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Fluorescence Lifetime Imaging (FLIM)

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Research topics – Fluorescence decay, photon counting and timing
See Laser Analytics Group website: http://laser.ceb.cam.ac.uk/

Introduction

FLIM is an imaging technique that captures the fluorescence decay rate (lifetime) of fluorescent molecules (fluorophores) in every pixel of the image. Every fluorophore has a characteristic fluorescence lifetime. Some, indeed most, fluorophores are sensitive to environmental parameters such as pH, temperature, ion concentration, interactions with other molecules, etc. and changes in these cause the fluorescence lifetime to change. Thus, the fluorescence lifetime can act as a molecular sensor. Because the lifetime responds only to changes in the immediate environment of a fluorophore, FLIM produces an image providing information on the molecular scale.

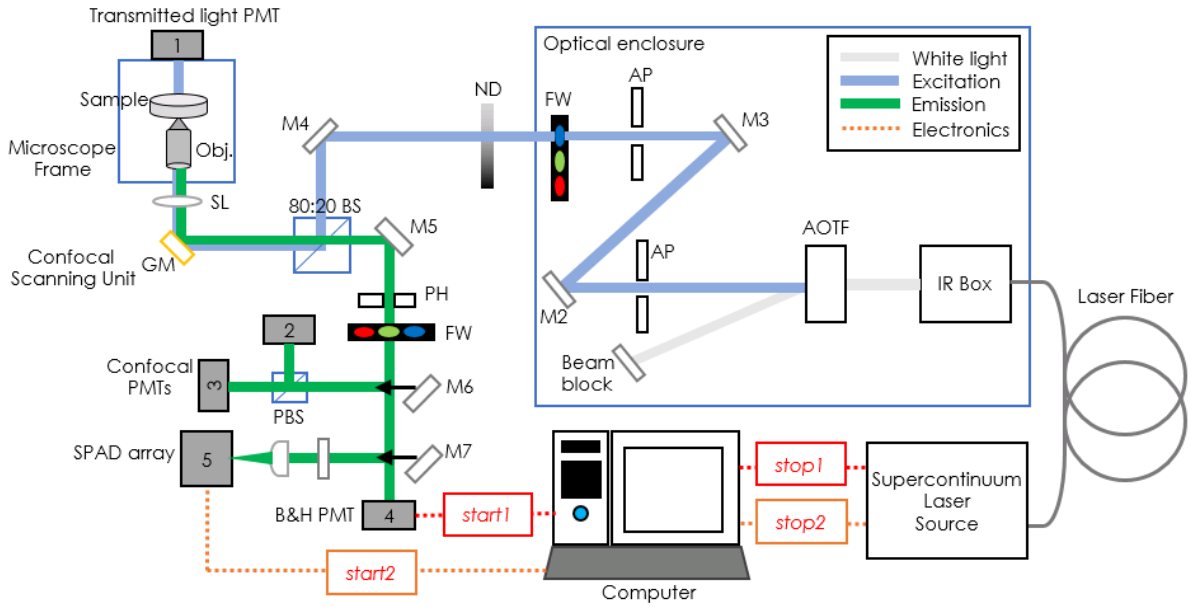

Fig 1: FLIM setup in the Laser analytics group, utilizing a supercontinuum source, five different detectors and associated electronics for single photon timing.

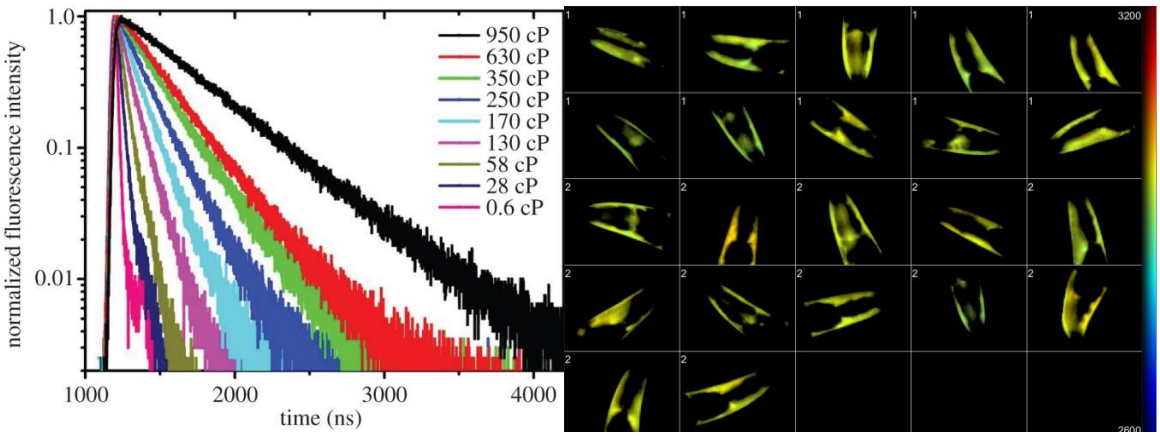

Fig 2: (left) Fluorescence decay curves of BODIPY rotor dyes change significantly at different viscosities. BODIPY can therefore be used as a sensor of viscosity in cells using the FLIM technique, adapted from [1]. (right) fluorescence lifetime images of fluorescent *C. elegans* worms that can be used as models of neurodegenerative diseases.

For more information, please refer to two review articles on FLIM [2,3] and an article on the use of supercontinuum sources in FLIM imaging [4].

1. Levitt, J. et al. Membrane-Bound Molecular Rotors Measure Viscosity in Live Cells via Fluorescence Lifetime Imaging. *J. Phys. Chem. C* **113**, 11634–11642 (2009).
2. Suhling, K. *et al.* Fluorescence lifetime imaging (FLIM): Basic concepts and some recent developments. *Med. Photonics* **44,** 3–40 (2015).
3. Becker, W. Fluorescence lifetime imaging - techniques and applications. *J. Microsc.* **247,** 119–136 (2012).
4. Schlachter, S., Elder, A., Frank, J. H., Grudinin, A. & Kaminski, C. F. Spectrally Resolved Confocal Fluorescence Microscopy with a Supercontinuum Laser. *Microsc. Anal.* **22,** 11–13 (2008).

Aims of the practical

- Selecting wavelengths from supercontinuum sources to match spectral properties of commonly used fluorophores
- Introduction to confocal laser scanning techniques and setting up a microscope for imaging
- Understanding single photon detection and timing, with associated electronics
- Understanding fluorescence decay curves and instrument calibration
- Measuring nanosecond fluorescence lifetimes of the temperature sensitive dye RhodamineB.

Questions

- What is fluorescence decay and how can this property be exploited in a useful way?
- Discuss biological or physical applications of FLIM.
- What is the principle behind time correlated single photon counting and how is it used to accurately measure the arrival time of single photons?

- Discuss the use of supercontinuum sources for FLIM imaging. What are the requirements for the excitation source? What are advantages and disadvantages? Would you use coherent or incoherent supercontinua as illumination sources for TCSPC experiments and give reasons for your answer.
- How are lifetime data analysed?
- What are current limitations of the technique? How could the technique be improved in terms of spatial and temporal resolution?

Experimental details and procedures

1. Investigate and discuss the different components necessary: Fianium Whitelase supercontinuum source, acousto-optic tuneable filter, confocal scan unit, Olympus microscope frame, photon detection systems, electronic triggering and software.
2. Use a *Convallaria* sample to get an appropriate focusing position and gain familiarity with the microscope and imaging.
3. Discuss photon counting rates and effect of photon pileup.
4. Use ErythrosinB dye with a very short 90ps lifetime to obtain the instrument response function (IRF).
5. Use RhodamineB at various temperatures to obtain the temperature dependence curve and verify with previous measurements.
6. Transfer data and analyse lifetime data using FLIMfit software.

Safe use of lasers in this practical

1. The supercontinuum laser used in this microscope is a **Class 4 laser**. Class 4 lasers pose an eye hazard both if the beam is viewed directly **and** if a diffuse reflection of the beam is viewed (e.g. if the beam reflects from a piece of white paper). Hence Class 4 beams must be fully enclosed when they are used for microscopy, by having a light-proof illumination enclosure and also by covering the sample during imaging. Supercontinuum lasers are particularly hazardous for two further reasons: (a) the beam contains a considerable amount of invisible infrared light, which could be a hazard even if the visible light were removed, and (b) the visible part of the laser beam covers the full visible spectrum, so laser goggles that allow the beam to be visualised must necessarily allow through some reflected light from the supercontinuum beam.
2. The first part of the beam path in the system (see Fig. 1) is a small enclosure (labelled IR box in the schematic) which contains two infrared-absorbing mirrors. After this box, the supercontinuum beam contains only visible light, making it safer to work with during system development and imaging.
3. The second part of the beam path in the illumination enclosure (see Fig. 1) is an optical filter (AOTF) which only allows a narrow wavelength range of the supercontinuum light to proceed to the microscope. The rest of the beam is absorbed in a beam dump. Therefore, after the end of the illumination enclosure, the laser light is low power (typically 1 mW or less is measured at the objective lens). It is not possible for high laser power (or infrared light) to reach the microscope stage, so this system is safe for use even if the sample is not covered (but it will be covered to prevent background light affecting the measurement).
4. Laser beams should be fully enclosed during normal use, to prevent any chance of direct exposure to laser light.
    a. The light source enclosure should be closed during the actual measurement.

   b. The microscopy specimens should be covered with a light-proof black box before the illumination shutter is opened.

5. **The illumination part of this system must not be opened or aligned by students on this practical** (it may only be aligned by the demonstrator). If this enclosure is open, then the following safety measures must be taken. (1) Thorlabs LG2 (green) laser goggles must be worn by all persons in the laboratory bay: these goggles will absorb any infrared light in the supercontinuum beam and makes this part of the beam eye safe. (2) The supercontinuum light source should be set to the lowest power at which the visible light can be seen, to minimise the risk from high-power laser light. (3) Care must be taken to minimise stray reflections and to terminate the beam on beam dumps; there must be no loose optical components near this beam path.