Light sheet fluorescence microscopy: Advancing biological discovery with more dimensions, higher speed, and lower phototoxicity

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TO SEE THE WORLD IN A GRAIN OF SAND

Fluorescence microscopy has proved to be essential for seeing life in a grain of a cell. Classic epifluorescence microscopes, such as wide-field microscopes and laser scanning confocal microscopes, have been widely used for biology research over the past few decades. The goal of fluorescence microscopy is to observe the structure and function of cellular events across time and three-dimensional (3D) space with spatiotemporal resolution as high as possible. However, conventional epifluorescence microscopes illuminate the entire 3D volume of the sample while detecting signals only from the 2D focal plane, thereby leading to low photon utilization and high phototoxicity issues that notably prevent high-resolution imaging of live biological specimens (Figure 1A). It becomes increasingly noteworthy that the massively emerging biomedical applications are posing significant challenges to current fluorescence microscopy techniques.

In recent years, a famous paradigm shift for the optical microscopy field has been made in the form of light sheet fluorescence microscopy (LSFM). LSFM can generate a thin laser sheet by introducing an additional illumination path to selectively illuminate the specimens in the vicinity of the focal plane and then collect the fluorescence emission of the illuminated plane using an orthogonally placed detection path, as shown in Figure 1B. Through the selective optical sectioning of biological samples, LSFM eliminates the out-of-focus excitation, which is apparent in epifluorescence microscopes, thus yielding high-contrast images with notably reduced phototoxicity, especially when the fluorescent samples are thick or densely labeled. Meanwhile, by consecutively imaging the sample plane by plane with a z-scan, LSFM can rapidly obtain a 3D volumetric image in the subsecond timescale. Therefore, it reduces the illumination dose by over three orders of magnitude and achieves one to two orders of magnitude higher temporal resolution at the same time. LSFM enables both the detection of fluorescent signals from deep regions of large-scale samples with high efficiency and multicolor long-term imaging of live specimens at high speed. Diverse advanced LSFM modalities have been developed in the past decades to allow rapid, mild imaging of various types of biological specimens, such as our double-ring interference light sheet microscopy for imaging live cells with superior axial resolution.[1] Being selected as Method of the Year 2014 at *Nature Methods* demonstrates LSFM's superior performance and high efficiency.

However, LSFM still has its limitations, especially when facing the challenge from geometry. Most conventional LSFM systems contain two orthogonally placed objectives, one for generating the laser sheet illumination and the other for detecting the fluorescence signals. The requirement for the precise positioning of the two objectives greatly limits the types of specimens that can be

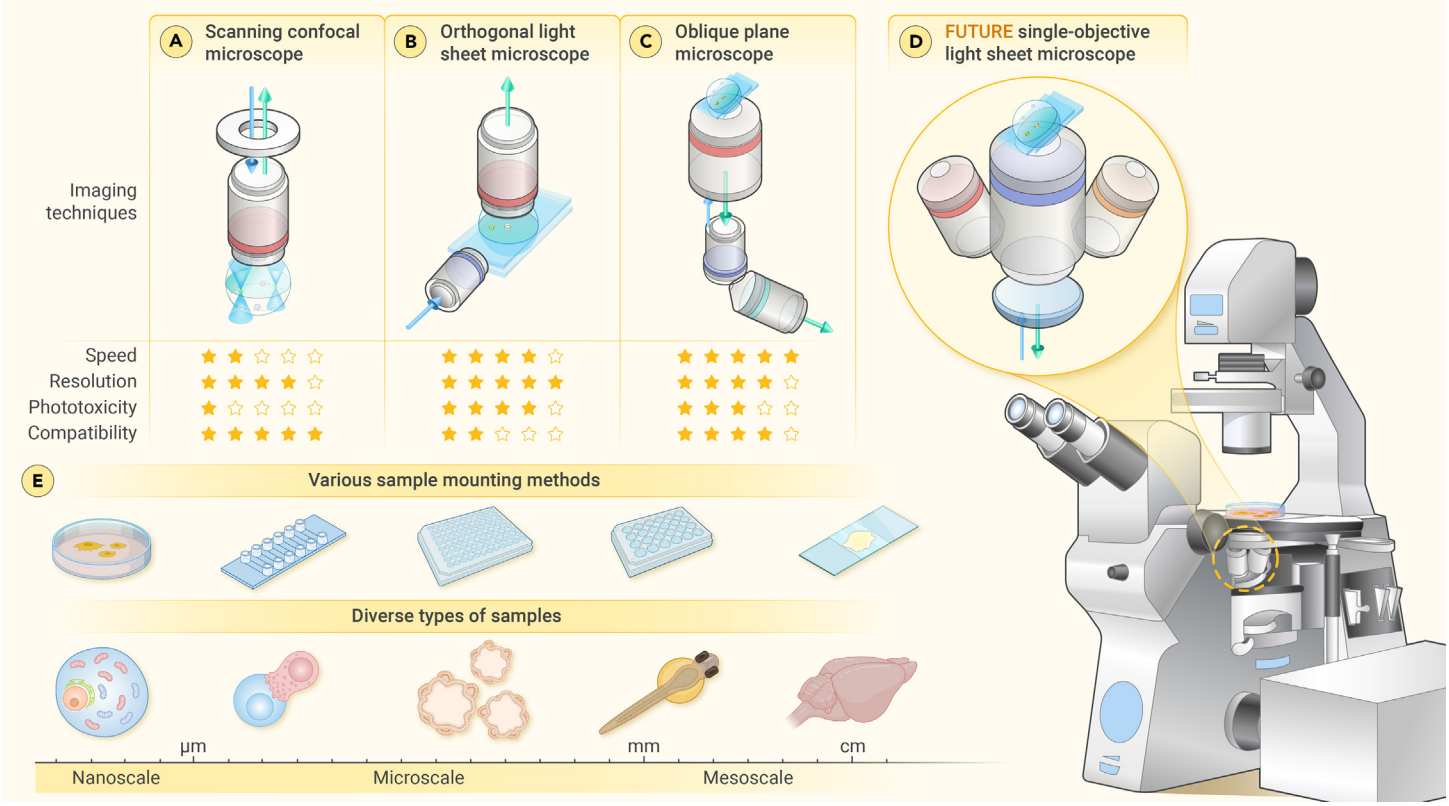

Figure 1. Designs and comparative performances of scanning confocal and different light sheet fluorescence microscopes (A–C) Schematic diagrams illustrating the principle of scanning confocal fluorescence microscope, orthogonally placed light sheet fluorescence microscope and oblique plane microscope using the same objective for fluorescence illumination and detection. Their imaging performances in terms of speed, resolution, phototoxicity, and compatibility are qualitatively compared via star rating. (D) Next-generation single-objective light sheet fluorescence microscope should be capable of efficient, high-resolution, and intelligent 3D imaging of diverse biological samples. (E) With a user-friendly open-top design and readily switchable imaging magnification, it should be compatible with various mounting methods and able to handle samples ranging from single cells at nanometer resolution to whole-mount tissues with centimeter size.

mounted onto the systems. As a result, many practical applications are further impeded by specific sample mounting methods or the inherent structures of biological organisms, which prevents imaging from two orthogonal directions. Furthermore, the coordination of two objectives makes further speed improvement difficult. This challenge has spurred the development of new instrumental designs to overcome the geometric limitations including LSFM, which uses the same objective for both signal excitation and collection. This so-termed single-objective LSFM technique was first reported by C. Dunsby with the introduction of oblique plane microscopy (OPM) in 2008.[2] It bypasses the restrictions from orthogonal arrangement with using off-axis remote focusing to enable light sheet excitation and fluorescence signal collection by the same objective, as illustrated in Figure 1C. The single-objective modality not only facilitates the imaging of diverse samples but also allows easy implementation of high-speed light sheet sweeping. In 2015, Elizabeth M.C. Hillman et al. proposed swept confocally aligned planar excitation (SCAPE) microscopy, an upright-mode OPM, demonstrating rapid, gentle, and high-resolution 3D live imaging on spontaneous neuronal firing in the intact brain of awake behaving mice and freely moving transgenic *Drosophila* larvae.[3] Despite being user friendly, the need for remote focusing with the oblique setup makes it difficult for OPMs to collect large-angle fluorescence, leading to a loss of numerical aperture (NA). As a result, OPMs suffer from suboptimal spatial resolution and fluorescence utilization, which are important to subcellular imaging and single-molecule detection. The more recent developments of OPMs are propelling single-objective LSFM techniques toward higher performance and broader applicability. For example, DaXi utilized a high-NA glass-tipped remote focusing objective to collect signals from large angles, achieving nearly lossless resolution in high-speed 3D imaging of challenging specimens mounted with standard methods.[4]

While we believe that these OPM-type single-objective LSFMs indeed advance biomedical discovery, several drawbacks still existed in current OPM implementations that prevent its more widespread applications. Due to the requirement for employing remote focusing, OPM needs to work at a high-NA status to ensure sufficient fluorescence collection. However, high NA often leads to a very small field of view, making it relatively hard for OPM to work with the histological/pathological imaging of large specimens. Meanwhile, directly working at low to middle magnifications is even more challenging to OPM, owing to the drastically reduced detection efficiency of the remote focusing under a small NA, making sufficient NA capability unattainable. It should also be noted that while OPM is convenient at the front end by using the same objective for both laser sheet generation and signal detection, a minimum of four additional lenses is required at the detection path to realize remote correction for aberrations arising from the oblique plane. Given the fact that this nominal single-objective mode is even more complicated than those orthogonal LSFMs, it often leads to low fluorescence utilization at the back end of detection. These limitations actually impose a glass ceiling for OPM in terms of versatility, performance, and applicability.

We envision that the circumvention of oblique plane correction for single-objective LSFM is crucial for further improving the resolution and efficiency of fluorescence detection. Such a new paradigm shift will be expected to overcome the challenges associated with current OPM working at low or middle magnifications and allow for the convenient switch of detection objectives, just like what we can do in commercial epifluorescence microscopes. In an ideal case, a single-objective LSFM should be as convenient as a confocal microscope, allowing switchable detection at variable magnifications for imaging samples of various types and sizes on the same system (Figure 1D). Meanwhile, it should be able to retain much higher speed and lower phototoxicity than confocal microscopes. Moreover, it is anticipated to enable integration with super-resolution methods such as structured illumination microscopy (SIM) to break the diffraction limit.[5] Furthermore, a hyperspectral imaging strategy specifically tailored for single-objective LSFM would be valuable to expand the spectral dimension from a maximum of four colors to over ten colors, potentially benefiting the desirable multiplex imaging of pathological tissues, mapping of embryo development, or study of the intracellular interactions. These creative instrument designs for truly versatile, authentic single-objective LSFM in conjunction with emerging event-trigged automatic imaging schemes will together facilitate high-throughput, high-resolution, smart 3D imaging and analysis of diverse samples ranging from single cells at nanometer resolution to whole-mount tissues with centimeter size (Figure 1E). For tumor immunobiologists, the ability to combine macro-level tissue imaging with micro-level single-cell imaging will allow researchers to study tissue structures and individual cell behaviors simultaneously. Additionally, the rich spectral data provided by hyperspectral imaging can be used for quantitative analysis of immune cells and their microenvironment's chemical compositions and physical states. This is crucial for developing precise diagnostic tools and personalized treatment plans. For neurobiologists, *trans*-scale imaging will aid in the precise study of the onset and progression of neurological diseases. For example, by observing the brains of patients with Alzheimer's disease at different scales, researchers can identify disease-related macrostructural changes (such as brain atrophy) and microstructural changes (such as amyloid plaques and neurofibrillary tangles), thereby developing more effective diagnostic and therapeutic methods. We firmly believe that these paradigm shifts will strongly push LSFM into the mainstream and establish themselves as essential tools for advancing biological discoveries.

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DECLARATION OF INTERESTS

The authors declare no competing interests.