

SPECIAL SECTION GUEST EDITORIAL

Multiphoton Microscopy

Multiphoton microscopy (MM) was not commonly known until Goppert-Mayer¹ introduced the theoretical prediction of two-photon absorption in the 1930s. MM technology became popular and was met with interest among 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 multiphoton technology by Bio-Rad Laboratories has created much awareness of the usage of this technology in biomedical imaging. Currently, there are many leading light microscopy centers and laboratories involved in developing new MM technology for implementation in various biomedical applications. Since 1990, many papers have been published in peer-reviewed journals and presented at conferences, and books have been published focusing specifically on MM technology and its applications in the biomedical sciences.³⁻⁸ We believe that the future is headed toward easier-to-use equipment, lower cost, and higher sensitivity, which will allow the user greater flexibility 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 multiphoton absorption in a biological sample. Absorption takes place throughout the illumination area in one-photon microscopy (confocal or wide-field), but in multiphoton microscopy it occurs only at the diffraction-limited spot (focal volume). For example, for the enhanced green fluorescent protein (eGFP), the one-photon excitation wavelength is 488 nm (continuous wave), and for two-photon excitation a 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.

This special section covers a number of topics in 17 papers written by many leading scientists on technology development and applications from a single cell to tissues. Dickinson et al. clearly demonstrate how the Zeiss spectral imaging system can be used for measuring the multiphoton excitation spectra for different fluorophores used in single and multilabel experiments for studying the influence of the biological environment on nonlinear excitation. There is great interest among biomedical scientists in applying the fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging (FLIM) methodology for various biological applications. In this special 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 to 10 nm, and (3) the donor emission dipole moment and the acceptor absorption should be in a particular orientation (about $\kappa^2 = 1-4$). Due to the spectral overlap there is a 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^{9,10} or by having an emission filter with a lower bandwidth. Wallrabe et al. compare 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 demonstrate the mathematical-based correction essential for extracting the FRET signal in tissue about 100 μm 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. demonstrate the *in vivo* association of promyelocyte (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 tissue by following the change in lifetime of the donor molecule. FLIM measurements are independent of changes in fluorophore concentration and excitation intensity but dependent on environmental changes in the biological systems. A FLIM technique does not require any spectral bleed-through correction and it allows monitoring of multiple protein pair's interactions. Krishnan et al. provide 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 into any commercially available multiphoton microscopy system for FLIM imaging to characterize the amyloid-beta plaques (Bacsikai et al.) and to study *C. elegans* embryos and primate histology specimens (Eliceiri et al.). Gratton et al. use time- and frequency-domain FLIM methodology to determine the ion and oxygen concentration in cells and the quantitation of FRET signals 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 is elegantly described by Cannone et al. using custom-build two-photon excitation microscopy. Werlein and Madren-Whalley describe how the cell behaves in the presence of sulfur mustard using multiphoton microscopy. Samkoe and Cramb describe the importance of two-photon excitation photodynamic therapy for clinical treatment to localize the photosensitizer in order to treat age-related macular degeneration. Two-photon flash photolysis (TPFP) is used to release effector molecules from caged precursors with high three-dimensional resolution. Soeller et al. describe 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 is demonstrated by Pons et al. for membrane potential measurement. Koenig and Riemann use SHG and multiphoton microscopy together to obtain high-resolution four-dimensional optical tomography of human skin images.

Using the Monte Carlo simulation method, Deng et al. investigate how the image resolution and signal level are affected when imaging through inhomogeneous turbid media. On the other hand, Dong et al. characterize the two-photon

point spread functions of water and oil immersion objective lenses in a turbid medium.

Finally, Iyer et al. describe a novel approach for compensating dispersion effects that arise when acousto-optic beam deflection of ultrafast laser pulses is used for multiphoton laser scanning microscopy.

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