TECHNICAL NOTE 07

TECHNICAL

The Advantages of Femtosecond Pulsed Lasers in Multi-Photon Microscopy

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Introduction

It is now well understood that multi-photon imaging opens up new possibilities both for imaging deeper into samples than previously possible and in the study of living samples for much longer than previously possible. Both these advantages arise from the fact that the multi-photon excitation process is highly localised.

Theoretical analysis and experimental results both show that the full advantages of multi-photon fluorescence imaging are best achieved by use of a laser source emitting pulses in the range 80 – 250 femtoseconds, and with a pulse repetition rate in the range 50-100 MHz. This Technical Note reiterates the reasons why laser sources with these characteristics are preferred and presents both theoretical and experimental background in support of these arguments.

Ideal conditions for multi-photon excitation

Multi-photon excitation relies on creating conditions whereby there is a significant probability of 2 or more photons interacting with the fluorophore almost simultaneously (actually within approximately 10^{-15} seconds). The probability of this happening is only significant where the photon density is at its highest; that is at the focal point of the laser excitation. It is this characteristic which ensures the highly localised nature of multi-photon excitation, hence the experimental advantages described above.

Ideal conditions which maximise the experimental advantages of multi-photon excitation are achieved when;

1. Multi-photon excitation is maximised for any given average laser power.
2. There is an ability to image as deep as possible into the sample.
3. There is flexibility to use 3-photon excitation as appropriate.

Conditions only approach this ideal with very short (femtosecond) pulses.

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Absorption and Scattering of Light in Biological Tissue

All biological tissue absorbs and scatters light to some extent. Absorption is dominated by the presence of water in biological tissue. Scattering is caused by microscopic refractive index changes due to the presence of fine structure in the tissue and is usually the dominant process. The bulk effect of absorption and scattering is to attenuate light passing through the material according to the formula:

$$I = I_0 e^{-(\mu_a + \mu_s)z}$$

where μ_a is the absorption coefficient, μ_s is the scattering coefficient. I_0 is the initial intensity and I the intensity after the light has traversed a distance z in the sample. A measure of the importance of scattering in any particular sample is the scattering length defined as $1/\mu_s$

Absorption in biological tissue is primarily due to the presence of water except in tissues with a high haemoglobin content and vascularisation, such as tumours or in tissue with pigmentation. Absorption is of concern if it leads to local heating of the sample. This question has been considered by Denk et al (1995) who conclude that '*heating effects during high repetition rate pulsed illumination are generally negligible…*'. The reason for this is that in point scanning systems the heating is concentrated into a small volume from which it can diffuse away rapidly before the same spot is re-illuminated.

Scattering in biological tissue is generally more important than is absorption, with the scattering coefficient being as much as 100 times larger than the absorption coefficient (*see Table 1*). All imaging systems must contend with the effects of scattering which reduces both the intensity of excitation light reaching the focus and the intensity of fluorescence escaping from the sample. *Figure 1* shows the calculated magnitude of both of these effects for a typical biological material.

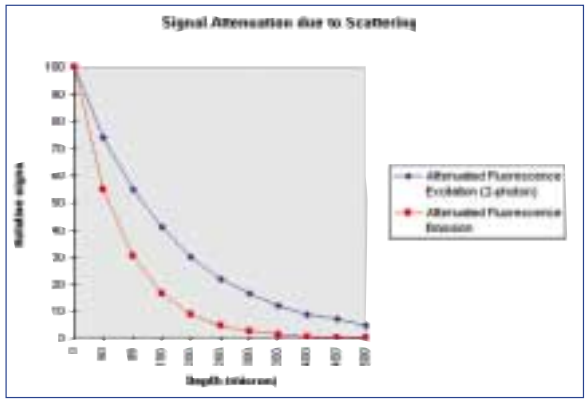

Fig 1

The negative effects of scattering on the detected, emitted signal can be countered by use of a specifically-designed non-descanned detector system (see Bio-Rad Technical Note 5). This specialised detector configuration makes it possible to collect and image fluorescence emission even if it has been scattered as it escapes from the sample. It thus becomes feasible to image deep into the sample where scattering effects in conventional confocal render useful imaging impossible.

The effect of scattering on excitation is such that more and more efficient excitation is needed to achieve satisfactory performance with increasing depth. It is thus the case that to achieve the depth imaging advantage of multi-photon fluorescence one requires a significantly higher laser power for deep imaging than would be used near the surface. The important consequence of this is that the available power from the laser may be what limits the achievable depth of imaging. This is the subject of the remainder of this Technical Note.

Table 1: Measurements of the absorption and scattering coefficients of some typical tissue samples at selected wavelengths.

Tissue	Wavelength λ nm	Absorption Coefficient μ_a mm^{-1}	Scattering Coefficient μ_s mm^{-1}	Scattering Length microns	Reference
Water	700 800 900 1000 1050	0.00067 0.0022 0.0065 0.041 0.013	–	–	Kou et al Svoboda et al
Excised Aorta	633		17 – 31	59 – 32	Keijzer et al
Normal Cortex	674 956	<0.02 <0.02	12.5 10.6	80 94	Bevilacqua et al
Skin (dermal tissue)	650 700 800 900 1000	0.028 0.019 0.011 0.014 0.035	27.5 23.5 19.3 16.6 15.6	36 42 52 60 64	Simpson et al
Skin (subdermal tissue)	700 1000	0.009 0.012	12.2 10.8	82 93	Simpson et al

Optimising Fluorescence Excitation in Multi-photon Microscopy

The preceding discussion has shown that it is an important requirement in multi-photon microscopy to maximise the fluorescence excitation for a given available laser power. As is well known, this is most directly achieved by using a laser source emitting short intense bursts of light at a high repetition rate. A typical laser source gives 100 femtosecond pulses at a repetition rate of 80 MHz. In multi-photon fluorescence excitation the intensity ($\mathbf{I_{f1}}$) depends on the laser pulse width, repetition rate and average power according to the following relationship;

$$\mathbf{I_{f1} = (P_{avg})^n / (\tau^{n-1} F^{n-1})}$$

where $\mathbf{P_{avg}}$ = average power, τ = pulse width, $\mathbf{F}$ = repetition rate, $\mathbf{n=2}$ for 2-photon excitation and $\mathbf{n=3}$ for 3-photon excitation.

The importance of working with femtosecond pulses is now clear. *Figure 2* shows the relative average power $\mathbf{(P_{avg})}$ required to give the same fluorescence signal for different pulse widths between 100 and 1000 femtoseconds. As the pulse width increases more average power is required, the increase being greatest for 3-photon imaging. This curve is plotted on the assumption that additional power is available from the laser up to the highest power required. As already discussed this is unlikely to be the case when imaging deep into tissue. For the real life case where one is using all the available laser power *Figure 3* shows that the fluorescence signal decreases rapidly with increasing pulse width such that for 2-photon excitation the signal is ten times smaller with 1000 femtosecond (1 picosecond) pulses than with 100 femtosecond pulses. For 3-photon excitation the reduction is a hundredfold!

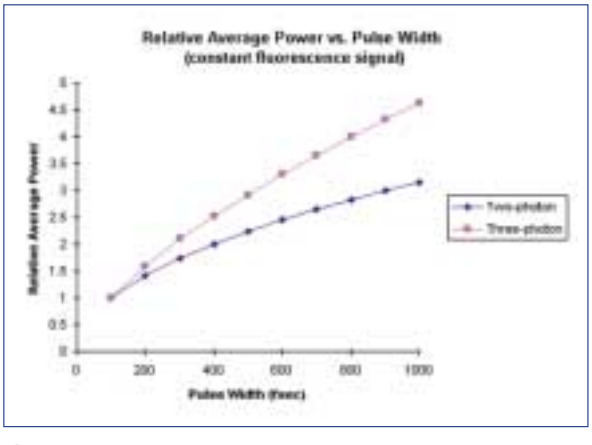

Fig 2

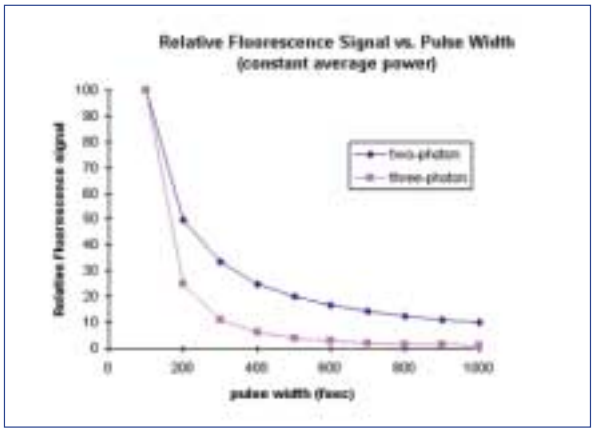

Fig 3

One can draw some important conclusions from these calculations. The most important is that for the excitation conditions needed for deep imaging with 100 femtosecond pulses it will not be possible to image anywhere near as deep using picosecond pulses from currently available laser sources. *Figure 4* shows the relative average laser power needed to image at different depths into a biological sample with 100 femtosecond, 1 picosecond and 4 picosecond pulses. For example if the relative average power for deep imaging with 100 femtosecond pulses is 10 times that needed at the surface, then with 1 picosecond pulses one will only be able to image to half this depth. This effect is even greater with pulses longer than 1 picosecond.

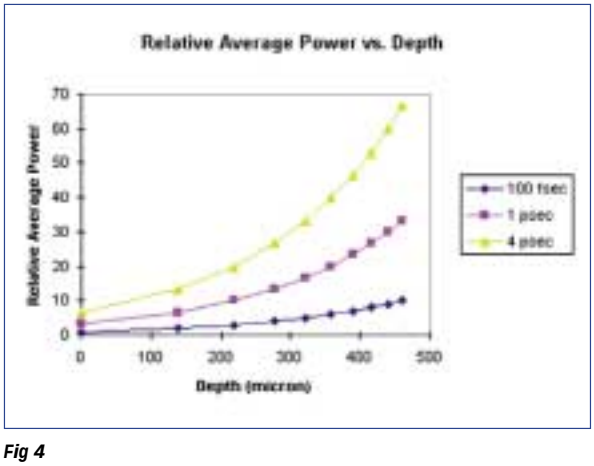

Fig 4

A second conclusion is that for 3-photon fluorescence which always requires higher average laser power on account of the relatively lower excitation efficiency, the arguments in favour of short pulses are overwhelming. Indeed there are no reported examples of 3-photon imaging with picosecond pulses.

Experimental Evidence

Svoboda et al have reported the need to use laser intensities approaching 200 mW when imaging rat neocortical neurons at a depth of 500 microns. In our own work imaging into skin (Brown, unpublished) useful imaging is possible up to the working distance of a high NA lens (several hundred microns) if the laser intensity is increased to approximately 10 times what is needed for imaging near the surface. The group working at Cornell University (Warren Zipfel, private communication) report that the full output of the TiSapphire laser (~500 mWatts) is required for some applications.

Summary

- Multi-photon imaging is able to image deeper into tissue than other methods because it can collect and image fluorescence emission which is scattered.
- To fully exploit the depth imaging advantage it is necessary to increase the laser power for deep imaging to compensate for attenuation by scattering of the intensity at the focus.
- A short pulse laser gives the greatest flexibility in use of the available power for deep imaging. A laser emitting 100 femtosecond pulses will be able to image approximately twice as deep as a laser emitting picosecond pulses if both have the same average power output.
- 3-photon imaging is feasible only with a femtosecond pulsed laser.

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