Since the invention of simple microscope by Leuwenhoek and Hooke in the 17th century, different types of light microscopy techniques (such as phase contrast, differential interference contrast and fluorescent microscopy) have been developed for biological research over the past few centuries. The main aim of these techniques is to improve the contrast of the microscopic specimen observation, eliminating unwanted background noise. Fluorescent microscopy occupies a unique niche in biological applications; fluorescent objects can be selectively excited and visualized, even in living system. (Lichtman and Conchello, 2005). The sensitivity of fluorescence detection is sufficiently high so that single fluorescent molecules can be detected in the presence of non-fluorescent molecules (e.g., water, amino acids, and lipids) (Eigen and Rigler, 1994).

Most of the biological samples need to be studied in their natural habitat. For example, neurons in intact brain tissue are especially challenging for fluorescent microscopy. In wide-field fluorescence microscopy, contrast and resolution are degraded by strong scattering in thick specimen (Denk and Svoboda, 1997). Confocal microscopy can overcome some of the effects of scattering, since the detector pinhole rejects fluorescence from off-focus locations (Conchello and Lichtman, 2005; Denk and Svoboda, 1997). However, scanning a single section excites, and damages the entire specimen. In addition, the pinhole also rejects signal photons emanating from the focus that are scattered on their way out of the tissue. Deep in tissue, confocal microscopy becomes unacceptably wasteful in terms of signal photons (Centonze and White, 1998; Conchello and Lichtman, 2005). Compensating for signal-loss with increase in fluorescence excitation will lead to phototoxicity and photobleaching of specimen. Therefore, wide-field and confocal microscopy are techniques that best for thin specimens applications, such as cultured preparations or the most superficial cell layer in a tissue (<20 mm) (Lichtman et al., 1987). Multiphoton microscopy (MP) has thus evolved as an alternative to conventional single-photon confocal microscopy and has been shown to provide several advantages to overcome the problem mentioned above for thick biological specimen. These include three-dimensionally resolved fluorescence imaging of living cells deep within thick, strongly scattering samples, and reduced phototoxicity, enabling long term imaging of photosensitive biological specimens.
Principles of Two-Photon Excitation

The phenomenon of two-photon excitation arises from the simultaneous absorption of two photons in a single quantitized event. Since the energy of a photon is inversely proportional to its wavelength, the two absorbed photons must have a wavelength about twice that required for one-photon excitation. For example, a fluorophore that normally absorbs ultraviolet light (approximately 350 nanometers wavelength) can also be excited by two photons of near-infrared light (approximately 700 nanometers wavelength) if both reach the fluorophore at the same time (Figure 1). In this case, "the same time" means within an interval of about $10^{-18}$ seconds. Because two-photon excitation depends on simultaneous absorption, the resulting fluorescence emission varies with the square of the excitation intensity. This quadratic relationship between excitation and emission gives rise to many of the significant advantages associated with two-photon excitation microscopy. In order to produce a significant number of two-photon absorption events (in which both photons interact with the fluorophore at the same time), the photon density must be approximately one million times that required to generate the same number of one-photon absorptions. The consequence is that extremely high laser power is required to generate significant two-photon-excited fluorescence. This power level is easily achieved by focusing mode-locked (pulsed) lasers, in which the power during the peak of the pulse is high enough to generate significant two-photon excitation, while the average laser power remains fairly low. In this situation, the resulting two-photon-excited state from which emission occurs is the same singlet state that is populated when carrying out a conventional fluorescence experiment. Thus, fluorescent emission following two-photon excitation is exactly the same as emission generated in normal one-photon excitation. Figure 1 presents a Jablonski diagram illustrating absorption of a single (ultraviolet) photon (Figure 1(a)) and the simultaneous absorption of two near-infrared photons (Figure 1(b)), producing the identical excited state.
In practice, the narrow localization of two-photon excitation to the illumination focal point ensures that no background fluorescence is produced, so pinhole is not required. This is the significant difference between multiphoton and confocal microscopy. Figure 2 illustrates the photobleaching pattern that arises in the x-z direction from repeated scanning of a single x-y plane (the image plane) in a fluorescein-stained gel. The laser of the confocal system excited fluorophores above and below the focal plane (Figure 2a), contributing to the bleaching observed in these extensive areas. In contrast, two-photon excitation occurs only at the focal plane, and bleaching is therefore confined to this area (Figure 2b).

![Figure 2. Excitation Photobleaching pattern for a) confocal microscopy; b) Multiphoton microscopy](image)

**Application of Multiphoton Microscopy**

Multiphoton microscopy is inherently optical sectioning, which provides deeper penetration (in thick scattering specimen) and reduces photo-toxicity and photo-bleaching (Grace et al., 2005). Therefore, it is ideal for probing sensitive live biological samples that cannot tolerate extrinsic fluorophores staining, or histological sectioning. Below are some examples of advantages of multiphoton applications in long-term time-lapse imaging and dynamic cellular imaging for photosensitive specimen.

**Long time lapse multiphoton imaging**

One of the major challenges neurobiologist faced in neuronal morphological study is to capture time-lapse images while minimize the photo-damage of neurons in thick and highly scattering brain specimen. The main reason for photo-damage of neurons in specimen is by the
process of imaging itself, for example, compensating fluorescent signal loss by high excitation power. Therefore, multiphoton microscopy which is having advantages of deeper optical penetration offer vast potential to capture long term imaging for thick brain specimen. One example of long time lapse multiphoton imaging is reported by Trachtenberg et al (2002) for studying synaptic plasticity in adult mice (Figure 3). They employed a method called “open skull” technique to gain optical access to the GFP expressing neuron in the mice brain by removing a circular portion of opaque skull. The 8-days repeated in vivo multiphoton imaging studies have suggested that experience-dependent plasticity of cortical receptive fields was accompanied by increased of synapse changes, which might underlie adaptive remodeling of neural circuits.

**Figure 3.** Long time lapse imaging of the same dendritic segment acquired over 8 day period in the barrel cortex of a mouse. Arrows indicate examples of dendritic spines that stable (yellow) or transient and semi-stable (blue, red).


**Dynamic cellular imaging**

Besides the morphological studies of neuron cells that located deep inside the thick brain specimen, their dynamic network activity can also be observed using multiphoton technique with a suitable fluorescent probes. However, imaging the neuron population activity post a significant challenge to present scanning technology because high acquisition rates are needed to measure such population activity simultaneously. One direct example is calcium imaging that probes calcium ions dynamic/oscillation in neuron cells. Calcium ions are important messengers in neuron that are responsible for several key activities such as signaling the release of neurotransmitter, gene expression and facilitate synaptic plasticity. There are two major sources that Ca\(^{2+}\) ions can enter into cytoplasm of neurons. First route, the Ca\(^{2+}\) ions enter through voltage operated Ca\(^{2+}\) channel from extracellular space. When a neuron becomes active, its
membrane undergoes depolarization and allows Ca\(^{2+}\) ions entry into cytoplasm. The second route for Ca\(^{2+}\) entry into neuron is from intracellular endoplasmic reticulum (ER) mediated by receptor operated Ca\(^{2+}\) channel. The intracellular store of Ca\(^{2+}\) can be released into cytoplasm when inositol triphosphate (IP3) is produced from G-protein coupled receptor on the cell surface by the action of phospholipase C (PLC). IP3 stimulates the receptor operated calcium channel on the ER, which causes the sudden increase of Ca\(^{2+}\) ions in cytoplasm.

The application of calcium imaging in Multi-photon was exemplified in Fan et al., (1999), where they utilized the famous protein-based Ca\(^{2+}\) indicators, “cameleons” for the testing of video-rate scanning Two-photon Excitation Fluorescence Microscopy. “Cameleons” is a genetically encoded calcium sensor and targetable to specific tissues and intracellular locations (Miyawaki et al., 1997), they have preceding advantages over the small molecule indicators. By utilizing the Fluorescence Resonance Energy Transfer (FRET) principle through cameleons, a ratio-metric imaging recorded using a video-rate scanning two-photon excitation fluorescence microscopy system that provides 30 frame/s of noninterlaced images of 512 X 484 pixels were obtained. With the implementation of video-rate scanning, a ratio imaging of calcium wave in HeLa cells expressing yellow cameleon-2.1 (Figure 4) was obtained.
Nikon A1R MP

Nikon’s A1R MP is a unique multiphoton imaging system featuring a high resolution galvanometer scanner and a high speed resonant scanner that is capable of frame rates from 30 fps at 512 X 512 pixels to as fast as 420 fps in band scan mode. New four channel non-descanned multiphoton detectors with higher sensitivity, reduced dark current and broad spectral range allow for real time unmixing of closely spaced probes for deep tissue and accurate, high contrast spectral imaging. This is especially important in multiphoton imaging because of the overlap of emission spectra of probes and autofluorescence, which is often unavoidable when using a single laser line.

The Nikon A1R MP also features a newly-developed, one click auto-alignment of the infrared femtosecond Ti:sapphire multiphoton excitation laser, allowing for fast set up, wavelength changes, GVD pre-chirping compensation and total ease of use (Figure 5). This is particularly important because when the multiphoton laser wavelength (or group velocity dispersion precompensation) is changed, the multiphoton laser beam positional pointing at the objective back aperture may also change, resulting in uneven intensity across the image, or a slight misalignment between the IR and visible laser light paths. Verifying the IR laser beam pointing and setting the alignment has traditionally been difficult. This device completely encloses the beam within the instrument from the laser to the objective lens – a huge improvement in the operating safety of multiphoton imaging systems as the main danger comes during the laser alignment.

Figure 5. A1R MP multiphoton laser beam alignment with a single click
Bright, high resolution imaging is provided by the newly introduced Nikon Lambda S (λS) objective series, featuring the highest numerical apertures (NA) for water immersion objectives yet. The CFI APO LWD 40X WI λS objective with NA 1.15 and a working distance of 610 microns incorporates Nikon’s new Nano Crystal Coat technology which the coat particle structure dramatically reduces stray reflection and boosts transmission over wide wavelength range. Ideal for high performance multiphoton imaging, this unique coating provides high transmissions over an expanded correction range from the violet through the near IR range.

The latest version of NIS-Elements C Version 3.2 software orchestrates all microscope control and multiphoton acquisition with the capabilities of acquiring data at high speed, control of various motorized devices. This includes fast piezoelectric Z focus positioners and XY translational stages, input/output triggers and flexible experiment design. High speed, high precision unmixing algorithms enable high contrast imaging.

References


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