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Faster fluorescence microscopy: advances in high speed biological imaging

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The past decade has seen explosive growth in new high speed imaging methods. These can broadly be classified as either point-scanning (which offer better depth penetration) or parallelized systems (which offer higher speed). We discuss each class generally, and cover specific advances in diffraction-limited microscopes (laser-scanning confocal, spinning-disk, and light-sheet) and superresolution microscopes (single-molecule imaging, stimulated emission-depletion, and structured illumination). A theme of our review is that there is no free lunch: each technique has strengths and weaknesses, and an advance in speed usually comes at the expense of either spatial resolution or depth penetration.

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Introduction

Fluorescence microscopy provides a unique combination of high contrast and molecular specificity that is well suited to a wide array of research areas, from cell biology [1] to neuroscience [2]. The last decade has seen explosive growth in new imaging techniques, tremendously improving the performance of fluorescence microscopes. These new methods make it challenging to determine which technique is appropriate for a given experiment, as many factors — including spatial resolution, imaging speed and the desired sample penetration — must be considered.

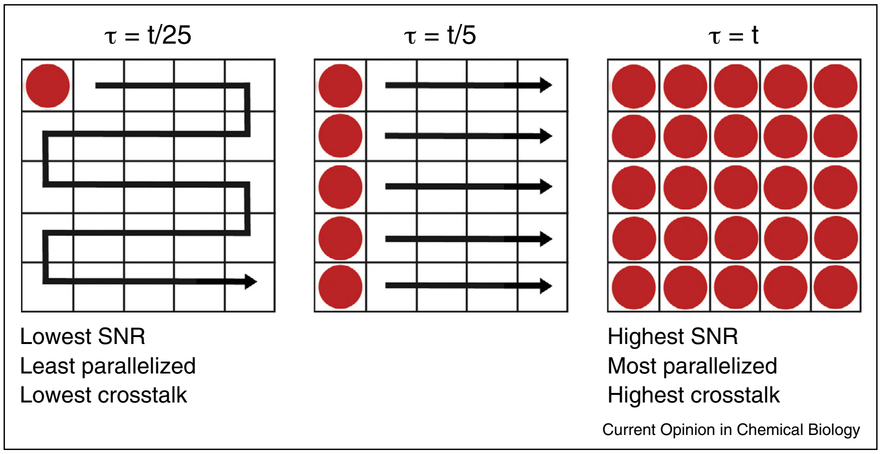
Although developments in hardware and brighter, more photostable fluorophores continue to result in faster and more sensitive imaging, there are still inherent speed limitations in fluorescence microscopy (Figure 1). Existing fluorescence microscopes can be broadly divided into two classes — point-scanning and parallelized systems. Point-scanning microscopes (such as laser-scanning confocal microscopy, LSCM) scan a single excitation focus through the sample, mapping the resulting fluorescence from each scan position to a unique pixel in the image. It is often assumed that the speed of point-scanning systems can be improved by simply increasing the scan speed, yet the resulting decrease in per-pixel dwell time lowers the total signal and degrades the image's signal-to-noise ratio (SNR). Increasing the illumination intensity compensates for this effect, but can also result in higher levels of photodamage and photobleaching (and at high intensities these processes can scale nonlinearly with intensity). Also, given the finite pool of fluorophores in the sample, above a certain illumination intensity effectively all fluorophores are excited and further increases in intensity are of no benefit. Higher speed, or higher SNR at the same speed, can be achieved by parallelizing excitation (i.e. using multiple simultaneous excitation foci to illuminate the sample). Widefield microscopy (illuminating the entire sample volume at once) exemplifies the highest degree of parallelization, thus offering the fastest image acquisition rates. However, this increased acquisition speed comes at a price, as any degree of parallelization results in 'crosstalk' between spatially distinct points in the sample, degrading optical sectioning and contaminating the in-focus signal with scattered light.

High speed imaging at the diffraction-limit

Point-scanners image large volumes much more slowly than parallelized systems, but in certain applications they are preferred. For example, when imaging deep into samples (especially when coupled with multiphoton excitation), robust performance in the presence of scattering is often as desirable as imaging fast. Additionally, when recording from multiple sites in live samples (as in functional imaging), scanning the entire volume is unnecessary and point-scanners can be advantageously used to sample arbitrary regions of interest ('random access scanning'). A major limitation of these systems has been slow scan speed in the axial direction, resulting from the need to move a relatively massive objective or sample chamber during refocusing.

One solution is to use a customized, light-weight mirror to rapidly translate the excitation at a location upstream of the sample, and then refocus this excitation at the sample plane [3]. Such 'remote refocusing' permits kHz scan rates over hundreds of microns in all three dimensions, enabling, for example, the study of neuronal activity in populations of neurons (Figure 2a–c). Other routes to high speed 3D scanning are to use acousto-optic scanning

Figure 1



Current Opinion in Chemical Biology

Effects of parallelizing excitation, *Left*: a single excitation spot (red) is scanned through a 5 × 5 = 25 pixel grid, illuminating one pixel at a time. This serial illumination is reminiscent of point-scanning confocal microscopy, and implies that for total frame exposure time *t* (the time required to illuminate all pixels), the per-pixel dwell time is only $\tau = t/25$. Note that in this case, there is no crosstalk as pixels are illuminated sequentially. *Middle*: excitation has been partially parallelized, so that an entire column of pixels is excited at once (similar to line-scanning confocal microscopy). The per-pixel dwell time is now $\tau = t/5$ for total exposure time *t*, increasing the signal-to-noise ratio (SNR) relative to the point-scanning case at left. Since multiple pixels are illuminated simultaneously, emission originating from spatially distinct regions in the sample (especially from outside the focal plane) may leak over to neighboring pixels, resulting in crosstalk and degrading optical sectioning relative to the point-scanning case. Another example of partially parallelized excitation is the spinning disk confocal microscope. *Right*: all pixels are simultaneously illuminated (similar to widefield microscopy), so for frame exposure time *t*, each pixel is also exposed for time $\tau = t$. SNR is maximized, but so is pixel crosstalk, and optical sectioning is worse than either the point- or line-scanning case.

technology to rapidly move the excitation focus [4•] or to increase the number of excitation foci (i.e. by parallelization). For example, multiplexing four pulsed two-photon (2P) beams that are offset spatially and temporally yields a 4× increase in speed, and was used to image neural activity in intact mouse brains [5].

Improvements have also been made to more highly paralellized systems, such as spinning disk confocal microscopy (SDCM). By increasing the interpinhole distance and utilizing two photon excitation, pinhole crosstalk can be reduced, permitting imaging at depths greater than 100 μm and enabling the tracking of microtubule plus-end binding protein EB1 25 μm into live drosophila embryos (Figure 2d–g) [6]. Another parallelized technique that can achieve high imaging rates is temporal focusing, in which a laser pulse is scanned across the sample on a picosecond timescale. In conjunction with an image-intensifier and a fast, scientific complementary metal-oxide-semiconductor (sCMOS) camera, this technique has enabled measurement of calcium activity at 80 Hz throughout 99 neurons in the brain of an adult nematode [7].

Most fluorescence microscopes, even those using multiphoton excitation, dose the imaging volume outside the focal plane causing unnecessary photodamage. Light sheet fluorescence microscopy (LSFM) solves this problem by illuminating only the sample plane currently in focus. Since an entire line or plane within the sample is illuminated simultaneously, excitation is parallelized and imaging rates and SNR are much improved relative to point-scanning systems.

LSFM has proved especially useful in developmental- and neurobiology. For example, LSFM on an inverted microscope base was used to follow the development of individual neurons at volumetric imaging rates of 0.5 Hz for periods exceeding 8 h in *C. elegans* embryos [8]. The spatial and temporal resolution of this inverted design has been recently improved by rapidly capturing and registering two perpendicular specimen views and performing joint-deconvolution on the result (Figure 2h–j) [9••]. LSFM has also been applied to embryogenesis studies in zebrafish and drosophila embryos [10–12] at speeds sufficient for following cellular development and neural activity, including a recent implementation that used multiple illumination objectives to obtain whole brain functional imaging of zebrafish larvae with cellular resolution at 0.8 s/volume (Figure 2k) [13].

Faster superresolution imaging

Super-resolution microscopes extract higher resolution spatial information than would be possible in standard 'diffraction-limited' systems (where resolution is typically limited to ~250 nm laterally, and ~500 nm axially). Regardless of their operating principle, all superresolution microscopes come with a hefty price. Since the pixel size in an image must be at least 2× as fine as the desired resolution (the Nyquist criterion), an *N*-fold increase in

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