Microscopie confocale à balayage laser

Semaine 10b
Stereo Imaging:

Stereo pair images can be created from a stack of confocal images by a technique known as “pixel shifting.”

In pixel shifting two separate 2-D projections of the data set are created by shifting adjacent image planes slightly out of registry creating a pseudo-left and pseudo-right projection.
(PC12 cell stained for microtubules)

Stereo Anaglyph

Two dimensional projection of focus series
Special topics -2

Photostimulation: One or more laser lines are used to stimulate the sample while the other lasers lines interrogate the it. (ref)

• Diffusion:
  1. FLIP—Fluorescence Loss in Photobleaching
  2. FRAP—Fluorescence Recovery after Photobleaching
  3. FLAP—Fluorescence Localization after Photobleaching
  4. FCS—Fluorescence Correlation Spectroscopy
  5. FCCS—Fluorescence Cross-Correlation Spectroscopy
  6. RICS—Raster Image Correlation Spectroscopy

7. Quantitative force measurements by optical tweezers and confocal imaging
8. Photo-thermal stimulation
Laser combiner
1. FLIP—Fluorescence Loss in Photobleaching

• Used to examine the movement of molecules inside cells and cell membranes.

• Continuous photobleaching of fluorophores outside of the region of interest. Monitor fluorescence intensity elsewhere.

• Determines continuity of membranes and organelles (e.g., compartments that are separated by lipid bilayers)
FLIP—Fluorescence Loss in Photobleaching

Specimen: HeLa cell, GFP (free), 488nm excitation (multi-argon laser)
Image acquisition time: 100ms/bleach time: 100s continuously, 405nm bleaching
FLIP—Fluorescence Loss in Photobleaching

FLIP plugins for ImageJ:

- [Quantitative fluorescence loss in photobleaching for analysis of protein transport and aggregation](https://doi.org/10.1186/1471-2105-13-296), *BMC Bioinformatics* 2012, **13**:296
- [FRAP/FLIP Analyser](https://www.embl.de/marvin/FRAPFLIPA/FRAP/FLIPAAnalyser) (EMBL)
2-(i) FRAP - Fluorescence Recovery after Photobleaching

\[ I_t = M_f \left( 1 - e^{-\tau t} \right) \]

\[ \tau_{1/2} = \frac{\ln(0.5)}{-\tau} \]
2. (i) FRAP-Fluorescence Recovery after Photobleaching
2. (i) FRAP - Fluorescence Recovery after Photobleaching
2. (i) FRAP-Fluorescence Recovery after Photobleaching

ImageJ plugins:

- SimFRAP FRAP simulator, HardinLab FRAP analysis,
- FRAP intensity measurement (FRAP analysis) and normalization (FRAP Norm).
- FrapCalc by EMBL (with instructions)
- Actinsim FRAPAnalyser standalone software (with manual)
3. FLAP-Fluorescence Localization after Photobleaching

- Molecules carry 2 fluorescent labels
- One is photo bleached, the other is not.
- Ratiometric imaging shows the photo bleached fraction of molecules within each pixel.
- Unlike FRAP/FLIP we can track photo bleached fluorophores.
3. FLAP-Fluorescence Localization after Photobleaching

$$I_{\text{rouge}} = \frac{I_{\text{CFP}}}{I_{\text{YFP}}}$$
4. FCS-Fluorescence Correlation Spectroscopy

- Non-imaging technique
- Monitors fluctuations in the fluorescence intensity corresponding to discrete changes to the number of fluorescent molecules in the detection volume.
- Fluctuations are the result of individual molecular diffusion
- Confocal microscopy is necessary to reduce the detection volume (and average number of molecules)

References: 1, 2, 3, 4, 5, 6
4. FCS-Fluorescence Correlation Spectroscopy

Average number of molecules in the focal volume

RMS fluctuation of molecules in the focal volume

Optimal $\langle N \rangle = 0.1$ and 1000

$\frac{<N>}{10^{-15}L} \approx 10^{-9} - 10^{-6}M$

or (1 nM – 1μM)
4. FCS-Fluorescence Correlation Spectroscopy

\[ G(\tau) = \frac{\langle \delta I(t) \delta I(t + \tau) \rangle}{\langle I(t) \rangle^2} = \frac{\langle I(t)I(t + \tau) \rangle}{\langle I(t) \rangle^2} - 1 \]

**ImageJ FCS plugins:**

Stowers Institute: [FCS Analysis](#), [FCS Simulation](#), [RICS analysis](#)

National university of Singapore: [ImFCS](#)

DeNovo: [FCS Express Image cytometry](#)
4. FCS-Fluorescence Correlation Spectroscopy

\[ G(\tau) = \frac{\langle \delta I(t) \delta I(t + \tau) \rangle}{\langle I(t) \rangle^2} = \frac{\langle I(t)I(t + \tau) \rangle}{\langle I(t) \rangle^2} - 1 \]

ImageJ FCS plugins:
Stowers Institute: [FCS Analysis](#), [FCS Simulation](#), [RICS analysis](#)
National university of Singapore: [ImFCS](#)
DeNovo: [FCS Express](#) Image Cytometry
4. FCS—Applications

1. Determination of diffusivity values

2. Determination of conformational states

\[ D = \frac{k \cdot T}{6\pi \cdot \eta_v \cdot R_h} \]

3. Time-varying FCS
4. FCS—Applications

4. Determination of mass-transport mechanisms

*Anomalous diffusion (crowded systems): eg protein diffusion within cells, diffusion through porous media. Is proposed as a measure of macromolecular crowding in the cytoplasm.

\[ G_{motion}(\tau) = \frac{1}{V_{eff}} \left( \sum \eta_i \langle C_i \rangle M_i(\tau) \right) \]

\[ M_i(\tau) : \text{Motility-terms} \]

Free 3D diffusion:
\[ M_i(\tau) = \frac{1}{\left(1 + \frac{\tau}{\tau_{d,i}}\right) \sqrt{1 + \left(\frac{\tau}{\tau_0}\right)^2 \cdot \frac{\tau}{\tau_{d,i}}}} \]

Free 2D membrane diffusion
\[ M_i(\tau) = \left(1 + \frac{\tau}{\tau_{d,i}}\right) \]

Active transport with velocity \( v_i \)
\[ M_i(\tau) = e^{\frac{-\tau}{\tau_0}} \]
5. FCCS-Fluorescence Cross-Correlation Spectroscopy (same as ccFCS)

- Measures FCS of 2+ different fluorophores
- Reports on interaction (correlation) between molecules

\[ G_{GR}(\tau) = 1 + \frac{\langle \delta I_G(t) \delta I_R(t + \tau) \rangle}{\langle I_G(t) \rangle \langle I_R(t) \rangle} = \frac{\langle I_G(t) I_R(t + \tau) \rangle}{\langle I_G(t) \rangle \langle I_R(t) \rangle} \]

Spectral bleed should be eliminated
6. RICS-Raster Image Correlation Spectroscopy (ref)

• Like FCS, but with spatial information
• Can be used in living cells
• Applicable to a larger range of molecular diffusivities than other correlation techniques
• Experiment time can be long (2 hours)
• There are other “ICS” measurements also: temporal ICS (TICS), spatio-temporal ICS (STICK), etc.
Some more links


• [Raster Image Correlation Spectroscopy and Number and Brightness Analysis](http://example.com), Methods in Enzymology, 518, 2013, Pages 121–144 Reviews RICS and “Number brightness analysis”
7. Special topics

**Optical tweezers** and CLSM

- Quantitative force measurements ([ref](#))
- Particle manipulation ([ref](#))
- Viscosity imaging ([ref](#))

Large particles

Small particles

Treated as a dipole in an EM field