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Short communication

Implementation and evaluation of a detector for forward propagated second harmonic signals

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Abstract

Second harmonic generation (SHG) is emerging as an alternative non-linear imaging method. The fact that most commercial multi-photon microscopes can be easily adapted to image SHG makes it appealing to explore the kind of sample information given by SHG. Here we describe an SHG detection implementation designed to optimize the collection of forward propagating light. A Hamamatsu H957-08 PMT is inserted at the back-focal plane of the condenser on an inverted Nikon TE300, and controlled by the existing electronics of a BioRad 1024MP system. Evaluation of the performance was done on common SHG generating preparations, KH_2PO_4 crystals and collagen. We concluded that positioning a detector at the back focal plane of the condenser provides a highly efficient detection system for second harmonic signals, with many advantages over a detector sited at the lamp housing.

Keywords: Second harmonic generation; Multiphoton microscopy; Collagen; Titanium–sapphire lasers

In the last decade there has been a significant increase in the application of multiphoton excited fluorescence (MPE) in the biological sciences (Denk et al., 1990; Cox and Sheppard, 1999; Feijó and Moreno, 2003). This is mainly due to two consequences of excitation in the near infrared: better penetration because of lower scattering and reduced phototoxicity due to lower photon energy. The fact that one laser can be tuned to a wide range of wavelengths and thus excite many different fluorochromes is an additional benefit. Microscopes used for MPE are in principle also suitable for imaging by second harmonic generation (SHG). Instead of using light to excite a fluorescent molecule, SHG requires a molecule with no symmetry center, which will generate the second harmonic at twice the frequency of the illuminating light. The emitted light can then be collected with a detection system similar to the fluorescence one except that the emitted light will have exactly half the wavelength of the excitation. Since the cross-section for this phenomenon is quite low, one will typically need several of these molecules ordered in a regular structure producing constructive interference to give a substantial emission.

The adaptation of this system to the microscope was first attempted in the 1970s (Hellwarth and Christensen, 1974; Gannaway and Sheppard, 1978) and was used in biological applications for the first time in the 1980s (Freund et al., 1986). Since there is no absorption in the imaging process, there is also no photodamage so photobleaching is not an issue. Some reported applications of this technique are visualisation of collagen (Freund et al., 1986; Cox et al., 2003a), and other endogenous proteins (Mohler et al., 2003) and measurement of membrane potential (Moreaux et al., 2000).

Due to the coherent nature of the process, the emitted light is propagated mainly forward, unlike the situation in fluorescence. Even when multiple SHG dipoles are arranged in an irregular array, the forward propagated wavefronts will be in phase whereas those propagated backwards are not. For many samples, therefore, the SHG signal will be propagated mainly forward and a suitable detector for this signal is required.

This detector needs to be positioned at a *pupil* or *back focal* plane (BFP). At this plane the scanned beam varies only in angle, not position, and therefore only at this plane

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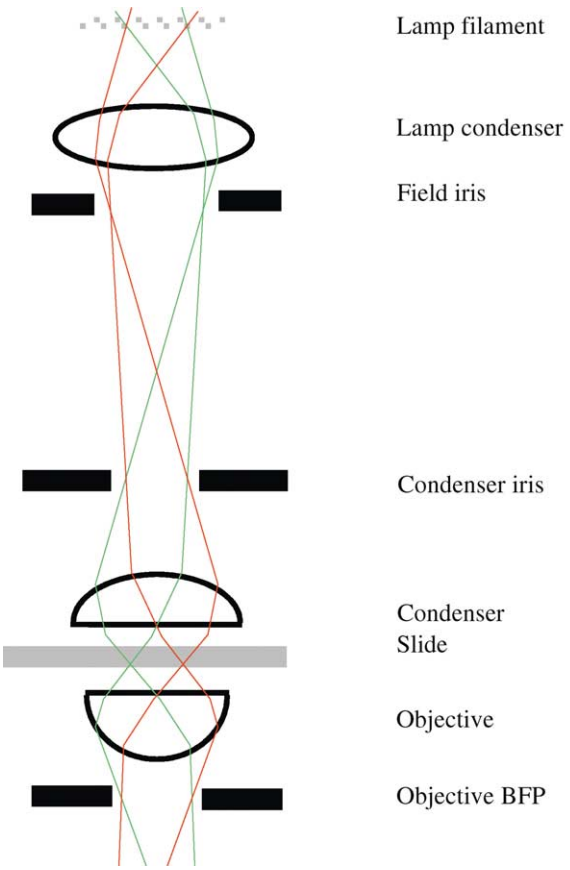

Fig. 1. Ray paths in an inverted microscope set up for Köhler illumination. Two separate positions of the scanning beam are shown (red and green). At the plane of the condenser iris (condenser BFP) the position of the beam is the same whichever spot is being scanned. At the plane of the field iris the scan pattern is imaged, and then at the lamp filament we have a BFP, and the beam position is again independent of the scan.

can one avoid vignetting. Fig. 1 shows a diagram of the various image and pupil planes in Köhler illumination for a scanning microscope. Two positions of the scanning beam are shown.

The plane of the filament of the widefield illumination lamp is a pupil plane, and most adaptations of commercial MPE microscopes to SHG imaging have used a modified detector originally intended for non-confocal imaging by transmitted light, and placed in a plane conjugate with the field iris of the tungsten illuminator. A mirror either directs the signal to the detector or allows the illuminator to be used for conventional wide-field imaging (Cox et al., 2002). Since a standoff piece is typically required to house the mirror, an additional transfer lens will also be required.

Such a layout has several disadvantages. It is difficult to exclude room light from the detector. There are several optical elements—typically uncoated—in the path, so losses will be substantial. Since this is a focussing system—an image of the scanning spot is formed at the plane of the field iris—the advantage of non-descanned detection in picking up scattered light which will not come to focus at an image is partly lost.

As Fig. 1 shows, the back focal plane of the condenser is from many aspects a more appropriate choice. It is the first BFP after the sample and therefore the closest place at which a detector can be positioned. With no intermediate image of the scanned spot, collection of scattered light will be maximised. We therefore set out to equip a MPE microscope with a detector positioned immediately behind the condenser iris. It is designed to require only minimal modification to the microscope, and not to prevent the operation of any existing microscope functions.

The microscope is a Bio-Rad MRC1024ES MP confocal microscope, equipped with a Coherent Verdi-Mira 900f titanium sapphire femtosecond pulsed laser and dual-channel non-descanned detectors, mounted on a Nikon Eclipse TE300 inverted microscope. A 0.85 NA condenser was fitted.

The photomultiplier used was a Hammamatsu H957-08 module, which combines an R928 side-window photo-multiplier tube (PMT) and its high-voltage power supply in one cylindrical housing. The active area of the Hamma-matsu R928 photocathode is a good match for the pupil size at the condenser BFP. A 15-V power supply is required for which we used a Lascar PSU203 but any stable 15-V dc supply would be adequate. The signal was taken to a Bio-Rad preamplifier which on a normal Bio-Rad end-window Thorn PMT is attached to the PMT base. The Bio-Rad MRC 1024 MP, when equipped with a two-channel non-descanned detector (NDD), has an external control box and amplifier unit equipped to handle three channels. This conveniently enabled us to integrate the new detector into the normal control system of the microscope by feeding the signal from the pre-amp to the spare channel of the NDD amplifier. Shielded coaxial cable was used for all signal connections. The wiring arrangements are shown schema-tically in Fig. 2a.

High-NA condensers on Nikon inverted microscopes are mounted on a tubular spacer and this was drilled to enable the cylindrical housing of the H957 to be slipped in. The metal of the spacer is thick enough for a grub screw to be fitted to retain the detector. (It is important to locate the grub screw at the base of the housing as it is not permissible to

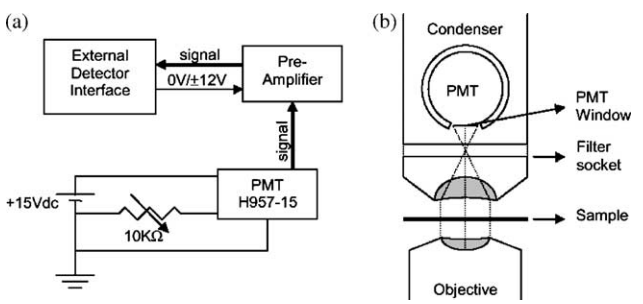

Fig. 2. (a) Schematic representation of the connection between the PMT power supply, the variable resistor, PMT, Pre-Amplifier and the external detector interface, on with the final signal was attached for producing a scanning image. (b) Scheme of the light path and the mounting positions of the devices.

Fig. 3. Photograph of our detector installed behind the condenser of the TE300. PMT—the Hammamatsu H957 photomultiplier module; S—locking screw to secure it in place; F, filter holder (originally holder for polarizer). The objectives are visible beneath the stage.

clamp the top of the housing.) Fig. 2b shows the arrangement in diagrammatic form and Fig. 3 in a photograph. The detector can be removed by slackening this one screw, returning the microscope to normal operation. In front of the detector is a filter slider, normally used to hold the polariser for differential interference contrast, which is a convenient place for barrier filters. It is also possible to drop a circular filter into the condenser housing above the iris, and this could be used for a permanently mounted infra-red blocking filter, since this would not hinder routine use of the microscope. Because the detector is totally enclosed it is much less susceptible to external room light than a detector at the lamp housing would be.

Several samples were used to test the detector and to compare the signals propagated forwards and backwards. Potassium dihydrogen phosphate (KH_2PO_4) crystals were ground in a small pestle and mortar and mounted in immersion oil. KH_2PO_4 crystals are very strong SH generators, and because they are of substantial size theory predicts that propagation of the SHG signal will be predominantly forward. Fig. 4 shows that the differences are clear. In the forward direction the image is around five times brighter. It is not possible to make any quantitative comparison since the two PMTs will have different characteristics but qualitatively this is striking, given that the forward signal is collected at a NA of 0.8 versus the NA 1.3 of the objective.

We propose in the future to fit an oil-immersion condenser which will both improve collection efficiency and reduce the entry of stray light, as in Cox et al. (2002). Campagnola et al. (1999) used an oil-immersion objective to collect the SHG signal, reporting that it was more efficient than a high NA condenser. Since an oil-immersion condenser would typically have far fewer glass elements than an objective of the same NA it is hard to see why this would be so. Possibly the smaller pupil size of an objective is beneficial in their layout since they project the light via a mirror and transfer lens to the detector. The limited working distance of an objective would require the sample to be between two coverslips and would severely restrict the imaging of thick samples. Furthermore the back focal plane of an objective is typically inside the lens barrel and thus inaccessible. Use of a detector matched to the pupil size of an oil-immersion condenser is a more effective geometry and imposes fewer limitations on the microscope.

Collagen is the predominant source of SHG in animal tissue and SHG imaging has already proven its value as

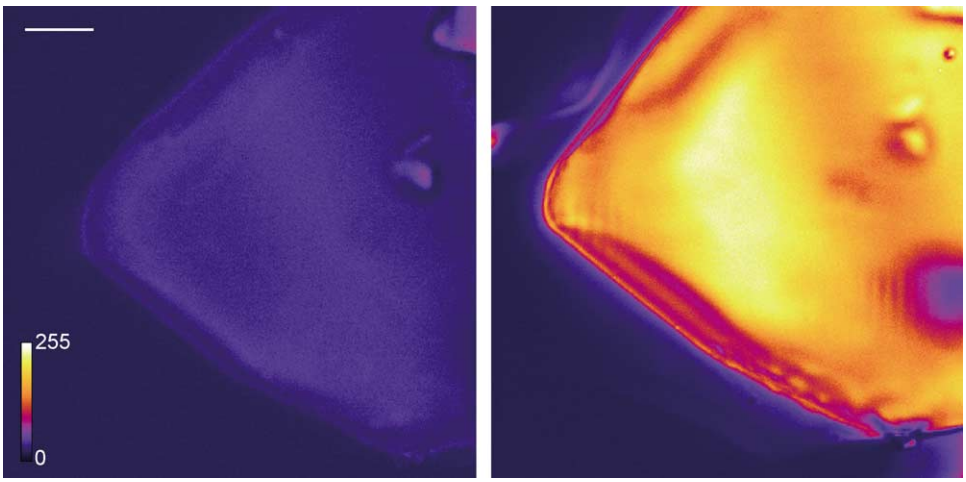

Fig. 4. Crystals of KH_2PO_4 mounted in immersion oil. Images collected by forward and backward detection. Band pass filter D455/30, for both back and forward directions, using 910 nm excitation. Scale bar 25 μm.

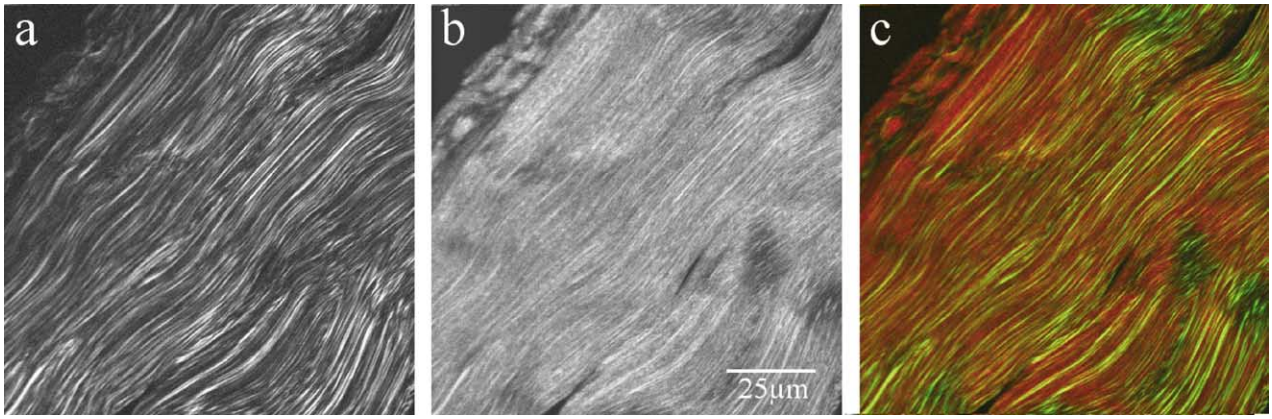

Fig. 5. Cryo-section of unfixed human Achilles tendon biopsy. (a) Forward propagated signal (no filtration other than 650SP to block infrared)—since there is very little auto-fluorescence in this material only SHG is seen. (b) Backward propagated signal, using 400BP60 filter so that only second harmonic is seen. Excitation 840 nm.

a mode of imaging the distribution of collagen in a wide variety of tissues (Cox et al., 2002, 2003a). In the context of our new detector it provides an interesting comparison between the signals propagated in different directions. Fig. 5 shows SHG signals from biopsy samples of human Achilles tendon, mounted in gelatine, detected in both directions. The difference between the two SHG images is quite striking, and is potentially a very useful tool. The highly crystalline type I collagen at the centre of each fibril produces a signal which has a stronger forward-propagating component than the more amorphous collagen (type I plus type IV) surrounding it. Hence, different collagen moieties within the tissue can be distinguished, without any staining. Similar behaviour has been described in purified collagen (Cox et al., 2003b).

Positioning a detector at the back focal plane of the condenser provides a highly efficient detection system for second harmonic signals, with many advantages over a detector sited at the lamp housing. Our implementation is low-cost and easy to implement and does not have any adverse effect on conventional microscope functionality—even the Bio-Rad photodiode transmission detectors remain in place and can be used when the PMT detector is withdrawn.

The transmission detector is also capable of picking up MPE fluorescence, with appropriate filters, and may be particularly useful when scanning deep into tissue. The far red or infrared exciting beam penetrates tissue extremely effectively (Denk et al., 1990) since it is scattered much less than the short wavelength light used in single photon excitation. The limiting factor therefore becomes the ability of the fluorescence to escape. Picking up the signal which passes through the tissue as well as that which returns through the objective lens may be a very useful bonus.

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