Zoological Studies 34, Supplement I (1995)

Zoological Studies

Recent Developments in Non-linear Laser Microscopy Illuminated by Two Photon Excitation

Watt W. Webb
Applied Physics and Developmental Resource for Biophysical Imaging and Optoelectronics, Cornell University, Ithaca, N.Y. 14853, USA

Non linear laser microscopy responds to the challenge of fundamental problems in biological science by providing the means for microscopic imaging of the dynamical microchemistry and for microscopic pharmacology in functional tissues and cells.

Simultaneous interaction of two low energy long wavelength (deep red) photons with a chromophore molecule adds their energies to excite molecular transitions that ordinarily absorb ultraviolet or visible light. The 100 femto second pulses of a strongly focused mode locked laser beam provide high peak intensity in the focal volume for a localized source of virtually simultaneous photons at about 10^{22} photons per second. Because two photon excitation is intrinsically limited to the focal volume there is no out of focus background, photobleaching or photodamage. Photoactivation of caged bioeffector molecules, such as neurotransmitters, can be localized to a submicron focal volume and released on microsecond to millisecond time scales for dynamical micropharmacology experiments. Because two photon excitation eliminates the need for confocal and ultraviolet optics it allows sharp, high efficiency fluorescence imaging deep within thick preparations such as 100μm thick hippocampal brain slices that strongly scatter light of short wavelength.

Ratio imaging of the calcium indicator Indo 1 using visible light of ~ 695 nm wavelength has routinely provided 3-dimensional (3-d) resolved imaging of cytoplasmic calcium at 4 images per second, and 3-d resolved linear scans at 1000 per second. True video rate 3-d resolved fluorescence imaging at 30 images per second has recently been obtained using two photon line illumination.

Instrumentation for two photon excitation laser scanning microscopy has been developed to a stage ready for adaptation to industrial production. The two photon excitation cross sections and spectra of many familiar fluorophores have been measured for the first time with the pleasing result that the optimum two photon excitation wavelengths are frequently far shorter than twice the one photon excitation wavelength as the simplest models had suggested. Therefore many, if not most, fluorescent markers and indicators that are commonly used in biological research can be excited within the convenient range of the commercially available titanium sapphire mode locked femtosecond pulsed lasers. However shorter wavelengths are preferred for some photochemical applications. The emission spectra of these fluorophores are essentially the same with two photon excitation as with one photon excitation.

Applications of two photon excitation in laser scanning microscopy that will be illustrated as time allows include dynamical 3-d resolved imaging of (1) cytoplasmic calcium waves, sparks and signals in several tissues, (2) neurotransmitter receptor function on cell surfaces by spatially resolved release of caged neurotransmitters, (3) nuclear distribution during embryonic development, (4) chromosome arrangements during meiosis, (5) NADH imaging of metabolic state, and others, Quantitation methods for micro pharmacology including localized calcium release, caged neurotransmitter release and 3-d diffusion measurements will be illustrated.

Some recent publications on biological applications of two photon excitation are listed for further reference to relevant literature.

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