TECHNICAL NOTE 03

TECHNICAL

Multi-Photon Fluorescence Microscopy from Bio-Rad

Introduction

Multi-photon microscopy is probably the most important development in fluorescence microscopy since the introduction of confocal imaging in the mid-1980's. It provides researchers with unique possibilities when imaging deep into live tissues and carrying out experiments over extended periods of time.

Bio-Rad, through its unique relationship with Cornell University, where multi-photon fluorescence was first developed, is pioneering the commercial application of multi-photon techniques. Having delivered the first commercial multi-photon system in 1996, and subsequently working with many of the leading researchers in the field, Bio-Rad has the knowledge and experience to ensure that system designs are optimised for multi-photon work and that installations are carried out quickly and effectively.

Basic Principles

In 1-photon fluorescence excitation, a single photon has sufficient energy to excite the fluorescence molecule from ground state to an excited state. The excited molecule then relaxes to a state from which it decays back to ground state with the emission of a longer wavelength photon.

In multi-photon fluorescence excitation, 2 or more photons, which individually have insufficient energy to excite the fluorescence molecule, interact co-operatively to achieve excitation. The excitation process depends on 2 or more photons arriving in a very short space of time (10^{-16} seconds). As in 1 photon fluorescence, the excited molecule relaxes to a state from which it decays back to ground state with the same emission wavelength spectrum as is produced in 1 photon excitation.

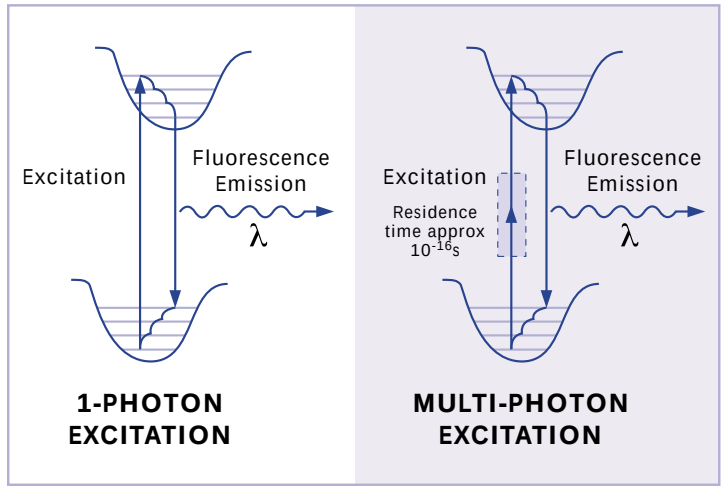

Diagram 1 *Principles of Fluorescence Excitation.*

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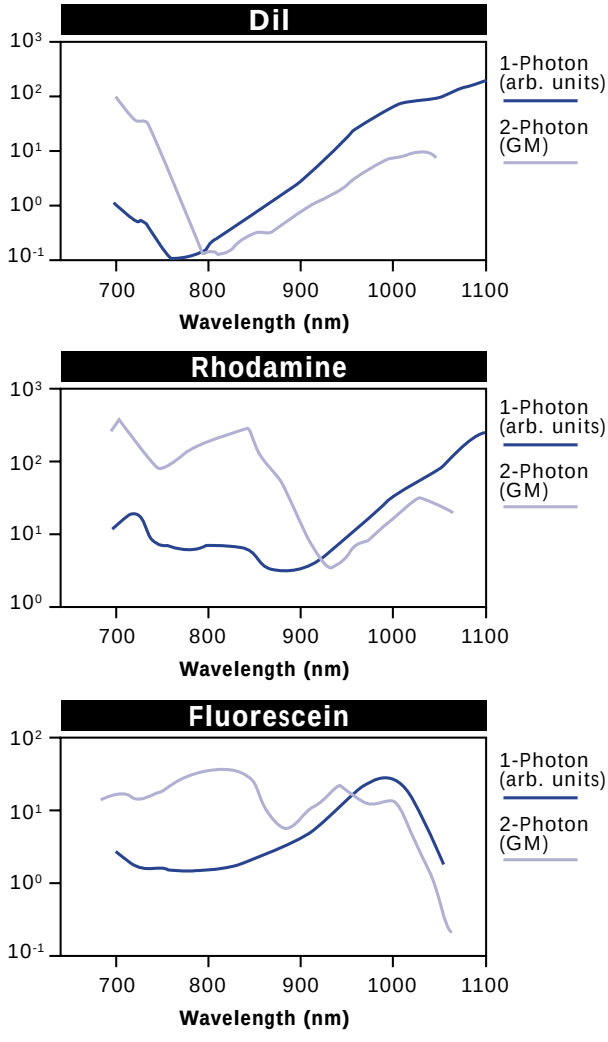
Lasers

A number of laser options are available for multi-photon work. The most commonly employed to date is a tuneable TiSapphire laser which operates in the range 690-1000nm. As a rule of thumb, the 2-photon excitation wavelength is somewhat less than 2 times the 1-photon excitation wavelength for a given fluorophore. Thus the TiSapphire laser, when operated for 2-photon excitation provides excitation wavelengths equivalent to approximately 350-500nm in conventional fluorescence. This covers a wide range of fluorophores commonly used in microscopy. It should be borne in mind however that excitation cross-sections in 2-photon and multi-photon excitation are typically broader than in conventional excitation spectra. This means that wavelengths and filters must be carefully chosen, particularly in two or three colour work to eliminate bleed-through.

All the recommended lasers for multi-photon work have a number of common characteristics; they operate with sub-picosecond, typically 30-200 femtosecond, pulses in order to achieve an adequate 2-photon or 3-photon excitation effect. Repetition frequencies are typically 80-100MHz and the average power is in the range 30-300mW. In choosing a laser for multi-photon work it is important to specify very short pulse widths. This characteristic enables very efficient 2-photon and multi-photon excitation without the need to use excessively high average powers which can be detrimental to the sample.

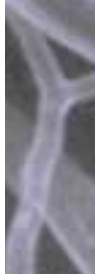
Laser developments for multi-photon work are proceeding very quickly at present. The open and flexible layout of the Bio-Rad MRC-1024MP ensures that new lasers can be accommodated as they become available and if they are appropriate for the work being done.

Fig 1 *Comparisons of 1-photon excitation spectra and 2-photon excitation spectra for 3 fluorophores. 1-photon spectra are plotted at x2 actual wavelength for easier comparison.*



Fluorophores for Multi-Photon Work

Multi-photon microscopy is still in its relatively early stages. This means that not all fluorophores or combinations of fluorophores have been tried experimentally. However, a large number of widely-used fluorescent probes have been reported as being used successfully and in principle any fluorphore that can be excited using 1-photon excitation can also be used with 2- or multi-photon excitation provided a suitable wavelength is selected and the appropriate power and pulse-width laser is used. Most multi-photon work to date has been with a tuneable TiSapphire laser operating between 690 and 1000nm and with 2-photon excitation. The 'Handbook of Biological Confocal Microscopy', 2nd Edition, Chapter 16, lists over 45 fluorophores which can be 2-photon excited in this range. Of this list, examples of flurophores which have been reported so far in multi-photon applications are:



AMCA	Bodipy
Cascade Blue	Calcium Crimson
Calcium Green	FM4-64
Calcium Orange	Coumarin 307
Di-I	Dansyl Hydrazine
DAPI	FITC
Flavins (auto-fluorescence)	Fluo-3
GFP (wild type)	GFP 5-65T
Hoechst 33258	Hoechst 33342
Indo-1	Lucifer Yellow
NADH (auto-fluorescence)	Rhodamine
Serotonin (auto-fluorescence)	TRITC

This list is growing all the time as new fluorophores are tried in 2-photon applications. In addition there is a growing list of fluorophores which have been used with 3-photon excitation and some researchers have shown work where 2 different fluorophores are used, (one being excited under 2-photon conditions and one under 3-photon conditions), both from the same laser excitation source.

Uncaging

Uncaging experiments involve the localised release of an agonist compound by the application of laser light. Caged compounds need UV wavelengths (ie. below 400nm) to activate them. This makes multi-photon microscopy particularly promising for these kind of experiments since uncaging can be achieved without exposing the sample to potentially damaging UV laser light.

Autofluorescence

One of the experimental possibilities with multi-photon excitation is the use of autofluorescence. Although some autofluorescence work can be done using conventional confocal excitation, these experiments are much more viable with multi-photon excitation because of the fact that fluorescent excitation is confined to the focal volume. Using multi-photon excitation also allows imaging much deeper into the sample and this is a major advantage in some kind of autofluorescence experiments; for example, experiments on skin tissue. Relevant experiments with multi-photon have to date, included observations using the autofluorescence of NADH and serotonin.

Bibliography

Multi-photon is a new and rapidly-growing technology. As such the applications and hence number of references are increasing all the time. On the back cover is a partial list of useful references at the time of going to print with this Technical Note. Additional information is available in the Bio-Rad Confocal Microscopy Bibliography, published annually and available from your local Bio-Rad Sales and Support Office.

Additionally, through a unique network of contacts, Bio-Rad specialists are often aware of the very latest work and thinking in multi-photon microscopy and are able to advise on system configurations.

Other Literature from Bio-Rad: MRC-1024 Brochure, Multi-Photon Microscopy Brochure

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