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SPOTLIGHT

Light sheet imaging comes of age

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Light-sheet fluorescence microscopy methods that minimize photodamage are increasingly penetrant in biomedical research and currently comprise three distinct platforms. In this issue, Fadero et al. (2018. *J. Cell Biol.* https://doi.org/10.1083/jcb.201710087) describe a fourth approach termed "LITE microscopy," which is useful for extended imaging of specimens from cells to organisms.

Light sheet microscopy has become a catchall phrase for multiple different microscopy approaches all of which rely on projecting a defined sheet of illumination orthogonal to the imaging objective. While there is harmony in the fundamental technologic concept, implementations are sufficiently diverse that there is significant confusion over the advantages and disadvantages of each method. In common with the confocal microscope, its incredibly impactful predecessor, light-sheet fluorescence microscopy (LSFM), has a complex early history. Implementation of either tool was limited by available supporting technologies; over time each microscopy has reemerged as an essential and powerful investigative tool. However, unlike confocal microscopy, which is essentially a single light rejection platform, light-sheet methods have evolved acutely into three parallel and clearly different technologies, each with fundamentally different applications. Here we clarify and define the application of each method and contextualize the importance of the new approach, which is a relatively inexpensive, minimally photoxic, and high-resolution method presented by Paul Maddox's group in this issue (Fadero et al.).

The confocal microscope can be traced back to either Paul Nipgow's invention of the "electronic telescope" in 1840, which sent pictures across the ether using radio waves and signals generated using multi-hole disc encoders (Maul, 2015), or Marvin Minsky's 1957 patent to build a stage scanning confocal microscope (Minsky, 1988). Similarly, the light-sheet microscope has origins that are over 100 yr old and are based on the "ultramicroscope" (Siedentopf and Zsigmondy, 1903). However, both technologies lay fallow until the development of fluorescence microscopy, the advent of lasers, and the ability to digitally encode and rebuild images using computers. The confocal microscope found its first truly successful commercial interpretation in the 1987 BioRad MRC 500, based on Brad Amos and John White's design and which used basic photomultiplier detectors, and the original IBM PC. On the other hand, the major limitations in the implementation of light-sheet methods have been the generally complex reconstruction/deconvolution essential to the approach (though uniquely not the case with the Maddox design) and the essential integration of modern, high speed cameras. Collectively these technologies are only now capable of collecting, delivering, and interpreting the massive data streams that the approach delivers.

The essential and unifying feature of all LSFM approaches is the delivery of a homogeneous sheet of spatially constrained intense light that selectively stimulates fluorophores within that volume. The emissions can then be collected by an objective focused on, though orthogonal to, the plane of propagation of the light sheet. To generate a 3D image the sample generally is moved through the light sheet using a piezo actuator and images are collected in series. Unlike confocal microscopy, which relies on a pinhole for light rejection, with light sheet imaging the sample is only illuminated in a single plane and hence photobleaching/phototoxicity are minimized, allowing either long term and/or very deep imaging with minimal detriment to the specimen or loss of signal (Adams et al., 2015).

The concept of light sheet sounds wonderfully simple but generating a "homogeneous sheet of spatially constrained light" is not trivial. The thickness of the sheet in the Z axis generally depends on the NA of the illuminating objective; however, propagating and maintaining the sheet in the XY axis is more complex. In essence, the distance the sheet can propagate is proportional to thickness. The thicker the sheet, the further in XY it will be maintained, making it possible to form a relatively thick (multi-micrometer) sheet that propagates for millimeters, potentially suitable for "macro" imaging, or thin sheets that propagate multi-micrometers. To generate truly thin sheets as found in the lattice light sheet approach, optical tricks that rely on light interference as well as a very high NA objective must be used. Because of these constraints the field becomes immediately divided by a triage point: Do you want to image large specimens (mouse brains) at low resolution or small specimens at high resolution?

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There is a second physical variable that must be considered when generating the light sheet. For light to propagate continuously without refraction, changes in refractive index (RI) through the sample must be minimal. When light passes from one RI medium to another, say from air (RI = 1) to water (RI = 1.33), it bends. Thus, any changes in RI will negatively affect the sheet, particularly as biological samples, while principally water, may have continuously variable RIs between 1.33 and 1.36. In addition, the light may be absorbed or scattered as it moves through the sample, further confounding the approach. Fortunately, for the large scale, lower resolution methods, which are largely used for anatomical studies of whole tissues such as mouse brain, the problem is solved using tissue clearing approaches that generate an optically homogeneous and transparent sample. Conversely, this is very much a fixed tissue approach, and although the light may become less scattered as it moves through the sample, there is still absorption (otherwise there would be no fluorescence) and some scattering. As such, it is necessary to deploy multiple axially coincident sheets entering the sample from multiple angles around the periphery, or to rotate the sample and integrate several images at each image plane to generate a homogeneous image.

Multiple sheets and/or sample rotation are at the heart of the home brew OpenSPIM approaches (http://openspim.org/Welcome_to_the_OpenSPIM_Wiki), which are truly simple and relatively inexpensive to build, or the more elaborate commercial macro platforms (LaVision; Bruker). The method can be used with living samples (2–3-d zebrafish embryos are perhaps the upper size limit), otherwise the tissue must be fixed and cleared. In addition, getting the sample into the imaging system may be somewhat complex (again to minimize RI changes). However, when used with the recognition that the resolution is practically limited, these methods continue to produce exceptional and penetrant science principally in developmental biology and anatomy, where we need to understand where things are and where they are going at a cellular or system level.

To move to the cellular/subcellular domain, a second technology (DiSPIM) developed by Hari Shroff's group (Kumar et al., 2014) cleverly uses two identical light paths orthogonal to each other to generate semi-synchronous image pairs. The satisfying part of the design is best understood by recognizing that although resolution in XY is limited by the NA of the collection optic, resolution in Z varies with $1/NA^2$. Lower NA lenses typically used in light sheet systems may have reasonable XY resolution but inevitably have relatively poor (micrometer to multi-micrometer) Z resolution. Because the Shroff design collects two sequential images orthogonal to each other, there is no Z axis image spread as computer post-processing allows integration of the two data such that the XY profile from one axis replaces the Z profile from the other image. By using very fast cameras and Z axis control, the device can collect very high-quality datasets; however, alignment is nontrivial. Importantly, this technology works within a spatially constrained volume and is designed specifically for living system imaging such as *Caenorhabditis elegans* or *Drosophila melanogaster*. Further, this approach relies on significant post-processing to generate data with high resolution. This solution can be home built but it is somewhat impractical to do so. It is commercially available from Applied Scientific Instrumentation.

However, as cell biologists, we need diffraction-limited resolution (a few hundred nanometers) or better to probe the inner workings of cells with minimal perturbation to physiology. Although the confocal microscope has proven absolutely invaluable when studying fixed materials, it is in essence a light rejection technique—the whole sample is illuminated and out of focus light is rejected by a pinhole conjugate to the focal plane, which leads to significant photobleaching and photoxicity. Further, the traditional dual galvanometer design is too slow to collect images from fast moving samples. The speed issue is partially solved using either resonant point scanners or multi-pinhole confocals coupled to sensitive cameras. However, the inefficiencies of pinhole collection and full field illumination cannot be avoided and although useful data can be collected the biology of the sample is almost always perturbed to some degree. Also, as we move toward using gene-editing methods rather than transfection approaches, appropriate levels of signal from fluorescent proteins may render the inefficiencies of the confocal confounding for truly low signal imaging. Multiphoton methods do avoid out of plane illumination but are highly phototoxic in the plane of illumination and prohibitively expensive to use if multiple lines (lasers) are needed concurrently. What is needed is a light sheet approach that offers an extremely thin light sheet focused over a limited lateral distance (50–100 µm) such that a diffraction limited image from a single optical plane within any cell type can be collected. The lattice light sheet approach developed by Planchon et al. (2011) uses a spatial light modulator to generate a defined Bessel beam profile that can be swept across a sample and imaged orthogonally. It is a truly elegant solution, and all the information needed to build the device is freely available. However, whereas the collection optics are extremely simple (no more complex than a basic fluorescence microscope), generating the Bessel beam is extremely complex to implement. The first commercial system has been sold by Intelligent Imaging Innovations, licensed from Carl Zeiss. The speed, sensitivity, and lack of phototoxicity of the device are extraordinary, but its cost is about twice that of a confocal microscope and it is certainly a complex device to align and use.

The "LITE" sheet approach developed by Fadero et al. (2018) team is designed as a reasonably priced, readily accessible high-resolution light sheet system. It is simple; the cleverness is the use of a barrel lens and an interference grating to generate a light sheet, which is delivered through air to the sample at an angle. The change in RI as the sheet enters the specimen chamber bends the sheet toward the imaging plane, though this is dependent on chamber design. It is high resolution, as lateral and axial resolution is equivalent to the physical constraints of the imaging optic, which can be a high NA oil lens. It is inexpensive; essentially if you have a functioning transmission internal reflection fluorescence or spinning disk confocal microscope with a single mode laser source, decent camera, and functional image collection software, adding the LITE sheet imaging is an approximately $50,000 add-on (available from Cairn or Mizar Imaging) or can be home built for much less. To put this in context, the LaVision and DiSpim systems cost between $200,000 and $400,000 depending on feature sets and the lattice light sheet approach costs about $700,000. Image collection with LITE is simple and

does not demand the complex image reconstruction software common to all the other approaches. Importantly, as with the approaches described above, the use of a light sheet dramatically reduces photobleaching/phototoxicity. This was demonstrated quite clearly in Fadero et al. (2018) by comparing epifluorescence quenching versus LITE sheet in early stage *C. elegans* embryos. The signal to noise ratio of LITE imaging remained higher than embryos imaged with epifluorescence and the rate of bleaching was much lower over a 9,000-frame series (Fadero et al., 2018). However, LITE sheet does not pretend to match the axial resolution of the lattice light sheet solution; and perhaps the most undeveloped and complex component of the design is the imaging chamber that needs to have an optically correct (#1.5 coverslip) side wall for the light sheet to pass through. Importantly though, this approach is a distinct light sheet method and does provide almost any cell biology laboratory the opportunity of integrating light sheet imaging into their technology repertoire.

In closing, although LITE sheet microscopy fills out the technology platform nicely, it is important to be cognizant of a unifying aspect of all the approaches. All are very fast data generators, so whichever platforms you implement, you must be ready to store, transport, reconstruct, and segment truly massive (terabyte and up) datasets. Ultimately, it is the computing technology rather than the optical platform that may the current barrier to integration of these imaging technologies into a contemporary research laboratory.

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