Multi-Photon Fluorescence Microscopy

Shigeru Hasegawa

Microscience Division, Nippon Bio-Rad Laboratories, 7–18, Higashi-Nippori 5-chome, Arakawa-ku, Tokyo 116–0014

Received for publication December 17, 1997 and in revised form April 21, 1998

Multi-photon microscopy uses a different principle in exciting fluorochromes from conventional epi-fluorescence microscopy and confocal laser scanning fluorescence microscopy. In multi-photon microscopy a femto second mode locked laser is used as an illumination light source which emits near infra-red light of high peak power (over kilo watt order) and short pulse (about 100 femto seconds at FWHM) at high repetition rate (about 100 MHz). Under illumination of very high intensity by laser scanning fluorescence microscopy, a non linear effect becomes

perceivable. Though near infra-red light is not usually absorbed by commonly used fluorochromes in conventional epi-fluorescence microscopy and confocal laser scanning fluorescence microscopy, intense near infra-red light is absorbed by those commonly used fluorochromes in multi-photon microscopy. This non linear interaction of near infra-red light with the fluorochromes under laser scanning microscope introduces unique benefits into the observations of biological specimens.

Key words: Two-photon excitation, Multi-photon excitation, Two-photon microscopy, Multi-photon microscopy, Non linear microscopy, Femto second mode locked laser

I. Introduction

Since multi-photon microscopy was first demonstrated by Denk *et al.* in 1990 [1], many groups of researchers has been developing this technology. The first commercial multi-photon microscope was introduced into the market in 1996.

Multi-photon microscopy offers particular advantages for *in vivo* imaging as the fluorescence excitation is confined to the focal region of the illuminating laser beam. Photo-bleaching and photo-toxicity are minimized due to the reduction in total fluorophore excitation compared to confocal or wide-field imaging so that live samples can be studied for a longer period of time. Multi-photon fluorescence microscopy can also image deeper into the sample than other fluorescence microscopy techniques. These advantages of multi-photon microscopy are particularly important in the study of live cells e.g. brain sections, cardiac cells and developing embryos.

II. Basic Principles of Multi-Photon Fluorescence Microscopy

In the normal process of fluorescence excitation, a molecule which absorbs a photon is excited to a higher energy molecular state and decays back to the ground state in such a way that light of a longer wavelength is emitted as shown in Fig. 1. A longer wavelength photon carries less energy than a shorter wavelength photon. So, if light of too long a wavelength is used, excitation will not occur and there will be no fluorescence emission.

In two-photon excitation, the illuminating light is of a long wavelength with insufficient energy to excite the molecule. However, excitation can occur provided a second photon interacts with the molecule within a sufficiently short space of time after the arrival of the first photon. What occurs is a cooperative process in which the energy of the two photons is combined. In this way one can use red light (e.g. 700 nm) to achieve the equivalent excitation energy of UV light (e.g. 350 nm). In cases where three photons act cooperatively, even shorter equivalent wavelengths can be reached (e.g. 233 nm) [3]. This is beyond what is normally possible in a light microscope.

In order for multi-photon excitation to occur the photons must interact with the molecule within a very short space of time of each other. This time interval is

Correspondence to: Mr. Shigeru Hasegawa, Microscience Division, Nippon Bio-Rad Laboratories, 7–18, Higashi-Nippori 5-chome, Arakawa-ku, Tokyo 116–0014, Japan.

Fig. 1. Fluorescence excitation (molecular transition). In conventional epi-fluorescence microscopy and confocal laser scanning fluorescence microscopy, fluorescent molecule is excited from its ground state to the upper state by absorbing a single photon whose energy is equal to the energy gap of the fluorescent molecule. The excited fluorescent molecule loses a part of its energy by a relaxation process then returns to the ground state by emitting the energy as a lower energy (longer wavelength) photon. In two-photon excitation process, two photons which have about half energy of the energy gap of the fluorescent molecule can cooperatively excite the fluorescent molecule. The following process after the molecule was excited is similar to the process of single photon process.

approximately 10^{-15} sec. Because the excitation efficiency depends on the probability of 2 (or more) photons interacting effectively simultaneously, there is a quadratic dependence (or higher) on laser illumination intensity.

This non-linear dependence of excitation efficiency on illumination means that if a focused laser beam is used, the fluorescence excitation is greatest in the focal region where the intensity is highest. It is in this way that multi-photon illumination causes the excitation to be restricted to a small focal volume.

III. Lateral and Axial Resolution

The lateral and axial resolution in a multi-photon imaging system is primarily determined by the excitation process. This follows directly from the quadratic (or higher) dependence of excitation on illumination intensity. Mathematically, one determines the resolution by calculating the product of the illumination intensity distribution (point spread function) with itself. This is different from what happens in a confocal imaging system where it is the product of the illumination and collection point spread functions which determines the optical resolution. The key difference in practice is that a multi-photon system restricts excitation of fluorescence, whereas a confocal system restricts collection of fluorescence. This is illustrated schematically in Fig. 2.

Williams *et al.* (1994) and others have derived theoretical expressions for the lateral and axial resolution in two-photon imaging [6].

To a good approximation;

$$\text{lateral resolution } (\Phi r) = 0.37\lambda/n \sin \Theta$$

and

$$\text{axial resolution } (\Delta z) = 0.32\lambda/n \sin^2 \Theta/2$$

Fig. 2. Laser scanning microscope optical layout. The most prominent differences between single-photon microscope and two-photon microscope are 1. Excitation occurs only at the focal point of the objective in two-photon microscope and 2. The confocal aperture is not needed any more in two-photon microscope.

Where λ is the excitation wavelength in air, n is the refractive index of the immersion medium and Θ is the half-angle of the objective focus. The numerical aperture of the objective is $n \sin \Theta$. For light of a wavelength of 700 nm and using an objective of numerical aperture 1.3, lateral resolution is approximately equal to $0.2 \mu\text{m}$ and a value for axial resolution of approximately $0.6 \mu\text{m}$ is achieved.

IV. Advantage of Using a Pulsed Laser

The fact that the excitation efficiency in multi-photon fluorescence goes as the squared (or higher) power of the illumination intensity has an important consequence. This is that it is advantageous to use a laser source in which the intensity output is bunched into short pulses. This is the short pulse advantage of multi-photon fluorescence.

Suppose that the laser output is a stream of pulses of width T_p , repetition rate F_p and average power P_{avg} . The fraction of time during which the laser is emitting (duty cycle) is $F_p T_p$. The peak power per pulse is therefore

$$P_{peak} = P_{avg} / (F_p T_p)$$

The average fluorescence intensity I_{fluor} (per second) is then given by

$$I_{fluor} = k(P_{peak})^2(F_p T_p) = k(P_{avg})^2/(F_p T_p)$$

where k is a constant that depends on the cross section for two-photon fluorescence excitation, the numerical aperture (NA) of the focusing lens and on the wavelength of the laser light.

The important conclusion of this analysis is that for a given average laser power and given repetition rate the fluorescence signal increases as the pulse width is reduced. This is the short pulse advantage. For three-photon excitation the advantage is even greater, such that the fluorescence signal increases as the inverse square of the pulse width.

V. Laser Light Sources for Multi-Photon Microscopy

There is great advantage in using a pulsed laser for multi-photon fluorescence microscopy. In practice it is soon clear that to obtain useful signal levels at acceptable average laser power a laser emitting sub-picosecond pulses is required, the optimum being in the region of 100 femtoseconds. This requires a mode locked laser. A further requirement is that the laser emits pulses at a high repetition rate. This is to ensure that there is the least possible 'dead' time between pulses when fluorescence excitation is not occurring. Since the typical lifetime of a fluorescent molecule in an excited state is 2–5 nsecs this suggests that a laser repetition rate of 200–500 MHz would be optimal. At present available lasers have a longer repetition rate of ~ 100 MHz, although in practice this gives satisfactory

performance (Fig. 3 [2]).

VI. Biological Applications of Multi-Photon Imaging

Multi-photon microscopy is particularly advantageous in the study of biological specimens due to the following features: 1. Minimizes bleaching and photo-toxicity effect; 2. Deeper Imaging; 3. Guaranteed co-localization of signals.

The first feature is due to the confined region of excitation of fluorochrome and specimen. Laser light is focused onto the specimen by objective lens, laser beam converges at the focal point and the intensity of laser light is maximized at this point in space. In addition, the excitation efficiency is proportional to the square of the excitation illumination intensity, consequently the excitation of fluorochrome occurs only at the focal point. The fluorochrome and specimen outside of the focal point does not interact with the excitation laser light. This means the fluorochrome out side of the focal point is not photo-bleached and the specimen is not affected from photo-toxicity. This feature minimizes the effect of photo-bleaching and photo-toxicity throughout a whole specimen then enables it to image live samples for extended periods of time.

The second feature is due to the longer wavelength of excitation laser light and the fact that multi-photon microscopy intrinsically does not need the confocal aperture which rejects the emitted light from the out of focus region. The longer the wavelength of the light is, the less scattered the light is. The scatter of the light is proportional to the inverse of the fourth power of the light wavelength. This enables the light of longer wavelengths to penetrate deeper into the tissue. Multi-photon microscopy can collect even the scattered emitted light from the excited focal point because multi-photon microscopy does not need confocal apertures any more. This means multi-photon microscopy can collect more

Fig. 3. Typical time-scales of mode locked laser pulses and the lifetime of a fluorescent molecule.

Table 1. *Fluorescent probes for multi-photon imaging.*

TiSapphire Laser	Nd: YLF Laser
Bodipy	AMCA
Cascade Blue	Bodipy
Calcium Crimson	Calcium Crimson
Calcium Green	Calcium Green (weak)
Calcium Orange	Congo Red
Coumarin 307	Cy-3
Di-I	DAPI (3-photon)
Dansyl Hydrazine	Di-I
DAPI	Evans Blue
FITC	FM4-64
Flavins (auto-fluorescence)	GFP (red-shifted, weak)
Fluo-3	GFPS-65T
GFP (wild type)	Hoechst 33258
GFPS-65T	Hoechst 33342
Hoechst 33258	Mitotracker Rosamine
Hoechst 33342	Nile JC-1
Indo-1	Nile Red
Lucifer Yellow	Oregon Blue
NADH (auto-fluorescence)	Propidium Iodide
Serotonin (auto-fluorescence, 3-photon)	Safranin
TRITC	Texas Red
	TRITC

Titanium Sapphire (Ti:S) laser and Nd:YLF laser are typical lasers used for multi-photon microscopy. Ti:S laser is a tunable laser in the wavelength range of 690 nm~1000 nm and Nd:YLF laser is a fixed wavelength laser of 1047 nm. In spite of the limitation of available wavelength of Nd:YLF, this laser can excite many kind of fluorescent probes.

efficiently the emitted light from the focal point than confocal microscopy. The combination of these benefits enables multi-photon microscopy to image deeper into tissue.

The third feature is due to the fact that a single laser wavelength is used as an illumination source and the region excited by this laser light is confined to a single focal point in space. For the multiple stained sample the multiple laser lights are usually used to excite the fluorochromes. In this confocal case the chromatic aberration of the objective lens becomes noticeable when imaging the deeper sections. The discrepancies between the focal points of multiple wavelengths of light become serious

when identifying the co-localization of fluorochromes in specimen.

Multi-photon fluorescence microscopy will find its application in the study of live cells from the features mentioned above, in particular in the three classes of application as follows [4, 5, 9]: Studies of cell development; Studies of neurons; Studies of cell physiology.

VII. Fluorescent Probes for Multi-Photon Imaging

Table 1 lists the fluorescent probes which so far have been used successfully for multi-photon microscopy. Although operating at a single fixed wavelength, the Nd:YLF laser can be used to image a wide selection of probes. Detailed measurement of two-photon excitation cross sections as a function of wavelength have been made by Xu, *et al.* [7, 8].

VIII. References

1. Denk, W., Stricker, J. H. and Webb, W. W.: Two-photon laser scanning fluorescence microscopy. *Science* 248; 73–76, 1990.
2. Denk, W., Piston, D. W. and Webb, W. W.: Two-photon molecular excitation in laser-scanning microscopy. In "Handbook of Biological Confocal Microscopy Second Edition", ed. by J. B. Pawley, Plenum Press, New York, 1995, pp. 445–458.
3. Maiti, S., Shear, J. B., Williams, R. M., Zipfel, W. R. and Webb, W. W.: Measuring serotonin distribution in live cells with three-photon excitation. *Science* 275; 530–532, 1997.
4. Svoboda, K., Tank, D. W. and Denk, W.: Direct measurement of coupling between dendritic spines and shafts. *Science* 272; 716–719, 1996.
5. Svoboda, K., Denk, W., Kleinfeld, D. and Tank, D. W.: In vivo dendritic calcium dynamics in neocortical pyramidal neurons. *Nature* 385; 161–165.
6. Williams, R. M., Piston, D. W. and Webb, W. W.: Two-photon molecular excitation provides intrinsic 3-dimensional resolution for laser-based microscopy and microphotochemistry. *FASEB J.* 8; 804–813, 1994.
7. Xu, C. and Webb, W. W.: Measurement of two-photon cross sections of molecular fluorophores with data from 690 to 1050 nm. *J. Opt. Soc. Am. B* 13; 481–491, 1996.
8. Xu, C., Williams, R. M., Zipfel, W. and Webb, W. W.: Multi-photon excitation cross-sections of molecular fluorophores. *Bioimaging* 4; 198–207, 1996.
9. Yuste, R. and Denk, W.: Dendritic spines as basic functional units of neuronal integration. *Nature* 375; 682–684, 1995.