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Tutorial Review

Light Sheet Fluorescence Microscopy (LSFM); Past, Present and Future.

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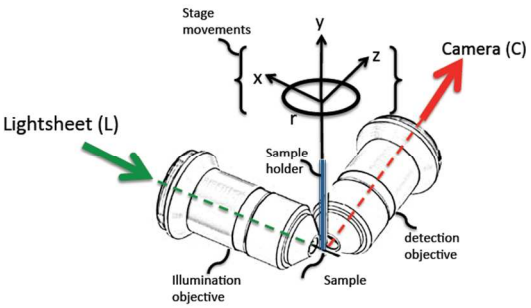
Light sheet fluorescence microscopy (LSFM) has emerged as an important imaging modality to follow biology in live 3D samples over time with reduced phototoxicity and photobleaching. In particular, the LSFM has been instrumental in revealing the detail of early embryonic development of Zebrafish, Drosophila, and C. elegans. Open access projects, DIY-SPIM, OpenSPIM, and OpenSPIN, now allow LSFM to be set-up easily and at low cost. The aim of this paper is to facilitate the set-up and use of LSFM by reviewing and comparing open access projects, image processing tools and future challenges.

1. Past

Laser scanning confocal microscopy (LSCM) has been the standard form of microscopy to extract cellular and subcellular information from biological systems for the last 20 years. LSCM relies on lasers of specific wavelengths exciting fluorophores and detecting their emission within a discreet volume by use of a pinhole. This pinhole-based optical sectioning comes at the price of unwanted excitation of fluorophores and phototoxic damage to biological material.

Lightsheet fluorescence microscopy (LSFM) couples observation and excitation volumes thereby reducing phototoxic damage. Another important advantage of LSFM is the speed of acquisition is much faster than LSCM allowing rapid events in biology to be investigated. The principle of LSFM is to use two objectives set perpendicular to each other; one to focus the lightsheet and the other to detect the fluorescence emission Voie et. al., 1993 (1), were the first to use LSFM to look at biological samples. They called the imaging technique OPFOS, Orthogonal Plane Fluorescence Optical Sectioning. Since the lightsheet is thin relative to the sample good sectioning is achieved. Figure 1 shows the basic arrangements of the components of a LSFM. Laser light is focused into a lightsheet using optical elements including a cylindrical lens (green arrow). The lightsheet is passed through an illumination objective to focus onto the sample. At right angles to the illumination objective sits the detection objective which relays the fluorescence to a camera (red arrow). The biological sample is held between the two objectives with its position controlled by a 4D stage (x, y, z positional and rotational angle, r).

(A)



(B)

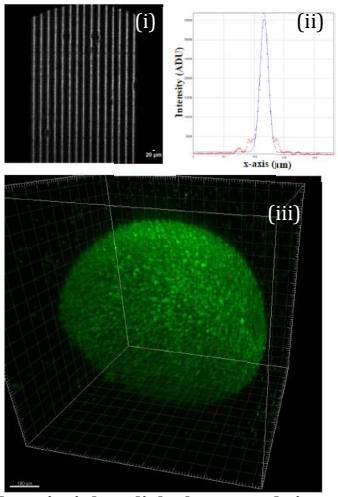


Figure 1. LSFM: principles, lightsheets and image. (A) The basic OpenSPIM set-up is shown with; objectives, lightsheet, sample and sample holder being controlled by the 4D stage. Lightpaths, red- illumination, green-detection. (B) Max intensity projection of the 488nm laser light sheet reflected from a 45 degree mirror at incremental positions along the x-axis. (i) This image shows that the lightsheets from the OpenSPIM setup have a near uniform thickness of 14.6 μm across the field of view. (ii) In this graph, the FWHM of the lightsheet, shown in (i), is calculated by Gaussian fitting using ImageJ. The intensity values are plotted (red circles) and the blue line is the Gaussian fitting. FWHM = 14.6 μm. (iii) This image shows ten angles (36 degree increment) fusion of a Zebrafish embryo with H2B-GFP labeled nuclei. The camera used was an ORCA Flash 4.0 using 25 ms exposure time with 1.5 μm z-step size using a Picard 4D stage. Scale bar = 200 μm

Figure 1B shows the calibration of a typical LSFM. Here an OpenSPIM set-up is used to characterize the size and uniformity of the lighsheets generated (panels (i) and (ii)). In panel (iii) a Zebrafish embryo is shown labeled with a nuclear protein H2B-GFP.

In 2004, Stelzer, Huisken and colleagues were the first to use LSFM to probe developmental biology with the imaging of Medaka and Drosophila embryos (2). In 2008, Stelzer, Keller and colleagues, developed digital lightsheets and used them to image Zebrafish development over 24 hours (3). These two

landmark studies have opened a new era in the understanding of biological systems in 4D (3D and time).

A number of excellent reviews on LSFM have been published. These include; history of LSFM (4), instrumentation (5-8) and biology (9,10). Here our focus is to discuss open access projects (DIY-SPIM, OpenSPIM and OpenSPIN), image processing, and future challenges, with the vision of helping biologists set-up and use their own LSFM.

microscopy by giving detailed instructions of building LSFMs, software for hardware control, user exchange and troubleshooting support. OpenSPIN and DIY-SPIM (hosted on an Institute website) rely more on direct contact with the developers – Emilio Gualda and Corrine Lorenzo, respectively. OpenSPIM focuses on low cost and minimalism while DIY-SPIM and OpenSPIN give options for more advanced imaging. All three systems can be built relatively cheaply in the range of 100,000 Euros. Table 1 and 2 show a comparison of the three build your own LSFM projects. Table 1 shows components that are in use in many set-ups, customised and commercial. This includes optics, lasers, Acousto-Optic Tunable Filters (AOTF), excitation

	DIY-SPIM	**OpenSPIM**	**OpenSPIN**	**Remarks**
Cylindrical lens	F = 100 mm	F = 50 mm	F = 50 mm	50 mm focus length cylindrical lens will have a shorter Rayleigh length and thinner light sheet when compared to 100 mm focus length cylindrical lens.
Illumination objective	10x Leica Air	10x Olympus water dipping	Nikon 4x or 10x Air (and water dipping options)	The use of air illumination objective lens allows the position of the Rayleigh length portion of the light sheet to be adjusted.
Detection objective	10x, 20x and 40x Leica water dipping	20x Olympus water dipping	Nikon 4x or 16x water dipping.	None
Galvo mirror	No	No	Yes (system can be operated in SPIM or DSLM mode)	Sample scanning is achieved by moving sample through the galvo generated lighsheet.
Beam expansion	30 mm and 200 mm (6.6x)	2 x (25 mm and 50 mm) (4x)	25.4 mm and 200 mm (8x) or 50 mm and 175 mm (3.5x)	None
Tube Lens	200 mm (Edmund)	180 mm (Olympus)	200 mm (Thorlabs)	None
Lasers	491 nm, 532 nm, 594 nm	488 nm	488 nm, 568 nm,647 nm and Ti-Sapphire laser.	Having more than one laser gives the possibility to image different labels and samples
AOTF	Yes	No	No	None
Excitation filter wheel	No	No	Yes	Custom designed using Thorlabs (metallic parts) but is controlled with an Arduino board with Motor shield Kit v1.1 from Ardafruit and a home made sensor
Emission filter wheel	Yes, Sutter Instrument	Yes, two manual slots for filters along the infinity space tube	Yes, Thorlabs.	None

Table 1. Comparison of open access projects: optics and lasers

For further information about open access projects go to website locations;
DIY-SPIM:
http://www.ip3d.fr/IP3D/SPIM/Entrees/2012/4/20_Do_It_Yourself_-_Build_your_own_SPIM_!.html
OPEN-SPIM: http://openspim.org/Welcome_to_the_OpenSPIM_Wiki
OPEN-SPIN: https://sites.google.com/site/openspinmicroscopy/

2. Present

A. Build your own LSFM

An important recent development of LSFM has been three open access projects, DIY-SPIM (11), OpenSPIM (12), and OpenSPIN (13), the objective of which is to allow easy implementation of this modality of microscopy. OpenSPIM and OpenSPIN, are developed on Wiki and Google webpages, respectively. In particular, OpenSPIM heralds a new era in communication in filters and emission filters with associated comments and remarks (Table 1). Particular components, the camera, software, sample chamber, environmental control (Table 2A), and stage (Table 2B) warrant further discussion. Currently (2014), the camera of choice for LSFM is the Hamamatsu Orca-Flash4 V2 which has been designed to incorporate a LSFM specific mode. This mode gives greater flexibility on how data is streamed and also reduces light scattering through the use of a rolling shutter. In addition, the larger field of view for the Orca-Flash4 sCMOS camera allows reduced image numbers needed to see the sample thus speeding-up the acquisition process. An important contribution of OpenSPIM and OpenSPIN is that the software used to control and integrate the microscope are open source, incorporating Micromanager, ImageJ and Fiji components. In addition, OpenSPIN includes Arduino with different shields for sample rotation, galvo scanning and filter wheel control, which can be used to obtain Optical Projection Tomography datasets. The choice of sample chamber is determined by the way in which the light sheet is aligned. DIY-SPIM and OpenSPIN use a moveable illumination objective for light sheet alignment while OpenSPIM uses mirrors and a telescope to control the light sheet. In the latter case the illumination objective and observation objective are integrated into the sample chamber and in contact with liquid. The possibility in DIY-SPIM to

adjust or change the illumination objective is a more flexible solution. Environmental control is an important consideration particularly for 3D cultures. An acrylic incubator box solution is probably the most straightforward. The OpenSPIM stage is simple and inexpensive but is not suitable for high speed imaging and precise repeatable positioning of the sample. We advise the use of stages from either Thorlabs or PI for time series experiments where precise positioning is required (Table 2B). The Thorlabs and PI stages are supported by Micromanager.

A

	DIY-SPIM	OpenSPIM	OpenSPIN	Remarks
Camera	CoolSNAP-HQ	Orca-ER	Orca-Flash 4.0	The Orca-Flash 4.0 has the highest effective number of pixels and has a LSFM specific mode
Sample chamber	UV lithography	Acrylic fabrication	Self-made – various options.	Water dipping illumination objectives attached to sample chamber
Software	Amiview, Labview	Micromanager/Fiji	Micromanager/Fiji – Arduino boards (to control Galvo and Filter wheel)	Softwares used of OpenSPIN and OpenSPIM are open access
Environmental control	Main elements enclosed in an incuabtor box	No	Under development. Perfusion chamber with temp. control	Environmental control allows the samples to survive for time lapse imaging.

B

	DIY-SPIM	OpenSPIM	OpenSPIN
Stage Company	PI	Picard	Thorlabs (Linear Stage) Astrosyn (Rotation Stage)
Model Number	M-232.17 with M-105.AP M-037.DG	USB 4D Stage	MTS50/M-Z8 Stepper motor as rotation stage
Linear Travel Range (mm)	17	9	50
Minimum Increment (μm)	0.1	1.5	0.1
Repeatability	0.2	1.5	1.6
Backlash	2	NS	<6
Rotation Resolution (degree)	34 x 10^{-6}	1.8	0.225

NS – not specified

Table 2. Comparison of open access projects: Other components
(A) Camera, sample chamber, software and environment control. (A) Stage specifications.

Rotation generates different views of the sample. So for LSFM it is essential to know how individual images relate to each other through a process know as bead-based registration (14). Following registration the images can be fused together to produce the final image. Full details on registration and fusion of images can be found on the OpenSPIM website (http://openspim.org/Operation under data processing) and Fiji (http://fiji.sc/SPIM_Registration) websites. An inherent limitation in LSFM is the problem of light scattering and absorption which generates streaking in the images. To overcome this problem a number of solutions have been put forward. (i) Multiview acquisition combined with a resonating mirror (15), (ii) noise removal by software (16), (iii) adaptive optics (17) and (iv) physics based image restoration (18)

B. LSFM improvements and variants

Starting with SPIM and DSLM (Table 3A) as the benchmarks for LSFM developed by Stelzer and colleagues a number improvements have been implemented in the last few years. Essentially these deal with correcting the artifacts that lead to image streaking and increasing the speed of image acquisition. Multiview approaches incorporating the latest developments in sCMOS cameras have dramatically increased acquisition speeds to a point where snapshots of developmental processes can be taken every 10-30 secs.

The different light paths used in SPIM and the improved LSFM variants are shown in figure 2.

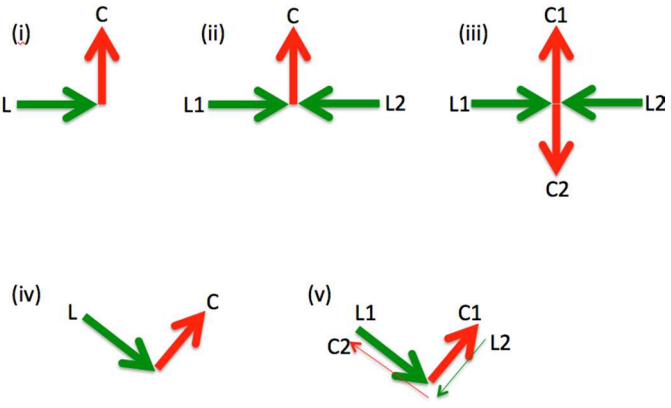


Figure 2. Lightpaths of LSFM variants
The figure shows the lightpath arrangments for LSFM variants. Green arrow – illumination lightpath, Red arrow-detection lightpath. (i) SPIM (2), (ii) Multiview with two illumination arms (15), (iii) Multiview with two illumination and two detection arms (19,20), (iv) inverted (i)SPIM (21), (v) Dual view (D) iSPIM (22). For (v) the same objectives are used for lightpaths one and two and this is shown by using smaller arrows for the second lightpath.

Table 3. LSFM, improvements and variant

A. LSFM	Feature	Biology	Reference
OPFOS	Lightsheets separating illumination and detection paths used for this first time	Ear structure	Voie et. al., (1993)[1]
SPIM (Figure 2i)	Lightsheets used to image Medaka and Drosophila	Medaka	Husiken et. al., (2004)[2]
DSLM	Digital lightsheets used to image Zebrafish development	Zebrafish embryos	Keller et. al., (2008)3
B. LSFM improvement			
mSPIM (Figure 2ii)	Improved imaging by using a resonant mirror and two side illumination where m stands for multidirectional	Zebrafish embryos	Huisken and Stanier, (2007)[15]
MuVi SPIM (Figure 2iii)	Multiview (MuVi) imaging reducing time needed for sample rotation	Drosophila embryos	Kricz et. al., (2012)[19]
SiMView (Figure 2iii)	Simultaneous multiview (SiMView) reducing time needed for sample rotation	Drosophila embryos	Tomer et. al., (2012)[20]
Besssel beam plane illumination microscopy	Thinner lightsheets with higher resolution for subcellular biology	Mammalian cells	Planchon et. al., (2011)[23]
iSPIM (Figure 2iv)	Inverted (i) high-speed imaging with sample presented in a dish	C. elegans embryos	Wu et. al., (2011)[21]
DiSPIM (Figure 2v)	Dual View (D) inverted (i) imaging with sample presented in a dish	C. elegans embryos	Wu et. al., (2013)[22]
RLSM	Reflected lighsheet microscopy producing thinner lightsheets for subcellular imaging	Mammalian cells	Gebhardt et. al., (2013)[24]
C. LSFM variant			
SPIM-FCS	SPIM with Fluorescence Correlation Spectroscopy (FCS)	Mammalian and Drosophila cells	Capoulade et. al., (2011)[25]
SPIM-FLIM	SPIM with Fluorescence Lifetime Imaging Microscopy (FLIM)	Mammalian cells	Greger et. al., (2011)[26]
DSLM-SI	Background removal by structured illumination (SI)	Zebrafish and Drosophila embryos	Keller et. al., (2010)[27]
IML-SPI	Individual molecule localization (IML)-SPIM allowing live 3D superresolution microscopy	Mammalian cells	Cella et. al., (2011)[28]
SPIM-STED	SPIM with Stimulated Emission Depletion (STED) increasing resolution by 60%	Zebrafish embryos	Friedrich et. al., (2011)[29]

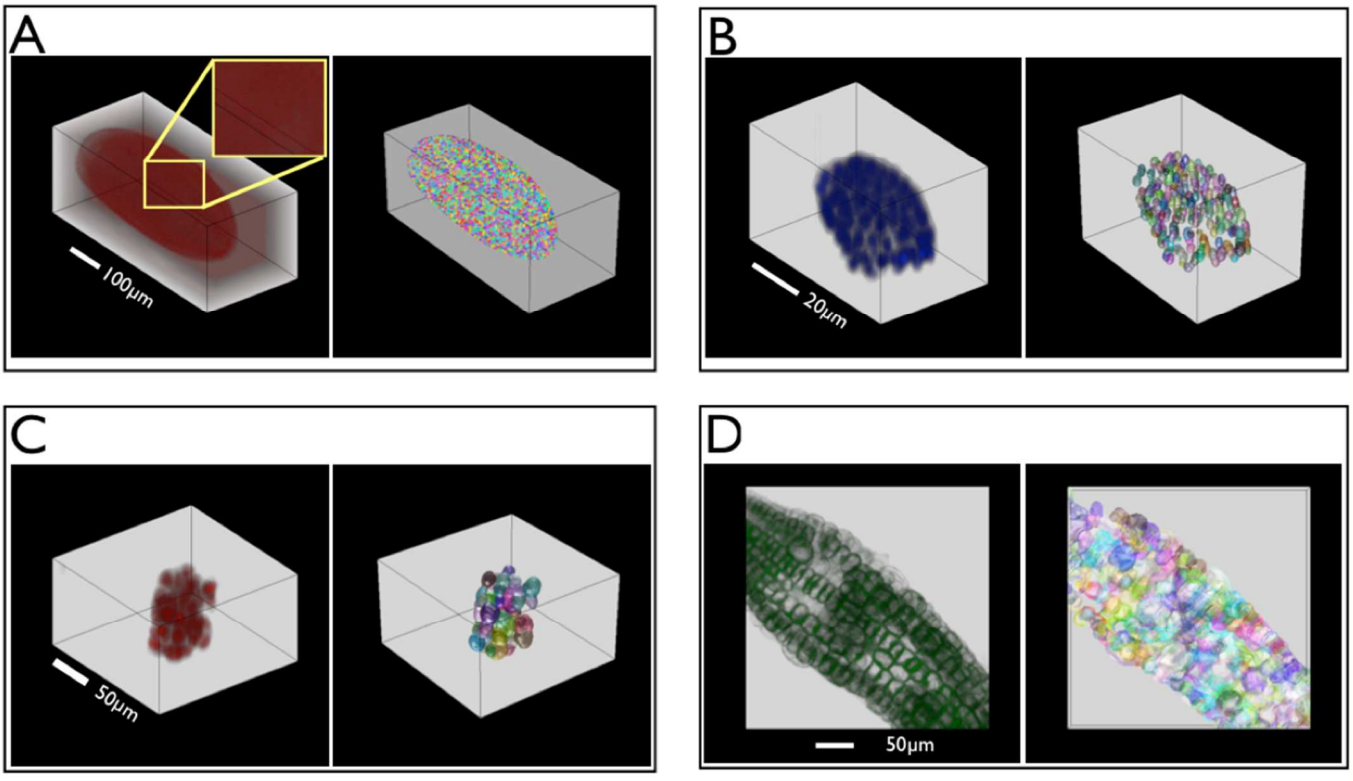


Figure 3. **EGVD based segmentation of LSFM data**
The image stacks were acquired by LSFM. The original image is shown on the left hand side of each Panel and the segmentation of the image is presented on the right hand side. Different colors identify different nuclei/cells. The segmentation in Panel (A)-(C) was based on the EGVD algorithm (30,31). Panel A: Drosophila embyro. Panel (B): C. elegans embyro. Panel (C). Neurosphere (Human). Panel (D): Arabidopsis root.

Embryo/3D culture	Size	Cell size (cell numbers)	Reference
Zebrafish	1 mm	20 μm (1000s)	Keller et. al., (2008)[3]; Huisken and Stanier, (2007)[15]; Keller et. al., (2010)[27]
C. elegans	0.1 mm	2 μm (100s)	Wu et. al., (2011)[21], Wu et. al., (2013)[22]
Drosophila	1 mm	5 μm (1000s)	Kricz et. al., (2012)[19] Tomer et. al., (2012)[20] Keller et. al., (2010)[27]
Arabidopsis root	0.5 mm	5 μm (1000s)	Costa et. al., (2013)[32]; Vermeer et al., (2014)[33]
Spheroid	0.3 mm	20 μm (1000s)	Pampoloni et. al., (2013)[34]
Neurosphere	0.1 mm	10 μm (100)	Lim and Ahmed, unpublished

Table 4. Biological systems imaged by LSFM

Bessel beams are formed by an annular illumination pattern and when scanned can form a virtual lighsheet. The Bessel beam approach can be used to generate thinner lightsheets (sub micron range) than SPIM or DSLM which is well suited for sub cellular imaging and cell biology. Bessel beams can be used in different modes depending on the application. Planchon et al., 2011 (23) introduced the two photon excitation (TPE) mode and structured illumination (SI) mode to give increased resolution and reduced phototoxicity and artefacts. For the latest development of Bessel beam plane illumination microscopy see section D.

LSFM has also been combined with the so called F-techniques and superresolution microscopy modalities (Table 3C). Bessel beams, Structured illumination Microscopy (SIM) and Photo-Activatable Localisation Microscopy (PALM), coupled with SPIM, will lead to substantial improvements in resolution. The development of SPIM-Fluorescence correlation spectroscopy (FCS) opens up a whole new vista of quantitative biology by defining cell wide protein diffusion rates and concentration maps on a rapid time scale (25).

C. Image processing.

Long-term high resolution time lapse LSFM generates vast amounts of data and significant challenges for both data handling and image processing. In order to extract quantitative information from these large LSFM datasets an automated segmentation workflow is a crucial step. A major issue in image processing of LSFM datasets is that the nuclei/cell segmentation and tracking are not accurate enough. Assuming we may achieve 95% accuracy for segmentation and tracking at each given time-point, after 20 time points only 36% of results will be reliable and just 13% after 40 time points. Thus new solutions are required for such a large-scale problem.

Segmentation. Segmentation determines which voxels in the image belong to which objects, e.g. nuclei or cell. The simplest segmentation analysis is to use voxel intensity thresholds of, for example, nuclear staining data. A number of approaches can be used for nuclear segmentation, including; simple thresholding (35), watershed approach (36,37), iterative voting methods (38), level set approach based on gradient flow (39) and flexible contour model (40). Cell segmentation is greatly facilitated by staining of the plasma membrane and seed finding. The watershed approach has been widely implemented for cell segmentation. One major problem with the watershed approach is over-segmentation. To overcome over-segmentation a few approaches have been proposed, e.g. rule-based merging (41) and marker-controlled Voronoi diagram (42), a level set formulation for Watershed segmentation (43) and preserving topology by simple point concept (44). Other approaches to address the problem of over-segmentation include multiple level set functions for individual cells (45) and topological dependence (46,31). The Evolving Genralized Voroni Diagram (EGVD) approach was also applied to 3D nuclei segmentation (30) and examples of EGVD based segmentation of LSFM data are shown in Figure 3.

Cell Tracking. Two basic point tracking techniques commonly used are centroid tracker (47) and correlation tracking (48,49). Other tracking approaches include the snake model with gradient vector flow (50), snake model with shape and size constraint (51,52), active contour approach for both segmentation and tracking (53), a K-mean Clustering and matching algorithm (54) and a Gaussian Mixture Model incorporating Markov chains (55).

Segmentation and cell tracking approaches used for LSFM. Different segmentation and tracking methods have been applied to LSFM data such as adaptive recursive analysis based on iterative thresholding (3), blob detection based on Laplacian of Gaussian (27), a constrained active contours approach (56). A pipeline of segmentation and tracking for the Drosophila embryo is proposed (19), which combines filtering, local thresholding and watershed approach. Tomer et. al. (20) have put forward a high speed pipeline, SiMView, for imaging nervous system development in Drosophila embryos. They acquire multiview images simultaneously over periods of secs which is fast enough to capture nuclear dynamics. Tomer et. al., (20) segmented their data using two independent methods; a Gaussian mixture model and the diffusion gradient vector field algorithm.

D. Biology

To date all the well known models for developmental biology have been imaged by LSFM and this includes; Zebrafish, Drosophila, C. elegans and Arabidopsis (Table 4). The establishment of the digital embryo has been a major accomplishment of LSFM. For example, in the Zebrafish embryo the cell divisions and movements that occur over the first 24 hours have been analysed (see for example, http://www.janelia.org/digitalembryo). Although improvements in imaging (contrast and resolution) and segmentation are still needed greater that 70% of all cells have been identified during Zebrafish embryogenesis. More recently, LSFM has been used to trace Zebrafish cell lineage formation by using the endoderm marker Sox17 (56). The ultimate aim of these experiments being to use LSFM in combination with genetic markers to create a complete lineage map.

Spheroids are the main 3D culture analysed by LSFM. For example, Lorenzo et al., (11) examined multicellular tumour spheroids with histone H2B to mark nuclei and monitor the effects of anti-cancer drugs on cell division. Bruns et al., (57) use LSFM to follow spheroids formed from CHO cells stained with membrane associated GFP and acridine orange. Pampaloni et al, (34) describe sample preparation and mounting conditions for a range of spheroids. LSFM was used on spheroids derived from T-47D cells as a model for breast cancer. The spheroids measured hundreds of microns, contain 1000s of cells and are imaged with the nuclear stain Draq5 or H2B, and a mitochondrial stain - Mitoview or Mitotracker. We have used mouse and human neural stem cells grown as neurospheres and obtained LSFM images using nuclear DAPI stains (30; Figure 3c). Clearly, LSFM is the method of choice to image 3D culture systems and in the future organotypic cultures such as reconstructed skin (see section 3).

The speed of image acquisition of LSFM has allowed new developments in imaging brain activity. Turaga et. al. 2008 (58) have used LSFM to examine brain activity in mice using calcium probes. More recently, Ahrens et al., (59) have used genetically encoded calcium probes (GCaMP5G) and LSFM to make recordings of 100,000 neurons (of the entire larvel Zebrafish brain) within approximately 1.3 secs. Similar work of brain activity in Zebrafish was also reported by Panier et. al., 2013 (60).

The current state of the art for imaging cell biology at high spatial and temporal resolution is the Bessel beam plane illumination microscopy

combined with Supersolution Sturctured Illumination (SR-SIM; 61). In this study Gao et. al., (61) examine the cell biology of chromosome and actin dynamics in thick samples as well as development of C. elegans embryos and the adult brain of Drosophila. It emerges that SR-SIM and Bessel beam plane illumination, on a variety of biological samples, gives low phototoxicity, high resolution and rapid time lapse revealing new insights to dynamic cell biology. Gao et. al., (62) have recently published a protocol for building and aligning their new Bessel beam plane illumination microscope.

3. Future

LSFM has developed to a very interesting stage where it is applicable to both cell and developmental biology at high resolution and with live samples. What are the areas for development of LSFM and what are the biological insights likely to be made in the near future? Here are some thoughts.

Image processing. For the open access project, and for LSFM in general, it is important develop a pipeline that includes image processing tools. In particular, for accurate 3D cell segmentation and for cell tracking. The challenge here is how to follow and distinguish 1000s of tightly packed objects (cells) that move in time, sometimes merge and/or divide. Robust cell segmentation and tracking tools would allow users to extract information from the LSFM image datasets. Pattern recognition software add-ons would also be interesting and facilitate single cell variation and population analysis to be carried out. Finally, visualization tools will be needed to present data, make spatial sense of it, and to generate models.

Data handling. The amount of data generated by LSFM is significant. The size of one image stack is 100s of GB and if this includes a long term time series data in the region of terabytes (TBs) can be generated. For example, data sets for high resolution imaging of Drosophila embryogenesis over 17 hours, with images acquired every 30 secs, generates 11 TBs of data. Two popular image data management systems are OMERO (63) and BISQUE (64). Both of these platforms are open source and web-based. This allows users to work on their data simply with an internet connection. To compliment these open data management projects it would be exciting to think about initiatives that focus on data access (OpenDATA) and processing tools (OpenPROCESSING) for LSFM. These two initiatives should be built on highly parallelized platforms to handle fast processing of large data sets. Perhaps, a GPU-like architecture with many processing units each with lower power may be suitable. The availability of data and algorithms has promoted the rapid growth of computer vision and the image processing field since 1980s. We advocate an open hosting of LSFM data and image processing tools through websites integrated with OpenSPIN and OpenSPIM.

Pattern Recognition. Pattern recognition is a very important advanced technique used in bioimage informatics that could be applied to LSFM datasets. Accurate segmentation of individual cells is usually, but not always, required for pattern recognition. A set of features such as shape, texture, or size, are extracted from each cell. Thus converting the image of each cell into a vector of numbers. Standard machine learning algorithms such as clustering and support vector machines can then be used for the final recognition task. The CellProfiler Analyst (65,66) is one of the more interface of online learning, whereby after some initial training, the user is prompted to grade the machine classification results for each new cell image. This grade is immediately feedback into the machine to improve subsequent classifications. An example of pattern recognition in practice is that of Peng et. al., (67) who use graph matching for the recognition of cells in C. elegans. Whereas most pattern recognition methods rely on a good segmentation as a preprocessing step, Gui-Mohammad et. al., (68) proposed a novel method in which image segmentation and pattern recognition are done simultaneously. Their segmentation results depend on their pattern recognition results and vice versa.

Cell lineage. The holy grail for developmental biology is to be able document and understand the origins of all cell types from the embryo to the adult. Starting with early cell fate decisions where pluripotent cells form the three germ layers (endoderm, ectoderm, and mesoderm) to the formation of multipotent stem cells and their progeny that define cell tissues. The establishment of the digital Zebrafish embryo demonstrates that this cell lineage analysis is possible. Essential for this analysis will be robust cell lineage markers (e.g. promoter-GFP gene fusions) that allow cell-types to be followed in space and time, from origin to final position in the tissue of choice. Two excellent examples of this approach are; (i) Schmid et. al., (56) who followed Zebrafish endoderm formation with the use of Sox17-GFP promoter fusion transgenic lines. (ii) Wu et. al., (21) who followed neurodevelopment in C. elegans using transgenic lines expressing H2B-mCherry and GFP-Ceh10p (a homeodomain transcription factor).

3D cultures. A major challenge for LSFM is to find novel ways to present biological samples for imaging. For 3D mammalian cell culture care has to be taken to maintain temperature, CO_2 levels and growth media. There are currently two formats for sample presentation in LSFM. The first is where the biological sample is suspended in an agarose plug or tube, between the illumination and detection objectives, within a media filled chamber (Figure 1, and used in all three open source projects). The second, the so called inverted iSPIM (and DiSPIM variant), uses a standard microscope platform where the illumination and detection objectives are held at right angles to each other above the stage (21,22). In iSPIM samples can be placed on coverslips, as per normal microscopy, using the stage as a support. One advantage here is that the conventional light path can be used to position the sample before use of the LSFM. Another advantage is that sample presentation is more flexible. iSPIM will have particular utility in high content set-ups where drug or toxicity screening is required. As mentioned in section 2D, LSFM has been used successfully to examine spheroids. In future experiments it will be important to use LSFM to image human organotypic cultures such as reconstructed skin, intestinal crypts and “mini-brains”. Thus LSFM together with assay development will allow the generation of a variety of novel in vitro models of human physiology and disease.

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