Workshop outline

- Confocal microscopy of living cells and tissues
- X-Z scanning
- Time series
- Bleach: FRAP, photoactivation
- Emission spectra and spectral deconvolution
- Ratio imaging
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Out-of-focus signal rejection by scanning confocal microscopy (epifluorescence)

- A field lens (L) and objective are used to focus a diffraction-limited spot.
- The field lens projects emitted light from this point to the infinite as a collimated beam.
- A pinhole (or diaphragm) is placed in the intermediate plane in front of the detector and rejects all but the signal originating from the focal point.
Comparison of sample illumination in conventional and confocal fluorescence imaging

Conventional microscope
(Widefield illumination)

Confocal microscope
(Illumination by a diffraction-limited spot)
Weaknesses of confocal microscopy

1. The luminous flux of excitation needed to detect enough signal is very high, which has the following consequences:
   - Photobleaching of dyes
   - Photodynamic damage to living cells

2. Speed limitation caused by point-by-point image formation (non-parallel scanning).
   
   E.g.: 512x512 pixel image, video rate = 40 msec/image (25 fps)
   --> each pixel is visited during only 152 nsec

3. No direct visual observation

4. Laser illumination limits the range of available wavelengths

5. Complexity and high cost of the instrument
Strengths of the confocal microscope

1. **Out-of-focus light** is not recorded.
   Moving the focus does not cause blur in the image but gradually cuts the parts of the object that are not in the focal plane: *optical sections* of the sample.

2. Because of the small dimension of the spot illuminating the focal plane, *scattered* and diffused light is minimized.

3. A set of *tri-dimensional* data can be recorded.

4. Scanning an object in the x/y direction (i.e. in the plane) and in the z direction (along the optical axis) enables visualizing the *object* from all sides.

5. Using image processing, optical sections can be superimposed and produce an *extended focus*, which can be obtained in conventional microscopy only by reducing the aperture and thus diminishing resolution.
Other possibilities offered by the confocal microscope

- (Very) Fast measurements by line scan (rather than frame scanning).
- Measurement of cell height or volume change by X-Z scanning (piezo focus).
- As laser light is normally polarized, excitation light that has not been absorbed by the sample can be collected by the condenser and be used to build a transmitted Nomarski image.
- By rapid change of laser light intensity using an acousto-optical modulator, it is possible to perform experiments of:
  - fluorescence recovery after photobleaching (FRAP) experiments to measure motility or diffusion of fluorescent compounds
  - photoactivation of caged compounds (flash photolysis)
  - photoactivation of photoactivatable proteins (e.g. Kaede)
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X-Z scanning

Single optical sections
X-Z scanning

After 3D reconstruction and rendering
X-Z scanning

Line scan along the X axis and rapid Z movement
X-Z scanning

Line scan along the X axis and rapid Z movement
X-Z scanning

Optimal using piezo Z-drive (speed and resolution)
X-Z scanning

Applications

Experimental

- direct and rapid measurement of specimen thickness
- XZ over time: dynamic cell volume measurement (e.g., swelling, regulatory volume decrease, etc.)

Technical / teaching

- direct assessment of point spread function (PSF)
- assessment of pinhole effect
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Time series

- Idea: recording of fluorescence signal over time
- Applicable for recording:
  - entire images, sub-regions, lines, points
  - in 2D, in 3D (z-stacks), spectra, etc.
  - which becomes "4D", "5D", "6D", etc.
- Acquisition of time series can be combined and synchronized with:
  - internal actions (e.g. bleach)
  - external events (opening of valves, stimulus, etc.)
"TIME SERIES" module of Zeiss LSM 510 Meta software
Frame averaging

Line averaging

Lateral mouvement
"TIME SERIES" module of Zeiss LSM 510 Meta software
to record only intensity in a region (!)
To visualize the results: Gallery
To visualize the results: Graphs
To visualize the results: Movies
To visualize the results: Movies

MAP GFP
(B. Riederer)
Stacks (3D) with time
"TIME SERIES" module of Zeiss LSM 510 Meta software
LINE SCAN: to go very fast...
LINE SCAN: to go very fast...

Each line is scanned very rapidly (in this example <1 msec)
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Fluorescence recovery after photobleaching (FRAP)

**Definition**

Rapid and localized photodestruction of fluorescence used as an experimental tool....
Photobleaching

- Weakening of fluorescent signal
- Poorly understood, but critical in fluorescence microscopy
- Transition to a triplet state, reaction with oxygen
- Quantum yield * of fluorescein: ~ 0.9

⇒ Quantum yield of bleaching of fluorescein: ~3 \(10^{-5}\)
⇒ The molecule will emit 30,000-40,000 photons in its photochemical life.
⇒ This is independent on the type of illumination (continuous or pulsed).
⇒ At low intensity of illumination, bleaching is not eliminated by only its kinetics is slowed down.

* Quantum yield:
  \[ Q = \frac{\text{Number of photons emitted}}{\text{Number of photons absorbed}} \]
FRAP - Fluorescence Recovery After Photobleaching

**Applications:**
- rates of diffusion and convection
- motility of proteins (e.g. in membrane, reticulum, etc.)
- protein interactions
- protein synthesis
AOTF (acousto-optical tunable filter)

- Working wavelengths: 340-4500 nm
- Speed of selection: < 1ms
- Angle of acceptance: ± 5° (i.e. laser)
- Efficiency of transmission/diffraction: 80%
- Maximum input power: 500W/cm²
- Selection of both wavelength and intensity

Measurement of diffusion by FRAP

Non-reversible

Reversible

A

fluorescein

GFP

500 kDa FITC-dextran

6 kilobase plasmid FITC

Fluorescence

B

GFP in cytoplasm

+ paraformaldehyde

200 ms

20 ms

GFP-AQP1 in cell membrane

+ paraformaldehyde

1 min

1 sec

Taken from Periasamy (2001) "Methods in cellular imaging", p. 115
"TIME SERIES" module of Zeiss LSM 510 Meta software
Example: Gap junctions in mouse astrocytes

Loading with BCECF AM

False color intensity LUT
Astrocytes functional gap jonctions

Percentage of recovery: 55%
Time to 90% : 17 seconds
Inhibition of gap junction using octanol

Loading with BCECF AM

False color intensity LUT
Inhibition of gap junction using octanol

Percentage of recovery: 7%
Time to 90%: 9 seconds
UV Photoactivation using the “Bleach” module

UV Photoactivation of caged ATP. Astrocytes loaded with calcium probe Fluo-4
UV Photoactivation using the “Bleach” module

Event markers
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Transitions from different vibrational levels define absorption spectrum of a molecule. A similar set of transitions from S1 to S0 define its emission spectrum.
Microscope with spectral detector:

Configuration Zeiss

Zeiss LSM 510 Meta

Detector:

array of 32 photomultipliers in parallel
Spectral detector: Zeiss configuration

- Emitted light (Polychromatic)
- PMT array (32 channels)
- Simultaneous recording of 8 channels (images)

Emitting Fluorescence

Wavelength (nm)
Possibilities offered by the spectral detector

Define band-pass "filters"  Spectral scanning
Emission spectra
Gallery by wavelength

492nm
566nm
513nm
566nm
534nm

545nm
556nm
566nm
577nm
588nm

599nm
609nm
620nm
631nm

Spectra in subregions of images
Spectra in subregions of images

Application example:
Demonstration of colocalization of two mitochondrial dyes, CoroNa Red and Mitotracker Green.
Spectra taken in one single mitochondrion

Possibilities offered by the spectral detector

Define band-pass "filters"

Spectral scanning
Create “Meta-channels”
Linear spectral deconvolution

Leica: “Spectral unmixing”
Zeiss: “Emission fingerprinting”, “Linear unmixing”
Spectral deconvolution

**EGFP**

**SYTOX Green**

**YFP**
Spectral deconvolution

Excitation 488nm Zeiss LSM 510 Meta

EGFP  SYTOX

YFP  Combined
See you next week…
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Ratio imaging

**Excitation ratio**: fixed emission, excitation with two wavelengths

**Examples**: indicators like Fura-2 (calcium), BCECF (pH), SBFI (sodium)

**Emission ratio**: fixed excitation, emission with two wavelengths

**Examples**: indicator like Indo-1 (calcium), SNARF-1 (pH)
Ratio imaging

**Emission ratio** : fixed excitation, emission with two wavelengths

**Examples**:
- organic probes like Indo-1 (Calcium)
- FRET probes (eg: cameleons)
BCECF : Sensitivity to ambient pH

Isosbestic point (440 nm)

BCECF = 2',7'-bis-(2-carboxyethyl) -5-(and-6)-carboxyfluorescein
pKa = 6.98
Example of ratio dye: Fura-2

- **Excitation**
  - High Ca\(^{2+}\)
  - 0-Ca\(^{2+}\)

- **Emmission**

- Isosbestic point

- Emission points:
  - 340 nm
  - 380 nm

Longueur d'onde (nm)
Comparison: single wavelength and ratio fluorescence changes

Rem: simulated plots
“Ratio” Module of Zeiss LSM software