Topic Introduction

High-Speed Two-Photon Imaging

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The small size of neuronal dendrites and spines combined with the high speed of neurophysiological signals, such as transients in membrane potential or ion concentration, necessitates that any functional study of these structures uses recording methods with both high spatial and high temporal resolutions. In this regard, conventional two-photon microscopy, in combination with fluorescent indicators sensitive to physiological parameters, has proved to be only a partial solution by providing near-diffraction-limited spatial resolution even when imaging structures deep inside light-scattering tissue. This is because the relatively slow beam-scanning methods used in most conventional two-photon microscopes severely limit the extent to which functional data can be recorded. Here, we detail developments to create high-speed two-photon imaging systems that overcome this limitation and discuss important considerations that must be taken into account when attempting to construct such systems.

INTRODUCTION

Techniques that increase the temporal resolution of optical sectioning imaging modalities such as two-photon microscopy are becoming increasingly necessary in experimental biology. Applications include tracking cellular and subcellular movements, monitoring for rare events, and recording fast temporal signals from small structures such as neuronal dendrites and dendritic spines. In this last application, the need for increased temporal resolution can be understood by the temporal dynamics of the signals of interest, such as back-propagating action potentials, dendritic spikes, or ionic transients, which occur on the order of milliseconds. Similarly, the micrometer and submicrometer sizes of these fine structures and their locations deep inside studied tissue require the use of high spatial resolution optical sectioning techniques such as two-photon microscopy.

Most high-speed two-photon imaging systems closely resemble, in their optical design, conventional two-photon systems, containing four primary components: the light source, the scanner, a base microscope, and a detector. However, the optimal choice for these components is primarily dependent on the scanning technique used. In this article, we will review several of these techniques, such as scanless spatial light modulator (SLM)-based microscopy, and we will discuss the considerations in choosing the additional elements needed to optimize them for effective use.

HIGH-SPEED TWO-PHOTON IMAGING SETUP

The four main components of most high-speed two-photon microscopes, whether they are designed for fast two-dimensional (2D) or three-dimensional (3D) imaging, are the near-infrared (NIR) laser

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light source, the laser scanner, an adaptable light microscope, and the photodetector (Fig. 1). Each of these elements is usually coupled to the next element in the optical path using relay optics, which could be a series of 1:1 telescopes. However, if the beam size needs to be adjusted to fill an aperture (such as the objective back focal aperture) or the scan angles need to be increased to fill the effective field of view, magnification or demagnification telescopes are used accordingly.

Light Source

Although two-photon excitation can be achieved utilizing a high-powered continuous-wave laser (Hell et al. 1998), such a scheme is not usually conducive to biological experimentation. As a result, modern two-photon microscopes use pulsed laser sources, which maintain moderate average power levels while ensuring sufficient peak powers for significant two-photon excitation. In this regard, since its introduction in 1986, titanium-doped sapphire (Moulton 1986), which has a very high gain bandwidth that allows for very short pulses as well as a large range of wavelength tunability, has become the preeminent gain medium for most two-photon laser sources.

However, the optimal laser for a two-photon microscope is not always the one with the shortest pulses. Indeed, the broad spectral profiles of transform-limited short pulses when combined with wavelength-sensitive refractive objects, such as lenses and optical fibers, or diffractive objects, such as gratings or acousto-optic devices (AODs), lead to two forms of distortion: spatial dispersion and temporal dispersion. These forms of dispersion can have detrimental effects on both image quality and efficient power use, unless their effects are minimized.

Spatial dispersion results because both refraction and diffraction are wavelength dependent. For the multiple wavelengths inherently present in a short pulse, this means that different wavelengths within the pulse will travel different paths, which leads to a less compact focus and a worsened spatial resolution. Because shorter pulses have larger spectral profiles, the degree to which the spatial resolution is reduced increases with the shortening of the temporal profile of the pulse.

Temporal dispersion results from the velocity differences between differing wavelengths of light when traveling through an optical medium, which result in pulse broadening and thus lower peak powers. In general, short pulses broaden to a greater extent than longer pulses, and in many optical layouts, a longer transform-limited pulse at the exit of the laser can lead to a shorter pulse at the image plane. This is increasingly true with long optical paths through material having a high index of refraction.

Although the amount of spatial or temporal dispersion introduced by conventional mirror-based two-photon microscopes is small, the value is significant for some high-speed two-photon imaging systems, particularly ones that use AOD-based scanning. In addition, although methods for both

FIGURE 1. Basic high-speed two-photon imaging system. Primary system components are a pulsed near-infrared (NIR) laser, various configurations of a beam scanner, an adaptable epifluorescence microscope, and a sensitive photodetector. Important optical path components include two telescopes acting as beam expander and scan angle magnifier, and a dichroic mirror to pass excitation light to the objective lens and direct fluorescence to the emission filter, which selects for the desired fluorescence band.

spatial and temporal dispersion compensations are available (Treacy 1969; Martinez et al. 1984; Iyer et al. 2003; Zeng et al. 2006), often the most practical and resource-efficient means for reducing the degree to which these dispersive mechanisms affect imaging is by selecting laser sources with slightly longer pulse widths and, hence, smaller bandwidths. This, in turn, will reduce both forms of dispersion, often negating the need for placing additional elements in the optical path for compensation. Similarly, when using 3D AOD-based scanning, in which spatial dispersion is inherently compensated by the scanning mechanism (Reddy and Saggau 2005), a resource-efficient solution can be to use a laser with higher average power to compensate for the reduction in peak power resulting from the anticipated temporal broadening of pulses.

The Scanning Mechanism

The crucial difference between various high-speed two-photon imaging devices is the scanning mechanism that is used (see Fig. 2). As mentioned above, in conventional two-photon microscopes, the primary limitation in temporal resolution is caused by the laser beam-scanning mechanisms, which use galvanometer-driven rotating mirrors (Fig. 2A). Given the inherent inertia of both the mirror and the galvanometer, there is a fundamental physical restriction on the highest speed at which these beam-scanning mechanisms can operate. Although higher speeds can be achieved by driving the galvanometers at their resonant frequency to minimize the rate-limiting inertia of the system (Fan et al. 1999), the increase in speed requires restricting the scan to linear or simple patterns. In general, restricting scan patterns to single scan lines allows for acquisition rates on the order of hundreds of images per second. However, when imaging highly irregular and sparse structures, such as neuronal dendrites, a line scan is highly inefficient because much time is spent visiting areas without relevant structures.

FIGURE 2. Beam-scanning schemes. (A–F) Two-dimensional scanners: (A) Point scanner uses two galvanometer-driven mirrors; (B) line scanner sweeps laterally to spread out the laser beam with a single galvanometer-operated mirror; (C) rotating polygonal mirrors are used to increase the scan speed in one dimension; (D) multifocal scanners use a microlens array to achieve multiple scan points; (E) scanless beam positioning uses an SLM to control one beam or multiple beams; (F) random-access scanner uses two acousto-optic deflectors to direct a single beam. (G) 3D random-access scanning system uses four AODs to position a laser focus in a volume without moving the objective lens.

2D Scanning Systems

Structural Imaging Systems

Several systems have been developed that increase the speed of conventional two-photon microscopes to video rate. In some of these systems, scanning an individual focal point is replaced with scanning entire lines of two-photon excitation (Fig. 2B) (Brakenhoff et al. 1996). Although these systems offer higher temporal resolution scanning, they suffer from a loss of resolution as well as a decrease in excitation efficiency. Other methods use a rotating polygonal mirror in place of one of the galvanometers (Fig. 2C) (Kim et al. 1999). This approach preserves spatial resolution; however, it restricts zooming capabilities. Still other popular approaches that resemble the Nipkow spinning-disk system in confocal microscopy, deterministically separate the input laser beam and use multiple foci of two-photon excitation to simultaneously excite several locations in a region of interest (Fig. 2D) (Bewersdorf et al. 1998). By dividing the excitation light into several beams, these systems suffer from reduced power for excitation at each individual focus and also require the use of an imaging detector (e.g., a sensitive high-speed camera) to separate the emission light from each excited focus. All these systems are designed primarily for structural imaging, in which raster scans are used by necessity. Thus, although they increase the temporal resolution over galvanometer-based systems, they do not overcome the primary limitation imposed by them. Therefore, similar to such systems, they are forced into simple linear-based scans that are highly time inefficient when functionally imaging fast signals on sparse structures.

Functional Imaging Systems

For applications in which functional imaging is of primary importance, scanless or random-access systems can be used. Scanless systems use SLMs to create several excitation foci (Fig. 2E) (Nikolenko et al. 2008), similar to the multifocal systems described above. However, unlike those systems, the SLMs in scanless systems allow the locations of the foci to be arbitrarily chosen by the user to match a complex structure such as a dendrite. These systems suffer from similar drawbacks to multifocal systems, including diminished excitation power at each foci as well as the need for a fast imaging detector to spatially separate the individual foci from each other.

Random-access scanning systems operate by using either a single AOD for fast single-axis imaging (Lechleiter et al. 2002) or dual AODs for x - y imaging (Iyer et al. 2006; Lv et al. 2006; Salome et al. 2006). AODs work by utilizing an acoustic wave traveling through a medium to set up a virtual diffraction grating, thereby diffracting the incident light by an angle θ , which is proportional to the frequency of the incident acoustic wave by

$$\theta = \frac{\lambda f}{v}, \quad (1)$$

where λ is the wavelength of the incident light and f and v are the frequencies and the velocities of the propagating acoustic waves, respectively. The advantage of such systems comes from the ability to change quickly from one acoustic frequency to a noncontiguous frequency. This makes it possible to use scan patterns that are no longer confined to raster, line, or trajectory scans. However, as mentioned above, utilizing AODs generates significantly higher levels of both spatial and temporal dispersions. As a result, either compensating elements have to be placed in the optical path (Iyer et al. 2003; Salome et al. 2006), or longer pulses, which reduce two-photon excitation efficiency, must be used (Iyer et al. 2006).

3D Scanning Systems

The systems mentioned above use high-speed scanning methods that were designed for fast lateral, or x - y , scans. To selectively move to different axial planes, such systems typically rely on moving an

objective lens via a stepper motor or moving the imaging detector. Although certain techniques manage to synchronize the movements of the lateral scanning mechanism and the stepper motor to create a scan pattern trajectory that visits most user-defined locations with sufficient temporal resolution to record physiological data (Gobel et al. 2007), all such systems are still limited by the inertia of the objective lens and its actuator.

One relatively inexpensive method to circumvent this problem is to use an axicon, which extends the depth of focus at the image plane (Dufour et al. 2006). This expands the axial range at which points of interest can be excited by a laser beam focused at a particular x - y location. Although this technique does offer the ability to visit several locations in different axial planes, it suffers from reduced available excitation energy at sites of interest because the power is spread over the increased depth of field. In addition, with this method, it is not possible to differentiate sites of interest that are located directly above or below each other.

Another technique that avoids the need for an objective lens actuator is 3D random-access imaging (Duemani Reddy et al. 2008). As with 2D random-access systems, this technique uses AODs to create scan patterns that can quickly change between several regions of interest. However, 3D random-access imaging uses four AODs to create a user-defined scan pattern in 3D (Fig. 2G). In such cases, two AODs are arranged such that chirped acoustic waves (which are defined by a continuously changing frequency over time) propagating within them are in opposing directions (Reddy and Saggau 2005). This arrangement allows for the generation of a cylindrical focus with a focal length, f_{AOL} proportional to the degree α at which the acoustic wave is being ramped and is determined by

$$f_{\text{AOL}} = \frac{v^2}{2\alpha\lambda}, \quad (2)$$

where λ and v are the wavelength and the velocity of the propagating acoustic waves, respectively. By utilizing two orthogonal pairs of deflectors, a full spherical focus can be generated. Lateral scanning is maintained in the x - y plane by manipulating the differences in the absolute frequencies between the two deflectors organized in the same direction. Although these systems show a greater temporal dispersion than 2D random-access systems, they are self-compensating with respect to spatial dispersion (Reddy and Saggau 2005). An example of dispersion-compensated fast imaging with 3D random-access scanning is shown in Figure 3 (Y. Liang, A.R. Faustov, and P. Saggau, unpubl.).

Using an SLM for 3D scanning without moving the objective lens would also be possible. However, its multibeam feature (Nikolenko et al. 2008) could not be straightforwardly utilized, because the imaging detector necessary for this scanning mechanism might not be able to separate fluorescence received from different axial distances. Therefore, SLM-based 3D imaging will most likely be limited to single-beam scanning, where AODs have been superior because of their ~ 10 times higher beam positioning speed.

Other methods for fast focusing, such as deformable mirrors (Zhu et al. 1999), fluid-filled lenses (Zhang et al. 2003), and electro-optic lenses (Shibaguchi and Funato 1992), have all been shown and are possibilities for offering fast 3D scanning when combined with either galvanometers or other 2D scanning methods. However, to our knowledge, none has yet been shown in two-photon imaging systems.

Microscopes

Regardless of the scanning method used, most two-photon imaging systems are constructed around adapted research microscopes. The type of microscope chosen depends on the biological task to be accomplished. For example, for optically studying electrophysiology, an upright microscope is used because it allows for easy patching of cells to be filled with the appropriate optical indicators. By adding an additional port to the microscope, the excitation and emission light for two-photon imaging can be separated from the wide-field illumination usually required for other gross visualization of tissues of interest.

FIGURE 3. Fast 3D imaging. Hippocampal CA3 neuron in organotypic culture, filled by patch pipette with two fluorescent dyes: structural label Alexa 594 (50 μM) and calcium indicator Oregon Green BAPTA-1 (100 μM). Current injection into the soma elicits three back-propagating action potentials. Calcium transients at user-selected sites measured simultaneously over an axial extension of 20 μm . Note color coding in micrometers relative to the physical focal plane of the objective lens.

The final element to any two-photon imaging system is the photodetector. The first concern about what type of detector is optimal is whether an imaging detector is needed or not. For single-beam laser scanning, such as that utilizing galvanometers or AODs, an imaging detector is unnecessary and either photomultiplier tubes or photodiodes can be used to detect the emission light. The benefits of each method are beyond the scope of this article. For scanning methods that require multiple beams, such as multifocal or scanless two-photon microscopy, or for those that image with a line instead of a point, an imaging detector, often a high-speed charge-coupled device camera, is a necessity.

CONCLUSION AND FUTURE PROSPECTS

High-speed two-photon imaging systems are becoming increasingly important in experimental biology. Nowhere is this more obvious than in the functional study of neuronal dendrites and their spines. Although most such systems are typically built with the same four components, optimizing these systems relies on choosing elements depending on the scanning methods used. Indeed, it is advances in high-speed beam scanning or the successful implementation of fast and effective scanless methods that will continue to advance the field of high-speed two-photon imaging.

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