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Imaging in focus

Light sheet microscopes: Novel imaging toolbox for visualizing life's processes

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ABSTRACT

Capturing dynamic processes in live samples is a nontrivial task in biological imaging. Although fluorescence provides high specificity and contrast compared to other light microscopy techniques, the photophysical principles of this method can have a harmful effect on the sample. Current advances in light sheet microscopy have created a novel imaging toolbox that allows for rapid acquisition of high-resolution fluorescent images with minimal perturbation of the processes of interest. Each unique design has its own advantages and limitations. In this review, we describe several cutting edge light sheet microscopes and their optimal applications.

1. Introduction

The resolution limit of a microscope dictates the minimum distance of separation between two point-emitting objects so that their airy discs are resolvable. However, it is important to note that this resolution assumes near-perfection of both the optics in the imaging system and the point emitter itself. Loss of contrast (also called signal-to-background, SNB, or signal-to-noise, SNR, ratios) will also significantly reduce the achievable limit of detection. The lower the SNR, the lower the contrast would be. The contribution of SNR to image quality can be under-appreciated; poor SNR can have a similar deleterious effect on object detection as the optical resolution of the system itself. In fluorescence microscopy, the focus of the detection objective is placed at the desired plane in the sample. The thickness of this focal plane (called depth of focus) varies depending on the numerical aperture (NA) and magnification of the detection objective; it can range from ~55 μm at 0.1 NA and 4× magnification to 0.19 μm at 0.95 NA and 100 x magnification. Photons originating from within this depth of focus will be collected and focused by the detection optics onto the camera. Photons originating outside of the depth of focus can still be collected by the detection optics but not focused at the correct plane corresponding to the camera chip, resulting in blurry images. A major pathway to improving SNR (and thereby image quality) is by reducing out-of-focus fluorescence.

2. Reaching for better SNR

In widefield fluorescence microscopy, the excitation pattern consists a relatively large field, significantly thicker than the depth of focus, in a cone shape both above and below the focal plane. This large illumination field unnecessarily excites fluorophores outside of the objective's depth of focus and causes premature photobleaching, significantly reducing SNR.

A mechanical solution for improving SNR was patented in 1957: confocal microscopy (Minsky, 1957). Confocal microscopes seek to eliminate the out of focus by placing a pinhole in an optically conjugate plane to the focal plane, thereby blocking photons originating outside of the focal plane of the objective. This method produces an "optical section" of better contrast and higher SNR.

Total internal reflection fluorescence (TIRF) microscopy uses optical principles to create optical sections. By taking advantage of the evanescent wave that penetrates into the aqueous medium following total internal reflection of the incident light at the glass coverslip, TIRF microscopy illuminates a few hundred nanometers from the interface, creating a thin optical section. TIRF microscopy generates data with superior SNR since the illuminated region is the same size or smaller than the depth of focus of the objective.

Although both confocal and TIRF microscopy improve SNR through effective methods, neither technique is without its draw-

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backs. Despite there being several "flavors" of confocal microscopy (e.g. point-scan, spinning disc, line-scan) it can be harsh on the sample and cause photobleaching. TIRF imaging can be performed at high speed but is limited to the region next to the coverslip-media interface. The search for a microscope capable of delivering high SNR and penetration depth for increased spatiotemporal resolution for biological applications continues.

3. The dawn of the light sheet microscope

Richard Zsigmondy and Henry Siedentopf invented the 'Ultramicroscope' in 1902 for the purpose of determining the size of colloidal particles in gold ruby glass using light scattering (Siedentopf and Zsigmondy, 1903). The unique change they made to the typical microscope was to separate the illumination and detection light paths by 90°. Incoming sunlight was reflected with a mirror oriented at 45° to the horizontal, which directed the light in a plane orthogonal to the detection optics. The orthogonal incident light caused scattering in their colloidal samples, which they could observe with their detection optic (i.e. looking down on the scattering). In 1993 Voie and colleagues developed the first modern light sheet system. The key improvement by Voie was to use a cylindrical lens to focus a collimated illumination laser source into a plane; this plane was significantly thinner than was previously achieved for light sheet microscopy (Voie et al., 1993). Voie was able to image nearly the entirety of a guinea pig cochlea with very high contrast due to the use of fluorescent dye and the 20 μm thick light sheet. Voie's work and improvement to the light sheet microscope served as a proof of principle that this technique could be suitable for biological imaging.

In 2004, Ernst Stelzer's group published work on the selective plane illumination microscope (SPIM) (Huisken, 2004). Their instrument also relied on a cylindrical lens. The authors demonstrated the benefit of light sheet imaging on a sample more relevant to the developmental biology community: a *Drosophila melanogaster* embryo. This work exemplified several benefits of light sheet imaging (e.g. high SNR, low photobleaching) but also showed that light sheet imaging could be used to capture events in a large sample over a long period of time (17 h in this work).

In the following years, incremental improvements were made to light sheet imaging, like deconvolution algorithms used during data processing (Lucy, 1974; Richardson and Richardson, 1972). However it was clear that light sheet imaging had shortcomings for developmental biology applications. A major drawback inherent in the technique is the thickness of the light sheet in Z (optical axis of the detection objective). Cylindrical lenses are unable to focus the sheet thinner than a few microns, which renders small intracellular details unresolvable. The other effect of using a cylindrical lens is highly varied illumination across the light sheet plane, particularly deep (>10 um) in a sample. An aperture is also necessary in the light path to center the laser on the cylindrical lens; this wastes light and essentially reduces the efficiency of the microscope (i.e. less laser light at the sample), which in turn requires longer exposure times to have acceptable SNR. Finally, reliance on a cylindrical lens may preclude the use of certain advanced optical components, such as galvo scanning mirrors, that could enable more sophisticated scanning/imaging modalities. The next generation of light sheet was dubbed the digital scanned laser light fluorescence microscope (DSLM) (Keller et al., 2008). To address many of the weaknesses of the cylindrical lens, DSLM, replaced it with a low NA objective lens, which could focus the light into a beam with a thin (~1 μm) diameter. This improved optical sectioning by scanning the focused beam with high-speed galvo mirrors, creating a virtual light sheet. This imaging modality still contained many benefits of conventional light sheet imaging (e.g. low photobleaching, high SNR) and significantly improved optical sectioning, and thereby Z spatial resolution. The virtual light sheet allowed for better control of laser characteristics, for example peak power, sheet thickness, and scan size, by varying the NA of the illumination objective. Finally, focusing the excitation light with an objective lens is a more energy efficient design for illumination. The improved illumination efficiency thus allows for reduced exposure time and reduced time per volume collected.

Shortly after the publication of DSLM, many other "flavors" of light sheet microscopy were published: objective coupled planar illumination (OCPI), highly inclined and laminated optical sheet (HILO) and oblique plane microscopy (OPM) (Huisken and Stainier, 2009). Each flavor was designed to address a particular shortcoming encountered in various biological applications. For example, when imaging embryos, consistent illumination and penetration of the light through the entire imaging field was challenging. Often, light sheet images would contain streaks or shadows due to optical aberrations. To address this issue, a variation of SPIM (mSPIM) was developed which utilized two excitation objectives that generate virtual light sheets separated by 180° (Huisken and Stainier, 2007). With computational improvements, this allows for better signal across the sample. Use of a virtual light sheet not only allowed for nuclear tracking during embryogenesis, but was of sufficient speed to perform whole-brain functional imaging in a zebrafish brain (Ahrens et al., 2013; Tomer et al., 2012).

The variations of light sheet instrumentation all sought to address the optimal balance of illumination light inhomogeneity, imaging depth, and spatial resolution. One methodology focused on increasing the number of "views" to cumulatively improve spatial resolution without sacrificing acquisition time via multiple angles on the sample. Multi-view light sheet imaging was started with Huisken's 2007 work (Huisken and Stainier, 2007; Schmid et al., 2013) and further expanded upon by Wu and colleagues for small, transparent samples (Wu et al., 2013, 2011) and by Keller and colleagues for larger specimens (Ahrens et al., 2013; Chhetri et al., 2015). As multiple light sheets are oriented so that the lowest resolution axis is orthogonal to each other for each sheet, when the images from the multiple views are combined computationally you can achieve isotropic resolution (Fig. 1A,B). Most recently this was done using four objective lenses that can both excite and detect emitted photons from the sample (Chhetri et al., 2015). In this work the authors were able to image large specimen volumes, up to 800 μm × 800 μm × 800 μm, with sub-second time resolution and 1.1 μm to 2.5 μm isotropic spatial resolution. Although the IsoView instrument significantly improved spatiotemporal resolution on large samples, it was not suitable for revealing intracellular details. An optical limitation of Gaussian light sheets necessitated a fundamental change in how the excitation light sheet is created and shaped.

4. Bessel beam-based light sheet microscopy

For nearly all fluorescent imaging technologies, excitation light follows a Gaussian power distribution. The longitudinal extent of the peak intensity in a Gaussian beam is directly tied to the intensity in its side lobes; this means there is an inherent trade-off with Gaussian light sheets in how long the light sheet is compared to how thin it can be. Increasing the beam power to appropriately illuminate the length of the sample can make the width of the light sheet significantly thicker than the focal plane of a high NA objective, thereby reducing Z spatial resolution and increasing out of focus fluorescence (i.e. background signal). To address this issue, a novel approach is needed for generating the excitation light. An unexpected solution came from the development of optical tweezers, through the use of a specialized lens that could generate a non-