FROM LOCALIZATION TO INTERACTION

BIOP COURSE 2015
Colocalization: The presence of two or more fluorophores on the same physical structure (in a cell).

http://www.olympusconfocal.com/applications/colocalization.html
MICROSCOPY
LOW PASS FILTERING

Object

Image

NA=0.8

NA=1.4

0.4 µm
COLOCALIZATION

• Two different molecules can never be at the same physical spot at the same time.

• Colocalization seen in images is coming from the low-pass filtering of the image formation in light microscopy.

• Colocalization is an artificial phenomenon and therefore always relative.
FÖRSTER RESONANCE ENERGY TRANSFER
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FRET is the non-radiative transfer of excitation energy from a donor fluorophore to a nearby acceptor. (Förster, Ann. Phys. 1948: 55-75)
ENERGY TRANSFER EFFICIENCY

\[ E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} \]

\( R_0 < R < R_0 \)

Energy transfer efficiency vs. distance \( r / \text{nm} \)
ENERGY TRANSFER
EFFICIENCY

\[ R_0 = \left\{ 0.211 \cdot \kappa^2 \cdot n^{-4} \cdot QY_D \cdot \varepsilon_A \cdot \frac{\int_0^\infty f_D(\lambda)f_A(\lambda)\lambda^4 d\lambda}{\int_0^\infty f_D(\lambda)d\lambda} \right\}^{\frac{1}{6}} \]

Förster distance \((R_0)\) is dependant on a number of factors, including:

- the fluorescence quantum yield of the donor in the absence of acceptor \((QY_D)\)
- the extinction coefficient of the acceptor \((\varepsilon_A)\)
- the refractive index of the solution \((n)\)
- the dipole angular orientation of each molecule \((\kappa_2)\)
- the spectral overlap integral of the donor and acceptor
ENERGY TRANSFER EFFICIENCY

<table>
<thead>
<tr>
<th>FRET pair</th>
<th>$\phi_D$</th>
<th>$\varepsilon_A$ (mM$^{-1}$ cm$^{-1}$)</th>
<th>$r_0$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECFP-Citrine</td>
<td>0.36$^c$</td>
<td>77$^d$</td>
<td>4.8</td>
</tr>
<tr>
<td>ECFP-Venus</td>
<td>0.36$^c$</td>
<td>92$^d$</td>
<td>5.0</td>
</tr>
<tr>
<td>Cerulean-Citrine</td>
<td>0.49$^c$</td>
<td>77$^d$</td>
<td>5.4</td>
</tr>
<tr>
<td>Cerulean-Venus</td>
<td>0.49$^c$</td>
<td>92$^d$</td>
<td>5.2</td>
</tr>
<tr>
<td>SECFP-SEYFP</td>
<td>0.58$^e$</td>
<td>101$^e$</td>
<td>5.4</td>
</tr>
<tr>
<td>EGFP-mCherry</td>
<td>0.60$^d$</td>
<td>72$^d$</td>
<td>5.4</td>
</tr>
<tr>
<td>TagGFP-TagRFP</td>
<td>0.59$^f$</td>
<td>100$^f$</td>
<td>5.7</td>
</tr>
<tr>
<td>mTFP1-Citrine</td>
<td>0.85$^d$</td>
<td>77$^d$</td>
<td>5.7</td>
</tr>
<tr>
<td>mTFP1-mOrange</td>
<td>0.85$^d$</td>
<td>71$^d$</td>
<td>5.7</td>
</tr>
<tr>
<td>Citrine-mKate2</td>
<td>0.76$^d$</td>
<td>63$^d$</td>
<td>5.8</td>
</tr>
<tr>
<td>Clover-mCherry</td>
<td>0.76$^g$</td>
<td>72$^d$</td>
<td>5.8</td>
</tr>
<tr>
<td>mTurquoise1-SEYFP</td>
<td>0.84$^c$</td>
<td>101$^e$</td>
<td>5.8</td>
</tr>
<tr>
<td>mTurquoise2-SEYFP</td>
<td>0.93$^c$</td>
<td>101$^e$</td>
<td>5.9</td>
</tr>
<tr>
<td>Clover-mRuby2</td>
<td>0.76$^g$</td>
<td>113$^g$</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Random interfluorophore orientation is assumed. Pairs are ordered by $r_0$.

$^a$Quantum yield of donor. $^b$Extinction coefficient of acceptor. $^c$Values from ref. 29. $^d$Values from ref. 47. $^e$Values from ref. 50. $^f$Values from ref. 15. $^g$Data from this study.

Lam et al. Nature Meth 2012 p1005
See also: Sun at al. Cytometry PartA 2013 p780
FRET APPLICATIONS IN BIOLOGY
PROTEIN-PROTEIN INTERACTION

FRET

Low/No FRET

No FRET

3 nm
FRET APPLICATIONS IN BIOLOGY INTRAMOLECULAR SENSOR

FRET APPLICATIONS IN BIOLOGY
INTRAMOLECULAR SENSOR

FRET efficiency is only apparent… but can be very precise as well…

The measured FRET efficiency (E) depends on a number of factors:

- $R_0$
- the affinity of the interaction between donor and acceptor
- the stoichiometry of donor and acceptor-labeled proteins
- the presence of unlabeled molecules
- the ratio of the number of labels per protein
- the saturation of the donor

⇒ The calculated FRET efficiency is an apparent FRET efficiency
FRET DETECTION

\[ E = 1 - \frac{\tau_{DA}}{\tau_{D}} \]
**FLIM MEASUREMENT**

**TCSPC**

Time-correlated single-photon counting (TCSPC)
Records times at which individual photons are detected by photo-multiplier tube (PMT) or an avalanche photo diode (APD) after a single pulse. The recordings are repeated for additional pulses.

- Histogram of the number of events across all of these recorded time points.

From *TCSPC Technical note*, PicoQuant
FLIM MEASUREMENT
PHASE MODULATION

Phase modulation of excitation light source (typically LED, laser AOTF)

\[ \tau \phi = \omega^{-1} \tan \Delta \phi, \tau_m = \omega^{-1} (M^{-2} - 1), \text{where } \omega \text{ is the frequency} \]

Advantage: camera-based detection ➔ fast lifetime image acquisition possible
In acceptor photobleaching, the acceptor molecule of the FRET pair is bleached, resulting in a brightening (unquenching) of the donor fluorescence.
ACCEPTR PHOTOBLEACHING

Median Filtering
Subtraction: Postbleach – Prebleach
Division: Subtraction/ Postbleach

\[ E_A(i) = 1 - \frac{F^D(i)}{F^{D\text{bleach}}(i)} = E \cdot \alpha_D(i) \]

An apparent FRET efficiency (product of the efficiency of the FRET pair and the amount of interacting donor) can be calculated
CROSS-TALK AND CROSS-EXCITATION

Channel 1: 460-500 nm

Channel 2: 520-570 nm

RGB Overlay

CFP only

YFP only

CFP+ YFP
Ratiometric imaging can only be done in samples with a fixed stochiometry of donor and acceptor (e.g. Cameleons).

In samples with variable stochiometries, the detected acceptor fluorescence has to be corrected for emission cross-talk and for cross-excitation.
SENSITIZED EMISSION DETECTION

Predetermined factors with pure samples of donor and acceptor:
Donor cross-talk : $R_D$
Acceptor cross-excitation : $R_E$

Required images:

<table>
<thead>
<tr>
<th>Donor channel</th>
<th>Acceptor channel</th>
<th>Acceptor channel</th>
<th>$F^{DA}_{corr}/F^A$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor excitation</td>
<td>$F^D$</td>
<td>$F^{DA}$</td>
<td>$F^A$</td>
</tr>
</tbody>
</table>
SENSITIZED EMISSION DETECTION

Predetermined factors with pure samples of donor and acceptor:
Donor cross-talk : \( R_D \)
Acceptor cross-excitation : \( R_E \)

\[
E_a = \frac{F_{DA}^{corr}}{F_A}
\]

\[
E_a = C \cdot E \cdot \alpha_A
\]

\[
F_{corr}^D = F_{DA}^D - F^D \cdot R_D - F^A \cdot R_E
\]

Donor cross-talk correction
Accepter cross-excitation correction

\( F_{DA}^{corr}/F_A \)
FRET DETECTION SUMMARY

- **Fluorescence Lifetime Measurement**
  - Absolute Energy Transfer Efficiency
  - Dedicated Hardware needed
  - Complex Data Analysis

- **Intensity based Measurements**
  - Sensitized Emission
    - Can be realized on wide-field and confocal systems.
    - Fast and suitable for live-cell imaging
    - Complex data analysis
    - Apparent energy transfer efficiency
  - Acceptor Photobleaching
    - Can be realized on any confocal system
    - Easy data analysis
    - Potential Artefacts with fluorescent proteins
DYNAMIC CELLULAR PROCESSES

- COPI-independent recycling?! (indicated by red arrow)
- Sar1
- COP I
- COP II
- ER
- ERES
- VTCs/ERGIC
- cis
- trans
- TGN
- Rab6
- Golgi
MOVEMENT OF PARTICLES

- No transport/movement of particles
- Transport/movement of particles from left to right and right to left (steady-state)
MOVEMENT OF PARTICLES

N=12
V=0

N=12
V=0

⇒ No transport/movement of particles
MOVEMENT OF PARTICLES

Transport/movement of particles from left to right and right to left
FLUORESCENCE RECOVERY AFTER PHOTobleaching (FRAP)

Bastiaens and Pepperkok (2000), TIBS 25/12
OVERVIEW

1) Introduction
2) FRAP principles
3) FRAP data analysis
4) Related techniques (FLIP, FLAP, Photoactivation, -conversion)
5) Possible limitations
6) New technology developments
SCHEMATIC OF A FRAP EXPERIMENT

I: Pre-bleach
II: Bleach
III: Post-bleach

Bleach pulse
Measure influx of labelled protein into bleached area
FRAP EXPERIMENT IN PRACTICE

1) Take a series of images before bleach (same settings as after the bleach)

2) Apply short local bleach

3) Take images after bleach until the recovery in the bleached area reaches a plateau
INTENSITY OF BLEACHING LIGHT

AOTF upregulation (0-100%):
- Linear

Zoom In:
- Exponential
  \[ 2^{zoomfactor} \]

Speed limitation due to switching of the scanfield
FRAP EXPERIMENTAL DATA

Kappel and Eils, Leica App.Letter 2004
DATA PROCESSING

1) Background subtraction
2) Correction for photobleaching during the measurement (whole cell or neighboring cell as reference)
3) Data normalization (alternative methods)
TYPICAL FRAP EXPERIMENT

-0.1
0.1
0.3
0.5
0.7
0.9
1.1

-300
-10
10
30
50
70
90
110

time / s

normalized intensity
TYPICAL FRAP EXPERIMENT

Normalized intensity vs. time (s)
FRAP PARAMETERS

Half Life ($\tau_{1/2}$)
Half Life \( \left( \tau_{1/2} \right) \)

\[
\tau_{1/2} = \frac{\ln 0.5}{-\tau}
\]

\[
f(t) = A \left( 1 - e^{-\tau t} \right)
\]
PARAMETERS OF EXPONENTIAL FIT

1) Mobile and immobile fraction

2) Recovery half-time

Estimation of diffusion coefficient (Axelrod et al.)

\[ D = 0.88 \cdot \frac{w^2}{4 \cdot t_{1/2}} \]

w: bleach radius

Assumptions:
- bleached area is disk shaped
- diffusion occurs only in 2D
BINDING REACTIONS

Diffusion: $D_f$
Binding: $k_{on}$
Unbinding: $k_{off}$
PURE DIFFUSION

- Circular bleach area
- Analytical Solution (Soumpasis)
- Recovery depends on bleach area

\[ f(x) = \exp \left( -\frac{\tau_D}{2 \cdot t} \right) \cdot \left[ I_0 \left( \frac{\tau_D}{2 \cdot t} \right) + I_1 \left( \frac{\tau_D}{2 \cdot t} \right) \right] \]

\[ \tau_D = \left( \frac{w^2}{D_f} \right) \]
f(x) = C_{equ}\left[1 - \exp(-k_{off} \cdot t)\right] 

\text{normalized intensity}

\text{time / s}

\text{k_{off} = 16 s^{-1}}

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\text{k_{off} = 16 s^{-1}}

\text{normalized intensity}

\text{time / s}

\text{k_{off} = 16 s^{-1}}

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f(x) = C_{equ}\left[1 - \exp(-k_{off} \cdot t)\right] 

C_{equ} = \frac{k_{on}}{k_{on} + k_{off}}
DIFFUSION VS. BINDING

Multiple populations with differing diffusion rates => multi-component equations

Phair and Mistelli, Nature Reviews MolCellBio, 2001
POTENTIAL FRAP ARTEFACTS

Problem:
Partial recovery:
Reversible photobleaching:
Non-diffusive behaviour:
Different values in consecutive measurements:

Potential explanation

e.g. immobile fraction, physical separation
fixed samples, variation of the bleach spot size
binding, active transport => modelling photodamage

FLUORESCENCE LOSS IN PHOTOBLEACHING

Phair and Mistelli, Nature Reviews MolCellBio, 2001
PHOTOACTIVATION PHOTOCONVERSION

Excitation at 488 nm

Irradiation at 405 nm

Other popular photomanipulatable Proteins:

Kaede
mEOS
DRONPA

But oligomerization especially when expressed in cells is often an issue.

Patterson and Lippincott-Schwartz (2002), Science 297:1873-1877
PHOTOACTIVATION
PA-GFP

Patterson and Lippincott-Schwartz (2002), Science 297:1873-1877
FRAP OUTLOOK

Curr. Opin. Cell Biology
FCS
FLUCTUATIONS ARE THE SIGNAL

Example of processes that could generate fluctuations:

- Diffusion
- Enzymatic Activity
- Phase Fluctuations
- Conformational Dynamics
- Rotational Motion
- Protein Folding
FLUORESCENCE CORRELATION SPECTROSCOPY (FCS)


http://www.cellmigration.org/resource/imaging/imaging_approaches_correlation_microscopy.shtml
What is Observed?

1. The Rate of Motion
2. The Concentration of Particles
3. Changes in the Particle Fluorescence while under Observation, for example conformational transitions
THE AUTOCORRELATION FUNCTION

\[ G(0) \propto 1/N \]

As time (tau) approaches 0

\[ G(\tau) = \frac{\langle \delta F(t)\delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \]
FRAP & FCS SUMMARY

• FRAP and FCS can be used to investigate the movement (e.g. Diffusion) of cellular components.

• FRAP can be realized on every commercial microscope.

• FRAP requires overexpression of the component to investigate.

• Photobleaching is toxic! Better: Usage of photo-convertebale variants.

• FCS requires a dedicated microscope setup.

• FCS works with (very) low concentrations.

• Data analysis is complex for FRAP & FCS.