A Colorful World – fluorescent 3D live imaging

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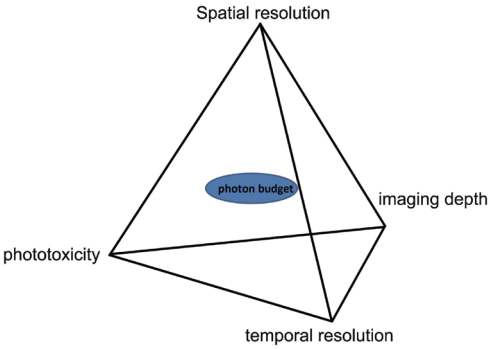
Introduction

Since the 17th Centuries when Anton van Leeuwenhoek improved microscope and successfully observed single-celled organism, the revolution of optical microscopy has been continuously promoting the development of modern cell biology by building up the fundamentals of microbiology. Optical imaging techniques provide much important information in understanding life science especially cellular structure and morphology because "*seeing is believing*. However, the resolution of optical imaging is limited by the diffraction limit, which is discovered by Ernst Abbe, i.e. λ/2(NA) (NA is the numerical aperture of the objective lens). For the last 100 years, biologists and optical scientists are unable to obtain a clear optical image of biological entities down to molecular level that are smaller than the diffraction limit (around 200-nm in lateral resolution). With the discovery of green fluorescent protein, the winners of Nobel Prize in Chemistry 2014, E. Betzig, W. E. Moerner and S. Hell, innovated super-resolution microscopic techniques. Such techniques enable biologists to visualize nano-sized fluorophores that are beyond the diffraction limit. These techniques do not physically violate the Abbe limit of resolution but exploit the photoluminescence properties and labelling specificity of fluorescence molecules to achieve super-resolution imaging. However, these super-resolution techniques limit most of their applications to fixed or dead samples due to the high laser power needed or slow speed for the localization process. Free from the suspected artifact caused by fixation process in sample preparation, live fluorescent imaging is more informative for biologists to elucidate the physiology of living specimens, especially for three three-dimensional (3D) imaging.



Challenges in the 3D Live Imaging

When it comes to live imaging, there are always tradeoffs between spatial resolution, temporal resolution, and phototoxicity based on the photon bank as shown to right. High imaging speeds are required to capture fast cellular process in order to avoid motion blurring in imaging. High spatial resolution is needed to resolve the structure details. High signal-to-noise ratios are crucial to have good quality imaging in order to perform computational image analysis. Long observation periods are needed to catch the biological process happening in the living specimens. Last but not least, low level of light dose is essential to reduce photobleaching of fluorescent probes and minimize phototoxicity to the samples. How to deal with the limited photon budget emitted from fluorophores to have the aforementioned tradeoffs inside live specimens efficiently is made even more difficult when the goal is to capture subcellular details. Established imaging technologies, such as widefield and confocal microscopy, have significant limitations for in vivo imaging of biological structure and function. Since they each illuminate the entire thickness of the specimen, even though high resolution information is obtained from only a single focal plane. This results in premature photobleaching and phototoxicity, limiting the duration of the imaging and altering the physiological state of the specimen. Thus, there is a great demand for technology which can uncover the complex biological phenomena that require 5D (x, y, z, t, λ) information in either a single- or multicellular context.

Novel Approach for bioimging in light microscopy

Widefield and confocal microscopes have been the main working platforms for biologists over the course of several decades. Widefield, or epi-fluorescence microscope provides fast imaging speed because of widefield

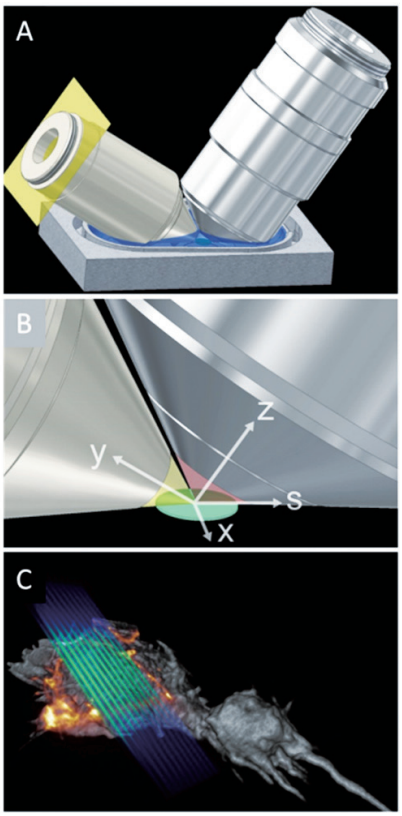
detection, but it suffers from the "out-of-focus" light resulting in the reduced contrast and poor axial resolution. By use of physically small pinhole, confocal fluorescent microscope can reject out-of-focus background to have optical section capability. In this point-scanning based optical system, a beam of laser light is focused into the specimen to excite the fluorescent markers of interest. The generated fluorescent signal at the focus of the objective is collected sequentially by scanning the laser excitation focus across the sample to reconstruct the entire 3D image. Obviously, it is time consuming for large sample or too slow speed to catch fast dynamics process occurring in the live observing subject. The major disadvantage of these two techniques is very inefficient use of laser excitation light and fluorescent probes, as well as potentially high photodamage to the sample. Later on, the discovery of two-photon excitation microscopy uses a special and pricing laser light source, ultrafast laser, to generate the fluorescent signal exclusively at the focal volume by nonlinearity. In addition to the background free, the near-IR wavelength is used to excite the fluorescent molecules so that the deeper penetration depth is allowed. However, in the conventional implementation as a point-scanning technique, it suffers from the same limitation in speed and signal-to-noise ratio as confocal fluorescent microscopes. For live imaging scanning, it is true that line scanning improves upon point scanning and plane scanning, in turn, improves upon line scanning with respect to the detection objective. To address this issue, a new fluorescent imaging technique should be invented for this purpose. A notable example is light sheet based microscopy. Unlike conventional fluorescent imaging based on the epi-illumination configuration, light sheet based microscopy uses a separate excitation lens perpendicular to the widefield detection lens to confine the illumination to the neighborhood of the focal plane. By combining intrinsic optical sectioning with widefield detection, light sheet microscopy allows fast imaging speed to record multimegapixel imaging of selected plane in a single exposure of the camera. Instead of point-scanning in confocal or two photon microscopes, an entire excitation plane formed by the laser illuminates the sample from the side. Thus, the photons emitted from fluorescent probes at this selected excitation plane is collected with an objective lens, orthogonal to the excitation light sheet. Although this idea is derived from very ancient thought, along with the rapid development of instruments such as computer, camera or objectives, the stunning performance has been demonstrated only recently. By selective plane illumination, light sheet microscopy substantially improves acquisition speed and signal-to-noise ratio, while minimal photobleaching and phototoxicity. Especial for 3D live imaging, some light sheet microscopy has been operated to collect high-resolution images rapidly and minimizes damage to cells, meaning it can image the three-dimensional activity of molecules, cells, and embryos in fine detail over longer periods than was previously possible; imaging three-dimensional (3D) dynamics for hundreds of volumes, often at sub-second intervals, at the diffraction limit and beyond.

Light sheet based microscopy

In light sheet microscopy, the light sheet could be a real one, existing simultaneously across the illumination plane (such as generated by cylindrical lens), or a virtual one, created by a serial scanning long Gaussian beam formed by an imaging objective with a low numerical aperture (NA) excitation across the pupil plane. A real light sheet typically requires much lower power so that it takes less photo damage at the cost of spatial resolution in all dimensions, whereas a virtual light sheet gives more control over the spatial and temporal resolution. The 100-year-old idea of light sheet illumination, once termed "ultramicroscopy". In ultramicroscopy, optical sections are generated by stepping the specimen through the illumination plane created by cylindrical lens. This method has been proved to image the cellular resolution in optical-cleaning tissue such as fixed mouse brains, allowing the resolution in around micrometer range for small objects (less than 2 mm). A modified version of ultramicroscopy was presented as selective plane illumination microscopy (SPIM), instead of cylindrical lens, the imaging objective is used to generate a light sheet with scanned Gaussian beam having thinner thickness around 2~10 um (in

ultramicroscopy the sheet thickness is ~ tenth of micrometer) to improve axial resolution and toward live imaging applications. SPIM has been demonstrated in a lot of applications, especially for understanding morphogenesis with live samples such as fruit fly, zebrafish or mouse with the acquisition rates on the order of 175 million voxels per second. This performance mainly benefits from the advances in high-speed camera technology, such as the recent progress in scientific complementary metal oxide semiconductor (sCMOS) technology. This technique has been proven to be a good tool for imaging embryos noninvasively in 3D over time at single-cell resolution; however, this commercial Gaussian light sheet microscopy uses too thick light sheet over cellular dimensions to benefit sub-cellular imaging, which results in the drawbacks of substantial out-of-focus excitation and poor axial resolution. For example, when imaging a 50-μm-diameter cultured cell, an optimized Gaussian light sheet diverges to a full-width at half-maximum (FWHM) thickness of ~ 3 μm. Because this is threefold greater than the depth of focus of a high-numerical-aperture (NA) detection objective, substantial out-of-focus excitation remains, and hence the benefits of background reduction and photobleaching mitigation that are the hallmarks of plane illumination are not fully appreciated. In response, a ultrathin "non-diffracting" Bessel beam plane illumination was introduced to light sheet microscopy to have less background, less photobleaching, less photodamage and better axial resolution. A virtual light sheet plane of submicrometer thickness is created by sweeping such a beam, which is well suited to non-killing high-speed 4D live cell imaging.

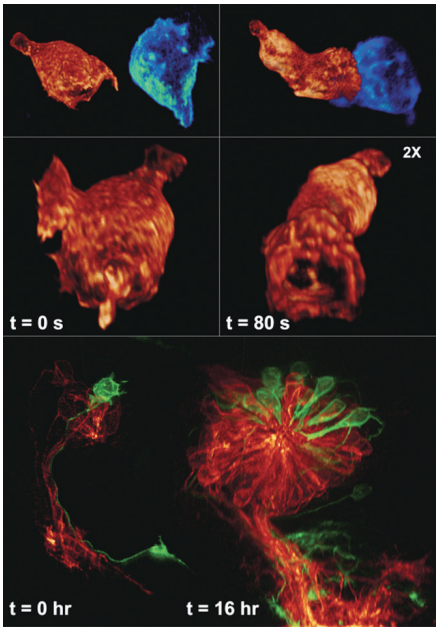
Next, an improved version of Bessel light sheet, lattice light sheet microscopy is invented for the better spatiotemporal resolution, especially for temporal resolution, to watch the dynamics happening inside the cell. Ultrathin light sheets from two-dimensional optical lattices that allowed us to image 3D dynamics for hundreds of volumes, often at subsecond intervals, at the diffraction limit and beyond. The approach begins with a 2D optical lattice. Optical lattices are periodic interference patterns in two or three dimensions created by the coherent superposition of a finite number of plane waves travelling in certain well-defined directions. Like an ideal Bessel beam, an ideal 2D lattice is non-diffracting in the sense that it propagates indefinitely in a direction y without changing its cross-sectional profile, which extends infinitely in x and z. In either case, this is accomplished by confining the illumination at the rear pupil plane of the excitation objective to points on an infinitesimally thin ring. In practice, it is necessary to replace this ring with an annulus of finite thickness, and to control the thickness of the annulus to confine the pattern in z, thereby producing a sheet rather than a block of light. However, this also reduces the extent in y over which the pattern is uniform, so there is a tradeoff between the thinness of the light sheet and its effective field of view in y.



To create these lattices, a fast switching spatial light modulator (SLM) will be used, conjugated to the sample plane and placed before the annular mask. The xz cross-sectional electric field amplitude of the desired theoretical lattice light sheet as a binary phase pattern on the SLM will be displayed. Incident laser light is then diffracted by the SLM, filtered by the mask, and focused by the excitation objective to produce a light sheet.

To apply such light sheets to in vivo imaging, in Fig. A, the lattice pattern will be conjugated to the rear pupil plane (yellow) of excitation objective (left) and an orthogonal detection objective (right) will be suspended from above with their ends dipped in a shallow media-filled and temperature controlled bath. At an angle (Fig. B) in the yz plane such that their light cones of excitation (yellow) and detection (red) lie on one side of a horizontally

mounted cover slip upon which the specimen rests. The lattice light sheet created in the xy plane at their common foci intersects the specimen obliquely (Fig. C). As the specimen is moved through the light sheet, the fluorescence thereby generated is recorded as series of 2D images on a camera. These are then assembled into a 3D image, and the process is repeated to build a 4D data set of cellular dynamics.



Applications of lattice light sheet microscopy

T cell motility and T cell/dendritic cell interactions (shown to the right) illustrates with high temporal resolution how a T cell (red) partially envelopes a target dendritic cell (blue) in a matter of seconds. A 2x zoom of the conjugated plane of the T cell is shown below, illustrating the opening that forms in 80 seconds. The rate of actin flow in 4D is reported therein, something that could not be ascertained with spinning disk microscopes. At a larger scale, the unprecedented resolution in a live vertebrate embryo how stem cells migrate and differentiate into neurons to form a complex network in the olfactory system (shown to the right) in longer period of time. The technique can cover for different length scales at space and time, which make it more attractive for the biologist.

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