JY Division Information
Fluorescence

Fluorescence Mapping using the Spex® FluoroMap

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Abstract

Fluorescence mapping, a technique for localizing fluorescent species' positions under the microscope, is a useful method for advancing many scientific fields, such as biological and materials-science studies. The Spex® FluoroMap system from Jobin Yvon (JY), a steady-state and lifetime spectrofluorometer with confocal microscope, can perform reliable and sensitive research-grade fluorescence mapping. Examples of the FluoroMap's ability to distinguish differences in fluorescence under the microscope are shown.

1 Introduction

Fluorescence mapping, that is, scanning the surface in the x-y plane of a sample under a microscope for fluorescence, has been gaining interest in recent years, especially in biological research, as well as for studies of various nanomaterials [1]. The technique lends itself readily to the study of intracellular materials and biological events, whether in vitro or in vivo. Variations of fluorescence mapping include steady-state scanning, spectral imaging [2], confocal fluorescence microscopy [3], scanning while distinguishing lifetimes of the various fluorophores under study [4], high-speed and -resolution three-dimensional microscopy [5], and two-photon scanning microscopy [6]. Among the systems studied via fluorescence mapping have been lipid bilayer phases [7], cell differentiation [8], breast cancer [9], single-molecule pH-sensors [10], photosensitizers in fibroblasts [11], fixation of tissues [12], light-harvesting complexes from Rhodopseudomonas acidophila [13] and green plants [14], and green fluorescent protein [15]. Jobin Yvon (JY), the leader in fluorescence spectroscopy, has developed a new fluorescence lifetime micro-mapping system, the FluoroMap (Fig. 1), that performs confocal fluorescence mapping in both steady-state and lifetime modes with ease and reliability.

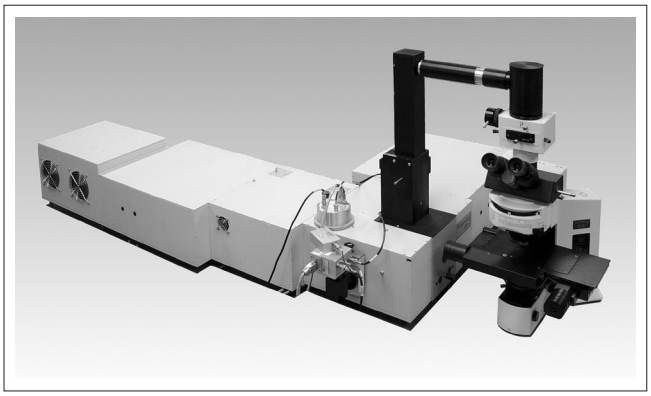

Fig. 1 Steady-state and Lifetime Spectrofluorometer, FluoroMap

2 Measurement Technique

2.1 System Configuration

In principle, the technique is simple: the sample is illuminated with an excitation wavelength, while sitting on a microscope stage that is reproducibly translatable in the x and y directions. The full fluorescence image is recorded, and the user chooses from which spots on the sample to take full spectra or lifetime measurements. The programmable stage moves to the desired areas, and the spectrofluorometer scans these areas for fluorescence emission. A diagram of the system is shown in Fig. 2.

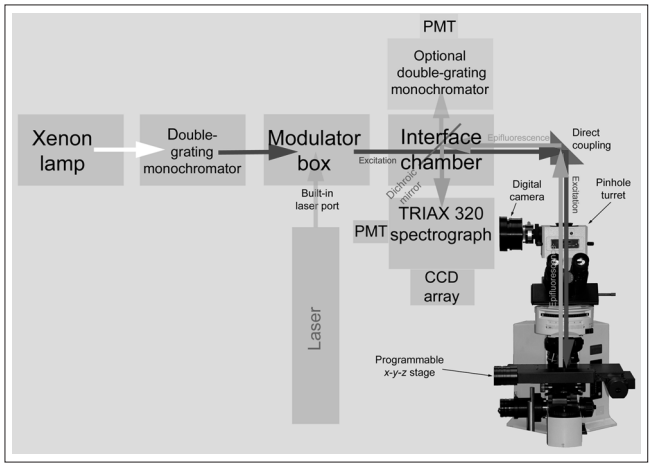

Fig. 2 Schematic of the FluoroMap's Optical Path

2.2 Steady-state Fluorescence Mapping

For steady-state fluorescence mapping, the system bypasses the Pockels-cell modulator. A continuous xenon lamp, providing an intense, broad spectrum from the UV to near-IR, is directed into a double-grating monochromator (a built-in laser port is also provided). Monochromatic light from the monochromator is fed across a spectrofluorometer-microscope interface into a multiple-pinhole turret of an Olympus BX51 confocal microscope. The user chooses the pinhole size. The incident excitation beam is directed onto the sample, resting on a programmable x-y-z microscope stage.

To view the sample's fluorescence directly, a binocular eyepiece and a digital camera are available. The user captures an image of the sample with the digital camera, and chooses small areas of the digital image over which the spectrofluorometer should record fluorescence spectra. A host computer directs the programmable stage to move to the desired areas of the sample.

Fluorescence from the sample is directed back up into the microscope. From the microscope, the resultant fluorescence travels across the interface back to the spectrofluorometer, is reflected from a dichroic mirror (an optical element which transmits certain wavelengths but reflects others) into a TRIAX 320 spectrograph, and captured with a CCD array or photomultiplier tube (PMT). Post-capture software may be used to analyze the fluorescence spectrum from each area of the image that was scanned.

2.3 Lifetime Fluorescence Mapping

For lifetime fluorescence mapping, a Pockels-cell modulator is moved into the optical path with the simple turn of a knob. The xenon source is filtered through the monochromator and directed into the modulator box. The modulator box sends the modulated monochromatic excitation beam across the interface to the microscope, through the pinhole turret, and onto the sample.

As with steady-state scanning, the user captures an image of the fluorescent sample with a digital camera, and chooses from an overlaid grid areas in which to determine fluorescent lifetimes. The host computer moves the stage to the desired areas, and captures the demodulated fluorescent response from the sample. The demodulated fluorescence returns across the interface to the spectrofluorometer, is reflected by the dichroic mirror into a double-grating spectrograph, and detected by the CCD array or PMT. Lifetimes are fitted using a non-linear least-squares algorithm.

An example of the intuitive software used with the FluoroMap is shown in Fig. 3. This Figure shows how to choose microscope parameters such as stage positioning, objective magnification, pinhole size, and file-naming for storage and later use.

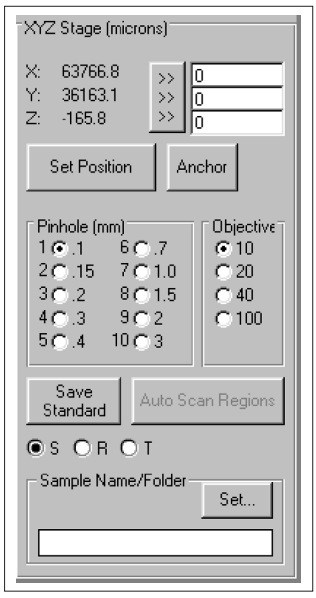

Fig. 3 Screen Capture of Window for Choosing Microscope Parameters for Spectral Capture

Top : position of the programmable stage;

middle : selecting pinhole and objective on the microscope;

bottom : sample type (Signal, Reference, or Third detector) and file-saving parameters.

3 Experiment and Results

To show the capabilities of the FluoroMap in biology and materials science, several experiments were performed.

3.1 Differentiation of Fluorescent Particles

First, an experiment that differentiates fluorescent particles in a mixture of dye powders was conducted. Small crystals of fluorescein (an orange powder; Matheson), rhodamine-6G (a dark-red powder; Eastman), and nile blue A (a greenish-blue powder; Aldrich) were mixed in roughly equal amounts and deposited on double-sided tape attached to a glass slide. Fig. 4 is an image of the mixture, taken using the FluoroMap with a 20 × objective and 0.4 mm confocal pinhole; portions of this area were studied using the FluoroMap's micro-mapping capability.

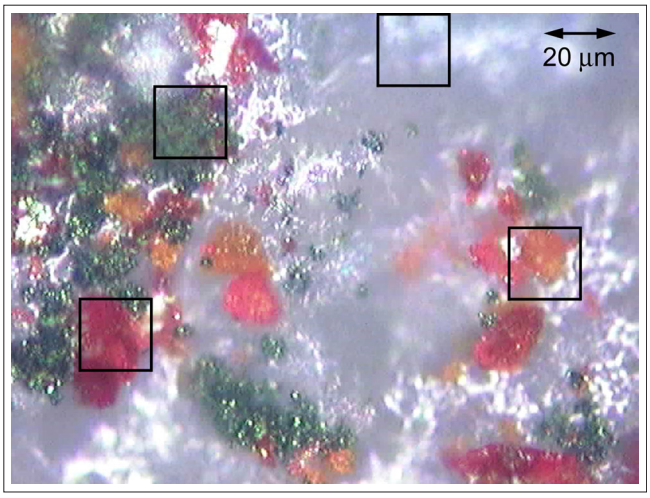

Fig. 4 Microscope Image of the Dye Powder

Mixture of fluorescein (orange), rhodamine 6G (red), and nile blue (dark green), under a 20 × objective. Gray and white areas are double-sided tape. The squares indicates areas scanned via fluorescence emission spectroscopy and presented in Fig. 6.

The actual areas studied using steady-state micro-mapping are illustrated in a screen-capture in Fig. 5.

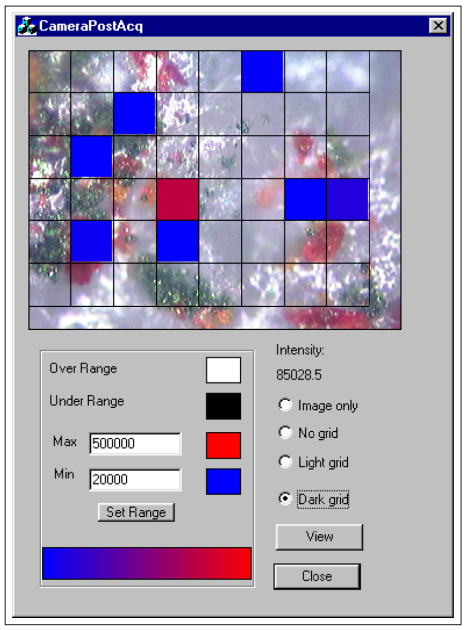

Fig. 5 Screen Capture Showing the Areas Scanned via Emission Spectroscopy

A red square indicates high fluorescence intensity; blue squares show lower fluorescence intensity.

A 450 W xenon lamp was the excitation source. The double-grating excitation monochromator was set to 420 nm, with entrance slit = 7 mm, intermediate slit = 3 mm, and exit slit = 1 mm. The emission spectrograph (TRIAX 320) was scanned from 470 to 750 nm, in steps of 2 nm, with entrance and excitation slits = 1.5 mm. The detector was an R928P photomultiplier tube with 950 V applied. The integration time per step was 2 s. Areas of the image were chosen based on the relative amounts of powdered material, plus one area was included lacking any powder. The colored squares are those for which emission spectra were taken.

Red indicates near maximum fluorescence intensity, while blue shows lower fluorescence intensity. In addition, a background scan with the microscope shutter closed was taken. All scans presented herein had the background subtracted.

Fig. 6 shows fluorescent spectrum of following four selected:

- An area mainly composed of rhodamine-6G
- An area with nile blue A
- An area predominantly of fluorescein
- An area of backing tape

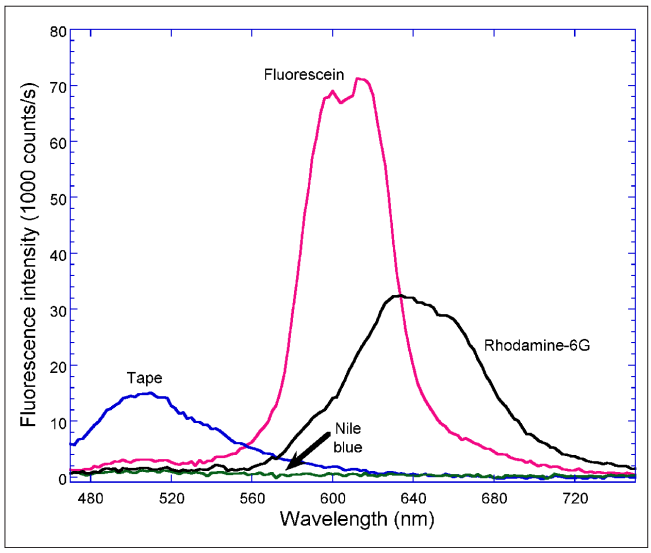

Fig. 6 Background-subtracted Emission Spectra of Four Areas of the Image

Excitation was at 420 nm. Other scan parameters are described within the text.

The red square in Fig. 5 indicated the highest fluorescence intensity of all areas examined on the image. Comparison to the image in Fig. 4 shows it is comprised of a crystal of fluorescein plus a crystal of rhodamine 6G. Because it is a mixture, the components cannot be isolated at the resolution presented here, and so it is not shown.

Four distinct spectra are visible in Fig. 6. By far the strongest spectrum is that from fluorescein (red curve), with a doublet near 600 and 620 nm, plus a weak signal from the backing tape at 510 nm. Second in intensity is the rhodamine-6G spectrum (black curve) with a peak near 632 nm and a broad emission near 656 nm. The support for the powders itself – double-sided tape (blue curve) – fluoresces near 510 nm. The nile-blue A scan (green curve) showed no significant fluorescence apart from a very weak signal from the tape at 510 nm. Among other reasons, variations in intensity can be ascribed to differences in quantum efficiency and a non-optimum excitation wavelength chosen for each material.

3.2 Variation Analysis on a Single Microscopic Crystal

A second steady-state experiment, to study various areas of a single microscopic crystal, was performed on a microscopic crystal of the fluorescent dye and photographic sensitizer 1,1-diethyl-2,2-cyanine iodide, or PIC (Aldrich). Here, with a higher magnification, four different spots on a single PIC crystal were examined (Fig. 7). The excitation monochromator was 420 nm (entrance slit = 7 mm, intermediate slit = 1 mm, and exit slit = 3 mm), and the emission was scanned from 550 - 700 nm (entrance and exit slits = 2 mm) with an increment of 2 nm, and an integration time of 1 s. Pinhole size was 0.1 mm, and a 100 × objective was used. Fig. 8 shows the background-subtracted results, showing that different areas of one PIC crystal produce different fluorescence spectra[16]. Note that spectra 3 and 4 are qualitatively similar, but spectrum 1 and spectrum 2 are different.

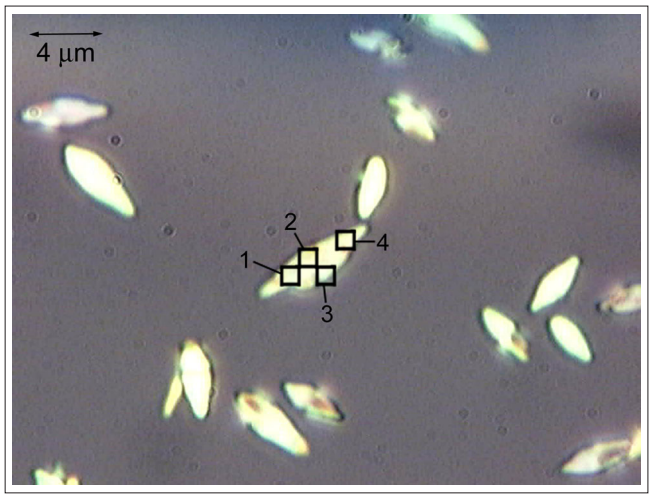

Fig. 7 PIC Crystals under a 100 × Objective

The squares indicates areas scanned via fluorescence emission spectroscopy and presented in Fig. 8.

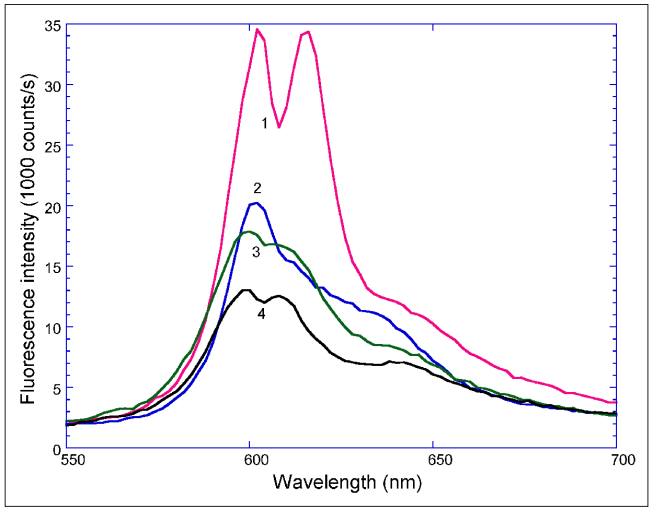

Fig. 8 Background-subtracted Emission Spectra of Four Spots (labeled in Fig. 7) on one PIC Crystal

Excitation was at 420 nm. Other scan parameters are described within the text.

3.3 Plant-cell Analysis

Finally, living cells from a spider plant were examined under the FluoroMap's microscope. Chlorophyll, a green compound, fluoresces red under short-wavelength illumination[17]. A leaf section under bright-field illumination, with a 40 × objective, is shown in Fig. 9a: the green areas are chloroplasts and the dark curved bands are cell walls. Chlorophyll resides in the thylakoids, stacked outer membranes that contain photosynthetic apparatus.

The distinct red fluorescence of the chloroplasts under excitation appears in Fig. 9b. In a "constant-wavelength analysis", the FluoroMap scanned the superimposed grid at 680 nm (Fig. 9b). The cells were excited with 457 nm light from an air-cooled Ar+-laser through a 0.1 mm confocal pinhole, and the excitation monochromator's slits were set to 2 mm bandpass, with an integration time of 0.1 s. Each square corresponds to an area of 2 µm ~ 2 µm.

When plotted as a three-dimensional graph (Fig. 10) of fluorescence intensity (height axis) versus the microscope stage's position, the peaks clearly correspond to the position of the chloroplasts.

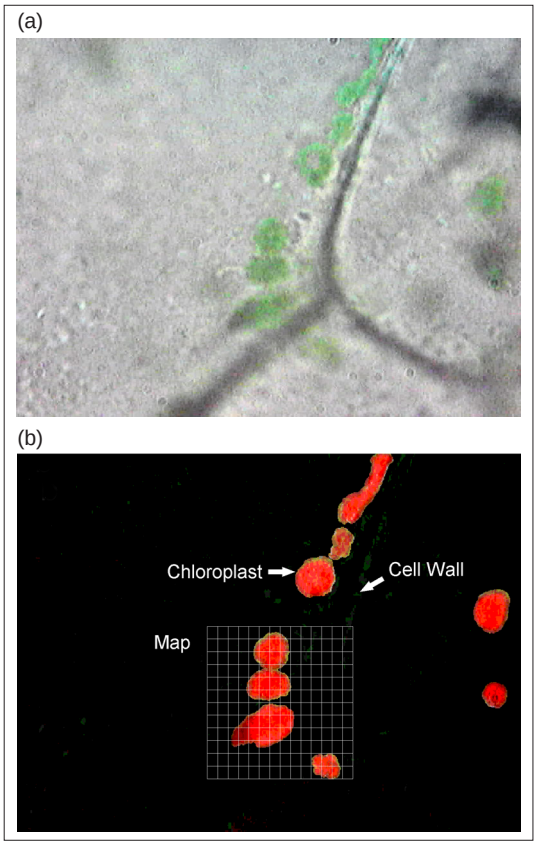

Fig. 9 Plant Cells under a 40 × Objective
(a) Bright-field Illumination of Green Chloroplasts and Darker Cell Walls
(b) The Same Area with Chloroplasts Fluorescing
The superimposed grid shows the area mapped by the FluoroMap. Each square in the grid is an area of 2 µm × 2 µm.

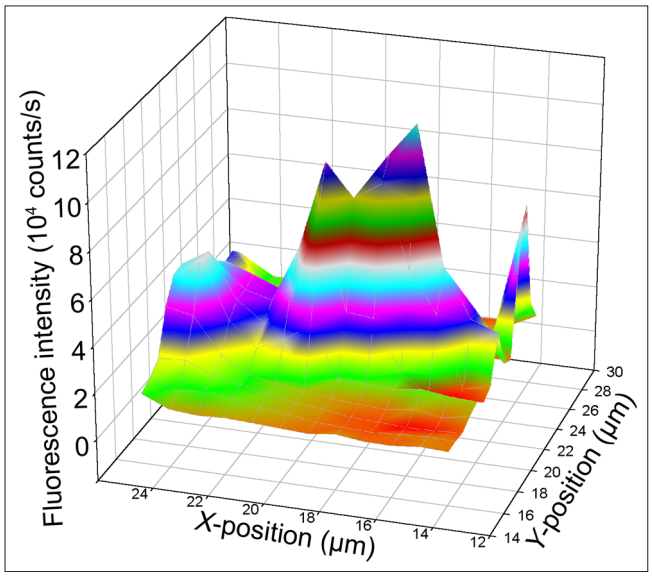

Fig. 10 Three-dimensional Plot of Fluorescence Intensity at 680 nm versus Stage Position (equivalent to position on sample)
Note that the peaks correspond to the chloroplasts in Fig. 9. Experimental conditions were: 0.1 mm pinhole, 457 nm excitation, 0.1 s integration time, and 2 nm bandpass on the emission monochromator.

4 Conclusion

The FluoroMap from JY, makers of the The World's Most Sensitive Spectrofluorometer, can distinguish microscopic areas via fluorescence mapping with ease and reliability. Both steady-state and lifetime data can be taken from microscopic biological samples rapidly and efficiently with the FluoroMap. Built around the research-grade Spex® Fluorolog®-Tau-3 lifetime spectrofluorometer, the FluoroMap revolutionizes the study of biological and biochemical fluorescence, as well as the rapidly changing field of nanomaterials.

References

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