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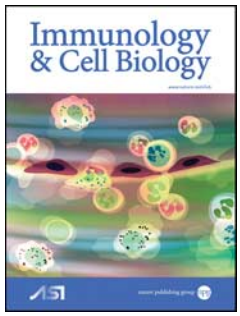

The reinvention of twentieth century microscopy for 3-dimensional imaging

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The reinvention of twentieth century microscopy for 3-dimensional imaging

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Introduction

Light microscopy has played a vital role in many scientific discoveries. It has, and continues to, transform our understanding of biology, providing major advances in the fields of embryonic development, cellular behavior, tissue and organ formation and many others. No other technique allows biology to be observed and studied quantitatively in both space and time over such a broad range, from the single molecule to whole organism. Importantly, microscopy allows us to move beyond the boundaries of what is possible from gene sequencing and biochemical assays, and accordingly, is essential for improving the prevention, diagnosis and treatment of disease.

In 2008 the Nobel Prize for chemistry was jointly awarded to Osamu Shimomura, Martin Chalfie and Roger Tsien in recognition of their discovery and development of green fluorescent protein. This pivotal discovery provided researchers the ability to directly visualise gene expression and the localisation of proteins in living organisms with enormous flexibility [1 2]. Less than a decade later, the 2014 Nobel Prize for chemistry was awarded to Eric Betzig, Stefan Hell and William Moerner for the development of super resolved fluorescence microscopy, further cementing microscopy as an essential tool for the life sciences.

The evolution of microscopy techniques is as dynamic as the processes it aims to visualize, and whilst the unrelenting pursuit of resolution has driven major technological advancement, it has typically been at the cost of removing the biology from its natural environment. Thus the Holy Grail for optical microscopy is to image with high spatio-temporal resolution in situ. To this end, methods enabling faster three-dimensional (3D) volumetric imaging of cells, tissues, organs or whole organisms will push the boundaries further and spearhead new discoveries.

A (very) brief history of confocal microscopy

In the early to mid-80's, a number of seminal papers were published, which would go on to form the basis of many advanced microscopy techniques used routinely today. A big improvement in the design of the fluorescence microscope at this time was the development of the confocal scanning laser microscope. In one of the earliest applications to the biomedical sciences, confocal microscopy was used to capture high level detail of nuclear stained chromatin[3]. This occurred with some delay after the first confocal had been patented in 1957 by Marvin Minsky [4]. Now ubiquitous, the confocal microscope described by Minsky had little utility in the life sciences until the development of adequate laser technology. Contributions from others, such as the introduction of photomultiplier tubes (PMTs) for low noise detection and galvanometer scanning mirrors [5 4] were instrumental in bringing confocal microscopy into mainstream use. Like many technologies, the confocal microscope evolved in parallel to the development of other related technologies; computers, detectors, optics, methods for spectral detection, and electronics, which were all required for the confocal microscope as we know it today to come to fruition. No other technology has been able to provide such high resolution, organ-level, 3-dimensional imaging; as demonstrated by studies on lineage tracing of mammary gland during development [6] or on immune cell interactions and regulation in the lymph nodes [7] and bone marrow [8].

Despite the enormous benefits of confocal microscopy to modern biomedical research, scanning point by point is slow and potentially damaging to live samples. Confocal microscopes equipped with resonant scanners attempt to ameliorate these issues, but often this is at the expense of sensitivity or image quality. Alternatively, spinning disk confocal microscopy, while fast and less phototoxic, lacks some of the optical sectioning capability of "conventional" confocal. Moving forward, low-throughput point scanning by confocal

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or multiphoton microscopy may gradually be replaced with some of the more modern technologies we are beginning to witness in the field.

The "Holy Grail"

Based on the challenges we face in biological imaging, the most ideal microscope we can imagine would enable ultrafast, three-dimensional, volumetric imaging of cells, tissues, organs or whole organisms with little to no phototoxicity[9]. It would accommodate environmental control for a wide range of samples and be suitable for intravital imaging. It would be an automated platform, capable of adapting in real-time to feedback derived from image data. A feedback loop would be combined with fast computing and deep learning to automatically identify a specific cell type, object, or event, and could be tailored to individual projects. Fast electronics would ensure the microscope could adapt to image data instantaneously. A combination of hardware (e.g. optics and beam path) and software would continuously adjust for spherical aberrations and could be trained to trigger specific protocols, such as switching from low to high magnification and executing an x,y,z,t scan. As a final stage in the process, experimental data would be automatically exported for analysis.

How close are we to such a system? Perhaps closer than we think.

Light-sheet based microscopy - A game changer for 3-dimensional microscopy?

Light-sheet or selective plane illumination microscopy (SPIM)

The last decade has provided a huge wave of innovation in microscopy platforms, and the rapid pace of development shows no sign of slowing. These developments have made a significant contribution to the advancement of science (as recognised by the 2014 Nobel Prize). In 2004, the team of Ernst Stelzer began to address some of the limitations of confocal microscopy in their publication of a new optical sectioning technique called Selective Plane Illumination Microscopy (SPIM) [10]. In confocal microscopy, the laser is focused to a point at the focal plane, but the light still penetrates areas of tissue above and below this region (see Table 1 for an overview of these methods). In SPIM, a thin sheet of light is created orthogonal to the detection axis, so that only areas of tissue within the focal plane are illuminated by the laser. This form of optical sectioning does not require a pinhole, so the sheet of light can be moved plane-by-plane through the sample, allowing volumes to be captured at very fast rates (up to 50 frames per second) and with a high signal-to-noise ratio (SNR). The unusual geometry of the objectives means that samples need to be mounted or suspended freely (as opposed to on a slide or coverslip), which permits multiple angles to be imaged sequentially if the sample is rotated (multi-view). This is ideal for specimens such as Zebrafish, *Drosophila* or other small organisms, which are relatively transparent; or for small specimens, such as spheroids, organoids or early stage mouse embryos [11]. The method also works well on cleared tissues or organs (e.g. kidney, lymph nodes, spleen, brain), anywhere from hundreds of microns up to millimeters in size (provided the refractive index is carefully matched). The axial resolution of light sheet microscopes can also exceed confocal when equivalent lower NA objectives are used (e.g. 10x/0.3), which makes them an attractive alternative for imaging large scale specimens [12]. Resolution can be further enhanced when multiple views are fused using a multi-view deconvolution algorithm, however without appropriate computing power, such processing of large datasets is extremely challenging [13].

Since its initial description, a number of different optical implementations have extended the functionality of the light sheet technology. In a beautiful example of what it means to "build your microscope around your biology", Tomer *et al*. demonstrated simultaneous multi-view imaging (SiMView SPIM) of a developing *Drosophila* embryo using a dual-camera and four objective adaptation [14]. In addition, two-photon excitation allowed them to reach greater depths and an automated real-time electronics control framework handled the massive amounts of recorded data. By building on the multi-view image processing techniques developed by Stelzer's group [13] and the high isotropic resolution achieved by the unique design of the dual-

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view plane illumination platform (diSPIM)[15], the Keller Lab developed IsoView SPIM[16]. The IsoView SPIM is a multi-colour, multi-view, deconvolved SPIM platform, which enables near isotropic resolution (as high as 0.42 mm in non-scattering media and around 1.1-1.7 microns in *Drosophila* embryo). In further developments, the same laboratory then developed the AutoPilot Framework; a potential *tour-de-force* in the world of 'smart' microscopes. This automated method for adaptive imaging improves spatial resolution and sensitivity, two to five fold, by continuously adjusting the light sheet to the optical properties of the biological specimen (e.g. refractive index or opacity) [17].

Movies of *Drosophila* during embryonic development or of whole-brain Ca^{2+} imaging with cellular resolution in Zebrafish, have provided unprecedented levels of detail and new opportunities to address questions in systems biology [18]. Light sheet microscopy is now the method of choice for 4-dimensional (3D + time), cellular imaging in smaller model organisms because it enables volumetric imaging with high spatiotemporal resolution and very low phototoxicity. What varies between light sheet systems is often the construction of the light sheet itself and the resulting impact this has on the system's versatility. Two outstanding variations to light sheet microscopy are detailed below, which address high-resolution subcellular imaging and intravital imaging in larger specimens, such as mice.

Lattice light sheet microscopy

Despite its obvious strength, earlier versions of light sheet microscopy were designed around larger specimens and were not easily adapted to cultured cells. Furthermore, these versions, like most laser-based microscopes, use a Gaussian-shaped laser beam, which has a diameter limited by diffraction. The trade-off is the thickness of the light sheet, which increases with the length of the beam as it propagates across the field of view (FOV). As the axial resolution is highly dependent on the radius of the beam, earlier implementations of this approach were unsuitable for high resolution imaging of cultured cells. In 2011, the Betzig lab adapted an alternative approach and used Bessel beam planar illumination instead [19]. In contrast to a Guassian-shaped beam, a Bessel beam is non-diffracting – ie it does not spread out as it propagates, this makes them attractive in the application of light sheet microscopy. In the first design of a Bessel beam light sheet microscope, the Betzig lab combined this method with structured illumination and/ or 2-photon excitation to give an isotropic resolution of 300 nm; thinner than would be achievable using a Guassian light sheet. In addition, Bessel beams proved to be less phototoxic, enabled faster acquisition speeds and improved SNR compared to confocal microscopy [19].

In subsequent implementations of the Bessel beam, the Betzig lab developed the highly innovative Lattice Light Sheet Microscope (LLSM). In this system, an array of scanned Bessel beams is used together with a spatial light modulator to create optical lattices [20]. The optical lattices are then dithered to create an ultrathin light sheet that can be scanned plane-by-plane through a cell or small specimen at very fast rates. The result is very high spatiotemporal resolution with very low levels of phototoxicity compared to spinning disk confocal microscopy, which is the gold standard for live cell imaging. The instrument can be operated in two different modes: either the diffraction-limited dithered mode (230 nm in *x* & 370 nm in *z*), which provides the highest speeds (up to 100 frames per second in multiple colours) or the super-resolution structured illumination microscopy (SIM) mode (150 *x* & 280 nm in *z*). Presently, the dithered mode has been the most commonly applied method because it enables multi-colour, volumetric imaging at very high speeds [20]. Neutrophils migrating through a 3D matrix [20], and T-cells dynamically interacting with a target cell during the formation of the immunological synapse [21] are two extraordinary examples showing the power of this microscopy technique.

The resolution, speed and non-invasive properties of the LLSM undeniably place it as the most exceptional method for live cell imaging currently available, yet it is not without limitations. Notably, the current design of the sample chamber does not allow for sterile conditions nor gas exchange, and restricts samples to only those that can be attached to a small coverslip. Undeterred, numerous labs around the world have built LLSMs, using detailed plans that have been generously distributed by the Betzig Lab at HHMI's Janelia Research Campus. Such wide and generous dissemination of the technology will undoubtedly facilitate ingenuity in the design of the hardware and applications, and should expedite developments.

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Swept, Confocally-Aligned Planar Excitation (SCAPE)

Both conventional light sheet and lattice light sheet microscopy are generating exciting biological observations in numerous fields over a wide range of resolutions and sample sizes. However, one of the key limitations of both technologies, is their departure from what would be considered "conventional" sample mounting procedures and geometries. Furthermore, neither technology is well adapted to intravital imaging of mice.

SCAPE is an emerging technology developed in 2015, which offers planar light sheet excitation through a conventional widefield/confocal microscopy orientation using only a single objective [22]. Using an oscillating polygonal mirror, light from a stationary incident beam forms an oblique light sheet at the sample with the emitted fluorescence collected via the same objective. The resultant fluorescence is de-scanned using a second facet of the polygonal mirror and is focussed to a stationary mounted camera. Due to minimal moving parts, (i.e. only the oscillating polygonal mirror is moved during acquisition), the technique is intrinsically fast. Full volumes can be acquired at a rate of up to 48 volumes per second, which is limited only by the camera frame rate. The resolution achieved is comparable to conventional light sheet microscopy, and large fields of view can be captured (600 x 1000 x 550 µm in x-y-z). This makes SCAPE an ideal method for whole body recordings of freely moving *Drosophila* larvae and for functional imaging in the brain of live mice [22]. The ability to monitor neuronal network activity in the brain of awake and freely moving mice with cellular resolution [23] or non-invasively across large-scale areas [24], has been a major goal for many years. SCAPE is therefore a nascent technology offering significant promise in delivering on three distinct fronts: spatial resolution, temporal resolution and field of view/penetration depth.

Data processing and analysis

With the increasing adoption of these technologies, there is also enormous expansion in demand on computational resources for quantitative microscopy. Inevitably, there are much greater requirements for network speed, disk space and processing capability owing to the size of image data (e.g. terabyte scale data). Directly integrating imaging equipment with scalable network storage, databases, and analysis tools, is expected to become essential in the near future. The Open Microscopy Environment (OME) project, and their image repository and database software (OMERO) is one of the leading players in this space. OME attempts to unify data analysis and handling by allowing an interface to over 140 proprietary image formats.

The ever-increasing need for image analysis processing capability has led to the routine use of hardware configurations that were once considered cutting edge. Researchers are increasingly moving to High-Performance Compute resources, either in house or cloud based, to provide the necessary computational power required to process the extremely large and complicated datasets being produced by new generation microscopes. Once again, the need for multi-disciplinary teams is apparent.

Concluding remarks

There are many questions in biology for which light microscopy is vital. When "seeing is believing", there is often a clarity of understanding achieved when dynamic processes or behaviour can be captured on camera. Furthermore, microscopy has an exhilarating ability to potentially expose novel or unique questions that were previously unimagined. How do stem cells and their progeny orchestrate *de novo* blood formation in the early embryo? What causes tumour cells to transition from a normal cellular control state and invade adjacent tissue? How do pathogens invade and overcome their host? What is the role of cellular positioning and interactions of the immune system during infection or disease? One of the prominent challenges is to map molecular, structural and functional processes over large tissue areas, to establish connectivity in the context of systems biology and to be able to zoom in and out of whole organs, without the traditional trade-offs in spatial and temporal resolution.

Excitingly, light sheet-based technologies are yet to reach maturity. Further innovation is expected to improve axial resolution and the depth of penetration, particularly in thick samples where this continues to

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be a prominent limitation. Adaptive optics provide a promising avenue for development, but the exact manner of their implementation into new or existing systems remains to be realized. At present, the evolution in this field of microscopy shows no signs of slowing. Swept along by this rapid pace, scientists are beginning to push for a more ‘open’ science framework, as innovative designs are increasingly shared amongst the research community. Indeed, the open-SPIM project is amongst the first of the "open access" systems attempting to demonstrate that an "open-source philosophy" can be as beneficial to hardware as it has been to the software development world.

As many scientists will inevitably desire the newest tools the instant they are published, waiting for commercialization is far from ideal. The rise of multi-disciplinary research teams involving biologists, physicists, and chemists is allowing for early adoption of such technology at a much faster rate than ever before. This will, in turn, drive future developments to the current systems- provided said developers embrace the "open-source philosophy".

Conflict of Interest

The authors declare no conflict of interest.

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Modality - Year introduced[#]	**Resolution x,y & z**	**Illumination & detection**	**Technological advance** ***Limitations**	**Application examples** *- recent & cutting edge*
Confocal laser scanning microscopy (CLSM) - 1985 [3] (first proposed by Marvin Minsky in 1957)	250 nm xy 500 nm z	- Single- focused beam of light, sequentially scanned in a raster pattern across a sample. – Pinhole in front of detector. - PMT, GaAsP, HyD and APD detectors	The pinhole rejects out-of-focus light and allows optical sectioning for 3D reconstruction. Allows atleast 4 channels to be simultaneously recorded. ***Slow scan speed, low SNR & limited penetration depths**	Well established method for a wide range of both live and fixed specimens.
Spinning disk confocal microscopy - 1986 ('flying spot technique first described in 1967) [25]	250 nm xy 500 nm z	Multiple point sources of light generated by an array of microlenses and pinholes on a very fast rotating disk. - CCD, EMCCD - sCMOS camera	Multi-point scanning technique for faster acquisition. Allows 2 channels to be simultaneously recorded. **Limited penetration depth, pinhole crosstalk will reduce z resolution in thicker specimens. Higher powered lasers may be needed.*	Well established method for live cell and intravital imaging because it enables faster frame rates and has lower levels of phototoxicity.
Multi-photon microscopy - 1990 [26]	290 nm xy 840 nm z	- Single- point source and femptosecond pulsed near infrared laser. - No pinhole - Non-descranned detectors, PMTsGaAsP, HyD	Pulsed near-infrared laser. No pinhole required as excitation limited to a small focal volume. Low background from scattered excitation light. **Slow scan speed, low SNR*	Well established technique for intravital microscopy anddeep tissue imaging.
Light sheet microscopy, also called SPIM - 2004 [10](Ultramicroscope first described in 1902)	1 μm xy 5 μm z based on 10x/0.3 [12] *Fast scan speed* Isotropic resolution of 1-1.7 μm with IsoView SPIM [16]	- Light sheet created using a Gaussian beam - Orthogonal – two objectives - sCMOS camera	Thin sheet of light, which is orthogonal to the detection plane. Scans plane by plane through the specimen. **Axial resolution is dependent on light sheet thickness. A larger FOV will have a thicker light sheet and vice versa. Limited penetration depth.*	Live Zebrafish – Embryonic development, whole brain neuron level Ca^{2+} imaging [18] Live Drosophila – Embryonic development [17 13 14] Live mouse embryonic development [27] Spheroids [28] Cleared organs/ tissues [29].
Lattice Light Sheet Microscopy - 2014 [20]	230 nm xy 370 nm z *(Dithered mode)* *Fast scan speed*	- Light sheet created using a patterned bessel beam - Orthogonal – two objectives - sCMOS camera	Ultrathin sheet of light, which is orthogonal to the detection plane. Scans plane by plane through the specimen. **Diffraction-limited, limited depth (< 50 μm), small FOV (50 μm^2), unusual geometry limits flexibility, difficulty to keep sterile during long-term experiments*	Single-molecule tracking Cell division Mitochondrial dynamics *C. elegans* (development) Immune cell interactions [21] Neutrophil motility [20]
Swept, Confocally-Aligned Planar Excitation (SCAPE) - 2015 [22]	2.9 μm x* 2.4 μm z 4.5 μm y *Depth of 100 μm in scattered media[22] *Very fast scan speed*	Angled swept light sheet in a single objective	Single objective and oblique sheet of light, very fast volumetric scanning. **Low axial resolution, diffraction-limited. Unavailable commercially.*	Beating heart in zebrafish embryos. Blood flow in mouse vasculature

Table 1. Comparison of laser scanning optical sectioning techniques

FOV, Field of view. [#]Approximate year when this technology was first demonstrated in cell biology.

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