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Fluorescence lifetime imaging microscopy (FLIM)

Performing FLIM using the inVia™ confocal Raman microscope

Fluorescent materials have a characteristic lifetime in which fluorescence is emitted after light interacts with the sample. Imaging based on the measurement of this lifetime, called florescence lifetime imaging microscopy (FLIM), can be incorporated into Renishaw's inVia Raman microscope to study the structure and composition of a material.

FLIM can be combined with the inVia Raman microscope to obtain a spatial image of the fluorescence lifetime. FLIM images can be generated alongside corresponding Raman images on the same coordinate system. This enables easy and direct pixel to pixel correlation. FLIM images can be obtained at least 10x faster than Raman images; this makes it an ideal technique to identify regions of interest for subsequent Raman measurement. FLIM is particularly applicable to research in cell biology and can be used for environmental sensing, monitoring molecular interactions, and fluorophore identification.

Renishaw has developed this system in conjunction with Becker & Hickl, an established vendor working on FLIM since 1993.

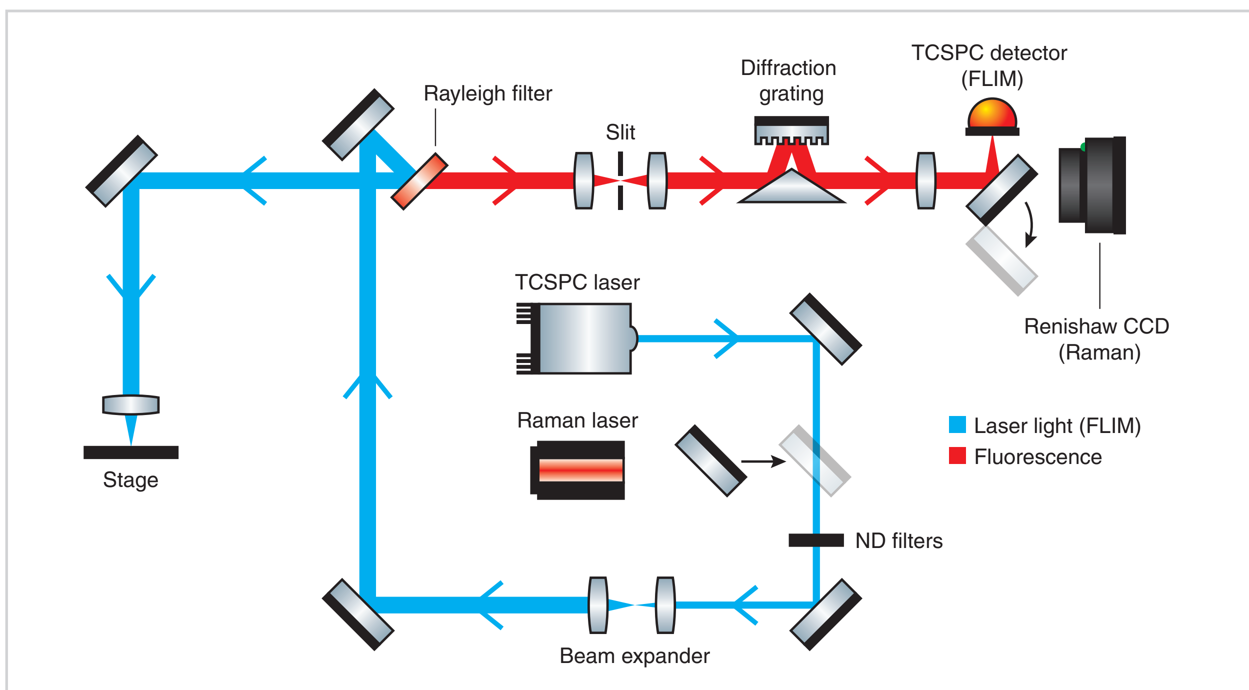

Figure 1. The Raman and FLIM beam-paths use the same optical arrangement and have parcentric beam paths allowing easy pixel to pixel correlation.

Integrating FLIM to the inVia Raman microscope

FLIM data is collected using a single-photon detector with associated timing electronics, a technique known as time-correlated single photon counting (TCSPC). A pulsed laser stimulates the fluorescence. The fluorescence lifetime is measured by determining the fluorescence arrival time at the detector relative to the excitation pulse. For imaging, the inVia microscope's high precision MS30 microscope stage moves the sample under the microscope objective. The MS30 enables correlated Raman FLIM images as large as 112 mm x 76 mm with user defined step size as small as 50 nm.

A diagram of the FLIM optical setup is shown in Figure 1. The optical path and mapping stage hardware are identical to that used for Raman imaging. Changing between FLIM and Raman imaging modes is therefore fully automated and is achieved by selecting the appropriate laser and detector. This selection is performed within the Renishaw WiRE™ software with no additional alignment required. Raman images can be acquired from the same area as the FLIM image using Renishaw's WiRE software. As the Raman and FLIM beam-paths are parcentric, the images can be precisely overlaid with pixel to pixel correlation.

The FLIM integration benefits from the ability to use the Raman system diffraction grating to control the fluorescence spectral range. This is achieved without the need for additional filters or hardware. Specific ranges and the total collectable range can all be selected purely in the software and does not require any additional alignment.

Example 1: FLIM and Raman imaging of a pharmaceutical tablet

Figure 2 shows FLIM and Raman images of a sectioned analgesic pharmaceutical tablet. The FLIM image was generated using a pulsed 405 nm laser with a 20 MHz repetition rate, and the Raman image was generated using a 532 nm continuous-wave laser. In this measurement, the Raman image reveals regions where aspirin, acetaminophen and caffeine are present, and regions where the Raman signal is dominated by auto-fluorescence (figure 2b - black represents areas where Raman bands are not visible). These auto-fluorescence regions are seen in the FLIM image and highlight the highly complementary nature of FLIM and Raman imaging.

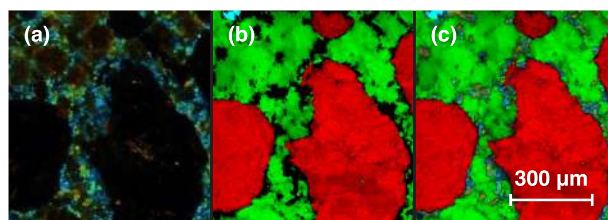

Figure 2. Combined FLIM/Raman images of an Anadin® tablet. (a) FLIM image only (b) Raman images only (aspirin – red, acetaminophen – green, caffeine – cyan) (c) Overlaid FLIM and Raman images.

Example 2: FLIM imaging of Convallaria majalis

A cross-section of a rhizome from Convallaria majalis (lily of the valley) was studied. Figure 3 shows the fluorescence lifetimes observed between 2-3 ns.

The circular structures shown in these images are vascular bundles, which transport water and nutrients to different parts of the plant. These structures have a shorter fluorescence lifetime than the main Convallaria structure. The high timing resolution of the detection electronics enables different components in the images to be distinguished with just a 1 ns lifetime range.

A FLIM image with a larger area of 1 mm x 1mm is shown in Figure 4. Such large area images are possible due to the rapid rate of the FLIM data acquisition, which can scan the sample at speeds of up to 4 mm/s. The inVia microscope also enables a high spatial resolution down to ~300 nm.

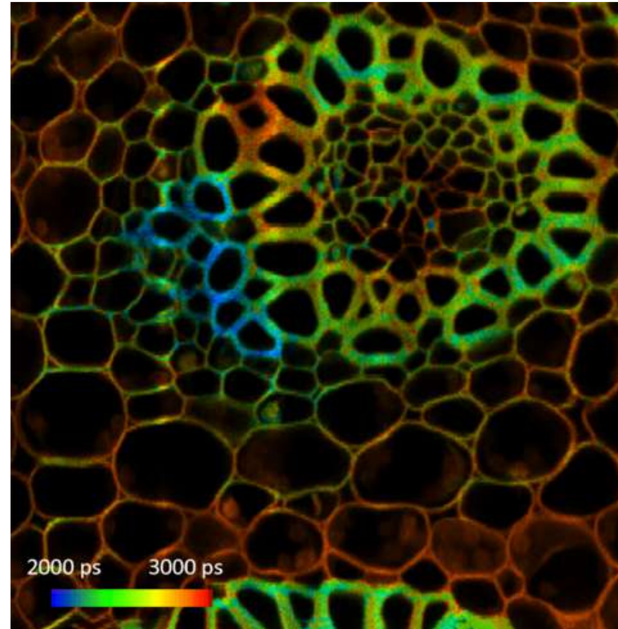

Figure 3. FLIM map of 0.25 mm x 0.25 mm region of a Convallaria majalis sample.

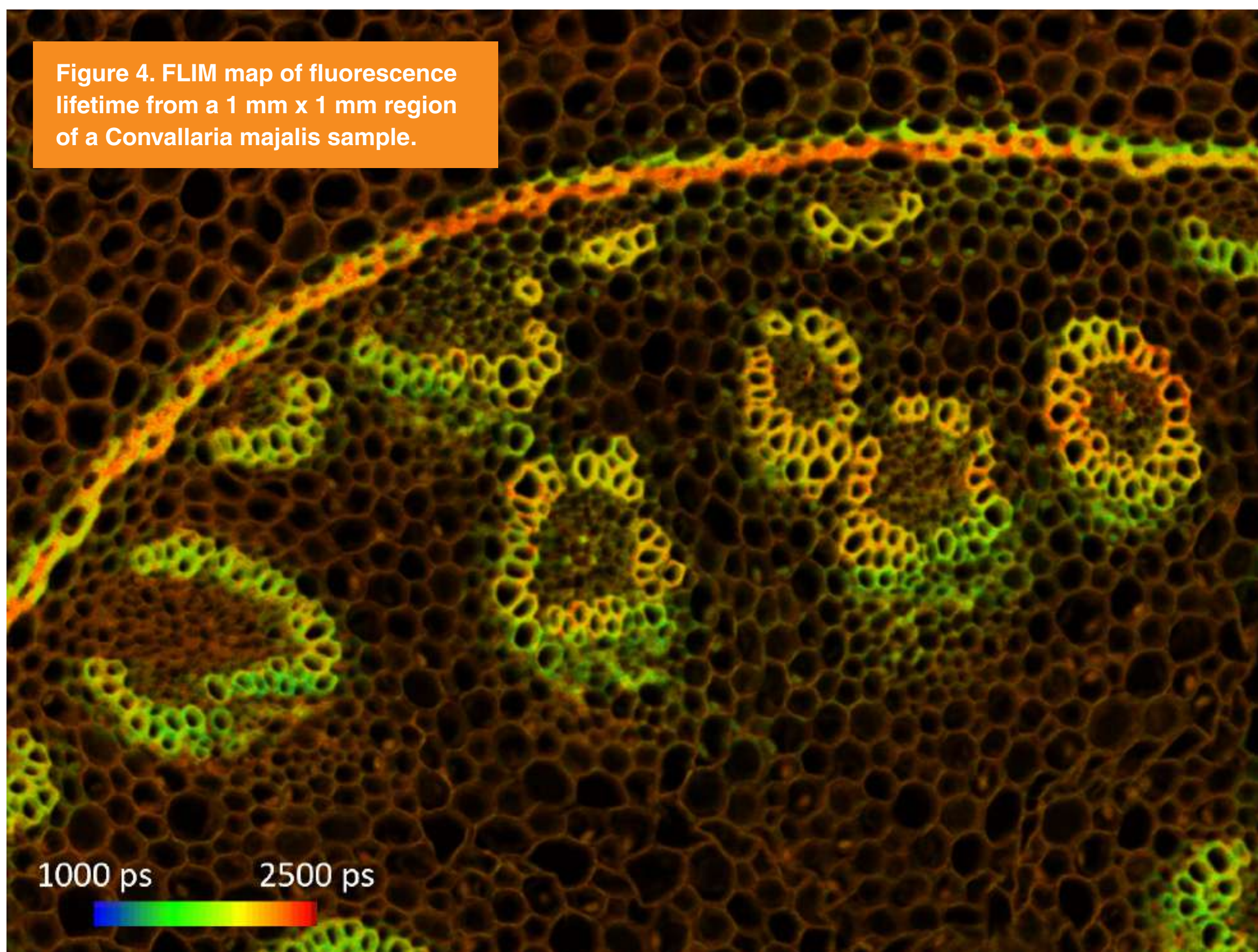

Figure 4. FLIM map of fluorescence lifetime from a 1 mm x 1 mm region of a Convallaria majalis sample.

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A combined system for increased flexibility

By adding a FLIM module to the inVia Raman microscope, you can directly correlate Raman and fluorescence lifetime images with ease. This enables you to analyse a range of structural and chemical properties at high spatial resolution, providing a key resource for a range of applications in biological imaging.

For more information on the inVia Raman microscope, please visit **www.renishaw.com/invia**

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