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CHARACTERISING THIN FILMS USING PICOSECOND LASER ULTRASONICS

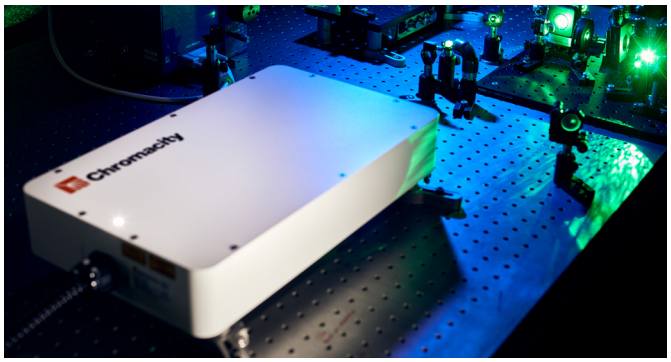
Advanced semiconductor metrology making use of the Chromacity 520

Introduction

Picosecond ultrasonics has become a widely used metrology tool in the semiconductor device industry. It provides an accurate method for the measurement of the thickness of thin films, can determine the quality of the bonding between a film and a substrate, and gives information about the mechanical properties. The overlay and alignment of a lithographically defined pattern on top of an underlying layer is fundamental to device performance.

There are a wide variety of optical techniques that are used to monitor this alignment in conventional production flows. Typically, either an ultraviolet, visible, or infrared light is coupled through a top photoresist layer or an etched hard mask to be aligned to the bottom layer. However, in some production flows there may be an opaque layer that interferes with the measurement. In such cases, conventional methods of alignment using light fail. To overcome this issue, extra patterning operations may be used to open areas around the alignment features, but these operations add significant process cost.

The picosecond laser acoustics (PLA) technique has been widely used in thin metal film metrology because of its unique advantages such as being a rapid, non-contact, non-destructive technology and its capabilities for simultaneous multiple layer measurement (even through opaque layers). Measuring velocity and thickness simultaneously for transparent and semi-transparent films offers the ability to not only monitor the growth process but offers insight into device performance.



Ultrasonic Technique

PLA is a well-established technique with an extensive publication history. Briefly, an ultra-fast laser generates light pulses of duration 100 to 200 fs with a repetition rate typically around 80 MHz. Each pulse is divided by a beam-splitting mirror to give a “pump” pulse and a “probe” pulse. The pump pulse is focused onto a selected area of the surface of the sample, and the absorption of the light results in the generation of a sound pulse. The sound propagates through the sample and when it reaches an interface is partially reflected back to the sample surface.

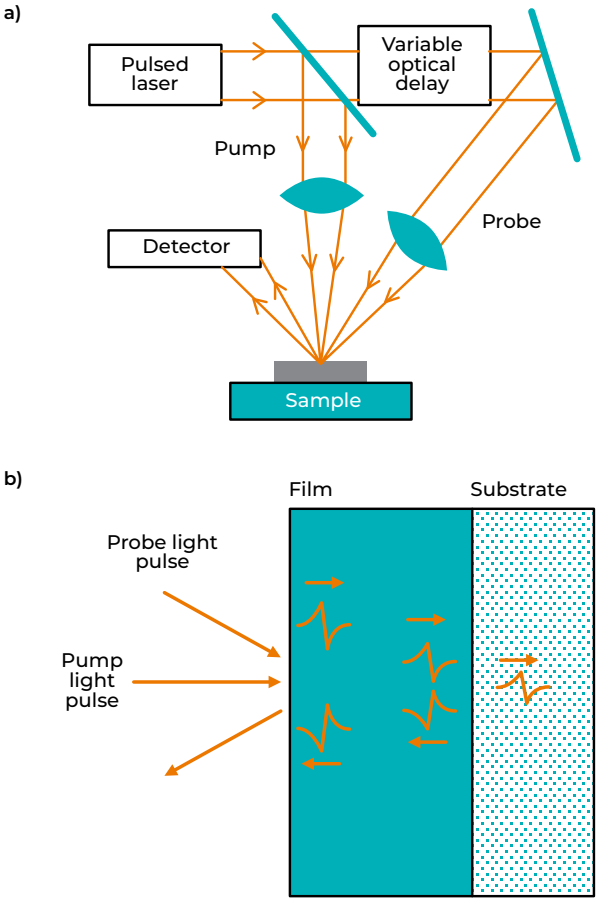


FIGURE 1. a) Block diagram of typical setup. b) A strain pulse is generated by the pump, is reflected at the interface between the film and the substrate, and is detected by the probe pulse when it returns to the free surface.

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The returning sound causes a small change in the optical reflectivity of the sample and this change is measured with the probe light pulse. The time of arrival of the probe pulse relative to the time of application of the pump is adjusted through the use of a variable optical path.

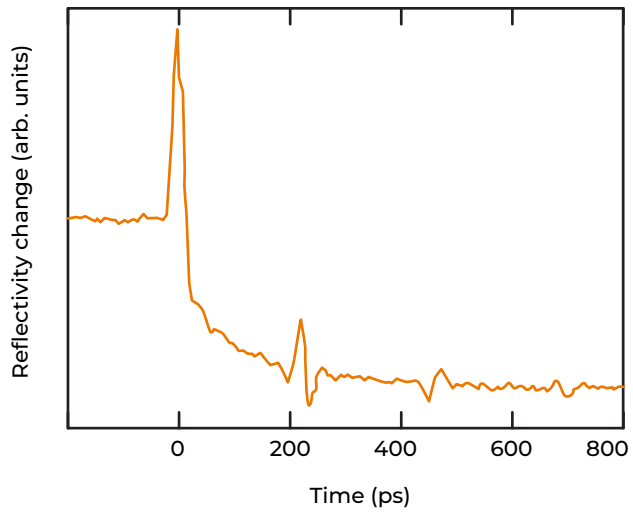
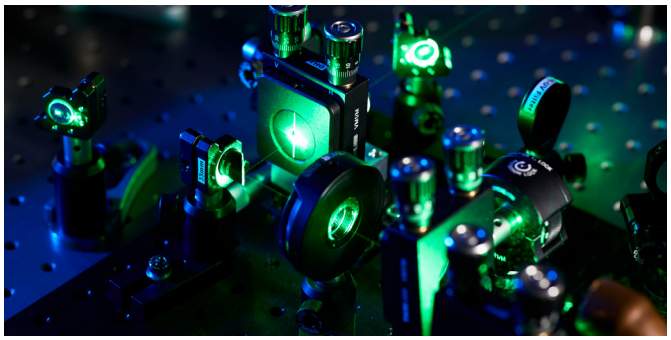


FIGURE 2. Example of acoustic echoes detected in a film of As_2Te_3 of thickness 2200 Å.* These echo signals can provide a high-resolution measurement of the thickness of the layer.



Technology Fit

One of the unique advantages of PLA technology is that it offers a first-principle approach to modelling the measured data. Thickness measurements rely on locating the echoes and transit time of the acoustic pulse through the films. Using the known speed of sound from literature, the thickness of a particular layer can be readily calculated without the need for additional calibration. Competing technologies like X-ray metrology require daily or weekly standards and cannot measure repeating layers in a multi-layer stack.

The PLA technique can measure metal films with thicknesses ranging from 50Å to 35 µm. Measurements take a few seconds per site and the high throughput allows mapping of a whole wafer in minutes.

Over the years the technique has been refined to the point that it has also show sensitivity to detect concentration changes by monitoring the changes in velocity of the sound wave.

Chromacity laser for ultrasonic technic

The Chromacity 520 offers characteristics in line with the requirements to execute PLA and flexibility to progress towards better metrology equipment performance:

Chromacity 520 core specifications:

Wavelength	520 nm
Pulse duration	100 – 200 fs
Repetition rate	50 – 200 MHz
Pulse energy	Up to 80 nJ if required

* C. Thomsen, H.T. Grahn, H.J. Maris and J. Tauc, Phys. Rev. B34, 4129-4138 (1986)

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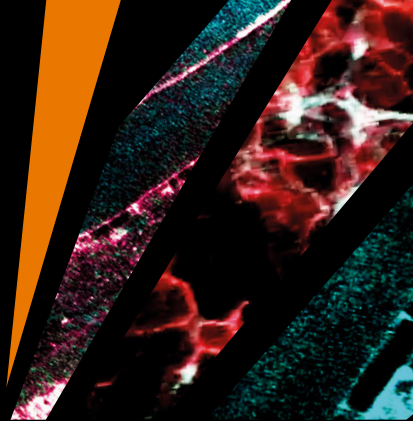

The Chromacity 520 offers characteristics in line with the requirements to execute PLA and flexibility to progress towards better metrology equipment performance.

Wavelength

Aluminium has largely been replaced by copper which has lower resistivity. It turns out that at the light wavelength generated by the first generation of compact ultrafast laser (800 nm), the piezo-optic coefficient of copper is extremely small. Thus, the change in optical reflectivity due to the returning sound echo is too small to measure accurately. To overcome this difficulty, it has been necessary to frequency double the light from these near-IR lasers. This is why fixed wavelength sources operating at ~520 nm are the ideal candidates for this technique.

Pulse Duration

To create an acoustic wave within a device the pump pulse must be of the order of 100 – 200 fs. This allows the creation of a kW peak power pulse within the device without generating unwanted thermal expansion. Shorter pulses, allowing thinner layer measurements, become critical considering the year-on-year reduction of film thickness. The Chromacity 520 offers stable and clean ultrashort pulses guaranteeing accurate results for metrology equipment measuring today's films but also the next generation to come.

Repetition Rate

Pulse repetition frequencies of 63 – 80 MHz have been cited in the literature. The higher the repetition rate the quicker the measurement. 80 MHz is the typical repetition rate used for the first generation of ultrafast lasers emitting at 800 nm. Chromacity technology platform allows an access to higher repetition rates (up to 200 MHz) that could, in the near future, be more appealing for PLA to reduce the time required to perform measurements, thus increasing the efficiency and cost of metrology equipment.

Pulse energy

Pulse energies for PLA are of the order of a few nJ at the sample plane (the literature indicates typically pulse energies ~0.5 nJ). This fits well with the high repetition rate nature of the Chromacity laser systems. With higher pulse energy, measurements of thicker films can be achieved, which is well within the Chromacity 520 capabilities. Our unique patented laser platform can achieve up to 80 nJ pulse energy (depending on repetition rate and pulse durations) with no compromise on the laser stability over time.

Chromacity 520 offers stable and clean ultrashort pulses guaranteeing accurate results for metrology equipment measuring today's films but also the next generation to come.

For more information about Chromacity capabilities for semiconductor applications: https://chromacitylasers.com/applications/lasers-semiconductor-failure-analysis/

For more information about the Chromacity 520 ultrafast laser: https://chromacitylasers.com/ultrafast-lasers/chromacity-520/

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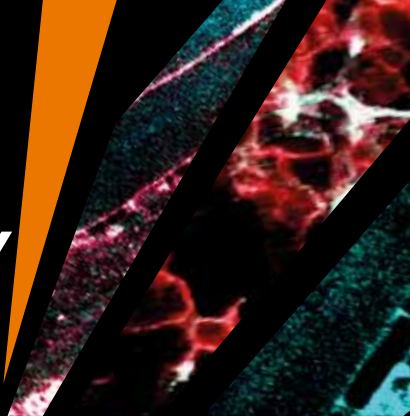
TWO-PHOTON FLUORESCENCE MICROSCOPY

with the Chromacity 1040



The Chromacity 1040 provides the perfect balance of peak-power, pulse duration and average power for generating images of biological samples through a two-photon non-linear process. In this application note, we illustrate the suitability of the Chromacity 1040 for two-photon microscopy, to image a wide range of cells with various labels.

Two-photon fluorescence microscopy

In recent years, two-photon fluorescence microscopy has become a powerful tool to study biological functions in vivo. In comparison to many standard imaging techniques it has become vital to optogenetic research as it has enabled deeper imaging in highly scattering brain tissue with reduced photobleaching and improved spatial resolution. The main advantages of two-photon fluorescence microscopy is that it enables excitation with a source which scatters less and only excites samples in the focal plane, thereby providing better spatial resolution. This requires a highly stable femtosecond laser, which provides the required combination of pulsewidth, pulse energy and average power to enable high-resolution imaging of biological samples. A range of samples have been imaged using the Chromacity 1040 to demonstrate its two-photon fluorescence microscopy capabilities.

Unlike solid-state lasers, which can produce beams with an elliptical cross-section, the Chromacity 1040's beam originates from a single-mode fibre, so it is perfectly symmetrical.

Imaging of invitrogen fluocells, kidney, liver and collagen

The Chromacity 1040 was used as the excitation laser to selectively highlight several different markers in a range of samples. Unlike solid-state lasers, which can produce beams with an elliptical cross-section, the Chromacity 1040's beam originates from a single-mode fibre, so it is perfectly symmetric. This makes the laser system ideally suited for coupling into commercial laser-scanning microscopes to provide excellent beam shape and high average powers at the sample plane.

Fig. 1 shows typical images acquired from the system when imaging; Fig. 1(a) - Invitrogen Fluocells - section of mouse intestine (cells approximately 5 µm in diameter) via excitation of SYTOX® Green; Fig. 1(b) demonstrates liver cells imaged using direct excitation of RFP, YFP and GFP using the same excitation laser (images overlaid); and Fig. 1(c) which shows kidney cells overlaid by collagen and demonstrate the ease with which samples can be differentiated using mT:mG as a marker and SHG from the collagen fibres (making it easy to differentiate between cell types).

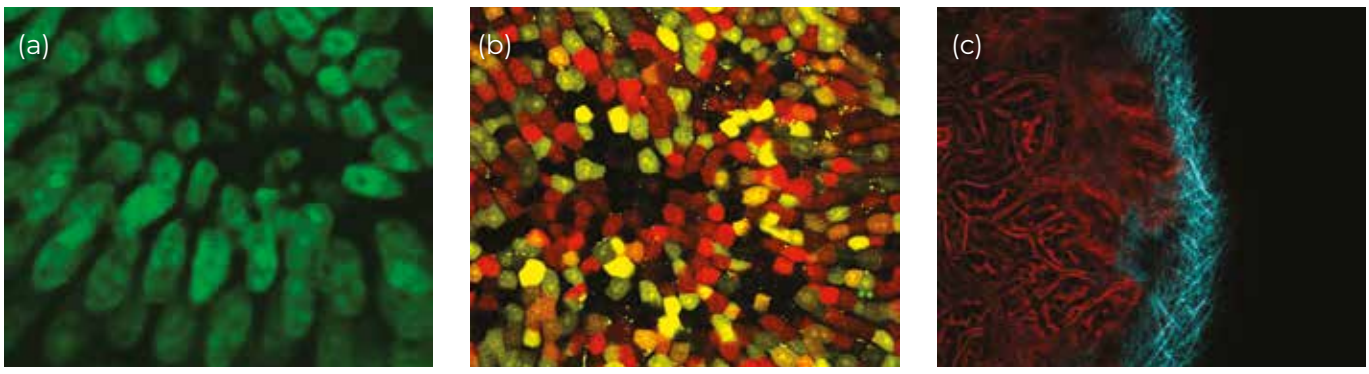


FIGURE 1. Two-photon fluorescence observed in (a) the nuclei of mouse intestine cells stained with SYTOX® Green, (b) liver cells visualized with RFP, YFP and GFP, and (c) kidney cells with mT:mG.

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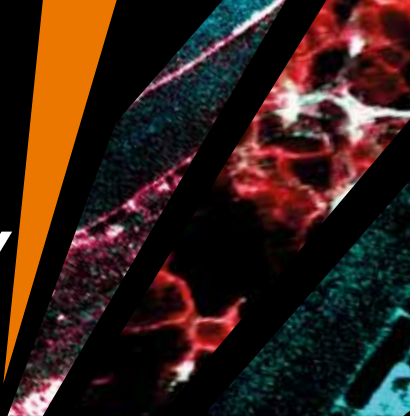
TWO-PHOTON FLUORESCENCE MICROSCOPY

with the Chromacity 1040



The power levels from the Chromacity 1040 were more than adequate for generating these fluorescence images, which were recorded with only 150 mW incident on the galvo-scanning mirrors.

The Chromacity 1040 produces a free-space beam ideally suited for coupling into microscope systems. Additionally, the output is horizontally polarized, making it a perfect source for two-photon polarization microscopy (including second harmonic generation microscopy). Unlike other systems on the market, the Chromacity 1040 is insensitive to small amounts of light reflected back from the microscope, so an optical isolator is typically not required between the laser and the microscope, thus minimizing the losses within the optical system.

Experimental setup

The images were acquired using a Nikon microscope, into which the beam was introduced via a pair of galvo-scanning mirrors. A Nikon 60x Plan Apochromat oil immersion objective (NA 1.4, working distance 0.21 mm) was used to image the samples. A 520 nm notch filter (Semrock Brightline FF01-520/15-25) was used to filter out the two-photon fluorescence signal, which was collected using a standard photo-multiplier tube (PMT). Fig. 2. illustrates the setup used and the Chromacity 1040 system.

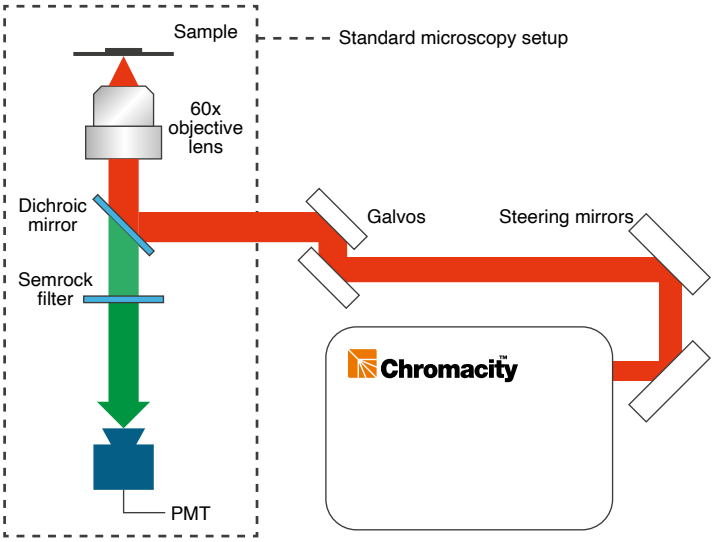


FIGURE 2.
Schematic representation of microscopy setup and the Chromacity 1040.

Summary

Including the Chromacity 1040 femtosecond laser as part of a multi-photon imaging system allows users to generate exceptionally clear, high-resolution images, as a result of its excellent beam quality, ultrafast pulses and high average power levels.

Acknowledgements

This work was carried out in partnership with ICFO - The Institute of Photonic Sciences in Barcelona and The Institute of Genetics and Molecular Medicine, at the University of Edinburgh.

The Chromacity 1040 is ideal for coupling into microscope systems, empowering users to produce clear and high-resolution images, as a result of its excellent beam quality, ultrafast pulses and high average power levels.

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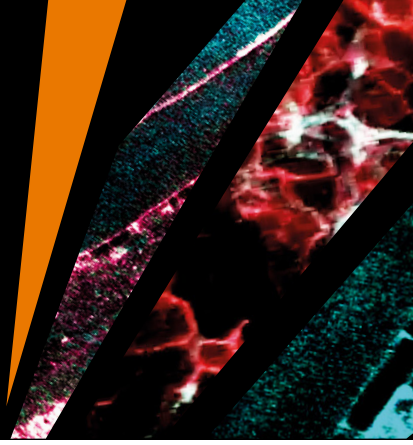
IMAGING OF SEMICONDUCTORS USING SECOND HARMONIC GENERATION (SHG) MICROSCOPY

Combining the Chromacity 1040 femtosecond laser with an Edinburgh Instruments RMS1000 Confocal Microscope



Second Harmonic Generation

Second harmonic generation (SHG) microscopy is an imaging technique where the sample's ability to generate second harmonic light creates contrast in the image. SHG is a non-linear optical process where two photons with the same wavelength interact in a material to generate a photon with exactly half the wavelength of the incident photons. The generation of a SHG signal depends on the symmetry of the molecular structures in present a sample, with SHG only occurring when there is a noncentrosymmetry in the excitation focal volume. This sensitivity to symmetry makes SHG imaging a powerful technique in materials science where it can be used to image the grain boundaries and layer stacking in 2D semiconductors through the changes in crystal symmetry.

Experimental Configuration

SHG requires a very high excitation intensity which is achieved using mode-locked femtosecond pulsed lasers. Traditionally this has been titanium sapphire lasers, but femtosecond fiber lasers are becoming an increasingly attractive and economical alternative. The Edinburgh Instruments RMS1000 Confocal Raman Microscope has external laser coupling ports that enable the optical coupling of mode-locked femtosecond lasers into the microscope. An example experimental setup for SHG microscopy with the RMS1000 is shown below. The sample is excited by the femtosecond laser and the SHG signal acquired on the RMS1000 CCD camera.

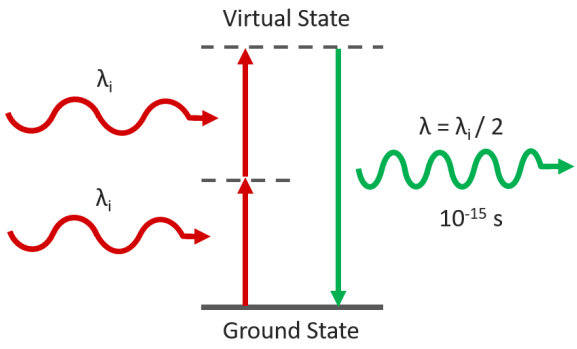


FIGURE 1. Schematic of the second harmonic process. This nonlinear optical process, causes two long wavelength photons to interact within a material to form new photons having twice the frequency of the input photons.

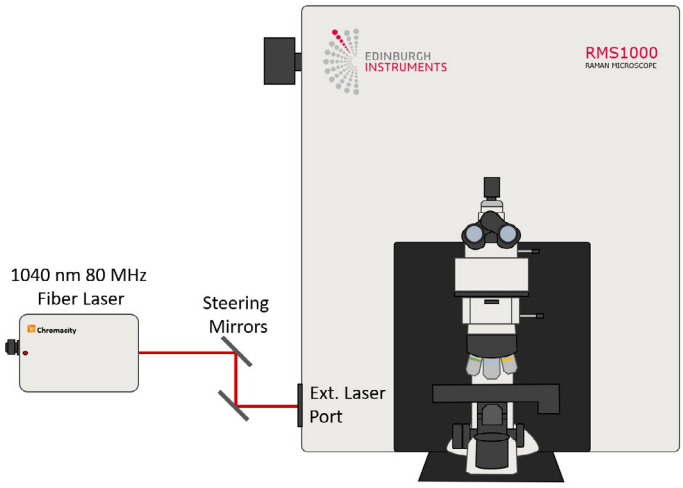


FIGURE 2. Example optical setup for SHG microscopy with the Edinburgh Instruments RMS1000. The laser excitation source is a Chromacity 1040 femtosecond fiber laser with an output wavelength of 1040 nm and a repetition rate of 80 MHz (Chromacity Ltd., UK).

SHG requires a very high excitation intensity which is achieved using mode-locked femtosecond pulsed lasers. Traditionally this has been titanium sapphire lasers, but femtosecond fiber lasers are becoming an increasingly attractive and economical alternative.

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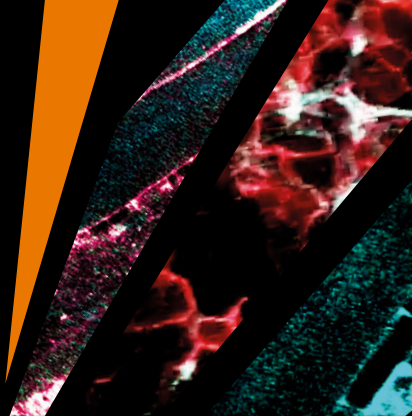
IMAGING OF SEMICONDUCTORS USING SECOND HARMONIC GENERATION (SHG) MICROSCOPY

Combining the Chromacity 1040 femtosecond laser with an Edinburgh Instruments RMS1000 Confocal Microscope



Examples of SHG Imaging with the RMS1000

WSe_2 Flake

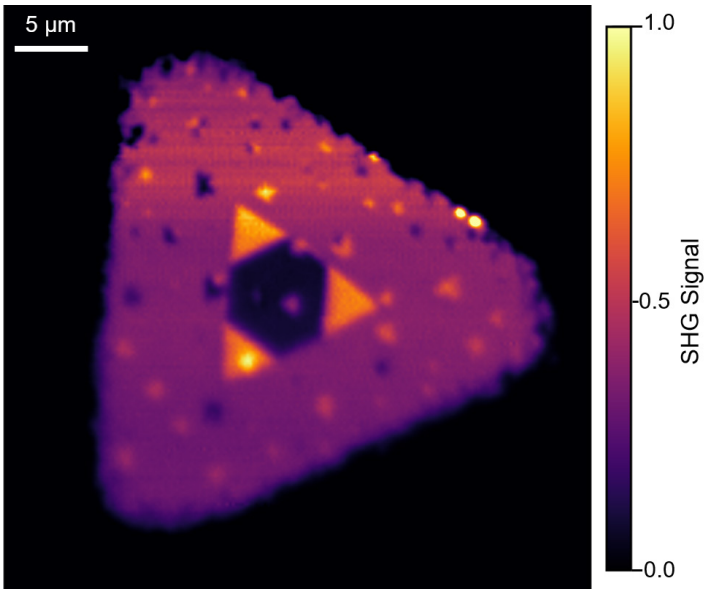


FIGURE 3. Second harmonic generation imaging of a WSe_2 flake grown using CVD. The SHG image reveals the presence of multiple layers in the centre of the flake. The main triangle is monolayer WSe_2 which has broken inversion symmetry (non-centrosymmetric) and therefore generates a SHG response. The three smaller bright triangles are bilayer WSe_2 where the second layer has the same crystal orientation as the monolayer and broken inversion symmetry is maintained so the SHG response of the layers is additive. The dark region in the centre is likely trilayer WSe_2 where the crystal growth is 180 degrees opposite to the monolayer and bilayer resulting in inversion symmetry and a significant decrease of the SHG signal.

WS_2 Polycrystal

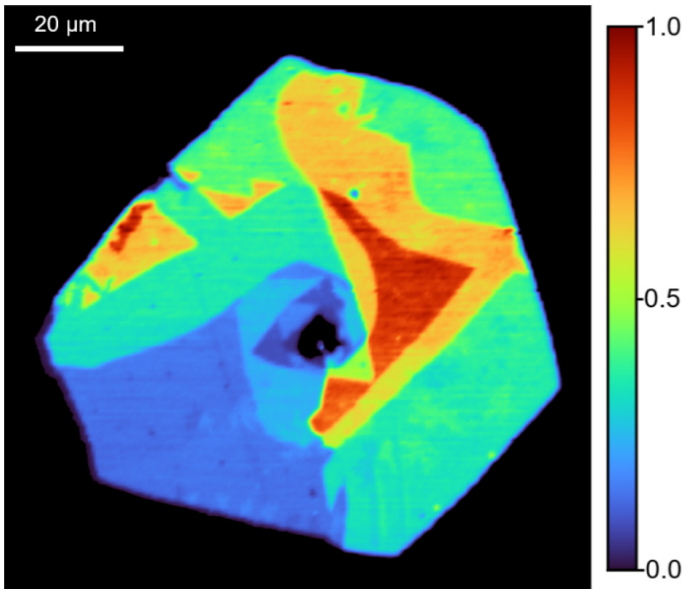


FIGURE 4. Second harmonic generation imaging of a WS_2 flake grown using CVD. The SHG imaging reveals that the flake is a polycrystal with each crystalline domain having a different SHG response due to differing number of layers, stacking symmetries and growth orientations.

Content courtesy of Edinburgh Instruments

EDINBURGH INSTRUMENTS

For more information about the RMS 1000 Confocal Raman Microscope: https://www.edinst.com/products/rms1000-raman-microscope/

For more information about Chromacity capabilities for life science applications: https://chromacitylasers.com/applications/life-sciences/

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Second-Harmonic Generation Imaging

using the Chromacity 1040

Second-harmonic generation imaging microscopy (SHG Microscopy, also known as SHIM) offers several advantages for live cell and tissue imaging. The ultrashort pulsewidth of the Chromacity 1040 is ideal for generating a second-harmonic response from a wide range of biological samples. This application note illustrates the suitability of the Chromacity 1040 for generating SHG images in starch and collagen fibres.

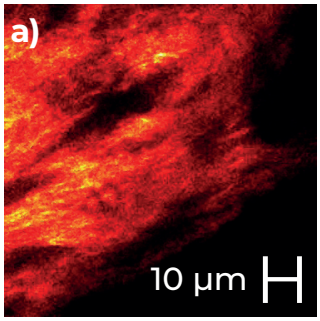
Second harmonic generation imaging

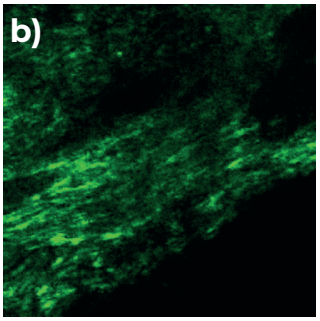
In recent years, SHG microscopy has proven its capability in the study of crystallized biomolecules such as starch, collagen and myosin. Unlike fluorescence-microscopy, SHG microscopy does not involve the creation of excited electronic states, so cell viability issues associated with heating and photo-bleaching are reduced. By using near-infrared wavelengths it enables the construction of 3-D images of specimens by imaging deeper into thick tissues. It enables the direct visualization of tissue structure (in situ) as it relies only on species present in the sample to provide a contrast. Imaging with external markers/labels normally only infer structural aspects as it relies on absorption whereas SHG microscopy signals stem from an induced polarization of tissue samples whose structural organization and molecular orientation are non-centrosymmetric, such as collagen and starch.

Collagen imaging

The non-centrosymmetric molecular structure of collagen makes it an ideal sample to image with SHG microscopy. Using a simple setup illustrated in Fig. 2 images of collagen fibres could be acquired in both the forward and backward directions. Fig. 1 demonstrates the typical images that can be generated by SHG Microscopy when using the Chromacity 1040.

Fig. 2 illustrates the imaging of collagen fibres overlaid on a liver sample which has been imaged using multiphoton microscopy. The SHG method enables accurate structural information to be detected using the Chromacity 1040 as an excitation source. The liver has been imaged using mT:mG and two-photon microscopy with a two channel detection system enabled the simultaneous acquisition of SHG images of the collagen and the two-photon fluorescene signal of the liver, before recombining as a single image.





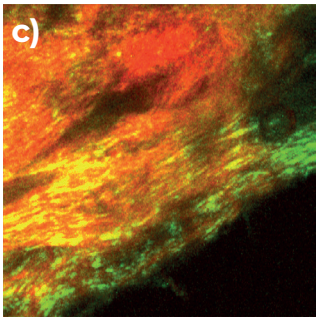


FIGURE 1. SHG images of collagen fibres. Images a) and b) are images of the same sample collected in the forward and backward directions respectively. Image c) is a composite showing the detail of the fibres. To acquire these images 300 mW of laser light was incident on the galvo-scanning mirrors.

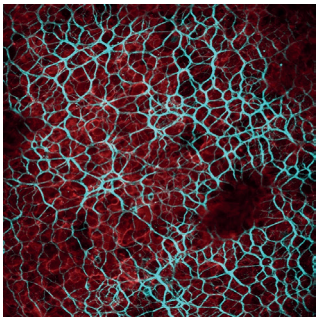


FIGURE 2. SHG image of collagen fibres on liver.

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Second-Harmonic Generation Imaging

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Experimental Setup and Starch Imaging

Starch, which is an important food source and a promising future energy candidate, has been shown to exhibit strong SHG response and is a relatively new tool/marker for plant research and other applications.

Fig. 3(a). illustrates how the Chromacity 1040 was used to generate an SHG signal in a solution of starch molecules. Unlike solid-state lasers, which can produce beams with an elliptical cross-section because of astigmatism in the laser cavity, the laser's beam originates from a single-mode fibre, so it is perfectly symmetric making it ideally suited for coupling into commercial laser-scanning microscopes.

The beam was introduced via a pair of galvo-scanning mirrors. A Nikon x60 plan apochromat oil immersion objective (NA 1.4, working distance 0.21 mm) was used to image the starch samples. A suitable filter was used to reject all wavelengths except the SHG signal, which was collected using a photo-multiplier tube (PMT).

Fig. 3(b) illustrates typical images acquired from the system in the forward direction. The power levels from the Chromacity 1040 were more than adequate for generating these fluorescence images, which were recorded with 300 mW incident on the galvo-scanning mirrors.

Summary

The Chromacity 1040 is an ideal source for an SHG Microscopy system, allowing users to generate exceptionally clear, high-resolution images, a result of the laser's excellent beam quality and high average power levels.

Multi-photon microscopy is also possible with the Chromacity 1040 nm laser. Contact us for more information about your microscopy requirements.

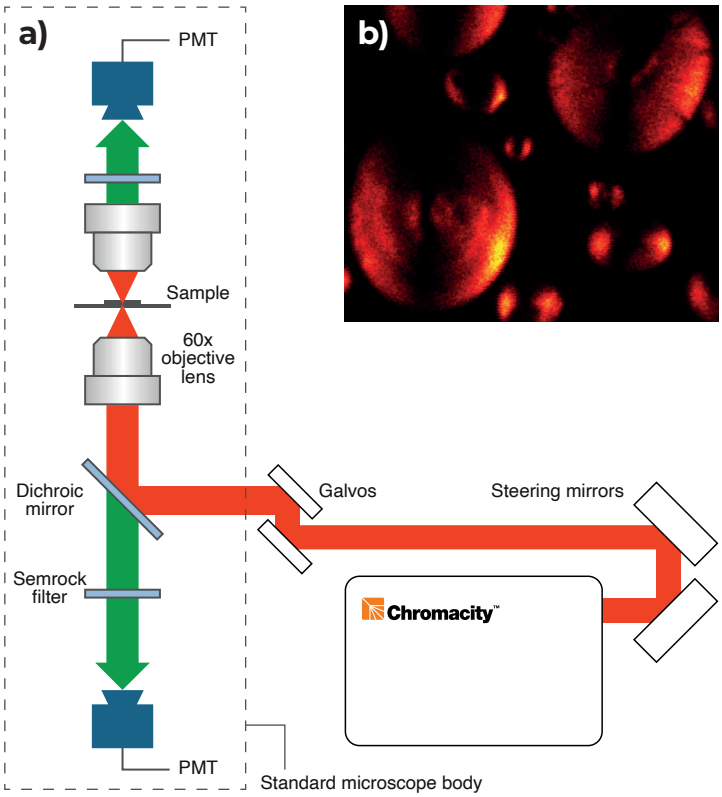

Figure 3. (a) Schematic representation of microscopy setup; (b) second harmonic generation observed in a sample of starch molecules. The field of view for this image is 100 x 100 µm. Only 300 mW of laser light was necessary to acquire these images.

Acknowledgement

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Two-Photon Lightsheet Microscopy

using the Chromacity 1040

Lightsheet microscopy is a new paradigm in two-photon imaging. Sectioned images can be generated in real time, and a high-density 3-D image stack can be recorded in seconds, representing a major advantage over traditional confocal laser-scanning microscopy.

Fluorescence excitation and detection are split into two separate light paths. Illumination of the sample is perpendicular to the detection axis. This allows for detection of the fluorescence signal only at the in-focal plane without the need for a pinhole or image processing. Images can be generated significantly faster than conventional confocal systems.

This application note describes an easy-to-build lightsheet microscope able to image a zebrafish embryo using the Chromacity 1040.

Two-photon lightsheet microscope

The Chromacity 1040 has excellent beam quality and its high average power makes it ideal for two-photon lightsheet microscopy[1,2]. The experiment described here combines the Chromacity 1040 laser with an open-source design for a lightsheet microscope[3]. Illuminating samples perpendicular to the detection axis makes the technique very photon efficient. The lightsheet microscope generates photons only in the focal plane and not in other layers of the sample. The technique is ideal for imaging deep within transparent tissues, or within whole organisms. Deep penetration is possible even within scattering tissues, as the samples are exposed to only a thin plane of light (0.5-5 µm of a sample can be illuminated), in contrast to epifluorescence microscopy. This means photobleaching and phototoxicity are comparatively less than in confocal, wide-field fluorescence, or multiphoton microscopy, making it possible to perform more scans per specimen. The technique also delivers high speed imaging with image acquisition up to 1000 times faster than those offered by point-scanning methods.

Two-photon lightsheet microscopy is a powerful tool for imaging embryonic development. zebrafish are often used as an alternative to mammalian species as they are vertebrates with a high degree of similarly in their early development (with respect to many mammals, including humans) and the roles of many biological processes and cell development is homologous across species with vertebrae. They are also mostly transparent and thus enable lightsheet imaging through the whole embryo enabling a stack to be created at different image depths enabling a 3-D reconstruction of the embryo. The specimen for this demonstration was a zebrafish embryo stained with eosin and fixed in agar gel inside a capillary. In the leftmost image (Fig 1.), the zebrafish head and tail are in focus and generating the strongest two-photon fluorescence signal.

As the zebrafish is translated through the lightsheet, these features move out of the sheet and become dark, while the yolk sac moves into the sheet and emits strong two-photon fluorescence.

This image stack illustrates how easy it is to generate detailed information of the entire embryo using a fast scanning technique.

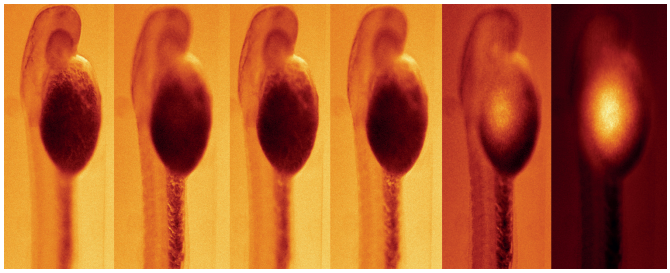

FIGURE 1. Zebrafish embryo image stack with 50-µm increments between the images.

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Two-Photon Lightsheet Microscopy

using the Chromacity 1040

Experimental setup

The sample was suspended in a glass capillary inside a cuvette on top of a stepper motor which allowed the sample to be rotated through 360°. The cuvette was fixed onto an optical rail, which also supported a x4 infinity corrected objective lens (Zeiss®) and a camera. A lens tube (Thorlabs®) was attached to the camera and used to support a 160 mm tube lens at its focal length from the camera sensor. This arrangement allows the camera to be moved freely to allow filters to be inserted between the tube lens and the 4x objective. An xyz stage (Newport®) with one axis motorised was used to suspend the stepper motor and sample combination.

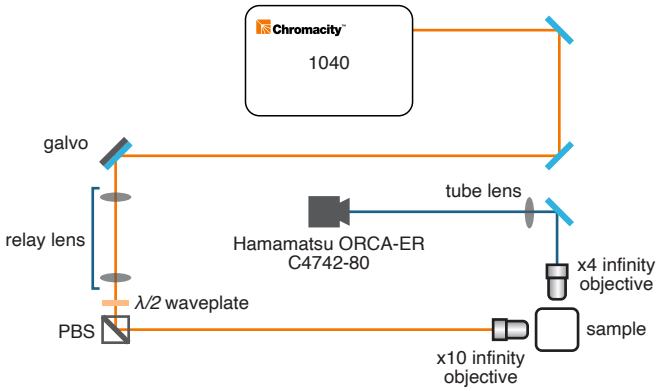

FIGURE 2. Two-photon lightsheet microscope layout.

Fig. 2 shows the layout, with the entire optical system on a 75 cm x 75 cm breadboard. Light from the laser is steered onto a galvanometer mirror (Thorlabs®) which is relay imaged using a pair of AR-coated lenses onto the pupil of a x10 infinity corrected objective lens (Mitutoyo®). Before entering the objective the light is attenuated using a half-wave plate and a polarizing beamsplitter, enabling adjustment of the illumination intensity to avoid camera saturation.

Summary

A two-photon lightsheet microscope is easily implemented using the Chromacity 1040 laser and readily available stock optical components.

The high stability and high average power of the laser means that two-photon lightsheet microscopy can be easily carried out using any fluorescent agents with some linear absorption in the 500 nm region, including RFP, YFP, GFP, Sytox green, fluorescein and many others.

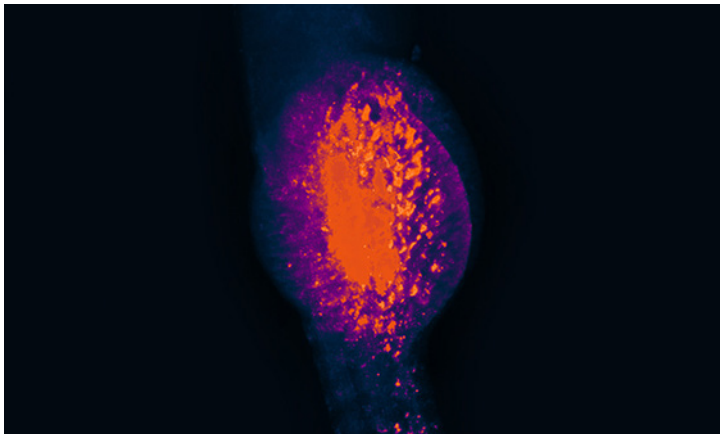

FIGURE 3. Image of zebrafish embryo when using a x4 objective.

Acknowledgements

We are grateful to the Institute of Genetics and Molecular Medicine at the University of Edinburgh for their collaboration in developing the lightsheet microscope.

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2. O. E. Olarte et al, "Image formation by linear and nonlinear digital scanned light- sheet fluorescence microscopy with Gaussian and Bessel beam profiles," Biomed. Opt. Express 3, 1492 (2012).
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TWO-PHOTON FLUORESCENCE MICROSCOPY (2P-FLIM)

Using the Chromacity 1040 femtosecond laser in line with an Edinburgh Instruments RMS1000 Confocal Microscope

Introduction

Two-photon microscopy is an imaging technique where two infrared photons are simultaneously absorbed to generate shorter wavelength fluorescence. Two-photon absorption is a non-linear process with the absorption probability scaling quadratically with excitation intensity. This results in the fluorescence being restricted to a much smaller focal volume than in one-photon fluorescence microscopy as the focal point is the only point in space with enough photon density for two-photon absorption to occur. This localized excitation combined with the lower absorption and scattering of the longer wavelength infrared excitation light makes two-photon microscopy ideal for imaging deep into thick biological samples.

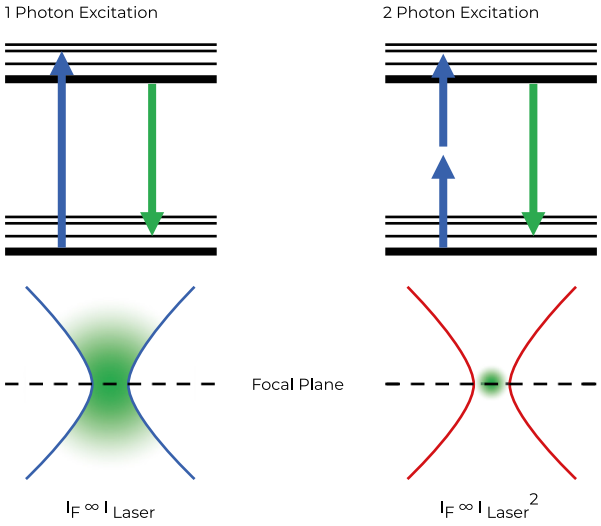

FIGURE 1. One-photon vs Two-photon Excitation.

The longer wavelength infrared excitation light makes two-photon microscopy ideal for imaging deep into thick biological samples.

Experimental Configuration

Two-photon absorption requires very high excitation intensities which can only be achieved with mode-locked femtosecond pulsed lasers. Traditionally this has been titanium sapphire lasers, but femtosecond fiber lasers are becoming an increasingly attractive and economical alternative. The Edinburgh Instruments RMS1000 Confocal Raman Microscope has external laser coupling ports that enable the optical coupling of mode-locked femtosecond lasers into the microscope. An experimental setup for two-photon spectral and lifetime microscopy with the RMS1000 is shown below, where the RMS1000 has been configured with both a CCD camera and a photon counting Hybrid Photodetector and time-correlated single photon counting (TCSPC) electronics for spectral and lifetime two-photon microscopy.

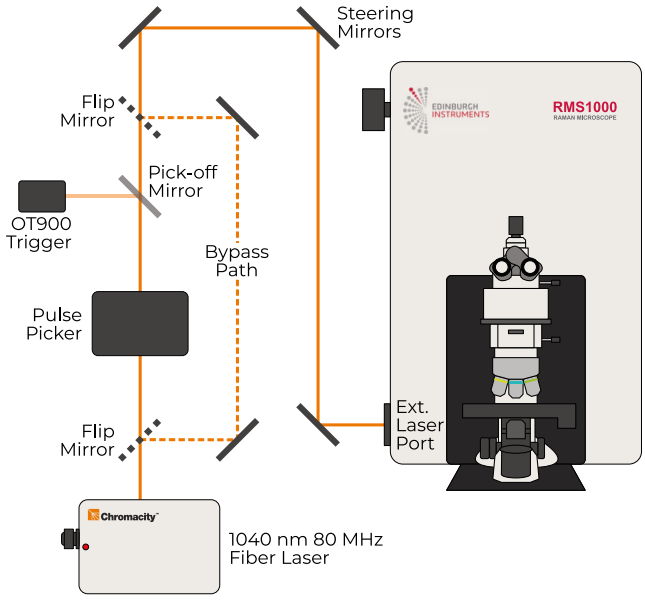

FIGURE 2. Example optical setup for two-photon microscopy. The RMS1000 is equipped with a BI-CCD camera for spectral fluorescence acquisition and a Hybrid Photodetector and TCSPC lifetime electronics for fluorescence lifetime acquisition. The laser excitation source is a Chromacity 1040 HP femtosecond fiber laser operating at 80 MHz. For lifetime imaging the output of the laser is pulse picked to the desired pulse frequency using the Pulse Picker pulseSelect (APE GmbH, Germany). A small fraction of the output is picked off into an Edinburgh Instruments OT900 optical trigger to trigger the TCSPC electronics. For spectral measurements the pulse picker is bypassed and the 80 MHz laser output is coupled directly into the RMS1000.

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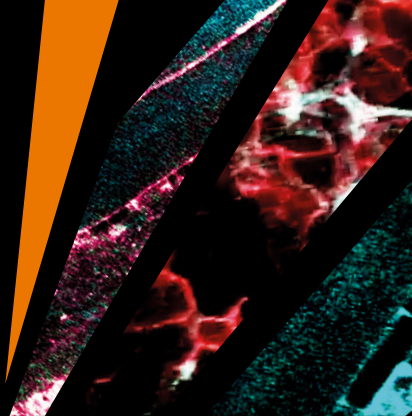

Examples of Two-Photon Imaging with the RMS1000

Kidney Tissue Section

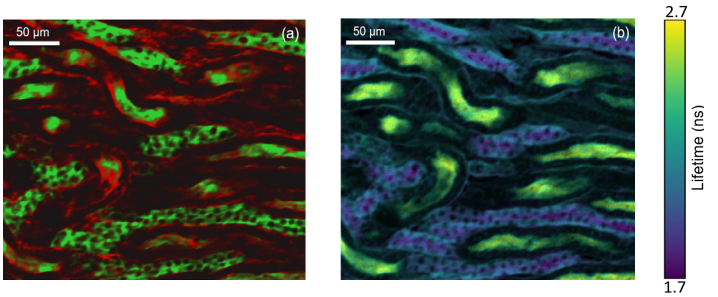

FIGURE 3. Two-photon intensity (a) and lifetime (b) imaging of a mouse kidney section. The glomeruli and convoluted tubules of the kidney tissue were labelled with green fluorescent Alexa Fluor® 488 while the filamentous actin present in glomeruli and the brush border was stained with red-orange fluorescent Alexa Fluor® 568. In (a) the Alexa Fluor® 488 and Alexa Fluor® 568 were excited by the 80 MHz 1040 nm femtosecond laser output and the green and red fluorescence simultaneously captured on the RMS1000 CCD camera. In (b) the 1040 nm laser was pulse picked to 20 MHz and the fluorescence decay measured on the RMS1000 Hybrid Photodetector using TCSPC.

Intestine Tissue Section

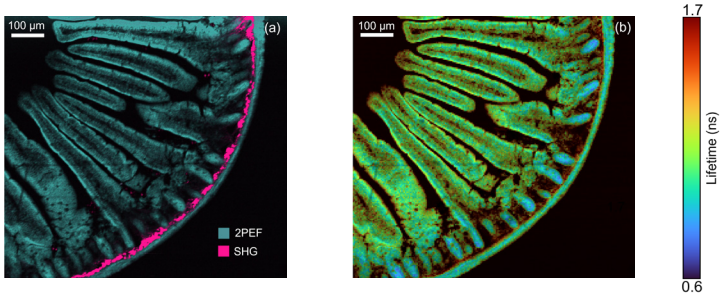

FIGURE 4. Two-photon intensity (a) and lifetime (b) imaging of a mouse intestine section stained with red-orange fluorescent Alexa Fluor® 568. In (a) the Alexa Fluor® 568 was excited by the 80 MHz 1040 nm femtosecond laser output and the two-photon excited fluorescence (2PEF) captured on the RMS1000 CCD camera. Two-photon imaging setups can also be used for complementary technique of second harmonic generation (SHG) imaging. SHG at 520 nm from collagen was simultaneously recorded on the RMS1000 CCD camera revealing collagen build up in the intestine wall. In (b) the 1040 nm laser was pulse picked to 20 MHz and the fluorescence decay measured on the RMS1000 Hybrid Photodetector using TCSPC.

Convallaria Rhizome

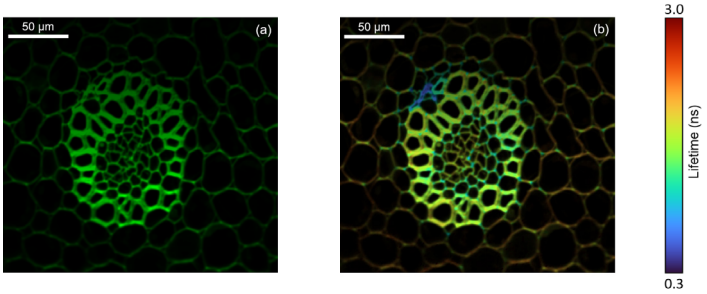

FIGURE 5. Two-photon intensity (a) and lifetime (b) imaging of a convallaria rhizome stained with acridine orange. The acridine orange was excited at 10 MHz using the pulse picked 1040 nm femtosecond laser output and the fluorescence decay measured on the RMS1000's Hybrid Photodetector using TCSPC. The integrated fluorescence intensity of the decay is shown in (a) and the fitted lifetime in (b), highlighting the increased local environment information that can be obtained from fluorescence lifetime imaging.

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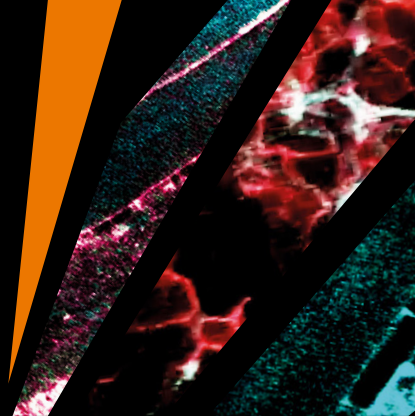
MULTIPHOTON IMAGING OF MOUSE INTESTINE

Using the Chromacity 1040 femtosecond laser in line with an Edinburgh Instruments RMS1000 Confocal Microscope



Introduction

Two-photon excited fluorescence (2PEF) and second harmonic generation (SHG) are complementary multiphoton imaging techniques for studying biological samples. Both imaging techniques utilise femtosecond pulsed infrared excitation light to generate shorter wavelength light to image the sample but operate via fundamentally different physical processes (Figure 1). In 2PEF, two infrared photons are simultaneously absorbed by a fluorophore promoting it to an excited state which then radiatively relaxes emitting shorter wavelength fluorescence. In contrast, SHG is not an absorption and emission process and instead the two infrared photons combine in a non-linear optical material with a particular symmetry to generate a new photon with exactly half the wavelength of the incident photons. Both techniques take advantage of the lower scattering and absorption of infrared light to enable imaging deep into tissue. In this application note, an Edinburgh Instruments RMS1000 Confocal Raman Microscope is used to image a tissue section of mouse intestine using 2PEF and SHG microscopy.

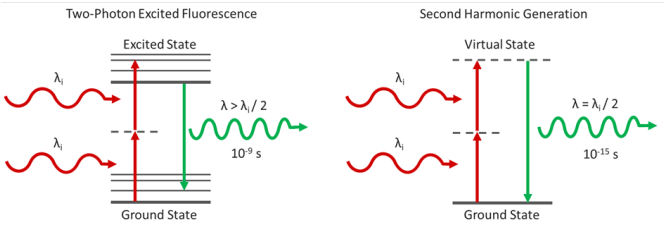


FIGURE 1. Two-Photon Excited Fluorescence and Second Harmonic Generation processes.

Experimental Configuration

The sample to be imaged was a section of mouse intestine tissue stained with Alexa Fluor® 568. The RMS1000 was equipped with a motorised XYZ stage and a 40x NA = 0.75 objective. For spectral imaging the RMS1000 was equipped with a back-illuminated CCD camera and for lifetime imaging; a photon counting Hybrid Photodetector and time-correlated single photon counting (TCSPC) electronics. 2PEF and SHG both require a very high excitation intensity, which is achieved using a mode-locked femtosecond pulsed laser. The RMS1000 has external laser coupling ports that enable the optical coupling of femtosecond lasers into the microscope. The optical setup for the femtosecond excitation source is shown in Figure 2. The laser was a Chromacity 1040 HP femtosecond fibre laser with an output wavelength of 1040 nm and an 80 MHz repetition rate (Chromacity Ltd., UK). For lifetime imaging the output of the laser was pulse picked to the desired pulse frequency using a pulseSelect pulse picker (APE GmbH, Germany). A small fraction of the pulse picker output was picked-off into an Edinburgh Instruments OT900 optical trigger module to trigger the TCSPC electronics. For spectral measurements, the pulse picker was bypassed, and the 80 MHz laser output coupled directly into the RMS1000.

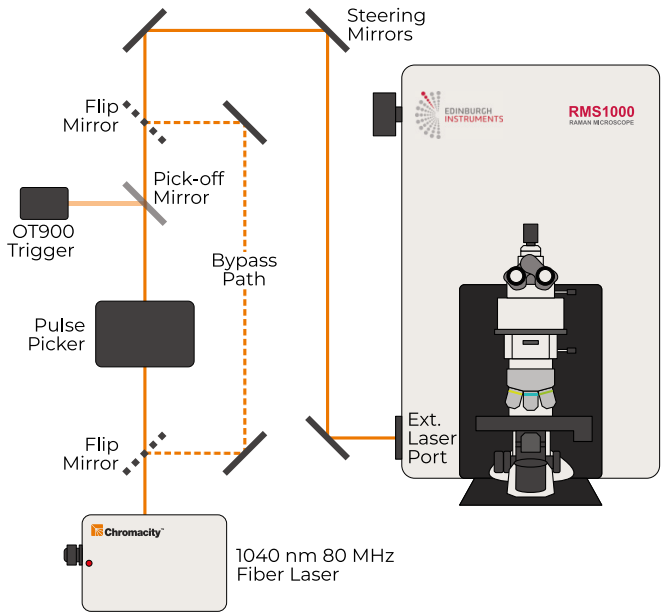


FIGURE 2. Optical setup for two-photon and second harmonic generation imaging.

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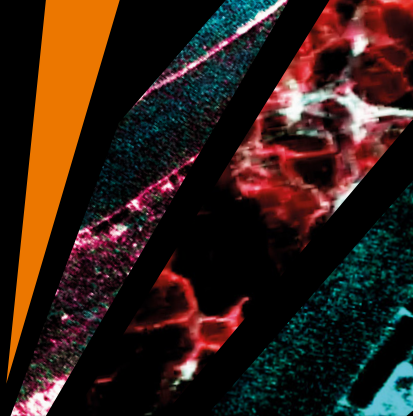

2PEF & SHG Spectral Imaging with CCD Camera

The intestine tissue section was first imaged spectrally using the CCD camera of the RMS1000. A 900 µm x 800 µm area of the sample was mapped with a spatial resolution of 2 µm. The sample was excited at 1040 nm, 80 MHz and the 2PEF and SHG signals acquired simultaneously using the CCD camera. The resulting multiphoton image is shown in Figure 3a. 2PEF at 630 nm from the Alexa Fluor® 568 dye is shown in teal and reveals the structure of the intestinal villi. The area shown in pink is SHG at 520 nm from fibrillar collagen near the intestinal wall. SHG only occurs from molecular structures that are non-centrosymmetric and fibrillar collagen is a common biological structure with this property, eliciting a strong SHG response. Each point in the image has a corresponding spectrum, and the spectra at two points A and B are shown in Figure 3b. At point A there is only 2PEF from the Alexa Fluor® 568 dye which has a broad emission centred at 630 nm, while at point B there is an additional sharp peak at 520 nm which is the SHG signal. The colour image in Figure 3a was obtained by plotting the intensity of the spectra at 630 nm and 520 nm for 2PEF and SHG respectively.

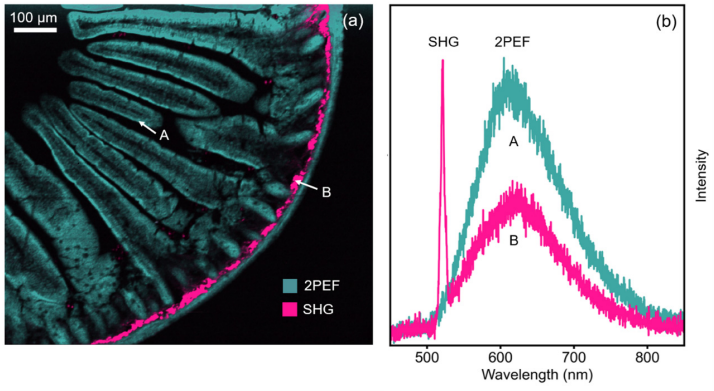

FIGURE 3. (a) 2PEF and SHG image of mouse intestine section stained with Alexa Fluor® 568 and (b) extracted spectra from two points in the image.

2PEF Lifetime Imaging with Hybrid Photodetector

Additional information can be obtained by two-photon fluorescence lifetime imaging. For lifetime imaging the repetition rate of the laser was lowered to 20 MHz using the pulse picker to ensure complete fluorescence decay between pulses. The same 900 µm x 800 µm area was mapped and the fluorescence decay at each point recorded using TCSPC on the photon counting Hybrid Photodetector. Each fluorescence decay was fit with an exponential model using the RMS1000 Ramacle® software and the resulting lifetime image is shown in Figure 4a. The lifetime image shows a decreased fluorescence lifetime in the intestinal crypts near the intestinal wall compared with the villi; an example of the increased information that can routinely be obtained from lifetime imaging.

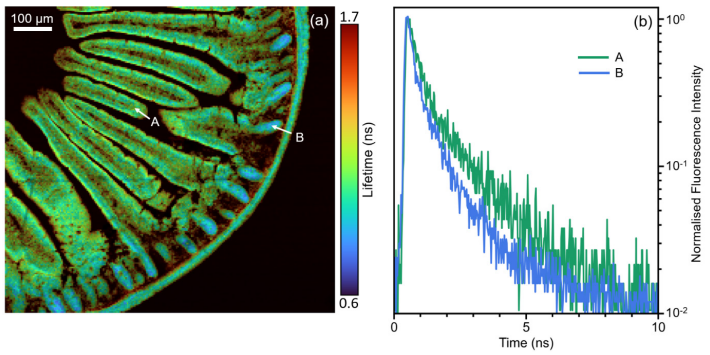

FIGURE 4. (a) 2PEF lifetime image of mouse intestine section stained with Alexa Fluor® 568 and (b) extracted fluorescence decays from two points in the image.

For lifetime imaging the repetition rate of the laser was lowered to 20 MHz using the pulse picker to ensure complete fluorescence decay between pulses

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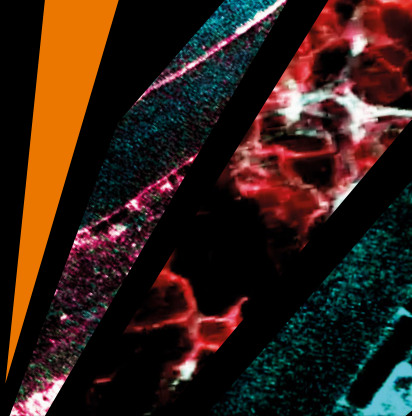

Conclusion

A section of mouse intestine was imaged using multiphoton microscopy with the RMS1000 Confocal Raman microscope. The RMS1000 can be equipped with the Chromacity 1040 femtosecond laser and TCSPC lifetime electronics for advanced spectral and time-resolved multiphoton imaging techniques such as 2PEF and SHG which augments its core Raman imaging capability.

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Cardiac Imaging

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In this application note, we compare the Chromacity 1040 and Ti:Sapphire two-photon lasers for enhanced fluorescence-based functional measurements at depth in the intact heart.

Summary

Multiphoton microscopy has been demonstrated as a versatile tool in the life sciences for many structural and functional applications both in-situ and in vivo, including electrophysiology [1], calcium signalling [2], blood flow [3] and immune cell trafficking in the heart in vivo [4]. Despite the analytic power of such devices, and their increased availability both as commercially and as a custom-built device, their uptake in biology labs remains relatively low in comparison to the sister technology of confocal microscopy. This is in part due to the size, cost, and perceived complexity of purchasing and maintaining the most crucial piece of equipment for two-photon microscopy; the laser.

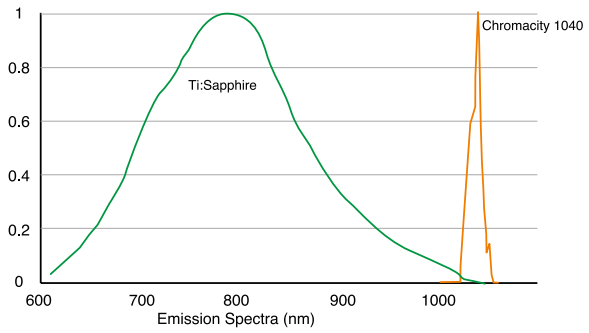

Figure 1. Emission Spectra of Ti:Sapphire and the Chromacity 1040.

While expensive, bulky Ti:Sapphire lasers remain the most commonly used laser for two-photon applications. There is an increasing need for low-cost, compact commercially available turnkey solutions capable of delivering higher average power at wavelengths >1000 nm, where the traditional tunable Ti:Sapphire system struggles to deliver. This is demonstrated by electrophysiological measurements in excitable tissues, particularly cardiac and neural tissue. This frequently involves recording membrane potentials and calcium transients via multiphoton processes. For this purpose, a broad range of voltage [5] and calcium- sensitive [6] fluorophores have been specifically developed. However, these processes are in the second IR imaging window (1 - 1.3 μm), wavelengths where the power of Ti:Sapphire systems fall off dramatically (Figure 1). Here we demonstrate the use of a low cost commercially available turnkey laser system, from Chromacity. It is capable of delivering high peak and average powers at the sample plane of a two-photon microscope.

Introduction

Two-photon imaging for in vivo applications has been shown to achieve high-resolution imaging and execute functional measurements within neuroscience [7]. Such functional measurements are also being performed in other excitable media such as cardiac muscle, which exhibits even greater absorption and scattering [8]. Despite the relatively high tissue penetration depths afforded by this technique there remain fundamental limits to the depth of imaging possible, which are a function of the detection characteristics of the microscope, the optical properties of the tissue being imaged, and more importantly the pulsed laser source. The increased requirement for deep-tissue in vivo imaging has also spurred the development of genetically encoded sensors for red-shifted imaging [9].

The signal-to-noise requirements for fluorescent electrophysiological recordings are much lower than that required to resolve a detailed structural image. Therefore, during purely electrophysiological measurements, the ability to deliver sufficient power to a confined region deep within certain tissue types becomes more important than resolving individual structural features. This is particularly true in electrically well-coupled tissue such as cardiac muscle, hence, the need for suitable optical powers being delivered to the sample plane.

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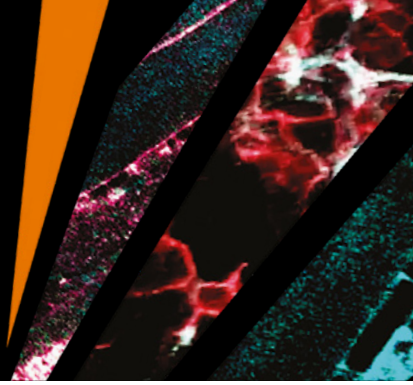

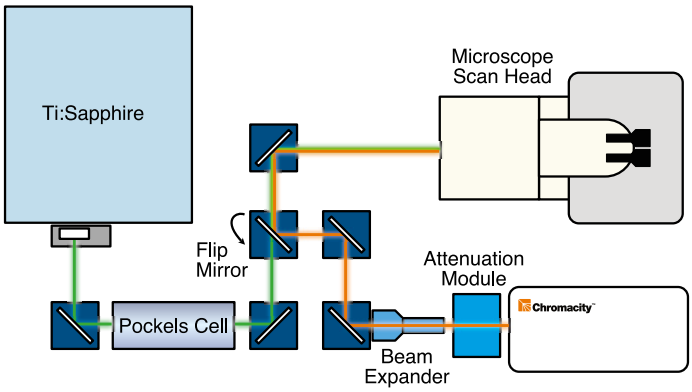

Figure 2. Laser and microscope optical setup. Beam path and intermediate optics for Ti:Sapphire and the Chromacity 1040 is shown above.

Optical Setup

A schematic of the optical layout is shown in Figure 2. An ultrafast ytterbium fiber laser from Chromacity capable of delivering 100 fs pulses at 80 MHz repetition rate, was coupled to a Zeiss LSM510 laser scanning microscope system. Laser output power was manually controlled using a Chromacity power attenuation module. Beam divergence was compensated for using a divergence - adjustable beam expander. The beam was focused into the LSM510 scan head. The use of a 90° flip-mount and mirror allowed the beam path to be switched rapidly between the Chromacity 1040 and a tunable Ti:sapphire.

Isolated perfused heart preparation

Hearts from three animals were used in the study. The whole heart preparation was setup and imaged as described in [10]. Hearts were bolus loaded with 25 µl of di-4-ANEPPS (2 mM stock) over a 10 min period. Imaging of cardiac structures was performed using a 40x mag, 1.0 NA objective lens. Two bi-alkali photomultiplier tubes (PMT) were used to capture the dual emission from di-4-ANEPPS. PMT 1 collected emitted light between 510-560 nm while PMT 2 collected the longer 590-650 nm emission band. A series of XY images were recorded using each laser to form an image stack (z-stack). Afterwards, rapid line scanning at 1 kHz was used to provide sufficient bandwidth to record optical action potentials. A ratio of both PMT signals was then taken to reduce motion artifact and baseline drift. This process was repeated sequentially, in 50 µm steps, at increasing transmural tissue depths from 50 to 250 µm below the epicardial surface.

Results

Pulse characteristics were determined initially using autocorrelation techniques. The autocorrelation for the Ti:Sapphire, yielded a pulse duration of 120 fs. The design of the Chromacity 1040 nm system features a movable diffraction grating, resulting in pre-chirping of the pulse to compensate for dispersion effects through the microscope optics and maximise the fluorescence signal at the sample plane. Once the beam was aligned with the microscope scan head the diffraction grating was adjusted to yield the maximal fluorescence from a test sample of 500 µm fluorescent beads.

Laser beam profiling measurements demonstrated that the beam waist of the Ti:Sapphire (Figure 3A) was roughly double that of the Chromacity 1040 (Figure 3B). As a consequence, the Chromacity 1040 beam diverged more rapidly than the Ti:Sapphire along its beam path. The Chromacity 1040 optical path therefore included a divergence adjustable beam expander. This yielded two photon- excited fluorescent signals with intensity matching the Ti:Sapphire at 1040 nm (PMT gain and average power at sample plane matched). This was confirmed from z-stack images taken through the left ventricular epicardial surface at 15 mW average power (Figure 4 and 5).

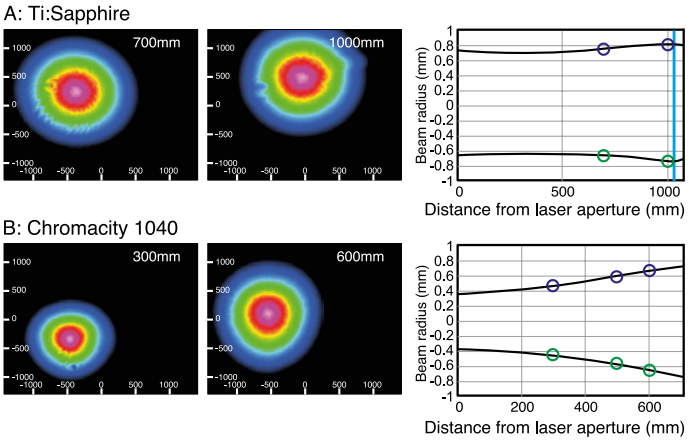

Figure 3. Laser beam profiles for Ti:Sapphire (A) and Chromacity 1040 (B). Blue lines on plots indicate relative position of focusing lenses for each laser beam path. Initial measurements showed the beam waist of the Chromacity 1040 to be narrower than the Ti:Sapphire but diverging much quicker. This divergence was compensated for in the final set up using a divergence adjustable beam expander.

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Due to the length of each scan, z-stacks for each laser were taken at adjacent regions of tissue to avoid any disparity in signal due to dye bleaching. The average signal from a defined imaging region was almost identical at each z-stack image plane for both lasers when power and wavelength matched (Figure 5B).

Optical action potentials recorded from a series of transmural depths with both lasers using rapid line scanning are shown in Figure 6A. Average power at the sample plane was relatively low (25 mW) close to the epicardial surface (50 µm z-depth).

Here both lasers performed equally well at 1040 nm, producing action potentials of comparable amplitude. From depths of 100- A 250 µm, power was increased on the Ti:Sapphire to maximum, yielding 60 mW at the sample plane. Figure 6B shows the drop in action potential amplitude with depth, to the extent that signal to noise was insufficient at 200 µm below the surface to record a usable action potential. Increasing the power of the Chromacity 1040 to 75 mW, 15 mW above the Ti:Sapphire power threshold at 1040 nm, allowed action potentials of sufficient signal to noise to be recorded as deep as 250 µm (data not shown), without damaging the tissue sample.

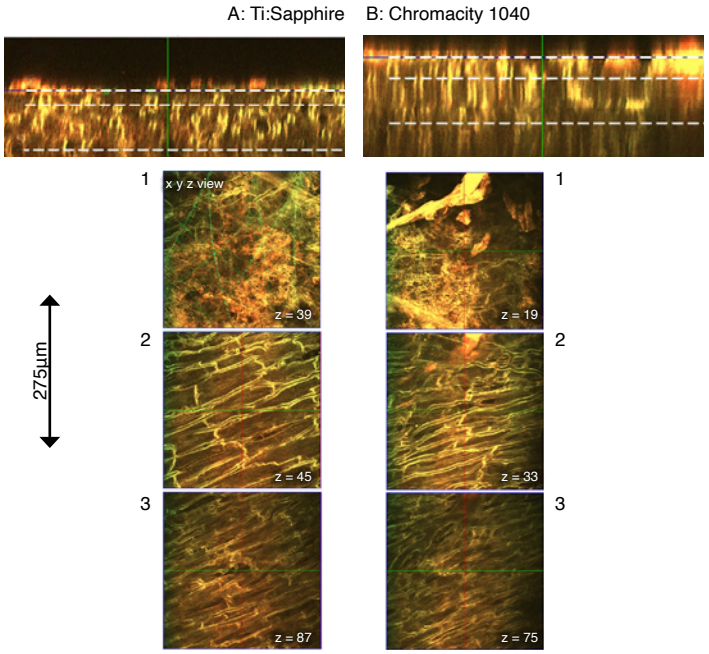

Figure 4. Z-stack structural images from adjacent regions of the epicardial surface of mouse left ventricle using Ti:Sapphire (A) and Chromacity 1040 (B). Average laser power at sample and PMT gain were matched in each. Top panels show y-z orthogonal view through the transmural wall, with x-y images from 3 separate planes (indicated by white dashed lines).

Discussion

Many system builders still opt for bundling microscopes with a tunable, solid state Ti:Sapphire laser which, while offering flexibility, is bulky, costly to purchase and requires regular maintenance. Advances in fiber lasers in recent years has seen a significant increase in the use of this technology as an efficient, low cost alternative to traditional solid-state lasers, albeit limited to single wavelengths. Low cost custom-built fiber lasers for two-photon applications have been demonstrated for as little as $13,000 [11], but remain within the realm of specialist users with the appropriate knowledge and skillset to construct them.

In our setup the Chromacity 1040 nm system can deliver >400 mW at the sample plane, whereas the Ti:Sapphire can only deliver 60 mW. While 400 mW is in excess of what biological tissue can tolerate, it is likely that increasing the power beyond 75 mW at deeper transmural tissue planes, shown here, would increase the range of imaging of the system even further than that reported while remaining within the tolerable energy limits for intact cardiac tissue.

The flexibility of this laser system is illustrated by the relative ease with which it was coupled into the existing microscope scan head, without any prior knowledge of the optics within. The footprint of the system is low, occupying almost 2.5x less optical-table space than the Ti:Sapphire and requiring only a single panel-mounted control unit. In addition, the system is passively air-cooled, eliminating maintenance costs and further reducing the overall footprint of the two-photon microscope.

In this experimental set-up, the Chromacity 1040 nm system can deliver >400 mW at the sample plane, whereas the Ti:Sapphire can only deliver 60 mW.

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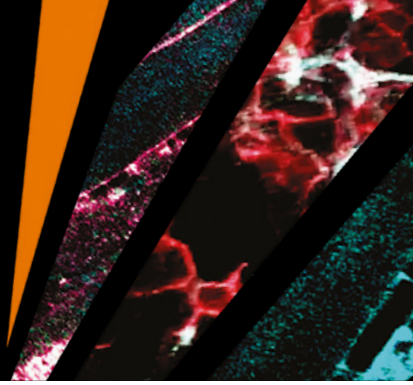

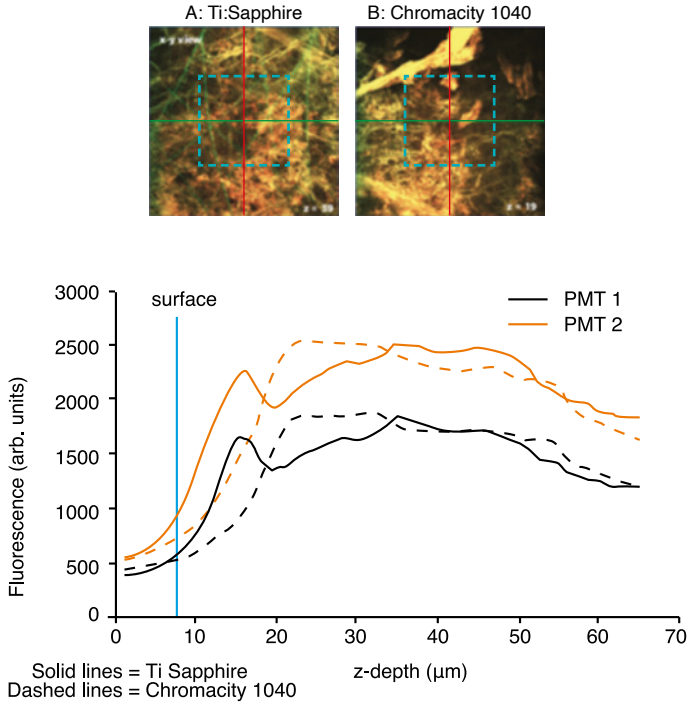

Figure 5. Mean fluorescence intensity with depth. Epicardial x-y surface images from adjacent regions using the Ti:Sapphire (A) and Chromacity 1040 (B). Mean fluorescence intensity in z- stack images for each PMT channel (red, PMT 1, black PMT 2) measured from the regions enclosed by the dashed blue box in A and B. Blue vertical line indicates which image plane denotes the absolute epicardial surface from each image z-stack. Both lasers at 1040 nm and PMT gain matched.

Conclusion

Significant improvements in transmural, deep tissue imaging of cardiac tissue can be gained through the use of a commercially available high-power ytterbium fiber laser at 1040 nm relative to existing Ti:Sapphire lasers. This laser platform offers ease of use and installation into a laser scanning microscope system and is suitable for new and existing microscopes alike.

Benefits

Comparing the Chromacity 1040 to the commercially available solid-state Ti:Sapphire at 1040 nm the Chromacity 1040 was able to perform equally well and in some cases better. The Chromacity 1040 was easily able to meet the high power demands of deep tissue optical electro-physiological imaging. In this short demonstration it enabled recording of action potentials from 250 µm, whereas the Ti:Sapphire was only able to reach 200 µm. The Chromacity 1040 was able to deliver >400 mW at the sample plane, way in excess of the 75 mW used to obtain the recording of the action potentials at 250 µm. While 400 mW is in excess of what biological tissue can tolerate, we believe that increasing the power beyond 75 mW at deeper transmural tissue planes would increase the range of imaging of the system even further than the 250 µm while remaining within the tolerable energy limits for intact cardiac tissue.

The Chromacity 1040 can be coupled into an existing microscope scan head with relative ease, without any prior knowledge of the optics within. The footprint is almost 2.5x less air table space than the Ti:Sapphire and requires only a single panel-mounted control unit. In addition, the system is passively air-cooled, eliminating maintenance costs and further reducing the overall footprint of the two-photon microscope.

For users wishing to purchase an entirely new system the fiber laser option reduces space considerably and brings the total cost for a complete basic laser scanning two-photon system down to between £100,000 - 120,000, close to the price of a single Ti:Sapphire laser alone. Those with an existing microscope who require more power for deep tissue imaging at wavelengths >1000 nm could also consider this as an option thanks to the compact size of the Chromacity 1040 system.

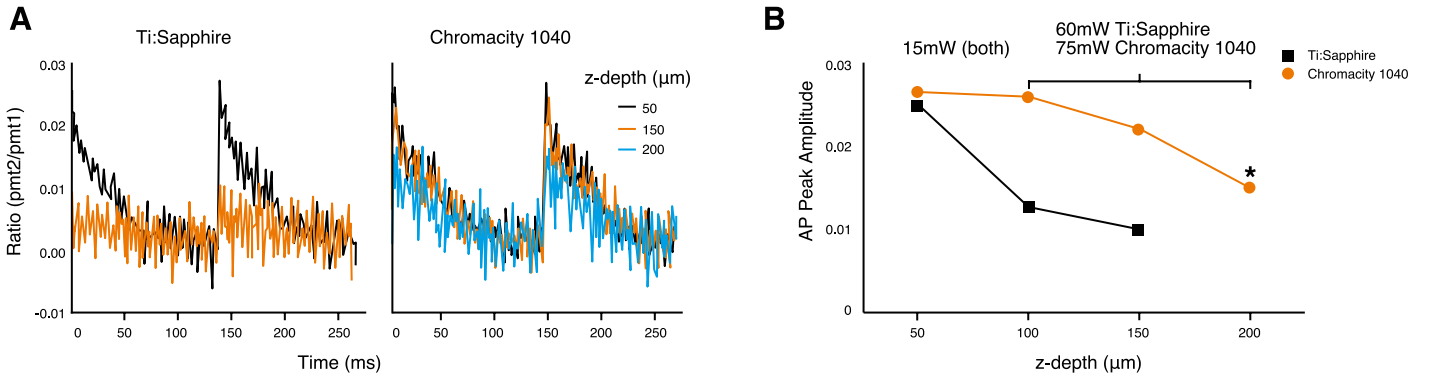

Figure 6. Higher average powers allow recording of deeper transmural optical action potentials. An optical action potential from 3 transmural layers (50, 150 and 200µm below epicardial surface) using the Ti:Sapphire (A, left) and Chromacity 1040 (B, right) at 1040nm (PMT gain matched). A ratio of the signals from PMT channels 1 & 2 was used to limit motion and baseline drift from photobleaching. (B) Max AP amplitude at each layer using the Ti:Sapphire (black squares) and Chromacity 1040 (orange circles). Ti:Sapphire power was maximal between 100-200 µm and was unable to resolve an action potential at 200 µm (indicated by *). Higher powers from the Chromacity 1040 overcame this problem.

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All procedures were approved by the Institutional Ethics Review Board and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

With thanks to Dr. Allan Kelly and QuantIC at the University of Glasgow.

Figure 7. Chromacity 1040.

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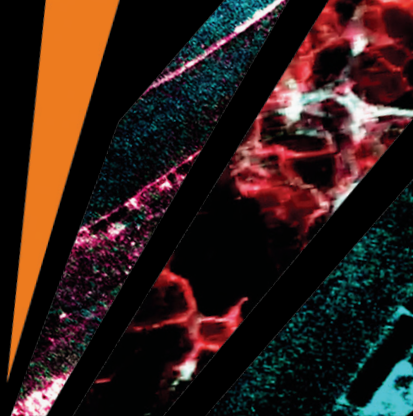
Heating of Hybrid Gold-Iron Oxide Nanoparticles in Biological Media

using the Chromacity 1040



In this application note, the Chromacity 1040 nm is used as an irradiation source for heating of hybrid gold-iron oxide nanoparticles in biological media.

Purpose

Hybrid gold-iron oxide nanoparticles have been shown to hold great potential for heat triggered drug delivery[1,2]. The magnetic core of the particles enables magnetic resonance imaging whilst the gold surface coating can act as a localised heat source after laser irradiation by exploitation of the surface plasmon resonance. Earlier work had reported heating properties in agar[3], inside cells[4] and in tumour bearing xenograft cadavers[4] after irradiation with a 1064 nm laser source. The purpose of this work was to test whether the Chromacity 1040 nm was A) useful for laser irradiation of these nanoparticles and B) more effective than the previous laser source.

Methodology

Agar phantoms

Hybrid nanoparticles were suspended in 2% agar phantoms at 5 $\mu g ml^{-1}$ and 50 $\mu g ml^{-1}$. The gels were irradiated for 60 seconds using the Chromacity 1040 nm and changes in heat monitored using an Optris PI640 thermal imaging camera (Optris, Germany). The subsequent 60 seconds following irradiation was also monitored to understand the cooling profile of the particles. Heat dissipation was deduced from the infrared images up to 12 mm from the point of irradiation. All reported thermal change is in respect to a control agar phantom containing no nanoparticles.

In vitro

Human pancreatic adenocarcinoma (BxPC-3) cells were seeded into 6-well plates (50,000 cells/ well) containing quartz coverslips (Alfa Aesar, USA) and incubated at 37 °C with 5 % CO_2. Hybrid nanoparticles (5 $\mu g ml^{-1}$ & 50 $\mu g ml^{-1}$) were incubated with the cells for 24 hrs. Cells were washed with phosphate buffered saline (PBS) four times and the coverslips removed from the plate. The cells were irradiated using the Chromacity 1040 nm for 60 seconds or 4 repetitions of 60 seconds with a 60 seconds cooling period between. After irradiation the coverslips were returned to the 6 well plate, fresh media added and incubated for 24 hrs. After this time, the cytotoxicity was measured using trypan blue exclusion as previously described. Cytotoxicity was measured in relation to control cells with no nanoparticles.

In vivo

Female Nu/Nu mice, five weeks of age (n=3), (Charles River, UK) were kept in pathogen-free conditions (weight of mice was 20–25 g). Human pancreatic cancer cell line BxPC-3 was cultured to 90 % confluence in RPMI 1640 supplemented with 10 % fetal bovine serum and 1 % penicillin streptomycin. The cells were washed twice with cold PBS and harvested with trypsin for 10 min at 37 °C. The cells were washed three times with PBS and resuspended in 50:50 media:PBS. The tumour cell suspension (3.0 × 106 cells in 100 µl of 50:50 PBS:media) was injected subcutaneously (s.c.) in the right flank of each mouse. When the tumour became palpable (approximately after one week), measurements in two dimensions with Vernier callipers were carried out twice a week and the volume of the tumours calculated according to Equation 1.

$$V = \frac{4}{3}\pi\left[\frac{(D1+D2)}{4}\right]^3$$

The aim of this experiment was to test if the Chromacity 1040 was A) useful for laser irradiation of these nanoparticles and B) more effective than the previous laser source.

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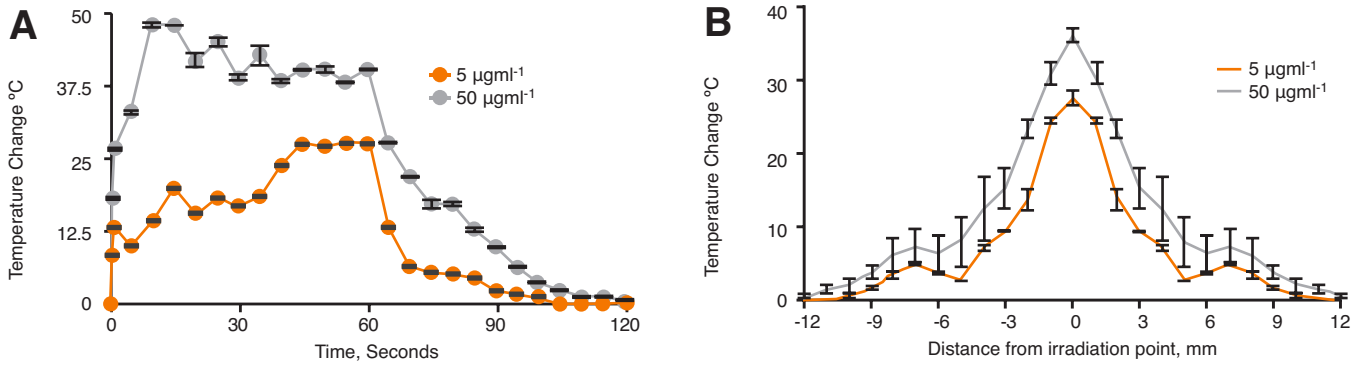

FIGURE 1. Infrared monitoring of hybrid nanoparticles in 2% agar after 60 s irradiation with the Chromacity 1040 nm showing A) Thermal change over 120 s and B) Heat dissipation from laser source at 60 s irradiation (n=3,±SD).

Once tumour volume reached a maximum of 0.9 cm^3 the mice were sacrificed and used for imaging immediately post mortem. Hybrid nanoparticle solution (0.2 $mgkg^{-1}$ or 0.02 $mgkg^{-1}$ 100 µl) was injected intra-tumorally (I.T.) using a 26 gauge needle (Vet-Tech, UK). The tumour was irradiated using the Chromacity 1040 nm for 60 seconds and temperature change and heat dissipation measured using an Optris PI640 thermal imaging camera (Optris, Germany). The ΔT data was calculated in relation to a tumour bearing mouse control containing no nanoparticles.

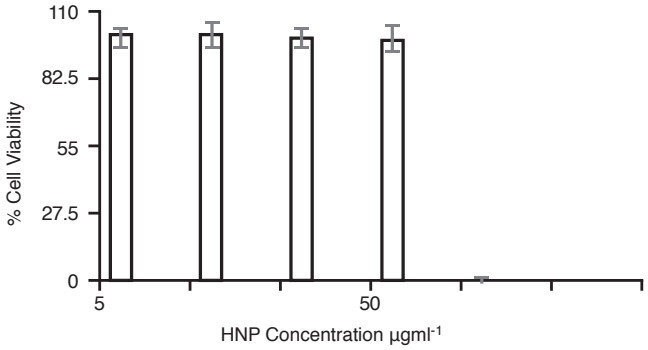

FIGURE 2. Cell viability of BxPC-3 cells containing 50 $µgml^{-1}$ and 5 $µgml^{-1}$ hybrid nanoparticles 24 h post irradiation (n=3,±SD).

Comparing previous work on hybrid nanoparticle heating shows that the Chromacity 1040 provides greater thermal rise in both agar and in tumours.

Results

The laser irradiation studies in agar phantom showed heating increase of up to 48 °C for the 50 $µgml^{-1}$ and 27 °C for the 5 $µgml^{-1}$ samples (Figure 1A). The maximum heating was achieved in both cases with a plateau in the graph being observed thereafter. The cooling profiles for both concentrations looked similar, whereby, heat was rapidly lost, followed by a slower return to ambient temperature. The heat dissipation studies (Figure 1B) showed that in both cases the heat spread from laser source was approximately 12 mm in each direction.

The cell viability was measured in BxPC-3 cells containing either 50 $µgml^{-1}$ or 5 $µgml^{-1}$ hybrid nanoparticles, 24 h post irradiation (Figure 2). Here it was observed that those cells containing the lower particle concentration, did not experience any adverse effect on their viability, even after the multiple irradiation cycles. However, those cells which contained the higher concentration of particles (50 $µgml^{-1}$) were no longer viable after both 60 s irradiation and multiple irradiations. This is probably due to the temperature increase inside the cells exceeding the threshold temperature (50 °C) after which cells can no longer survive.

FIGURE 3.
The Chromacity 1040.

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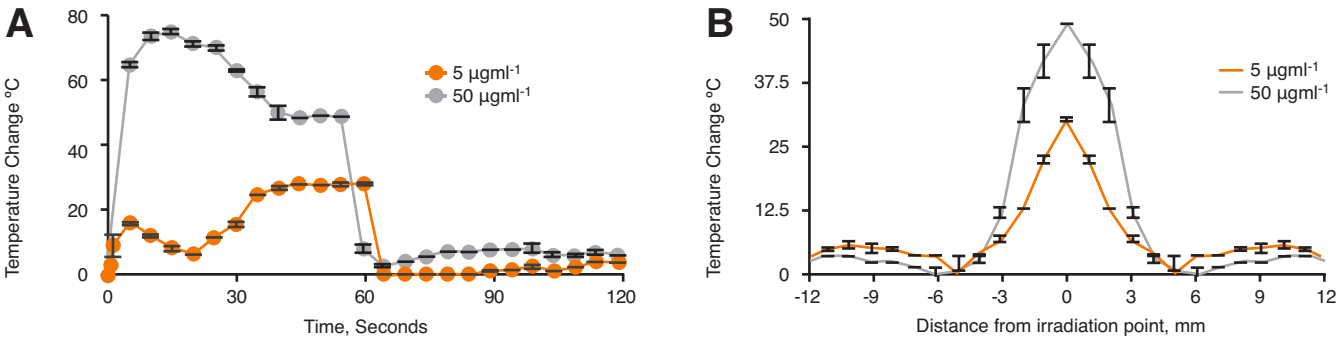

Figure 4. Infared monitoring of hybrid nanoparticles in tumor bearing nude mouse xenographs cadavers after 60 s irradiation with the Chromacity laser source showing A) Thermal change over 120 s and B) Heat dissipation from laser source at 60 s irradiation (n=3,±SD).

Thermal rise and heat dissipation was measured in a xenograft bearing cadaver. The hybrid particles were injected intra-tumorally at the equivalent concentrations dependant on mouse weight. Here the data showed that a greater increase in heat was observed compared to the agar phantoms with up to 80 °C heating increase at 50 µgml^{-1} and a 26 °C increase at 5 µgml^{-1} (Figure 4A). The reason for the difference to the agar data is not known. However, pancreatic cancer forms very dense solid tumours, and perhaps this has an impact on localised heating. Figure 4B shows the heat dissipation in the tumour tissue. Here we observed that at 5 mm radius from the irradiation point, the majority of heat had been lost with only small thermal changes beyond this point.

Benefits

Comparing previous work on hybrid nanoparticle heating shows that the Chromacity 1040 has major advantages compared to the previously used 1064 nm source.

Consistently, we observed that the Chromacity 1040 nm provides greater thermal rise both in agar and in tumours. Previously, at 5 µgml^{-1} no notable heating was observed compared to control samples. However, in this study in xenografts, those injected with 5 µgml^{-1} experienced 27 °C temperature increases. Additionally, there was an 8-fold increase in temperature at 50 µgml^{-1} compared with the 1064 nm irradiation study.

These important findings show that for biomedical application more than a 10-fold reduction in nanoparticle concentration could be administered in order to experience similar heating effects. This would not only render formulations more cost effective but also reduce any question over the toxic burden of nanocarrier or long-term accumulation. This capability would enable either localised tumour ablation or thermally triggered drug delivery which is highly localised to the laser irradiation point, thus minimum impact on surrounding tissue would be experienced.

Reproduced with thanks to Dr. Clare Hoskins and the Nanopharmaceutics group at Keele University.

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MEASURING PICOSECOND FLUORESCENCE LIFETIMES

Using a Chromacity 520 in line with an Edinburgh Instruments FLS1000 Equipped with a Hybrid Photodetector

Introduction

The measurement of fluorescence lifetimes provides a wealth of information on the sample under study, from charge-carrier lifetimes in semiconductors to the local environment of biomolecules in living cells. In many of these studies, short fluorescence lifetimes on a picosecond timescale are required to be measured.

In this application note, the measurement of sub 20 picosecond lifetimes is achieved by combining a Chromacity 520[2], ultrashort pulse laser source with an Edinburgh Instruments FLS1000 Photoluminescence Spectrometer equipped with a hybrid photodetector.

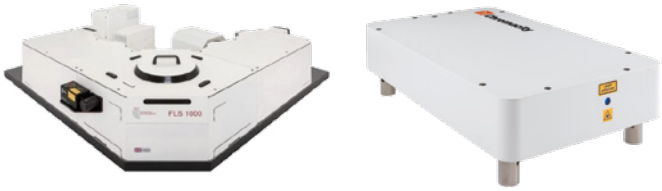

FIGURE 1. The Chromacity 520 was coupled into the FLS1000 Photoluminesce Spectrometer to narrow the IRF and drive performance.

Materials

The hemicyanine dye trans-4-[4-(dimethylamino)-styryl]-1-methyl-pyridiniumiodide (4-DASPI) was chosen to demonstrate the measurement of short lifetimes using the FLS1000. DASPI dyes are commonly used as short lifetime fluorescence probes and as a laser gain media. 4-DASPI was purchased from Sigma Aldrich® and dissolved in either anhydrous ethanol or distilled water.

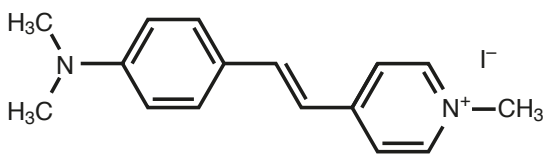

FIGURE 2. Molecular structure of 4-DASPI

Experimental Configuration

Fluorescent lifetimes in the FLS1000 are measured using the time-correlated single photon counting (TCSPC) technique. In an ideal TCSPC system, the temporal width of the excitation pulse and the temporal response of the detection system would both be infinitely sharp (delta functions). However, this is never the case since lasers have finite pulse widths and detectors have transit response times. The deviation from the ideal is characterised by the instrument response function (IRF) of the TCSPC system. The IRF can be thought of as temporal response that the TCSPC system records for an infinitely short fluorescence lifetime.

The measured fluorescence decay is convoluted with the IRF, and the Full Width Half Maximum also known as the temporal width of IRF ($FWHM_{IRF}$) determines the minimum lifetime that can be successfully measured.

A useful rule of thumb is that the minimum fluorescence lifetime that can be determined through reconvolution analysis is around 1/10th of the $FWHM_{IRF}$. It is therefore important to minimise the $FWHM_{IRF}$ when measuring short fluorescence lifetimes.

The FLS1000 is a fully modular spectrometer and can be configured to suit different research requirements. By changing the configuration of the FLS1000 or the light source, the width of the IRF can be adjusted to meet the minimum measurable fluorescence lifetime required in different research applications.

In this application note, the measurement of sub 20 picosecond lifetimes is achieved by combining a chromacity 520, ultrashort pulse laser source with an Edinburgh Instruments fls1000 photoluminescence spectrometer equipped with a hybrid photodetector.

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$$FWHM_{IRF} = \sqrt{FWHM^2_{detector} + FWHM^2_{laser} + FWHM^3_{dispersion} + FWHM^2_{electronics}}$$

FIGURE 3. There are four main components which combine to give the total width of the IRF.

FWHM Electronics

$FWHM_{electronics}$ is the broadening of the IRF due to the electronic jitter of the TCSPC timing electronics. This contribution to the IRF is small as every FLS1000 uses the advanced Edinburgh Instruments TCC2 counting electronics module which has a jitter as low as 20 ps.

FWHM Dispersion

$FWHM_{dispersion}$ is the temporal dispersion of the light after passing through the emission monochromator. The extent of the dispersion depends on the groove density of the monochromator grating and the width of the monochromator slits. The higher the groove density the larger the spectral and temporal dispersion. High groove density gratings are therefore optimal for high resolution spectral measurements but broaden the IRF.

Each monochromator in the FLS1000 can house up to three gratings simultaneously enabling an optional low groove density grating to be installed for measuring short lifetimes. The user can quickly switch between the spectral (typically 1200 gr/mm) and lifetime (300 gr/mm) gratings in the Fluoracle® operating software.

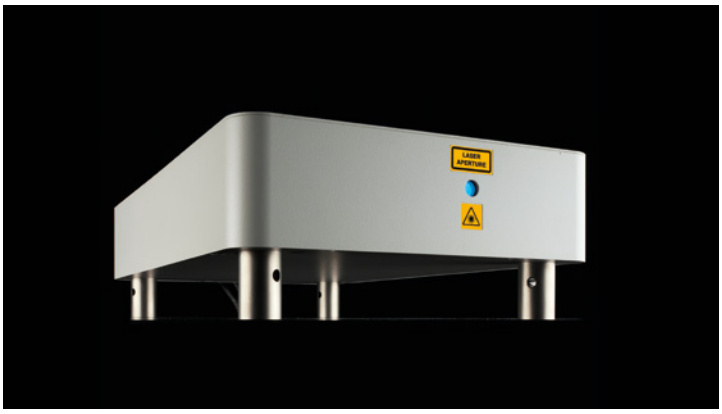

FIGURE 4. The Chromacity 520 is a compact, air cooled laser source making it easy to set up and use

For the measurement of 4-DASPI in the following section, the 300 gr/mm lifetime grating was used. To lower the temporal dispersion even further a subtractive double monochromator could be used, as found in the Lifespec II Spectrometer, which has zero temporal dispersion.

FWHM Laser

$FWHM_{laser}$ is the pulse width of the laser excitation source. Picosecond pulsed diode lasers are the most common light source for TCSPC measurements but are not ideal for all scenarios. For that reason the FLS1000 has laser ports to allow the user to couple the Chromacity 520 into the spectrometer. By making use of this ultrashort laser source it has been possible to narrow the IRF. In this instance the Chromacity 520 generated pulse durations of 150 fs for the measurement of 4-DASPI. To trigger the TCSPC electronics, a fraction of the laser pulse was picked off and directed into the OT900 Optical Trigger Module.

FWHM Detector

$FWHM_{detector}$ is broadening due to the transit time spread (TTS) of the detector and has the greatest influence on the overall width of the IRF.

The TTS is due to the photon-to-photon variation in the delay between the absorption of a photon at the photocathode of the detector and the electrical output pulse. The standard detector of the FLS1000 is the PMT-900 which is a conventional side window photomultiplier tube (PMT) which has a TTS of ~600 ps and limits the minimum lifetime to > ~60 ps. To measure shorter lifetimes the FLS1000 can be equipped with high-speed PMTs (HS-PMT) which have TTS of ~180 ps. For many years the only option for measuring shorter fluorescence lifetimes was using a microchannel plate PMT (MCP-PMT) which have excellent TTS of <25 ps but are expensive and fragile. Recently a new type of fast detector has been developed, the hybrid photodetector (HPD), which combines components of a traditional PMT with an avalanche photodiode to make a robust detector with a TTS as low as an MCP-PMT. For the measurement of 4-DASPI, the FLS1000 was equipped with an HPD detector. The combination of HPD detector, low groove density grating and 150 fs pulses from the Chromacity 520 laser resulted in a $FWHM_{IRF}$ of only 34 ps.

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Using a Chromacity 520 in line with an Edinburgh Instruments FLS1000 Equipped with a Hybrid Photodetector

Results

The fluorescence decay of 4-DASPI in ethanol is shown in Figure 5a. The decay was measured at the emission maximum (600 nm) and the IRF was measured at the laser wavelength (520 nm) using a scattering dispersion of colloidal silica (LUDOX). The decay was fit with a single exponential using revonvolution fitting in the Edinburgh Instruments FAST advanced lifetime analysis software. A single exponential decay models the fluorescence well and the lifetime of 57 ps is in good agreement to previous reports.[1]

It is known that when 4-DASPI is dissolved in water its fluorescence lifetime decreases fivefold,[1] and a solution of 4-DASPI in water was therefore also measured to provide a more challenging sample to test the short lifetime capabilities of the FLS1000. The fluorescence decay and fit of 4-DASPI in water are shown in Figure 5b revealing a lifetime of 11 ps which is in perfect agreement to the 11 ps lifetime previously reported in the literature.[3]

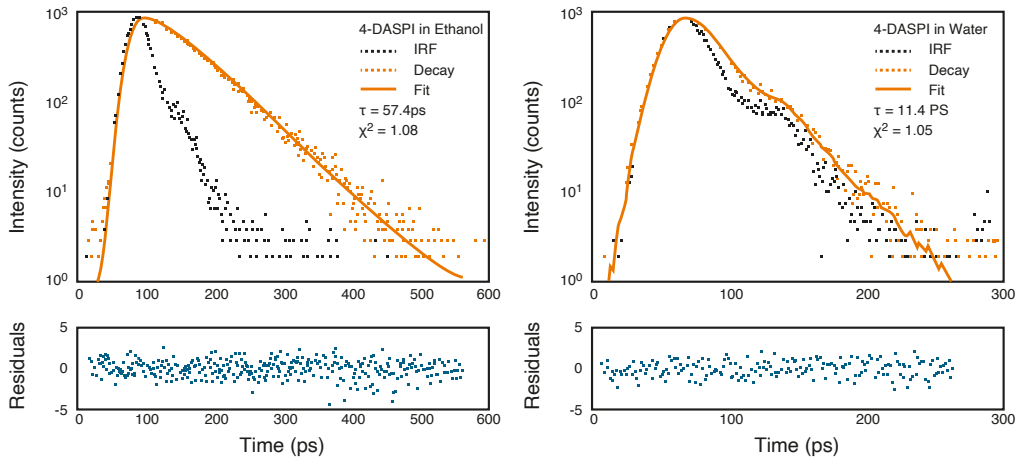

FIGURE 5. Fluorescence decays of 4-DASPI in (a) ethanol and (b) water measured using TCSPC.

Conclusion

The capability of the FLS1000 Photoluminescence Spectrometer, in line with the Chromacity 520 ultrashort pulse laser, to measure short picosecond lifetimes was demonstrated by measuring the fluorescence lifetime of 4-DASPI in ethanol (57 ps) and water (11 ps). Through customisation of the light sources, gratings and detectors of the modular FLS1000, the minimum measurable lifetime can be adjusted to meet the demands of a wide variety of different research areas.

Content courtesy of Edinburgh Instruments

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