Spectrally Resolved Confocal Fluorescence Microscopy with a Supercontinuum Laser

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BIOGRAPHY

Simon Schlachter received his BS (2004) and MS (2006) in electrical engineering from the University of Illinois at Urbana-Champaign, USA. He is currently pursuing his PhD at the University of Cambridge in the Laser Analytics Group working on the development of advanced optical microscopy techniques (http://laser.cheng.cam.ac.uk).

ABSTRACT

The advent of novel supercontinuum light sources is set to change the future of confocal microscopy, enabling wavelength- and lifetime-resolved imaging. Here we describe the development of a versatile confocal microscopy system which features full flexibility for wavelength excitation and spectrally resolved fluorescence detection. The system makes use of a compact, turn-key supercontinuum light source and a custom built prism spectrometer in combination with a commercial microscope frame. It greatly expands the parameter space accessible to the microscopist and permits the resolution and differentiation of features that are spectrally similar. Some of these capabilities are demonstrated with applications in plant and live-cell samples.

KEYWORDS

light microscopy, confocal microscopy, fluorescence microscopy, supercontinuum radiation, laser, spectral imaging, live-cell

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INTRODUCTION

Researchers are continually seeking to improve methods for the quantification of a fluorophore's local environment and functional state. This is being achieved by using hyperspectral, multi-modal imaging techniques. For example, combined measurements of fluorescence excitation and emission spectra as well as lifetimes provide a new capability for determining the photophysical state of the fluorophore. This state is affected by the local environmental properties of the fluorophore, such as pH, viscosity, and the presence of quenchers. Thus, by precisely measuring the photophysical state of a fluorophore, we inherently learn about the environment of that fluorophore. In biology this has led to a revolution: much of what we know about chemical pathways in living cells stems from data generated by sophisticated fluorescence microscopy techniques.

The advent of supercontinuum sources in the late 1990s provided for the first time a broadband excitation source having a continuous spectral range from the near-UV to the near-infrared with power densities that are on the order of 1 mW nm^{-1}. The use of acousto-optic tunable filters (AOTFs) in conjunction with this wide spectrum creates an ideal source for fluorescence microscopy excitation, capable of rapidly and continuously scanning through a wide range of wavelengths.

Access to a continuous spectrum is especially useful for several new classes of fluorophore. Of particular interest are the recently developed fluorescent monomeric proteins, such as the mFruit fluorophores. These fluorescent labels are small enough to avoid interfering with the protein(s) under investigation, yet have relatively high quantum efficiency, good photostability and a wide range of emission wavelengths [1]. The peak excitation wavelengths of these new fluorescent proteins, however, often lie between the fixed laser lines that are available with traditional fluorescence microscopes. For example, mCherry has a peak excitation wavelength of 587 nm, which is between the 543 nm and the 633 nm He:Ne lines. The supercontinuum laser coupled with an AOTF enables efficient excitation of the mCherry fluorophore at the peak absorption wavelength.

With such a flexible excitation source, it is important to have an equally capable detection system that can provide spectrally resolved measurements of the fluorescence emission over a wide bandwidth. Combining spectrally resolved excitation and detection systems opens new frontiers of imaging possibilities. For example, several fluorophores can be individually excited and detected whilst minimizing cross-excitation and separately resolving each fluorophore. The use of multiple fluorophores is becoming increasingly important in the study of the complex interactions of proteins or intracellular structures [2,3].

Additionally, spectrally resolved microscopy is useful for studying the Förster resonance energy transfer (FRET) phenomenon. Subtle spectral emission changes caused by FRET between two fluorophores can be fully quantified by the detector, and undesirable excitation of the acceptor fluorophore minimized by the highly specific supercontinuum-AOTF source [2].

We expect that spectrally resolved microscopy will become an indispensable tool as researchers seek to extract ever more information from fluorescence measurements.

In this article we describe the integration of

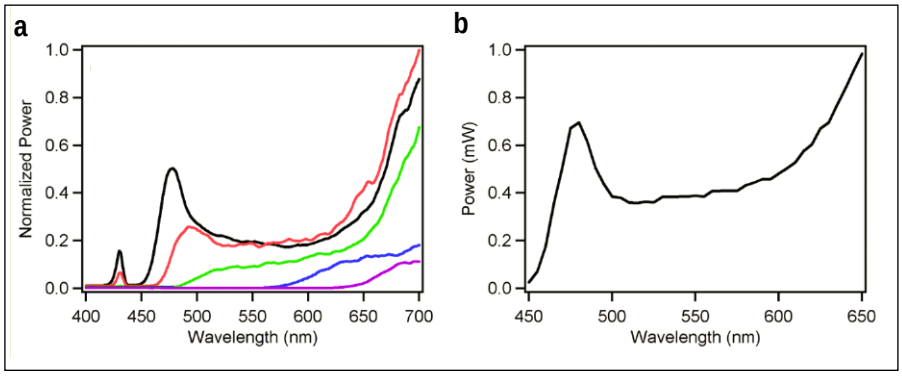

Figure 1:
(a) Visible spectrum from the Fianium SC450 supercontinuum laser with different pumping intensities.
(b) Power transmitted by an acousto-optical tunable filter for a single line with full incident laser power.

a supercontinuum laser into a confocal imaging microscope that was developed in the Department of Chemical Engineering at the University of Cambridge. The supercontinuum source is generated by a compact fibre laser-pumped photonic crystal fibre.

SUPERCONTINUUM SOURCE

The word 'supercontinuum' refers to the broad optical spectrum that is generated by pumping a nonlinear optical fibre with a short, high-intensity laser pulse. Supercontinuum generation involves a combination of nonlinear optical effects, including soliton generation, self-phase modulation (SPM), wave mixing, and Raman scattering. Their relative importance depends on the type of pump source.

Photonic crystal fibres are frequently used in the generation of supercontinua because of their highly nonlinear properties and the relative ease of coupling light into them [4]. Traditionally, high-peak power solid state pulsed lasers such as Nd:YAG and Ti:Sapphire have been used to pump these fibres. Recently, amplified mode-locked fibre lasers with short pulse widths and high peak powers have become available for supercontinuum generation [5]. The direct coupling of these fibre lasers into photonic crystal fibres provides an entirely fibre-based supercontinuum source that is robust, reliable, and can be packaged in a turn-key commercial system.

Our supercontinuum source (Fianium SC450, Fianium Inc.), uses a mode-locked fibre laser (λ = 1060 nm) as a master oscillator. The pulses produced by the master oscillator are optically amplified using a Yb-doped double-clad, pumped fibre. The amplified pulses propagate through a photonic crystal fibre in which the aforementioned nonlinear optical effects cause significant broadening of the pulse spectrum.

The visible portion of the supercontinuum spectrum is shown in Figure 1. The near infrared region of the spectrum is removed but is available for use with a different AOTF crystal.

Advances in supercontinuum sources continue to expand their output to shorter wavelengths, and currently wavelengths down to 400 nm are accessible [6]. This allows the vast majority of currently available fluorophores to be excited with a single source.

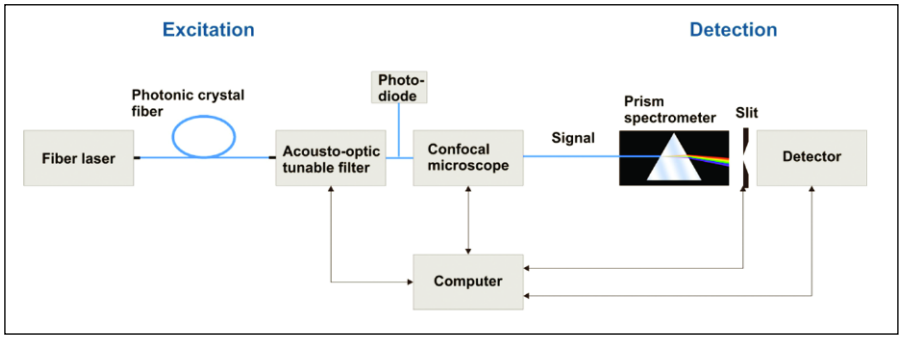

Figure 2:
Block diagram of a microscope system for spectrally resolved confocal fluorescence imaging.

SYSTEM DESIGN

Our group has built a microscope system for spectrally resolved confocal fluorescence imaging by coupling a supercontinuum laser and spectrometer to a commercial Olympus IX70 frame with an FV300 confocal scanning head. A block diagram of the microscope setup is shown in Figure 2.

The supercontinuum source described in the previous section is coupled to an AOTF that enables electronic selection of a 1nm-wide band of the supercontinuum spectrum. The AOTF consists of a TeO_2 anisotropic crystal that is driven by an ultrasonic transducer. The acoustic waves propagating in the crystal create a periodic modulation of the refractive index. This periodic modulation acts as a Bragg grating, causing the deflection of a narrow band of wavelengths that match the periodic spacing of the grating. The wavelength and intensity of the deflected beam are controlled by varying the transducer driving frequency and amplitude, respectively.

The output beam from the AOTF is coupled into a commercial Olympus FV300 confocal scan head, which directs the beam into the microscope objective lens via a pair of scanning mirrors. This confocal scanner is controlled through proprietary Olympus software that provides a wide range of options for sample illumination, including region-of-interest scanning, zoom-in, and Z stacks. Fluorescence emission from the sample is imaged onto the confocal pinhole and is subsequently coupled into a custom-designed, high-throughput spectrometer.

The prism spectrometer spatially disperses the fluorescence spectrum onto a photomultiplier tube (PMT). Two independently-controlled knife-edges placed in front of the PMT control which region of the spectrum reaches the PMT. The prism and lenses in the spectrometer are anti-reflection coated for the visible spectrum, resulting in 80% transmission of the spectrometer system. Both the spectrometer slit position and the AOTF driving frequency and amplitude are controlled via a single LabView interface, which is synchronized with the confocal scanner.

This allows us to quickly set up excitation scans, in which the excitation wavelength is rapidly swept across a given range while the sample is continually imaged. Emission scans are also possible, and the two can be combined in a joint excitation-emission scan where the fluorescence spectrum is sampled for each excitation wavelength at each pixel in the image, providing a large amount of hyperspectral data.

The entire system has been carefully calibrated to correct for the effects of variations in the excitation laser power, detection bandwidth, and detector sensitivity [7].

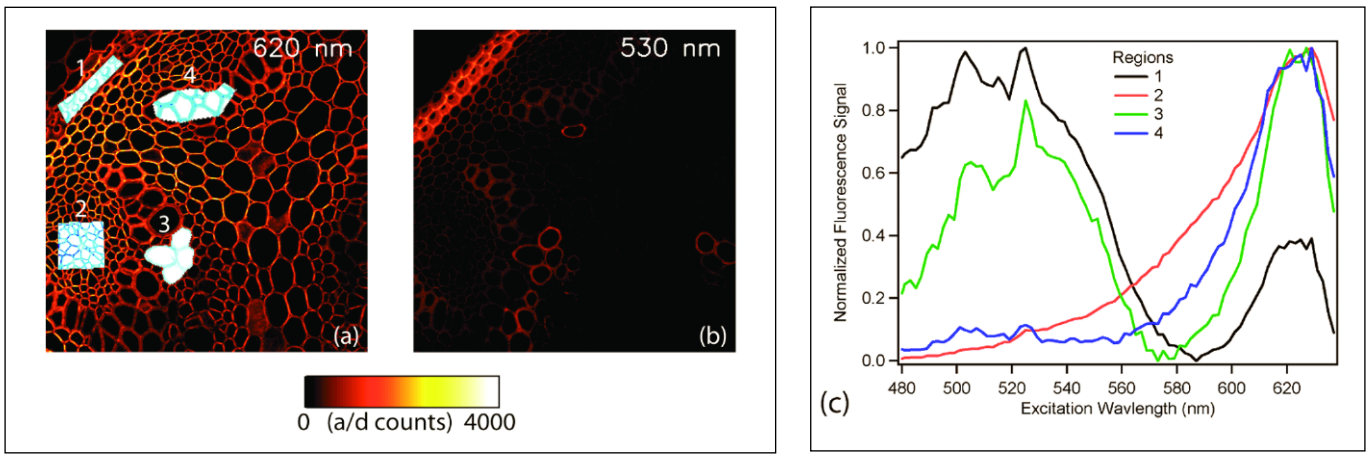

Figure 3:
Two images of Convallaria majalis taken at excitation wavelengths of (a) 620 nm and (b) 530 nm. Each image is 355 µm x 355 µm.
(c) A plot of fluorescence intensity versus excitation wavelength for several different structures within the sample (see (a) for locations).

RESULTS

To demonstrate the capabilities of this spectrally resolved confocal microscope, we imaged a fixed and stained section of *Convallaria majalis*. The sample was stained with safranin and fast-green dyes, which have excitation peaks at 530 nm and 620 nm, respectively.

We performed an excitation scan, ranging from 480 nm to 640 nm whilst continually acquiring 2D confocal images. Two images from this sequence, taken at 530 nm and 620 nm are shown in Figure 3. Different structures are evident at the two excitation wavelengths, indicating that the different stains bind preferentially to different structures within the sample.

A full excitation spectrum is available at each pixel in the image, and several of these spectra are shown in Figure 3c. As can be seen, the ability to resolve the entire excitation spectrum allows a very precise pixel-by-pixel differentiation of the multiple stains within the sample.

Though the microscope has been extensively modified, it still retains its original point-spread function, and thus the optical resolution of the instrument is unchanged. This is demonstrated in the high-resolution 2D and 3D fluorescence measurements in Figure 4. The imaged volumes in Figures 4 c-e are 92 µm × 138 µm × 18 µm with a voxel size of 0.46 × 0.46 × 0.70 µm^3. Features of the sample are still clearly resolved at this magnification, and the PSF of the microscope was measured to be 330 nm (lateral), 680 nm (axial) for a 490 nm illumination wavelength, in line with other commercial instruments.

To demonstrate that our instrument is fast enough to image live cells, we performed a fluorescence excitation scan of human U2OS osteosarcoma cells that had been transfected with a plasmid expressing an mCherry-Rad51 fusion protein. Rad51 is a DNA binding protein that is involved in DNA repair, and mCherry is a fluorescent monomeric protein described earlier. The excitation wavelength was varied between 537 nm to 607 nm in fourteen steps, with a 512×512 pixel image acquired for each step. The results of this excitation scan are shown in Figure 5. As expected, the excitation spectrum peaks at 587 nm, which is not accessible with gas or diode lasers. The closest commonly available laser line is the 543 nm He:Ne line, which from Figure 5d would result in a 60% decrease in fluorescence. Due to the overexpression of Rad51, long fibrils of DNA wrapped in fluorescent Rad51 protein appear in the nucleus of the cell. These fibrils are below the resolvable limit of the instrument and thus have been blurred by the PSF, which gives a good idea of the resolution of the instrument.

FUTURE DEVELOPMENTS

We will shortly be adding the capability for spectrally resolved fluorescence lifetime measurements to our confocal microscope. The supercontinuum source is ideally suited for lifetime imaging, because it produces 5 ps pulses at a 40 MHz repetition rate. Furthermore, the AOTF allows the excitation power to be quickly and accurately varied, and thus with the addition of a time-correlated single-photon counting (TCSPC) card to the PMT output, it is straightforward to integrate fluorescence lifetime imaging into the setup. The wavelength selectivity of the detection system and the excitation source will be preserved, so that the lifetime of the fluorophore can be determined at different spectral ranges.

As an additional imaging modality, we are also adding a second detection channel, polarized orthogonal to the first, to allow anisotropy imaging. Our instrument will then be capable of eight dimensional imaging (x, y, z, t, lifetime, lambda excitation, lambda emission and anisotropy). With these additions we hope to create a truly multi-modal confocal microscope, capable of quantifying all aspects of fluorescence emission.

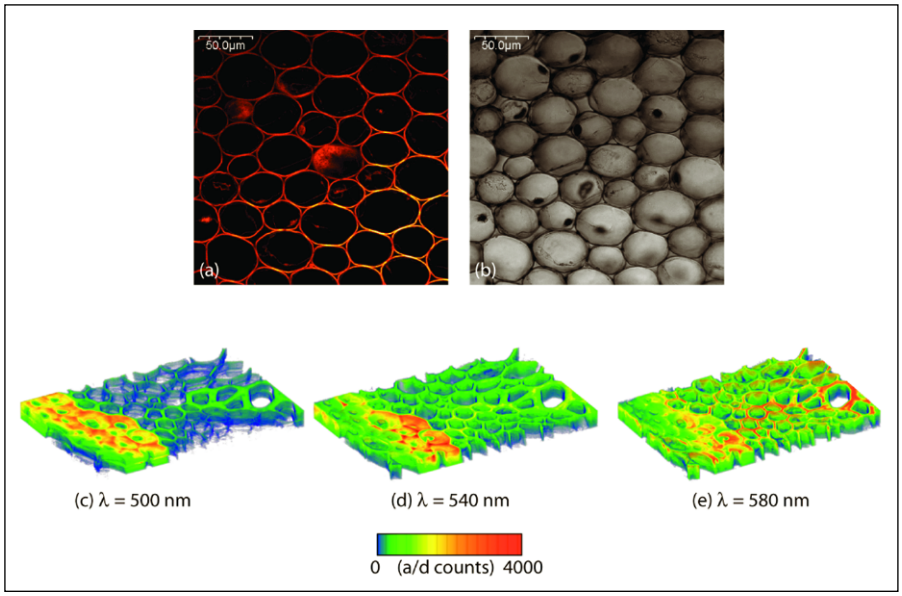

Figure 4:
High-resolution confocal (a) fluorescence and (b) broadband transmission images of Convallaria rhizome. The colour scale for (a) is the same as in Figures 3 a,b. (c-e) Comparison of 3D confocal fluorescence measurements of Convallaria with excitation wavelengths at 500, 540, and 580 nm. The imaged volume is 92 µm x 138 µm x 18 µm.

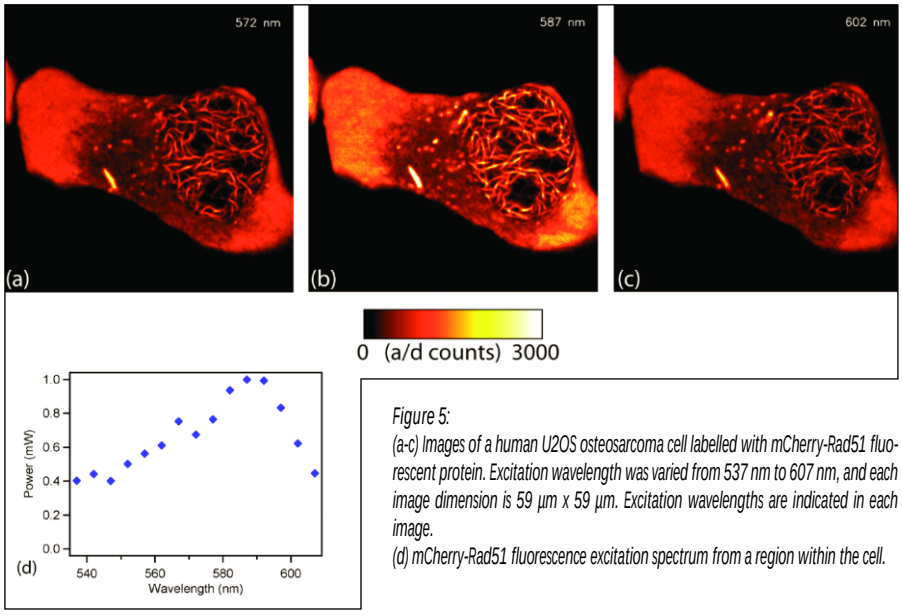

Figure 5:
(a-c) Images of a human U2OS osteosarcoma cell labelled with mCherry-Rad51 fluorescent protein. Excitation wavelength was varied from 537 nm to 607 nm, and each image dimension is 59 µm x 59 µm. Excitation wavelengths are indicated in each image.
(d) mCherry-Rad51 fluorescence excitation spectrum from a region within the cell.

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