The Benefits of Non-Descanned (External) Detectors in Multi-Photon Microscopy

Written by: Graham Brown, Bio-Rad Microscopy Division, Hemel Hempstead, UK

- Multi-photon excitation is inherently confocal
- Scattering, which is dependent on tissue type and imaging depth, is a major factor in sensitivity and image quality
- Non-descanned (external) detectors collect all emitted light and significantly enhance imaging in deep tissue or faintly fluorescent samples
- The performance of multi-photon microscopy is significantly enhanced by the use of external detectors, irrespective of scanhead optical configuration

Introduction

The key feature of multi-photon microscopy[1,2] is that excitation and hence fluorescence emission only occurs at the focal plane of the exciting laser beam (*Figure 1*).

In conventional single-photon microscopy (A) excitation (blue) is present above and below the plane of focus causing labelled structures in this region to fluoresce (*Figure 1*). A sub-resolution green fluorescent object is depicted in the focal volume. In the case of multi-photon microscopy (B), ultra-short pulses of infrared excitation (grey) can produce fluorophore excitation in the focal volume where the photon density is sufficiently high. Excitation does not occur to labelled structures above or below the plane of focus.

Since multi-photon excitation generates an image at a single plane within the sample all of the emitted light can be collected and it is therefore unnecessary to re-focus the emitted light back through confocal apertures.

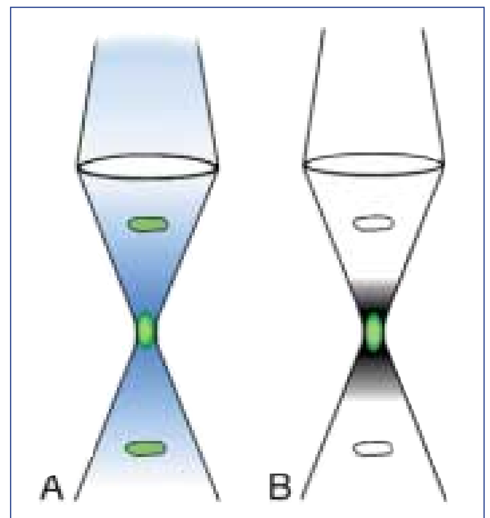

Fig 1 Single-photon (A) and multi-photon (B) excitation.

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Light scattering and its effect upon sensitivity

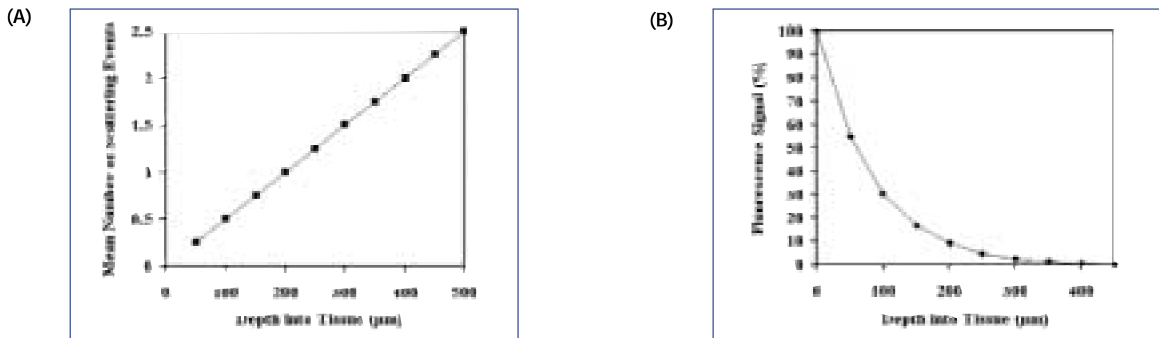

Fig 2 **(A)** Relationship between the number of scattering events and depth of imaging in aortic tissue. **(B)** Signal attenuation with imaging depth due to scattering events.

All biological tissue scatters light. The degree of scattering depends on the depth of imaging and the type of tissue. *Figure 2A* shows the number of light scattering events (at a wavelength of 500nm) as a function of depth, in this example of aortic tissue[3]. Because of this scattering, a significant percentage of the emitted fluorescence cannot be imaged in a confocal system (see below). *Figure 2B* shows how the fluorescence signal is attenuated due to scattering as a function of depth into the tissue. At a depth of as little as 100 µm 70% of the fluorescence signal is lost due to scattering within the sample.

These calculations clearly demonstrate that scattering is the primary cause of signal loss when imaging deep into tissue and emphasise the importance of collecting as many scattered photons as possible. Hence, in order to efficiently image from deep into tissue and/or low signal samples, a detector which has been optimised to collect as much emitted light as possible – particularly scattered light – must be employed. Non-descanned detectors are designed and positioned in the system so that this is ensured.

How external detectors collect more scattered light

In a confocal microscope, the detectors are placed beyond the confocal apertures. These apertures are necessary to eliminate out-of-focus light which would otherwise ruin the image quality. In order to make this work, emitted light from the focal point is 'descanned' back via the scanning system so that it passes back through the confocal aperture and is collected.

In multi-photon microscopy, the emission only comes from the focal point hence the emission is inherently confocal.

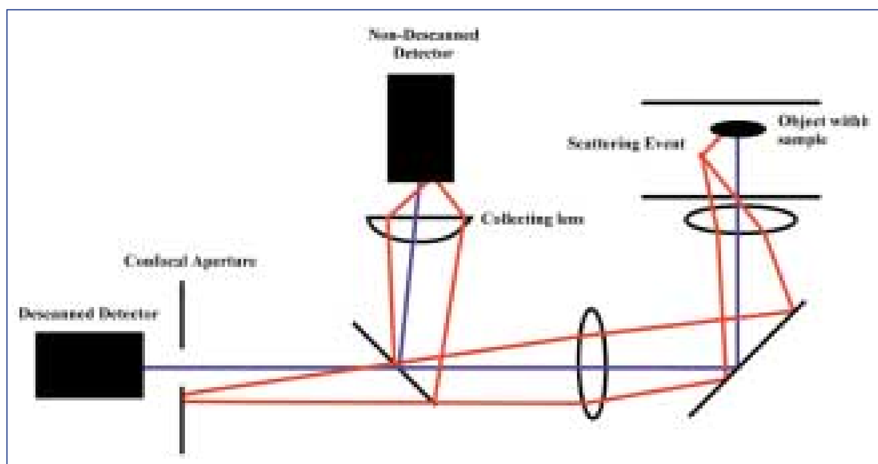

Fig 3 Diagram of the emitted light path from a scattering sample showing the advantage of non-descanned detectors.

Descanning the emitted signal is therefore unnecessary and non-descanned or external detectors can be used to significantly enhance performance.

Light emitted ballistically (blue) would be collected through the confocal aperture. Light that undergoes scattering events (red) is lost to the conventional descanned detector as it does not pass through the confocal aperture. By designing the optics of the non-descanned detector to collect all of the emitted light, ballistic and scattered, sensitivity is significantly enhanced without loss of image quality.

The Bio-Rad external detector unit

On the Bio-Rad MRC-1024MP system the non-descanned detectors are positioned external to the scanhead and as close to the sample as possible. The configuration of these external detectors is optimised to collect the maximum possible emitted light, including scattered light (*Figures 3, 4 & 5*).

Fig 4 (right) The position of the external detector unit, and electronics control unit, in relation to a typical Bio-Rad multi-photon system, in this case an MRC-1024MP installed on a Zeiss inverted microscope. Other configurations are also available.

Optical design

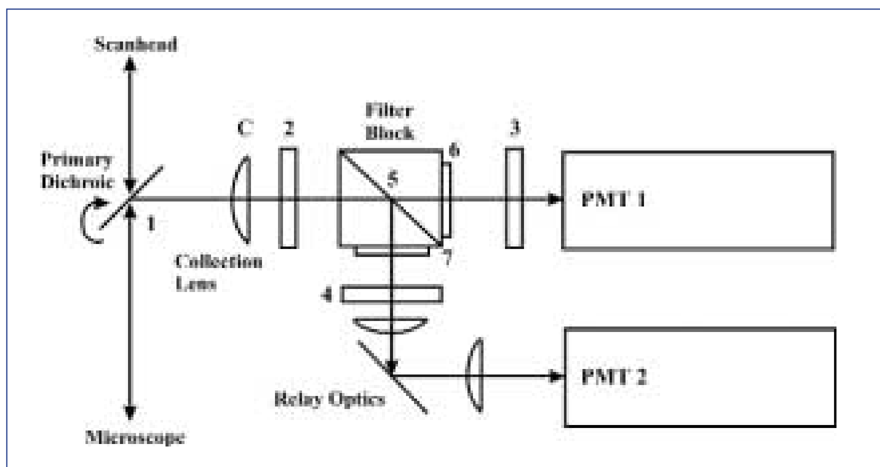

Fig 5 External detector schematic.

1	=	Primary dichroic transmitting infra-red light to the sample and reflecting emitted fluorescence light to the non-descanned detectors
C	=	Collection lens positioned to gather the emitted light
2,3,4	=	Blocking filters selected to remove any reflected infra-red light
5	=	Filter block dichroic splitting light into PMT1 and PMT2
6,7	=	Emission filters to further select the wavelength of light entering the PMTs
PMT 1,2	=	Photomultipliers.

The external detectors are designed to offer maximum experimental flexibility with virtually all components being user-interchangeable. *Figure 5* is a schematic of the external detector unit showing its key components.

Filter Options

The table below illustrates the filter options available.

	1	2	3	4	5	6	7
Green/Red (Fluorescein, GFP/Rhodamine)	670nm UVDCLP	-	HQ575/ 150nm	HQ575/ 150nm	550nm DCLP	D605/ 90nm	D525/ 50nm
Blue/Red (DAPI/Rhodamine)	670nm UVDCLP	-	HQ575/ 150nm	E625nm SP	490nm DCXR	D605/ 90nm	D455/ 30nm
Blue/Green (DAPI/Fluorescein)	670nm UVDCLP	E625nm SP	-	-	490nm DCLP	D525/ 50nm	D455/ 30nm
UV/Visible (Serotonin/Fluorescein)	670nm UVDCLP	-	-	-	UV400nm DCLP	HQ575/ 150nm	UG11/IR
UV (Serotonin)	670nm UVDCLP	UG11/IR	-	-	-	-	-
Indo-1	670nm UVDCLP	BGG22	-	-	UV440nm DCLP	460nm DCLP	D390/ 70nm

Key Features

- Moveable primary dichroic which can be positioned to allow the use of either descanned (internal) or non-descanned (external) detectors. This can also be replaced by a shorter wavelength dichroic so that a descanned detector and the non-descanned detectors can be used together.
- Key optical elements are all user exchangeable allowing maximum flexibility for future applications and experimental designs.
- Bio-Rad unique prism enhanced photomultipliers provide maximum sensitivity over a wide range of collection wavelengths. For more specialised applications, a blue sensitive photomultiplier can be fitted.
- Control of gain and black levels is provided through an external electronics unit.
- A third electronic control channel is provided for the integration of an additional 'custom' detector. This can be positioned by the user providing greater flexibility in experimental design.
- Photon counting, a particularly effective technique in detecting very low light levels, is retained with the external detectors. This technique, a unique Bio-Rad feature, digitally processes the output signal at low light levels increasing the signal to noise ratio.

Examples of image enhancement from non-descanned detectors

Increased sensitivity at depth

As discussed above, the key benefit of external detectors is increased sensitivity in samples which scatter the emitted light (i.e. almost all biological samples). A comparison between internal and external detectors is illustrated in *Figure 6*, which shows a four-fold increase in signal using external detectors when imaging a 10% intralipid sample. (Courtesy of Warren Zipfel, Cornell University).

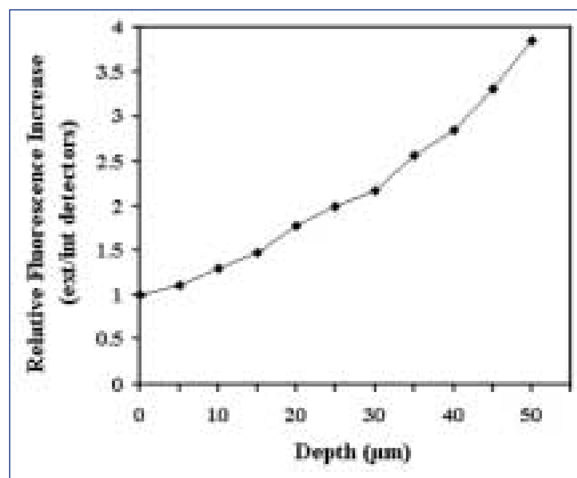

Fig 6 Relative increased performance of external over internal detectors on increasing depth of imaging.

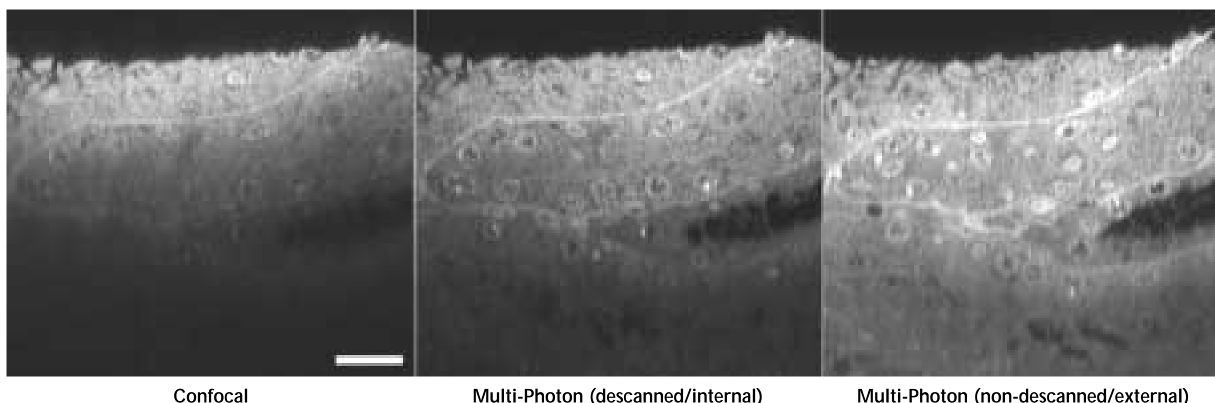

Fig 7 Comparison of imaging penetration depth with different imaging modes.

The figure shows XZ profiles through an acid fucsin stained, monkey kidney pathology sample imaged through a depth of 140 µm with confocal microscopy at 2 µW of 532 nm light, multi-photon microscopy at 4 mW of 1047 nm light detecting the emission by descanned/internal and non-descanned/external detectors. Scale bar represents 20 µm (Courtesy of Victoria Centonze Frohlich, Deputy Director, IMR University of Wisconsin, Madison).

This example shows that far more information can be obtained at depths of over 100 µm into the sample using the external detectors, revealing details that are not visible using internal detector collection.

Conclusions

As the majority of biological samples scatter light it therefore becomes critical to capture as much of this scattered light as possible. The inherent confocality of the multi-photon process allows detectors without pinholes to be located outside the scanhead, close to the sample. These non-descanned detectors are designed to collect scattered light for greater sensitivity in multi-photon imaging, allowing deeper optical sectioning and detection of faint fluorescent signals.

References

1. *Two-photon laser scanning microscopy.* DENK, W., STICKLER, J.P. AND WEBB, W.W. (1990) Science **248**, 73-76.

2. *Two-photon molecular excitation in laser scanning microscopy.* DENK, W., STICKLER, J.P. AND WEBB, W.W. (1995) Handbook of Biological Confocal Microscopy. Ed. Pawley, J.B. Plenum Press, New York.

3. DURKIN et al. (1994) Applied Optics **33**, 414-423.

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Microscopy Division

U.S. (800) 4BIORAD **California** (510) 7411000 **Australia** +61 (2) 99142800 **Austria** +43 (1) 8778901
Belgium +32 (9) 3855511 **Canada** +1 (905) 7122771 **China** +86 (10) 62051860 **Denmark** +45 (39) 179947
Finland +35 (8080) 42200 **France** +44 0800 909190 **Germany** +49 (89) 318840 **Hong Kong** +852 (2789) 3300
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The Netherlands +31 (31) 8540666 **New Zealand** +64 (9) 4433099 **Singapore** +65 (272) 9877 **Spain** +34 (1) 6617085
Sweden +46 (8) 6275000 **Switzerland** +41 (1) 8095555 **United Kingdom** +44 (0)181 328 2000

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