall thickness is below 1 mm. 3. The cells–gel mix (matrigel or collagen type I) is poured into the agarose chamber. 4. The mix is allowed to gel at 37 °C and 5% CO_2 for 30 minutes inside the agarose chamber. **(b)** The polymerized 3D gel inside the agarose chamber is inserted into and firmly supported by a polypropylene holder, which can be maintained in a conventional Petri dish. **(c)** The cells, the culturing material and the holder are prepared for mounting in the SPIM's stage. **(d)** The whole unit once inserted into the SPIM can be moved along the detection system's optical axis to generate three-dimensional image stacks as well as rotated to generate stacks of images along different directions. Long-working distance objective lenses, e.g. the water dipping Carl Zeiss 40×/0.8 with a working distance of 3.61 mm, work very well. Brightfield illumination is particularly useful to locate the cells inside the gel.

One approach is illustrated in Figure 3. Several types of matrices are commercially available for three-dimensional cell cultures. They are extracted either from living systems (e.g. Matrigel) or from synthetic systems (e.g. Puramatrix). Matrigel reproduces the mechanical and biochemical characteristics of natural ECM and consequently exerts an environmental pressure on the cells that is close to a physiological situation. For many years it has been known that MDCK cells cultured in a collagen gel or Matrigel for 7–10 days form hollow cysts, consisting of a monolayer of 50–100 polarized cells [43,44]. MDCK cells also undergo a branching tubulogenesis when exposed to hepatocyte growth factor (HGF) [43,45]. MDCK cells thus represent an interesting model system for investigating the morphogenesis of epithelia [44]. In order to test both our instruments and our understanding of the underlying biology of cyst and tubules formation, we cultured MDCK inside Matrigel or collagen type I matrices. We observed the structures with SPIM using the agarose chamber approach illustrated in Figure 3. Some of our results are shown in Figure 4.

Figure 4



Madin-Darby Canine Kidney (MDCK) cells form hollow clusters when grown in Matrigel. **(a)** Cysts were obtained by culturing MDCK cells in Matrigel for 7–10 days. The agarose chamber containing the cells as shown in Figure 3 was put into a conventional Petri dish. After several days in culture, the sample was mounted within the SPIM-holder and imaged. The average diameter of mature cysts is 50–60 μm. The image was recorded in brightfield illumination. Carl Zeiss W 10×/0.3. **(b)** Single MDCK cyst imaged with the SPIM in brightfield at a higher magnification. A small cluster of cells (delimited by the dotted white circle) is visible inside the hollow cavity of the cyst. Carl Zeiss W 40×/0.8. **(c)** Single slices through a cyst at 10 μm spacing. Each section has a thickness of 10 μm. **(d)** Maximum projection of 29 slices with 1 μm spacing rotated to different angles. The three-dimensional spatial arrangement of the small cell clusters inside the lumen can be readily visualized. Labeling: Syto61 (absorbance 628 nm, emission 645 nm).

Conclusions

As Karl H Popper noted more than 40 years ago [46], the sciences, in particular the natural sciences, evolve by breaking a dogma and developing a new one. Alexis Carrel broke the rules of physiology by growing myocardia and sarcoma cells in tissue cultures, applying techniques formerly devised by others [47,48]. His ideas induced a revolution in his time but entered the mainstream and helped to address fundamental cellular phenomena. Nowadays, with technical advances in tissue engineering, chemistry and microscopy, the obstacles hindering us from growing and observing cells in their natural tissue environment are becoming smaller. Nevertheless, it is still a challenge to take advantage of fifty fruitful years of cell biology research and move into the third dimension.

SPIM has the potential to contribute substantially to the emerging field of three-dimensional cell biology. It provides a suitable imaging technology for investigating large and complex cellular systems at high resolution with extremely low photobleaching and hence extremely low phototoxicity. The applicability of SPIM ranges from cells grown in ECM-like matrices to organs and whole organisms [16••]. Light-sheet-based technologies enable researchers to visualize growth, migration and morphologic changes in the context of a physiological environment.

Not surprisingly, the same techniques can also be applied at the molecular level, since a cell's cytoskeleton and in particular its microtubule asters are three-dimensional structures. Nevertheless, most conventional approaches to cell biology and biophysics employ two-dimensional

methods and essentially two-dimensional imaging techniques [49]. However, the focus of biological studies up to now has mainly been on comparative analyses of protein function [50•]. With the possibility of reducing spatial and temporal constraints, new experiments can be designed that yield insights into three-dimensional structural dynamics in an explicitly quantitative manner.

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