Latin American Posgraduate Program in Biophysics (POSLATAM)

IV POSLATAM Course
Third South American Workshop
International Gregorio Weber Conference
in

New Trends in Advanced Fluorescence Microscopy Techniques

Buenos Aires, December 12 to 17, 2011

Course description: This course aims to provide a solid background in the theoretical concepts and practical skills in modern fluorescence spectroscopy and microscopy with applications in biological research. Topics will be introduced in lectures, and experimental and computer labs will give the student hands-on practice. The course will introduce novel concepts and applications on fluorescence and discuss different microscopy and image analysis techniques.

Goal: To acquire the fundamental tools for the analysis and design of quantitative fluorescence microscopy experiments.

COURSE PROGRAM

Basic Definitions and Principles of Fluorescence
A short history of fluorescence . Basic spectral properties. Jablonski Diagram and Stoke´s shift. Excitation and emission spectra. Polarization/anisotropy. Fluorescence lifetime. FRET: Forster resonance energy transfer. Multi-exponential decays. Time-domain lifetime measurements. Frequency-domain lifetime measurements. Static and dynamic quenching, Transients. Anisotropy decays. Energy transfer-distance distributions. Time-dependent spectral relaxation. Excited state reactions

Fluorescence probes
Naturally occurring fluorophores in proteins. Tryptophan derivatives. Enzymes cofactors extrinsic probes. Covalent and non-covalent attachments *in vitro*. General labeling protocol for extrinsic labeling. Characterization after the labeling. Fluorescent proteins (e.g. GFP). Ds Red fluorescent proteins and derivatives. Labeling DNA. Nick translation. Commercially labeled dUTP. Labeling membranes Analogs of fatty acids and phospholipids. Di-alkyl-carbocyanine and di-alkyl-aminostyryl probes. Other nonpolar and amphiphilic probes. Laurdan, Prodan, Bis ANS Laurdan Generalized Polarization (GP). Quantum dots. Ions indicators. How do we choose the correct probe for ion determination. Probes for pH determination. Labeling “in vivo”. Mechanical incorporation. Electroporation. Microinjection. Biolistics. Genetic incorporation. GFP-fusion proteins. FlAsH-EDT2 labeling.

Instrumentation for fluorescence spectroscopy
Steady-State spectrofluorometers. Light Sources: lamps, lasers, laser diodes, LEDs, synchrotron radiation. Monochromators. Optical filters. Polarizers. Detectors: photomultiplier tubes, avalanche photodiodes, micro-channel plates. Instrumentation for time-resolved fluorescence.

Introduction to Microscopy
History of microscopy. First microscopes and microscopists. Resolution limit of the human eye. Evolution of the compound microscopes. Modern microscopes. The objective, a key component. The 3 classes of objectives. Numerical aperture. The resolution limit.

Fluorescence microscopy
Epi-fluorescence microscopes. The filter cube. Light sources. Filters. Charge-coupled devices (CCD) detectors. Primary features of CCDs. Types of CCD detectors. Improvements in interline CCDs. Noise as a function of incident camera illumination. Parameters for maximizing sensitivity. Photobleaching. Confocal detection. Light pathways in confocal microscopy. Airy disk and Pinhole size. The “Pinhole” as a “spatial filter”. Laser Scanning Confocal Microscopy. From spot to image. From plane to 3D image. Multi-photon excitation. Two-Photon, scanning microscopes. Laser technology needed for two-photon excitation. Advantages of two-photon excitation. Applications in Biophysics.

Fluctuation Correlation Spectroscopy(FCS)
Measuring the internal dynamics in cell. Methods based on perturbations and on fluctuations. The fluctuation-dissipation principle. Fluctuations in fluorescence signals. The autocorrelation function. The effects of particle concentration on the autocorrelation curve. Effect of shape on the (two-photon) autocorrelation functions. Blinking and binding processes. The effects of particle size on the autocorrelation curve. Two channel detection: Cross-correlation. Removal of detector noise by cross-correlation. Two-color cross-correlation. Experimental concerns: excitation focusing & emission collection. Biophysical applications.

The Photon Counting Histogram: Statistical Analysis of Single Molecule Populations
Fluorescence trajectories. Photon count histograms. Contributions from the detector noise and from the profile of illumination. Contribution from several particles of same or different brightness.

Raster Image Correlation Spectroscopy (RICS)
Raster Scanning. Temporal information hidden in the raster-scan image: the RICS approach. The RICS approach for diffusion. Space and time relationships. How to setup the laser scanning confocal microscope. Common errors in RICS. How we go from solutions to cells. How to subtract immobile features from images. Cross-correlation RICS. Experimental issues.

The Number & Molecular Brightness (N&B) Method
Existing methods to determine protein concentration and stoichiometry of protein complexes in cells. The Number and Brightness (N&B) analysis. How to distinguish pixels with many dim molecules from pixels with few bright molecules. Calculating protein complexes from images. Selecting the dwell time. What contributes to the variance. Identification of mobile and immobile molecules. Brightness and number of molecules can be measured independently. Cross N&B analysis. The co-variance principle and the derivation of the ccN&B method.

Fluorescence Lifetime Imaging Microscopy (FLIM) and the Phasor approach
Time domain and frequency domain in time resolved fluorescence. Fluorescence lifetime imaging. Evaluation of Förster Resonance Energy Transfer by FLIM. The challenges of FLIM. The phasor space and the universal circle. The algebra of phasors. How to identify components. How to distinguish two multi-exponential components from FRET. The fractional intensity calculator and the FRET calculator. Examples of FLIM analysis using phasors. The pitfall of “conventional” FLIM analysis.

Particle tracking
Single particle tracking: The position of one particle as a function of time. Image-based tracking techniques. Finding the position of a particle with nanometer precision. Pattern-recognition tracking method. In vivo tracking experiments: molecular motors. How the motor moves along a filament. Understanding the stepping mechanism. Minimizing the tracking error. Obtaining quantitative information from trajectories analysis. The mean square displacement (MSD). Determination of the mechanism of motion. Detection of multiple populations. 3D particle tracking. Image-based 3D methods. Particle tracking in a 2-photon microscope. Real-time 3D tracking of viral particles in live cells.

PRACTICAL ACTIVITIES

Computer training

A. Fluorescence Correlation Spectroscopy (FCS)
- Exercise 1. Simulation of single point FCS.
- Exercise 2. Simulation of monomer-dimer and global analysis. Autocorrelation function (ACF) and photon counting histogram (PCH) analysis.
- Exercise 3. Simulation of two color experiments: cross correlation analysis of heterodimer formation.
- Exercise 4. Simulation of fluorescence kinetics: FRET fluctuations

B. Line scanning, Image Correlation Spectroscopy (ICS) and Raster Image Correlation Spectroscopy (RICS)
- Exercise 1. RICS simulations
- Exercise 2: Simulation using circular scanning
- Exercise 3. Line scan analysis of adhesions
- Exercise 4. RICS analysis of cell images
- Exercise 5. Number and Brightness (N&B) analysis in line scanning and in cells

C. Fluorescence Lifetime Imaging (FLIM).
- Fluorescence Lifetime Imaging Tutorial.
- Phasor Properties.
- Two State Equilibria.
- Quenching.
- Analysis of FRET from CFP and CFP-YFP expressing cells

D. Particle Tracking with SimFCS
- Introduction
- Simulation settings for different particle motion scenarios
- Tracking Particles
- Analyzing Trajectories (Basics)

E. Biosensors: FRET intensity image calculation in SimFCS

Experimental training

LAB 1. Effect of solvent environment on ANS fluorescence.
Determination of excitation and emission corrected spectra of ANS in ethanol, aqueous buffer and bound to BSA.
Polarization (anisotropy) measurements on the 3 samples
Discussion of the obtained results.

LAB 2. Covalent labeling of a protein with a fluorescente probe
Labeling Bovine Serum Albumin (BSA) with FITC (Fluorescein Isothiocynate)
Polarization measurements on FITC free and bound to BSA
Discussion of the obtained results

LAB 3. Basic Fluorescence Microscopy
A. Setting up the microscope.
B. Analysis of the images
C. Images treatment
Discussion of how these parameters affect the images
1. Lookup tables (LUTs)
2. Contrast, offset.
3. Filters of images
4. Achieving sub-pixel resolution by fitting a Gaussian: deconvolution

LAB 4. Basic Confocal Microscopy

A. Set up the microscope (dichroic mirrors, filters, laser power, etc) to take an image of a specimen of fixed cells labeled with a fluorescent probe.

Explore the changes in the image with the following variables:

1. Pinhole size
2. Laser power
3. Pixel size
4. Scanning speed
5. Detectors Gain and Offset
6. Spectral bandwidth (lambda-scanning)

B. Obtain a Z-stack (series of images in different planes in the z axis) of the cells:

1. Discuss how the parameters influence the final images and the 3D information obtained.
2. Change the pinhole size: How does it affect the reconstruction in 3D? What is the ideal pinhole size for a given Z-stack? What is the relationship between the pinhole size and the distance between the image planes? What is the importance of the pinhole size for FCS measurements in a one-photon excitation set up?

LAB 5. Fluorescence Correlation Spectroscopy

-Autocorrelation

-Photon Counting Histogram (PCH)

-Cross-correlation

- Experiment 1: Calculating concentrations: volume calculations
- Experiment 2: Instrument and sample artifacts.
- Experiment 3: Multiple Species: PCH & Autocorrelation
- Experiment 4: Two Color FCS / cross-correlation

Course coordinators

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Instructors

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Confirmed Invited Lectures (International Gregorio Weber Conference).

Luis Bagatolli, MEMPHYS - Center for Biomembrane Physics, Denmark.

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Target audience

The course is designed to be taken by both life sciences and physical sciences Ph.D. students. It is specifically designed to accommodate the different academic backgrounds of the students enrolled in the different programs. The course is also appropriate for advanced undergraduate students who have sufficient laboratory training. Undergraduates should (and Ph.D. students may) consult the instructors to check that they have adequate preparation for the course.

Applicants should go through a selection procedure. (Maximum number of participants in the experimental training module: 20). Lectures and computer training activities are open to all, but pre-registration is required.

Grading

There will be one exam (take home, but worked on independently) at the end of the course.