Light Sheet Microscopy Comes of Age

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Modern developmental biology is essentially about observing complex three-dimensional processes *in vivo* with sufficient spatial and temporal resolution, whilst ensuring minimal perturbation to the sample. Currently the most common samples observed are transgenic animals with cells or whole tissues, labelled with one, or more, of the wide range of fluorescent proteins now in routine use.

Using conventional epifluorescence microscopy techniques, which employ the same lens to both illuminate the sample and to collect the fluorescence, leads to a number of problems when imaging these intact samples. First, since the whole specimen is irradiated, significant photodamage can cause death of a critical percentage of cells, or even whole samples. Second, emitted fluorescent light doesn't only come from fluorophores located in the focal plane, but also from above and below. Thus one has a distribution of intensity of fluorescence along the optical axis with the maximum on the focal plane. In addition to these issues fluorescence itself has some limitations, the most important of which are degrading of fluorophores and absorption of the excitation beam not only by fluorophores but other organic compounds within the specimen's body. Confocal microscopy does provide optical sectioning but still excites fluorophores outside the focal plane leading to these potential cyto-toxic events and also out of imaging plane bleaching. In the last decade, Light Sheet Fluorescence Microscopy (LSFM) techniques have emerged as the most promising answer to the challenge of imaging *in vivo* with optical sectioning at high resolution with minimal perturbation to the sample. The technique has established its ability to reduce bleaching and phototoxicity, provide optical sectioning and satisfactory resolution, both lateral and axial, and be capable of high speed imaging [1].

History

The LSFM method takes its origins from the Ultramicroscopy technique which was first introduced in 1902 by Richard Zsigmondy and Henry Siedentopf who observed small gold particles using sunlight projected through a slit aperture [2]. This approach showed signal to noise at a better level than that of existing techniques and made it possible to observe at the level of single gold particles. Zsigmondy was later awarded the Nobel Prize in Chemistry for his study in ultramicroscopy while the technique was used to plot particle movement and provide evidence of Brownian motion by Jean Perrin. It should be remembered that this was long before the invention of the laser as a bright light source and also modern electronic cameras and data analysis methods.

However, development of the LSFM technique was abandoned due to this lack of suitable technology for almost a century until 1993 when, Arne Voie and David Burns at the University of Washington, combined planar illumination and fluorescence microscopy to introduce Orthogonal-Plane Fluorescence Optical Sectioning, which they employed for imaging and mapping a guinea pig inner ear cochlea. The resulting images obtained appeared to be comparable to those obtained with high-sensitivity tomography (such as confocal microscopy) but with a higher ability to resolve soft tissues. The method itself included all the elements now associated with recent LSFM setups, that is a laser, cylindrical lens, specimen chamber, perpendicular illumination and movement of the specimen to obtain a 3D stack of images.

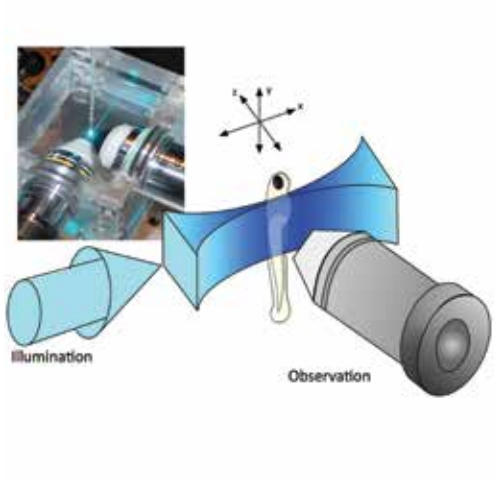

Figure 1: Close up of the focal volume in a SPIM system

In 1994 the Confocal Theta Microscope was developed in Ernst Stelzer's laboratory in an attempt to enhance axial resolution of confocal microscopy by using perpendicular illumination and detection optics, which led to a 3-4 times improvement in axial resolution, and a similar advance in imaging speed having removed the requirement to raster scan the sample.

Ten years later in 2004 the Stelzer group published a breakthrough paper introducing Selective Plane Illumination Microscopy (SPIM) and its application to *in vivo* imaging of embryogenesis of the common fruit fly *Drosophila melanogaster* and the muscle of Medaka fish *Oryzias latipes* using green fluorescent protein (GFP) markers. The timing of this was perfect and has led to an explosion of new techniques and improvements within LSFM. Genetically encoded fluorescent markers were well established, electronic cameras were rapidly advancing in speed, sensitivity and reducing in cost, and high performance personal computing was readily available, though perhaps a full appreciation of the quantity of data that can be generated was not initially considered. SPIM became commercially available in 2013 but another, unique feature, has been the development of open source projects which began in 2012.

Method

In its current configuration SPIM is a fluorescent-based imaging technique the main concept of which is to illuminate a specimen from the side in a well-defined volume using a thin sheet of light, while the axis of detection is orthogonal to the illumination (Figures 1 & 2) [3,4,5]. The illumination plane, the light sheet, is aligned to be at the focal plane of the imaging objective and the specimen is located at the point at which these two axes intersect. The thin sheet of light, typically around 1-2 microns in depth, is created in the simplest configuration by using a cylindrical lens such that the back aperture of the excitation objective (of a reasonably low NA ~0.3) is illuminated with a line of light. This means that the full numerical aperture of the lens

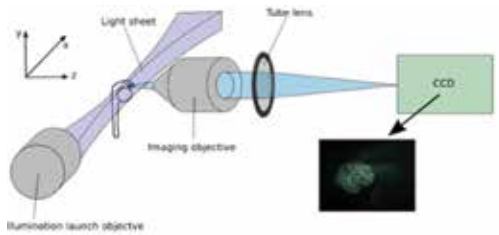

Figure 2: Basic SPIM configuration showing the orthogonal arrangement of the imaging and illumination objective

is used in one axis (causing tight focusing). In the other axis, by limiting the extent of the light filling, little focusing power is added by the objective lens leading to an extended sheet of light. In this configuration the optical properties of the objective is deliberately being "abused" in one axis to create the sheet. The thickness of the sheet, and the area over which it is "thin", can be varied depending on

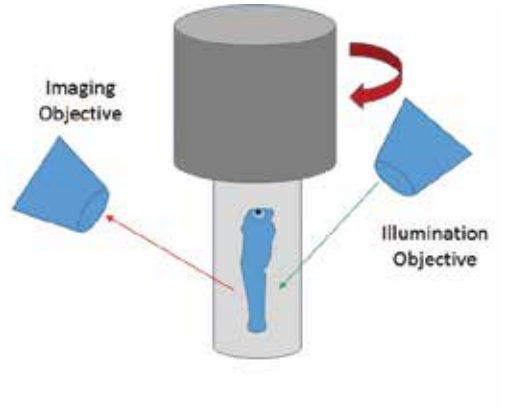

Figure 3: Typical mounting for a conventional SPIM imaging system with the ability to rotate the sample to place the area of interest in the optimal position

extent of the sample, with a thinner sheet only being possible over a smaller field of view, before the focused beam starts to diverge and hence cause an increasing thickness in the sheet. Typical thickness range from 1 micron, for a field of view of around 100 microns, up to 5 microns for a 500 micron field of view. The lateral resolution of the image is determined by the observation objective, the one perpendicular to the excitation, and this typically has a numerical aperture of around 0.8 to provide sufficient working distance to the sample and a lateral resolution of around 400 nm. As an alternative to this cylindrical lens configuration one option is to produce the sheet by rapidly scanning a laser beam up and down as a single line such that in a single camera exposure a sheet is produced. This has the advantage of producing a more uniform illumination and using a modern s-CMOS camera a rolling shutter can be employed, synchronised to the illumination, such that only photons from the illuminated line are detected, helping to reduce background by rejecting scattered light. An even more sophisticated version entails using a spatial light modulator to impose extra spatial control on the beam to give extended depths of focus such as using a Bessel light beam.

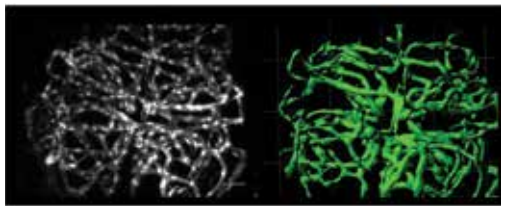

Figure 4: Live, anaesthetised 4dpf kdrl:GFP zebrafish larvae, marking endothelial cells, were mounted head-down and imaged dorsally through the fore-brain region. Axial slices of 1µm were taken across 200µm depth, and (a) a maximum intensity projection (fiji) or (b) segmentation followed by 3D rendering (Amira) were performed. Scale bar represents 50µm

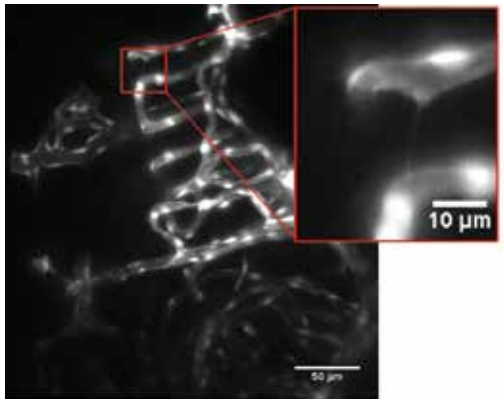

Figure 5: The same fish as in Figure 3 was imaged through the median plane of the gills. Axial slices of 1um were taken across 200µm depth, and a maximum intensity projection was performed. An endothelial cell projection involved in vascular development can be clearly seen between vessels (enlarged red box)

3D stacks of images are then acquired simply by moving the specimen along the detection axis through the excitation light sheet thus recording a single optical section in one exposure. This again illustrates one of the advantages for *in vivo* imaging, compared to conventional confocal imaging, that of only exciting the optical section you are imaging thus reducing the out of focus bleaching and minimising photo-toxicity in the sample. Most of the SPIM setups use water immersion illumination and detection objectives so the specimen is placed in a medium-filled chamber. This results in a high numerical aperture with sufficiently long working distances to enable the two objectives to be placed at right angles to each other.

Sample preparation and mounting

The development of SPIM has coincided with a significant increase in the use of Zebrafish as the biological model of choice and this synergistic growth has helped both fields develop rapidly. A major reason for the growth in the use of Zebrafish, beyond cost, ease of genetic manipulation, speed of growth and ethical considerations, is that somewhat surprisingly they have proved to be an excellent model for human disease and development. They are now accepted as an outstanding model of humans in such major organs as the brain, eyes, heart, kidney, liver and spinal cord. One ability currently being studied is that even as a mature adult Zebrafish have the ability to repair their heart muscle. That is, they can grow new operational muscle tissue when heart tissue is damaged within two weeks, while human hearts grow only scar tissue after the equivalent damage from a heart attack. The other crucial factor in relation to their use in SPIM is that they are basically transparent or can be made so through the use of chemicals to reduce melanocyte formation or genetic variations such as "Casper" which have no pigment. Other frequently used models in SPIM now include C elegans, Drosophila and Arabidopsis for root development.

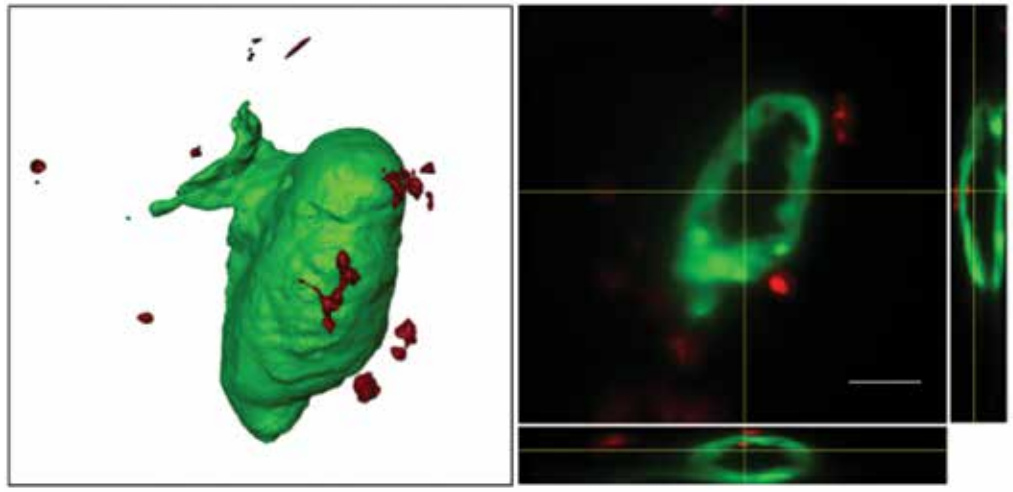

Figure 6: Live, anaesthetised 3dpf cmlc2:GFP;mpeg:mCh zebrafish larvae, marking cardiomyocyte cells in green and macrophages in red, were mounted head-down and the heart imaged using optically-gated heart synchronisation software, designed and implemented by Jonathan Taylor. Axial slices of 1µm were taken across 100µm depth, and (a) segmentation followed by 3D rendering (Amira) was performed and (b) orthogonal views taken (fiji). By effectively 'freezing' the motion of the live, beating heart, location of macrophages with respect to the cardiac tissue can clearly be resolved. Scale bar represents 50µm.

Due to the requirement of having two objectives mounted with a common focal volume at right angles to each other, conventional microscope slides are not normally suitable for sample mounting. In developmental biology the mounting method has to fulfil three important priorities. First, the way the specimen is prepared and mounted can't impede the development and growth conditions must be maintained as close to normal as possible, second, the mounting should, be mechanically stable and third, it should, as far as possible, not affect the quality of imaging.

The most common method of mounting in LSFM is embedding the specimen in low melting agarose or another gelling agent such as gelatine, gelrite, etc. The specimen is "mixed" with one of these agents and then carefully "pumped" into a cylindrical capillary that is in turn placed in front of an objective lens normally in a water bath (Figure 3). Refractive indices of the gelling agent and capillary must be close to that of water in order not introduce excessive light scattering or distortions. This method has some possible disadvantages such as a mounting potentially affecting specimen development due to compression forces especially with a relatively high concentration of gelling agent. An alternative approach is to place the sample in a plastic tube with the same refractive index as water and FEP tubing has proved to be a low cost solution to this challenge. The mounted sample is then normally placed into the SPIM imaging field vertically so it is suspended in space. This means that the illumination

and detection optics are maintained in the plane of the table. Work is also underway to develop high throughput systems of imaging in which the samples are in effect "flowed" past the imaging volume frequently using micro-fluidic systems.

Figures 4, 5 & 6 illustrate a range of 3D reconstructions of Zebrafish organs.

Recent SPIM Innovations

iSPIM

Inverted SPIM (iSPIM) was introduced in 2011 by Wu et al. and as the title indicates it provides a more traditional way of mounting the sample by placing on a horizontal coverslip while illumination and detection optical axis are aligned above at an angle of 45° to the coverslip plane. This approach has several advantages; one of which is that the sample is less likely to subside compared to mounting inside a vertical capillary as it is already in a resting state [6]. However, the optical system is more mechanically complicated as it has to extend vertically from the table holding the sample, though it does make the high throughput screening mentioned above a more viable option.

mSPIM

The idea behind multiview SPIM (mSPIM) is to achieve simultaneous multiple views of the sample to increase speed and image quality. This general approach can be divided by three common techniques: (i) dual-excitation SPIM (deSPIM), (ii) rotation SPIM (rmSPIM), and (iii) dual-view SPIM(dvSPIM) [6].

The objective of the deSPIM method is to illuminate the sample from two opposing sides, rather than the conventional one, but still using one detection objective. The aim being to reduce visible striping artefacts caused by shadows and scattering in large or opaque live tissues. By aligning the focal points of the illumination objectives one can achieve significant benefit either in field of view or in axial resolution. The obvious disadvantage is the requirement for the two counter propagating beams to be spatially overlapped with high precision.

The rmSPIM technique maintains the advantages of the deSPIM approach but offers more accurate reconstruction by trading off temporal, for improved spatial, resolution and better signal to noise. An rmSPIM system consists of one detection lens and one illumination lens, but introduces a rotating sample holder which offers up to four orthogonal views. Final volume data sets are obtained after deconvolution and generally have superior resolution to conventional SPIM and improved contrast and reduced striping artefacts, though the method loses in terms of temporal resolution capability as the sample needs to be rotated after each acquisition.

Finally, the dvSPIM modality is similar to a basic iSPIM setup except both objective lenses are used to direct the excitation beam and collect emitted fluorescent light from the sample simultaneously. Orthogonal views obtained from two objectives working together are then reconstructed in a similar manner to rmSPIM. This method is much faster than rmSPIM and offers higher axial resolution. However, finding objectives with high numerical apertures and sufficiently long working distances is a challenge.

SPIM has also been utilised with two-photon excitation (2P-SPIM). 2P-SPIM incorporates a femtosecond-pulsed laser and deSPIM bidirectional illumination. This technique improves both imaging depth and speed compared to regular SPIM [7]. The main disadvantage of this modality is the high cost of an ultrafast laser.

Improved Imaging at Depth using Adaptive optics

As with any imaging modality as one explores the sample more deeply this increasingly adds aberrations to the image and SPIM imaging is no different. However, there are two potential sources of aberrations leading to a loss of contrast and resolution. The excitation beam can be distorted on its way into the imaging volume. This is most normally seen through a loss of excitation due to absorption in the sample and is partly overcome using the line scanning method described earlier. There are also detection aberrations, as a result of the fluorescent light travelling through the rest of the sample and sample holder on its way from the excited fluorophore to the objective lens. Whilst the sample holder geometry is known, thus it is possible to model and hence deconvolve from the image, the sample contribution is not very predictable due to its shape, size, orientation etc. The two most significant aberrations in this case are astigmatism and spherical. These aberrations clearly have an increasing affect with deeper optical sectioning. Adaptive optics are an efficient method to correct aberrations and achieve final images of higher quality and this has now demonstrated significant advances in beam scanned microscopy and more recently SPIM [8,9].

Active optics can be segregated in two groups: (i) spatial-light modulators (SLM) and (ii) deformable mirrors (DM). A SLM is a device used for wavefront correction. In essence, it is a liquid-crystal display with the ability to change intensity and/or phase of the incoming beam by manipulating the refractive index of every pixel. There are two different sub-types of SLM which vary the way the image on the modulator is created and changed: electrically addressed SLMs and optically addressed SLM. Both have been used to alter the incoming light beam in a SPIM system. In general SLMs are slower than DMs however, their main limitation is in the wavelength range over which they operate efficiently and thus they are less suitable for use on the broad spectrum of light emitted by the flurophores. Their main advantage is that the phase can be much more accurately controlled than on a DM.

DMs are mirrors whose surface can be deformed to achieve correction of an aberration. Such mirrors are usually more preferable than SLM due to their high reflectivity, speed, achromaticity and relatively low cost. The complexity in their use is to control their shape to remove the sample induced aberrations. This is now most normally achieved using an image optimisation method in which various shapes are applied to the mirror and an image metric used to determine which shape, or combination of shapes, maximises the overall quality of the images. Whilst for many SPIM applications, where the organ is near the surface, such advanced optical methods are not required but as SPIM is increasingly used on older, and hence larger, samples then such correction becomes more important.

One of the most recent methods based on the beam control of the excitation beam is super-resolution Lattice Sheet SPIM. This approach improves upon the Bessel beam light sheet microscopy technique. It is intended to reduce the intensity of the outer lobes of the beam with destructive interference. In essence, a 2D lattice of Bessel beams is created and then destructive interference is induced by manipulating the spacing between the beams using a SLM [10]. This kind of microscope can be operated in two modes: dithered mode and super-resolution SIM mode. In the dithered mode only one image is captured at each Z position while the beam is scanned rapidly along the X axis. This mode can provide only diffraction-limited resolution. In SIM mode N images are captured at each Z position as lattice light sheet is stepped along the X axis in N equal parts of the lattice period. This method allows users to obtain reconstructed 3D images with resolution higher than diffraction limit in all axes.

Recent Biological Insight Enabled by SPIM

Refractive Index Measurements in the Developing Eye

Detailed below are two recent examples in which SPIM, or LSFM, has helped to provide an insight into a biological process, which is clearly the aim of all advanced biological instrumentation. The first example illustrates SPIM's advantage of having orthogonal illumination and detection to measure the development of the Zebrafish embryo eye lens refractive index (RI) over three days of growth. In these embryos the eye lens starts developing around 16 hours post fertilisation and

achieves visual response on the retina 4 days post fertilization (dpf) providing fish with ability to catch prey 5 dpf after exhaustion of its yolk supply. As the lens grows its local refractive index also changes due to expression of lens crystallins, The resulting refractive index is not uniform - becoming higher at the centre and decreasing towards the edges. This has been developed in order to reduce optical aberrations from this highly curved lens (around a 200 micron sphere) and is present in many animal lenses including our own. The uniform RI eye lens would focus peripheral rays at a shorter distance than para-axial ones. Such a gradient refractive index (GRIN) can be manipulated using gene editing technologies which may potentially lead to new optical products with significantly reduced optical and chromatic aberrations.

In our recent experiments we monitored eye growth by directing single lines of light into the lens and observing the bending of the ray using orthogonal observations [11]. The lens, and surrounding tissue, were labelled with fluorescent dye and thus the beam path could be observed and subsequently accurately measured and then fitted to a geometric ray trace to estimate the RI. As the radius of curvature of the lens increases during development so does RI. We then used two chemicals which are already known to alter crystallin expression within the lens and hence alter its local optical properties. Optimally the ratio of the effective focal length to the distance from the centre of the lens to the retina should equal one and indeed it moves in this direction as the lens ages and through the use of the drugs this value can be increased or decreased depending on local changes in the refractive index.

Controlled Cell Ablation using SPIM and KillerRed©

KillerRed© is a genetically encoded, light activated photo-toxic compound. Although it has previously been used to ablate cells in petri-dishes it has now been used successfully in a full animal model. The protein was linked to a renin promoter and thus only produced in cells which also produced renin. When these fish were subjected to light at around 561nm in theory only the renin producing cells should have been killed. However, using a conventional epi-illumination system 90% of the samples died. Using a blue light sheet in the SPIM system we excited GFP within the fish so the anatomy could be accurately located and then in the area of the kidney an additional lightsheet at 561 nm (yellow) was activated leading to an area of localised cell death with 100% survival of the samples. The accuracy of the ablation was further enhanced using light introduced through the imaging arm to produce a "dagger of light" using a Bessel beam which provided the ability to target individual cells within the live animal whilst simultaneously imaging the GFP expressing cells using the conventional SPIM configuration (12). This work opens up new possibilities to study aberrated development and biological repair processes.

Conclusion

SPIM is relatively new and very rapidly developing fluorescent microscopy method the main feature of which is illuminating the sample with a very thin sheet of light and detecting the emitted fluorescent light perpendicular to the direction of excitation. This approach offers minimal phototoxicity and photobleaching, allowing specimens to be intact and alive through the whole experiment, providing imaging advantages such as spatial and temporal resolution, optical sectioning and with modern cameras a large dynamic range. These particular qualities have made SPIM very attractive for use in modern biology, especially in developmental biology. SPIM offers a number of different techniques within the general approach each of which has its own imaging criteria trade-off and should be picked according to each particular experiment and its goals. Challenges still exist though, such as data storage and analysis which is a very important aspect due to very large stacks of data produced from a single experiment. Typical 24 hour imaging runs generate at least 2Tb of data for each sample. Rapid imaging variations in which neuronal calcium is imaged at high speed [13] produce files that are even larger and this is potentially the largest barrier to the greater use of SPIM [14]. It is also interesting to view the way Open SPIM has developed, allowing the highly powerful tool to be used without the need to pay for expensive dedicated microscopes and increased multidisciplinary intereactions between physicsts and life scientists. SPIM has already proven itself as an efficient and very promising new imaging technology. Its popularity will only increase as its obvious advantages of high-speed 3D imaging for extended periods of time are fully appreciated.

Acknowledgments

SPIM images courtesy of Dr Charlotte Buckley, Queen's Medical Research Institute, Edinburgh University.

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