Multiphoton induced spectral imaging for biological studies

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Introduction

The use of wide field epifluorescence and laser scanning confocal microscopy in biological applications provides excellent location information of organelles within the sample. The addition of fluorescence lifetime imaging to the confocal microscope set up has started to provide valuable information on the chemical environment of fluorophores of interest by analysing the excited state properties of the molecules under observation [1,2]. We and others have used these unique biophysical and photo-physical properties on a range of biological probe molecules to obtain further information on protein signalling in live cells [3].

As well as the temporal dimension of fluorophores the different wavelengths of emission provides another way of studying several biomolecules in unison to unravel complex molecule-to-molecule interactions. Thus the application of spectral imaging (SI) when coupled to confocal and multiphoton microscopy yields a unique form of microscopy [4]. In the SI a complete spectrum or some spectral information (such as wavelength broadening, spectral line shifts and Forster resonance energy transfer) is obtained at every pixel in an image plane. The unique combination of SI with multiphoton microscopy has several advantages including;

i) The nature of multiphoton excitation provides an inherit 3D spectral imaging of the specimen.

ii) A single excitation wavelength is able to excite several probe molecules through 2- or 3 photon processes to generate an excited state emission from several fluorphores.

iii) The use of near infrared light (700- 1200 nm) in biological samples is less cyto-toxic compared to UV or visible light providing deeper penetration of the light due to reduced absorption by cells and tissues.

iv) The rate of photo bleaching is reduced in multiphoton excitation and samples can be observed for longer periods of time. This latter point opens up the technique to applications in tissue and organ studies such as detection of disease of skin, pathological changes and forensics to name a few future application of the technique.

We have recently combined our established confocal and multiphoton techniques with Spectral imaging and present here a summary of our feasibility studies.

Experimental

Our multiphoton induced Spectral imaging consists of high repetition rate (75 Mhz) ultra short (180 fs) near infrared laser light (700 to 980 nm) from a titanium sapphire laser (Mira), pumped by a 8W 532nm diode laser (Verdi) both from Coherent (UK) Ltd. The output from the Mira laser is used to pump an optical parametric oscillator laser to give high repetition rate, sub picosecond laser in the visible (550-650nm) (APE laser, Germany). The output of the laser is directed into the back of an inverted microscope (TE2000U, Nikon Ltd) through a set of beam expander lenses and an XY galvanometer (GSI Lumonics, USA). The laser light is reflected off a dichrioc mirror and raster scan across the sample using a ×60 water, na 1.2 objective. The emitted fluorescence is collected by the same objective and separated from the excitation light using a BG39 filter or cupper sulphate solution. The fluorescence is either focused onto a fast photomultiplier tube for time resolved excited state fluorescence measurements or onto the front entrance slit of a spectrograph (Acton, USA). A custom written software scans the laser light using the galvanometers and also logs the fluorescence spectra at each XY coordinate or pixel. Thus each pixel has an associated complete fluorescence spectrum to make up the image. Since the excitation method is a multiphoton processes, there is no need to incorporate a pinhole within the emission path to obtain a confocal type imaging (figure 1).

To demonstrate the technique, fluorescent beads were purchased from Molecular Probes and mounted on to glass slides (F-24633) and used without further treatment. RBL-2H3 cells growing over a 3 day period were added to, 10 µL of 1mg/ml of Hoechst (Sigma) solution. Following 10min of incubation, the cells were washed and fresh phosphate buffer saline solution added prior to observation.

Results and discussion

We have used the new multiphoton induced Spectral Imaging (MISI) technique to obtain complete spectral profile at every pixel within our sample images. Figure 2a,b shows fluorescent beads stained with a green dye on the outside with a blue fluorophore in the inside. Using our new MISI technique, we are able to excite both the outer green fluorophore and inner blue dye at 750 nm and to obtain complete spectra of both dyes. Figure 3 shows rat RBL cells growing under physiological condition and stained with Hoechst dye. A complete spectrum at each pixel was obtained following multiphoton excitation at 750 nm. Both samples were imaged with a pixel dwell time of 60 microseconds and laser power of 3.2 mW at the sample in a single scan pass.

Although both the green and blue fluorophore loaded into the microsphere are unknown, it is likely that the green component is a fluoroscein derivative since the spectrum obtain in this work matches closely that of a known fluoroscein sample ran under identical conditions [5]. Furthermore, the one photon excitation of the loaded blue dye was quoted to be 365nm which is different to that of the green dye at 505 nm with emissions as 405 and 515 nm respectively according to the Molecular Probe web site [5]. This demonstrates the usefulness of the technique for

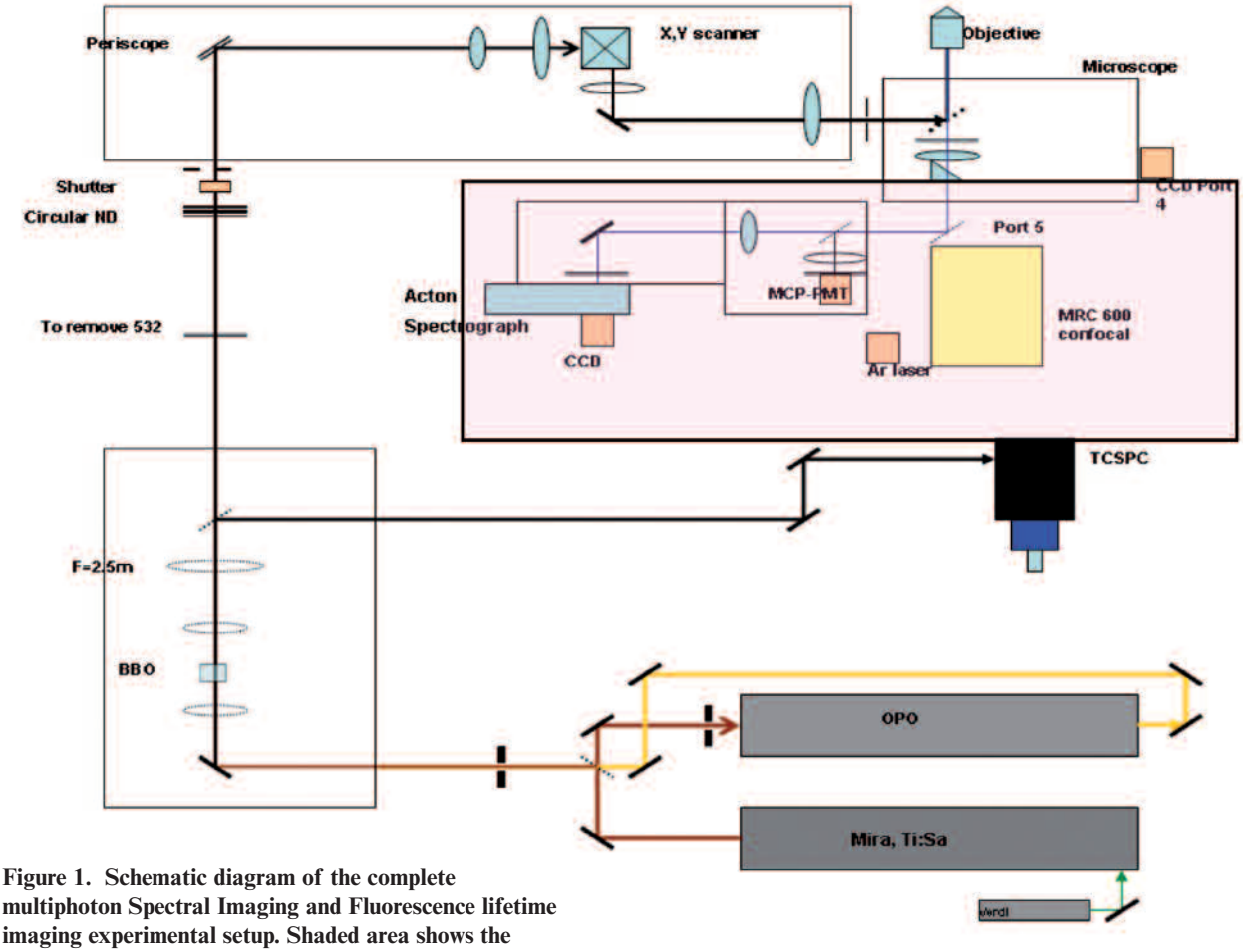

Figure 1. Schematic diagram of the complete multiphoton Spectral Imaging and Fluorescence lifetime imaging experimental setup. Shaded area shows the spectral imaging addition.

analysis of unknown samples and also offers the additional advantage of imaging as well.

Since Hoechst dye only stains a cell nucleus by interchelating the minor grove of double stranded deoxyribonucleic acid (DNA), the image in figure 3b only shows the cell nucleus and not the cell cytoplasm. During normal one photon excitation, both cell cytoplasm and nucleus will be observed as the one photon excites in a cone of radiation. This is not the case in multiphoton excitation process whereby only the submicron femtolitre volume is excited. Thus excitation and emission outside the plane of focus are not observed[4].

Conclusions

The new Multiphoton Induced Spectral Imaging (MISI) method demonstrates the feasibility of using multiphoton excitation with the added benefit of obtaining the emission spectrum from individual pixels to constitute an image. The use of multiphoton to induce the excitation has several benefits over the one photon method as described

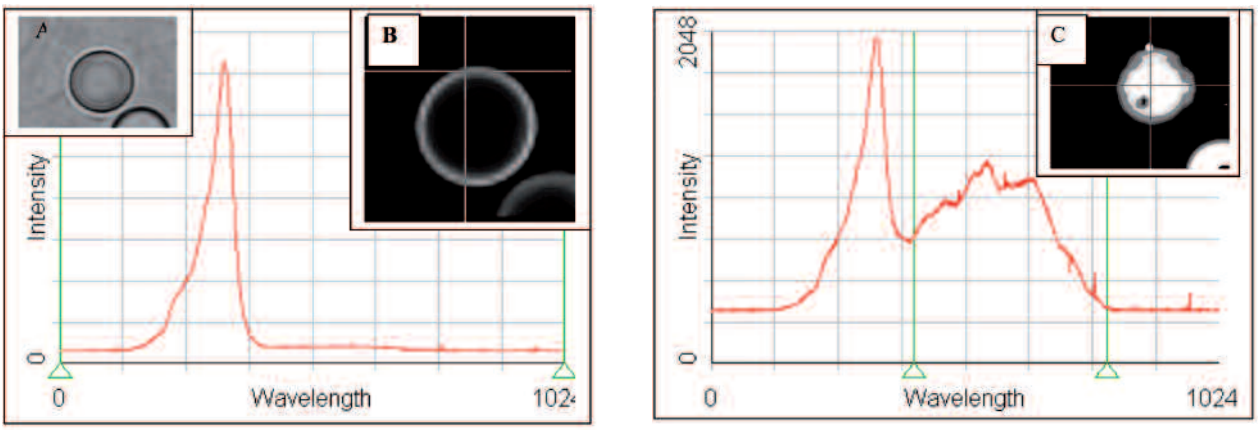

Figure 2. a) White light image of fluorescent beads. b) Spectral image of the same beads as figure 1 with associated spectra at the selected pixel where marked. c) Regions of interest may be selected depending on the spectral region of interest.

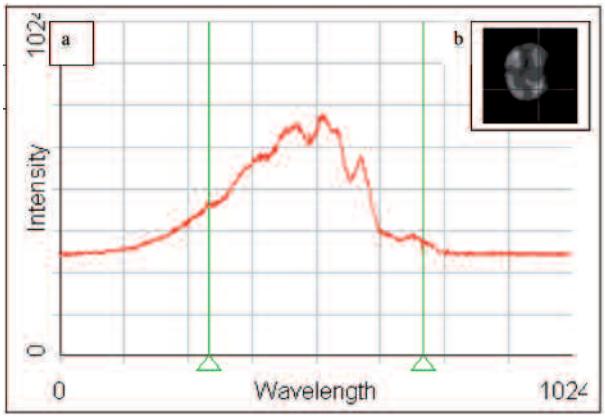

Figure 3. a). White light image of RBL-2H3 cells under physiological conditions. b) Spectral image of the same cells as figure 3a with associated spectra at the selected pixel where marked of typical Hoechst nuclear stain.

above and the spectral component allows identification of individual fluorophores to be identified within a multi-fluorophore system. Furthermore, this technology can be readily adapted to include the addition of optical trapping of samples and imaging using both fluorescence and Raman imaging that in combination will give a much higher degree of chemical specific information [7].

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