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Ammasi Periasamy, Ph.D.
Alberto Diaspro, Ph.D.

MULTIPHOTON MICROSCOPY

INTRODUCTION

Multiphoton Microscopy (MM) was not commonly known until Goppert-Mayer[1] introduced the theoretical prediction of two-photon absorption in 1930s. The MM technology became popular and met with the interests of biomedical scientists after the introduction of high-speed lasers and more importantly because of the contribution made by Watt Webb's group from Cornell University[2]. It's also important to mention that the commercialization of this multi-photon technology by Bio-Rad Laboratories has created much awareness in using this technology for biomedical imaging. Currently, there are many leading light microscopy centers and laboratories involved in developing advanced MM technology for implementation in various biomedical applications. Since 1990, many papers have been published in peer-reviewed journals, presented in conferences and books published focusing specifically on MM technology and its applications in the biomedical sciences[3-6]. We believe that the future is headed towards easier-to-use equipment, lower cost and higher sensitivity, which will allow greater flexibility on the user in imaging multiple fluorochromes simultaneously, while collecting images from a single cell to tissue.

In brief, MM requires an infrared femtosecond pulsed laser wavelength to create multi-photon absorption in a biological sample. Absorption takes place throughout the illumination area in one-photon (confocal or wide-field), but in multi-photon it occurs only at the diffraction limited spot (focal volume). For example, for the enhanced green fluorescent protein (eGFP), one-photon excitation wavelength is 488 nm (CW, continuous wave), and for two-photon excitation 870 nm (pulsed IR laser) excitation wavelength is used. The multiphoton excitation microscopic images have better signal-to-noise ratio compared to the confocal images because of considerably less light scattering, autofluorescence, and photobleaching.

The special section on MM in this journal covers number of topics written (17 papers) by leading scientists working on technology development and applications from single cell to tissues. Dickinson et al clearly demonstrated how the Zeiss spectral imaging system can be used for measuring the multiphoton excitation spectra for different fluorophores used in single and multi-label experiments for studying the influence of the biological environment on non-linear excitation. It has been a great interest among biomedical scientists to apply the fluorescence

resonance energy transfer (FRET) and fluorescence lifetime imaging (FLIM) methodology for various biological applications. In this MM section there are about 9 papers covering FRET and FLIM for various biomedical applications.

For FRET to occur there are three important conditions (1) the donor emission spectrum should overlap the acceptor absorption spectrum by at least 30% (2) the proximity between donor and acceptor fluorophore should be within 1-10 nm, and (3) the donor emission dipole moment and the acceptor absorption should be in a particular orientation (about κ^2=1-4). Due to the spectral overlap there is large amount of donor bleed-through in the acceptor channel (FRET channel) along with the sensitized emission and this can be removed by mathematical correction[7-8] or by having an emission filter with a lower bandwidth. Wallrabe et al compared the usage of various emission filter bandwidths for FRET imaging to a mathematical correction that removes the spectral bleed-through in order to study the clustered distribution of receptor-ligand complexes in endocytic membranes. Mills et al clearly demonstrated the need for mathematical based correction in extracting the FRET signal in tissues about 100 microns deep to study the interaction between the proapoptotic protein BAD and the prosurvival protein Bcl-xl within traumatic axonal injury following traumatic brain injury. On the other hand, LaMorte et al demonstrated the *in vivo* association of promyleocyte (PML) homodimers within their corresponding nuclear body using the two-photon spectral FRET imaging technique. This FRET spectral imaging technique does not require any algorithm to correct the spectral bleed-through.

FLIM allows quantitative measurements of protein associations in living cells and tissues by following the change in lifetime of the donor molecule. FLIM measurements are independent of change in fluorophore concentration and excitation intensity but dependent on the environmental changes in the biological systems. A FLIM technique does not require any spectral bleed-through correction and it allows monitoring multiple protein pair interactions. Krishnan et al provided an excellent review of the basics of FLIM and the implementation of FLIM technology for various biological applications. They specifically demonstrate how a streak-camera-based system could be used to monitor the change in donor lifetime for the mitochondrial caspase activity induced by oxidative stress. A Becker-Hickl board can be integrated to any commercially available multiphoton microscopy system for FLIM imaging to characterize the amyloid-beta plaques (Bacskai et al) and to study *C. elegans* embryos and primate histology specimens (Eliceiri et al). Gratton et al used time and frequency domain FLIM methodology to determine the ion and oxygen

concentration in cells and the quantitation of FRET signal for distance measurements in the nanometer range.

It is always of great interest among biomedical scientists to detect specimens at the single molecule level, which has been elegantly described by Cannone et al using custom build two-photon excitation microscopy. Werrlein and Madren-Whalley described how the cell behaves in the presence of sulfur mustard using multiphoton microscopy. Samkoe and Cramb described the importance of two-photon excitation photodynamic therapy (TPE-PDT) for clinical treatment to localize the photosensitizer in order to treat the age-related macular degeneration (AMD). Two-photon flash photolysis (TPFP) is used to release effector molecules from caged precursors with high three-dimensional resolution. Soeller et al described the Ca^{2+} cage for heart muscle cells and in mouse oocytes cytosol and inside a nucleolus using TPFP. Another imaging technique called second harmonic generation (SHG) microscopy was demonstrated by Pons et al for membrane potential measurement. Koenig and Riemann used SHG and multiphoton microscopy together to obtain a high-resolution four-dimensional (4D) optical tomography of human skin images.

Using the Monte Carlo simulation method, Deng et al investigated how the image resolution and signal level are affected when imaging through inhomogeneous turbid media. On the other hand, Dong et al characterized the two-photon point spread functions (PSFs) of water and oil immersion objective lens in a turbid medium. Finally, Iyer et al described a novel approach for compensating dispersion effects that arise when acousto-optic (AO) beam deflection of ultrafast laser pulses are used for multiphoton laser scanning microscopy (MPLSM).

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Guest Editors

Ammasi Periasamy, Ph.D.
Alberto Diaspro, Ph.D.

Selected Reading

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