Fluorescence techniques
- Single-molecule optical microscopy
  - Principle and instrumentation
  - Localization precision
  - Applications to biomolecule conformational changes
  - Applications to biomolecule diffusion and cell dynamics
- Fluorescence Resonant Energy Transfer (FRET)
  - Förster model
  - Applications
- Fluorescence lifetime imaging (FLIM)
  - Time-domain and frequency-domain implementations
  - Applications
- Fluorescence recovery after photobleaching (FRAP)
- Fluorescence correlation (FCS)
  - Principle
  - Applications
Single-molecule signatures

Diffraction-limited emission spot
Signal intensity

D. Casanova, S. Türkcan, LOB

For organic fluorophores: Photobleaching steps

For Quantum Dots (QDs): Blinking

For single-photon sources: Photon anti-bunching
CdSe/ZnS QDs, lifetime 20 ns

Hanbury-Brown-Twiss setup


Visualization of single ATP turnovers in myosin using TIRFM

BDTC-S1: head portion (S1) of myosin which contains both the actin-binding site and the catalytic (ATP hydrolysis) site modified with a biotin-binding peptide (BDTC)

When Cy3-ATP (or Cy3-ADP) is bound to the myosin head, it appears as a bright fluorescent spot.
When it is free, it undergoes rapid Brownian motion away for the surface and is not visible as a discrete spot.

T. Yanagida group, Osaka University
ATP synthesis: Oxidative phosphorylation

Why oxidative phosphorylation?
Phosphorylation because a phosphate group is added to ADP
Oxidative because oxygen is used in the process

NADH-Q reductase : NADH déshydrogénase ou complexe I
Q : quinone ou ubiquinone
Cytochrome reductase : complexe bc1 ou complexe III
Cytochrome oxidase : Cytochrome c oxidase ou complexe IV (CcO)
Functioning of ATP synthase

When dissociated from a proton gradient, ATP synthase acts as an ATPase (counterclockwise rotation).

Protein Data Bank

John Walker, Medical Research Center, Mitochondrial Biology Unit, Cambridge, UK
http://www.mrc-mbu.cam.ac.uk/research/atp-synthase

Modified from

F1 portion:
3 α and 3 β subunits
Rotating γ subunit induces conformational changes of α and β subunits

Red: open state
Orange: loose state
Pink: tight state

When dissociated from a proton gradient, ATP synthase acts as an ATPase (counterclockwise rotation).
Rotation of the γ-subunit of F$_1$-ATPase

In the presence of 20 mM ATP
No rotation without ATP

K. Kinosita Jr group, Yokohama
Rotation of the $\gamma$-subunit of $F_1$-ATPase

K. Kinosita Jr group, Yokohama
Myosin V motion on actin filaments

In vitro

P. Selvin’s group, University of Illinois
Myosin V motion on actin filaments

Oxygen leads to fluorophore oxidation and photobleaching

-> Oxygen scavenging conditions:
Glucose + glucose oxidase + catalase

Obtained 10000 photons in 0.5 s

-> localization precision: <1.5 nm !!

Immobile myosin without ATP

Low ATP concentration (300 nM ATP) led to decreased stepping rate and discernable steps

Alternating 52-23 nm steps are compatible with a dye located at 6.5-7 nm from the midpoint in the direction of motion

-> myosin V walks hand over hand

Cy3 or bisiodoacetamidorhodamine labeling

Measuring distances: Beyond the Rayleigh criterion
For two identical, self-luminous, in-focus point sources emitting unpolarized, incoherent light separated by a distance $d$, the fundamental resolution measure (FREM) depends on the number of photons

$$\delta_d := \frac{1}{\sqrt{4\pi A_0 (t - t_0) \Gamma_0(d)}} \cdot \frac{\lambda}{n_a}$$

Resolution as a function of the distance $d$

$$\Gamma_0(d) := \int_{\mathbb{R}^2} \frac{1}{r_{01}^2 + r_{02}^2} \left( \frac{x + d/2}{J_1(\alpha r_{01})J_2(\alpha r_{02})} \right) \frac{J_1(\alpha r_{02})J_2(\alpha r_{01})}{r_{01}^3} \left( \frac{d}{2} \right) dx dy,$$

For an experiment where the Rayleigh criterion gives: 290 nm

After adding pixellisation noise, background fluorescence (Poisson) noise, and detector readout noise (Gaussian), the practical resolution measure (PREM) is obtained.

Ultrahigh-resolution multicolor colocalization of single fluorescent probes

QDs emitting at 540 nm (green) and 620 nm (red)

DC1, DC2: dichroic mirror
PH: pinhole

S. Weiss group
T. Lacoste et al., PNAS 97, 9461 (2000)
Myosin V walks hand over hand
one color per head

Green images shifted vertically
by 12 pixels (660 nm) for clarity

QD labeling

Localization precision: \( \sim 6 \text{ nm} \)

Subunit counting in membrane-bound proteins

- GFP labeling, 1:1 stoichiometry

Cyclic nucleotide gated channel

Ulbrich & Isacoff, Nature Meth. 4, 319 (2007)
Counting the number of proteins bound to single nanoparticles

\[ Y_{1-x}Eu_xVO_4 \]

luminescent nanoparticle

NP channel

(617 nm)

Alexa channel

(519 nm)

NP channel

Alexa channel

Count number

Time (s)

Number of emission spots

Initial count number

LOB & PMC, Ecole Polytechnique
Observing protein synthesis one molecule at a time

Fusion protein of Venus (a YFP variant) and a membrane protein (Tsr)

Principle: observation by immobilization

Sunney Xie group, Harvard University
Observing protein synthesis one molecule at a time

Every 3 min observe produced proteins, then photobleach the Venus molecules.
-> proteins are produced in random bursts, a variable number is synthesized each time

One mRNA copy gives rise to each burst; lifetime 1.5 min
Average number of proteins produced per burst: 4.2

Sunney Xie group, Harvard University
Dual-color detection and dual-polarization detection

Measure simultaneously 2 different fluorophores and their 2D orientations (-> rotational dynamics)

T. Schmidt group, Leiden Univ., The Netherlands
Single-molecule observations to study diffusion
Example: Confined motion of a membrane receptor

S. Türkcan, LOB, Ecole Polytechnique
Single-molecule observations to study diffusion
Analysis using the mean square displacement (MSD)

Two-dimensional mean square displacement (MSD)

\[
\text{MSD}(\Delta t_n) = \text{MSD}(n\delta t) = \text{MSD}_x(n\delta t) + \text{MSD}_y(n\delta t)
\]

\[
= \frac{1}{N - 1 - n} \sum_{j=1}^{N-1-n} \left\{ [x(j\delta t + n\delta t) - x(j\delta t)]^2 + [y(j\delta t + n\delta t) - y(j\delta t)]^2 \right\}
\]

\[\Delta t_n = n\delta t\]

Analysis using the mean square displacement (MSD)

**Case a:** directed diffusion with a constant drift velocity \( v \)

\[
\text{MSD}(\Delta t) = 4D\Delta t + v^2(\Delta t)^2
\]

**Case c:** free Brownian diffusion inside an infinitely high square well potential

\[
\langle x^2 \rangle(t) = \frac{L_x^2}{6} - \frac{16L_x^2}{\pi^4} \sum_{n=1 \text{ (odd)}}^{\infty} \frac{1}{n^4} \exp \left\{ -\frac{1}{2} \left( \frac{n\pi \sigma_x}{L_x} \right)^2 t \right\}
\]

\[
\langle y^2 \rangle(t) = \frac{L_y^2}{6} - \frac{16L_y^2}{\pi^4} \sum_{n=1 \text{ (odd)}}^{\infty} \frac{1}{n^4} \exp \left\{ -\frac{1}{2} \left( \frac{n\pi \sigma_y}{L_y} \right)^2 t \right\}
\]

\[
\sigma_x^2 = 2D_x, \quad \sigma_y^2 = 2D_y, \quad 4D = 2D_x + 2D_y
\]

\[
L_r^2 = L_x^2 + L_y^2.
\]

In all cases, the initial slope gives the diffusion coefficient \( D \).


Diffusion of glycine receptors revealed by single-QD tracking

Red: synaptic boutons
Green: QDs – glycine receptors

Extrasynaptic motion
Motion at the synapse

Free diffusion
Confined diffusion

Diffusion of GABA receptors in nerve growth cones revealed by single-QD tracking

Alternating directed and Brownian motion

White-light transmission

Imaging of QDs labeling GABA receptors


Red: QDs
Green: Microtubules
Blue: Nucleus

Use a speed correlation index to determine the portions of the trajectory corresponding to directed motion.
Photothermal tracking of 5-nm gold nanoparticles

Track 5-nm gold nanoparticles with photothermal heterodyne imaging

**Advantage:**
Very small particles are detectable
No blinking
No photobleaching

**Disadvantage:**
It is not a wide-field technique

**Solution:**
Follow a single nanoparticle using a **triangulation procedure**
- Locate a NP
- Measure 3 data points around this position at the apices of an equilateral triangle
  - Determine \((x_t, y_t, S_t)\)
  - Measure 3 new data points after recentering the equilateral triangle
  - Determine \((x_{t+\Delta t}, y_{t+\Delta t}, S_{t+\Delta t})\)

B. Lounis, L. Cognet & D. Choquet groups, Univ. Bordeaux
Analyzing trajectories using the inference approach

Let us consider free Brownian motion + a force

Equation of Motion

$$m \frac{dv_x}{dt} = -\gamma v_x - \nabla_x V(x) + \sqrt{D\gamma^2} \xi(t)$$

Friction Potential Thermal noise

This means that the system relaxes quickly:

$$\tau = \frac{m}{\gamma} \approx \frac{10^{-22} \text{ kg}}{10^{-6} \text{ kg/s}} = 10^{-16} \text{s}$$

The associated Fokker-Planck equation is:

$$\partial_t P = -\frac{1}{\gamma} \nabla \cdot (F P) + D \Delta P$$

For constant $D$ and $F$, the solution is:

$$P(r, t \mid r_0, t_0) = \exp \left[ -\frac{(r - r_0 - F\Delta t / \gamma)^2}{4D\Delta t} \right] \frac{4\pi D\Delta t}{\Delta t = t - t_0}$$

For a trajectory $T$ starting at $r_0, t_0$ and ending at: $r_N, t_N$

$$P(T \mid D, F) = P(r_N, t_N \mid r_{N-1}, t_{N-1}) \cdot \ldots \cdot P(r_2, t_2 \mid r_1, t_1) \cdot P(r_1, t_1 \mid r_0, t_0)$$

Analyzing trajectories using the inference approach

-> When you know F and D, you can calculate the probability to go from the space-time point \((r_0, t_0)\) to the space-time point \((r, t)\)

We are in the inverse situation: We know that the molecule went from the space-time point \((r_0, t_0)\) to the space-time point \((r, t)\) and want to calculate the probability that this happened for a certain value of parameters F and D.

**Probability of observing the trajectory T**

**Prior probability = constant**

**In our case:**

\[ P(Q | T) = \frac{P(T | Q) \times P_0(Q)}{P(T)} \]

\( P(Q | T) \): Posterior probability of having the parameter values Q given the observation of the trajectory T

\( P(T | Q) \): Probability of observing the trajectory T given the parameter values Q

\( P_0(Q) \): Prior probability of having the parameter values Q

\( P(T) \): Normalization Constant

Analyzing trajectories using the inference approach

Trajectory: confined motion of a membrane receptor

Map of the forces felt by the receptor

Different types of membrane compartmentation

Cytoskeleton boundaries

Lipid rafts

Variants for single-molecule spectroscopy

Wide-field

Prism TIRF

Objective TIRF

Comparison epi-fluor.

Narrow field

Scanning near-field optical microscopy SNOM

Confinement in microfluidic channels Width smaller than the diffraction limit 15-100 nm

A cylindrical lens introduces astigmatism.

Without cylindrical lens, no difference between +z and -z

3D tracking using a cylindrical lens

Without cylindrical lens, the z-position can be determined from the FWHM of the intensity distribution, $\sigma$

$$z = \pm \frac{z_r}{\sigma_0} \sqrt{\sigma^2 - \sigma_0^2}$$

$z_r$: focal depth
$\sigma_0$: diffraction-limited FWHM for a point source at focus

With cylindrical lens, the z-position can be determined from the width, $\sigma_r$, and the ellipticity, $\varepsilon$, of the intensity distribution:

$$z(\sigma_r, \varepsilon) = \begin{cases} 
\frac{z_r}{\sigma_0} \sqrt{\frac{\sigma_r^2}{\varepsilon^2} - \sigma_0^2} - \gamma, & \varepsilon < 1 \\
-\frac{z_r}{\sigma_0} \sqrt{\frac{\sigma_r^2}{\varepsilon^2} - \sigma_0^2} + \gamma, & \varepsilon > 1 
\end{cases}$$

$\gamma$ is the axial astigmatism of the lens

With cylindrical lens:
- Improved axial accuracy
- Lateral accuracy only slightly worse

3D tracking of QDs inside live cells using a cylindrical lens

Cylindrical lens
f=10 m

exposure time: 6 ms
x, y localization precision: 43 nm
z localization precision: 130 nm

Fluorescence techniques
Fürster resonant energy transfer (FRET)
Förster resonant energy transfer (FRET)

In the presence of resonant energy transfer:
- decrease of donor emission
- increase of acceptor emission
- decrease of donor lifetime

Energy transfer depends on the 6th power of donor-acceptor distance
-> spectroscopic ruler

Prerequisites:
- Overlap between donor emission and acceptor absorption spectra
- Extensively used in biology to observe distance changes (2-10 nm):
  - Interaction between biomolecules
  - Conformational changes

T. Förster, Annalen der Physik 2, 55 (1948)
Förster resonant energy transfer (FRET)

Nonradiative induced dipole – induced dipole interaction

Point dipole approximation
A harmonically oscillating electric dipole $\rho_D$ produces an electric field:

$$\vec{E}_D(\vec{r}) = \frac{1}{4\pi\varepsilon_0} \left[ \frac{k^2}{r} (\vec{r}_0 \times \vec{p}_D) \times \vec{r}_0 + \left[ 3\vec{r}_0(\vec{r}_0 \cdot \vec{p}_D) - \vec{p}_D \right] \left( \frac{1}{r^3} - \frac{ik}{r^2} \right) \right] e^{ikr} \quad \vec{r} = \vec{r}_0 r$$

In the near field ($r<<\lambda$):

$$\vec{E}_D = \frac{1}{4\pi\varepsilon_0} \left[ \frac{3\vec{r}_0(\vec{r}_0 \cdot \vec{p}_D) - \vec{p}_D}{r^3} \right] e^{ikr}$$

Coupling with another point dipole $\vec{p}_A$

Interaction energy

$$V = -\vec{p}_A \cdot \vec{E}_D = -\frac{1}{4\pi\varepsilon_0} \left[ \frac{3(\vec{r}_0 \cdot \vec{p}_A)(\vec{r}_0 \cdot \vec{p}_D) - \vec{p}_A \cdot \vec{p}_D}{R^3} \right] e^{ikR}$$

Fermi’s golden rule gives the transition rate from the donor excited state to the acceptor excited state:

$$k_T = \frac{2\pi}{\hbar} \left| \langle i | V | f \rangle \right|^2 \rho_f \propto \left( \frac{1}{R} \right)^6$$

$\langle i \rangle = \langle D^* | A \rangle$ Initial state

$\langle f \rangle = \langle D | A^* \rangle$ Final state

$\rho_f$ Density of final states
Förster resonant energy transfer (FRET)

Jablonski energy level diagram

$D^* \rightarrow A \xrightleftharpoons[k_T]{k_T} D - A^*$

$D^* \rightarrow A \xrightleftharpoons[k_T]{k_T} D - A^*$

$k_D^D = k_f + k_{n.r.}$

$k_D^D$
Energy transfer rate and efficiency

\[ E = \frac{k_T}{k_T + k_D} \]

**E:** efficiency of energy transfer (or FRET efficiency), defined as the probability for the excited donor to return to its ground state by energy transfer to the acceptor

\[ k_T = k_D \left( \frac{R_0}{R} \right)^6 \]

\[ R_0 \sim 5-7 \text{ nm} \]

Distance measurements: \(~2-10 \text{ nm}\)

\[ J = \int \frac{\varepsilon_A(\lambda)}{f_D(\lambda)} \lambda^4 d\lambda \left/ \int f_D(\lambda) d\lambda \right. \text{ in } \text{M}^{-1} \text{ cm}^{-1} \text{ nm}^4 \]

\[ \varepsilon_A(\lambda) \text{ and } f_D(\lambda) \text{ are normalized over wavelength } \lambda \]

\[ \kappa^2 \text{ depends on relative dipole orientation } \]

\[ q_D \text{: donor quantum yield, } n \text{: refraction index} \]

\[ R_0 = 0.21 (J q_D n^{-4} \kappa^2)^{1/6} \text{ (in Angstroms)} \]

\[ \kappa^2 \text{: donor-acceptor dipole orientation product} \]

\[ \tau_D = \frac{1}{k_D} \]

- **Förster, Annalen der Physik 2, 55 (1948)**
**Measurements of energy transfer efficiency**

Rewording the FRET efficiency definition: $E$ is the donor fraction de-excited via energy transfer to the acceptor

$$E = 1 - \frac{I_{DA}}{I_D}$$

$I_D$ : donor emission intensity in the absence of the acceptor

$I_{DA}$ : donor emission intensity in the presence of the acceptor

**Intensity measurements: beware of pitfalls**

$->$ In ensemble measurements, intensities must be normalized for concentration.

$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$

$\tau_D$ : excited-state lifetime of the donor in the absence of the acceptor

$\tau_{DA}$ : excited-state lifetime of the donor in the presence of the acceptor

The lifetime approach is concentration independent.

Alternatively:

$$E = \frac{I_{AD}/q_A}{I_{AD}/q_A + I_{DA}/q_D}$$

$I_{AD}$ : FRET-induced acceptor emission intensity

$q_D(A)$ : Donor (acceptor) quantum yield

**Beware of direct acceptor excitation**

**FRET donor-acceptor pairs**

A large variety of FRET pairs exists:
- Dye→dye (ex. Cy3→Cy5)
- Fluorescent protein→fluorescent protein
- Nanoparticle → dye
- ....

**Quantum dot → dye**

![Diagram of a quantum dot](image)

**Table 1.** Overlap Integrals, Quantum Yields, and Calculated Förster Distances for MBP-Coated QD–Cy3 Pairs

<table>
<thead>
<tr>
<th>donor–acceptor pair</th>
<th>overlap integral, $I \times 10^{13}$ (cm$^3$/M)</th>
<th>quantum yield, $Q_0$</th>
<th>Förster distance, $R_0$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>510–Cy3</td>
<td>3.86</td>
<td>0.190</td>
<td>47.3</td>
</tr>
<tr>
<td>530–Cy3</td>
<td>7.01</td>
<td>0.153</td>
<td>50.4</td>
</tr>
<tr>
<td>555–Cy3</td>
<td>8.91</td>
<td>0.239</td>
<td>56.5</td>
</tr>
</tbody>
</table>

H. Mattoussi group, Naval Res. Lab
Energy transfer from a QD donor to multiple organic acceptors

For 1 donor interacting with n acceptors:

\[ E = \frac{nR_0^6}{nR_0^6 + r^6} \]

H. Mattoussi group, Naval Res. Lab
QD-peptide-dye conjugates to monitor proteolytic activity

Thrombin cleaves the peptide attaching the QD to the acceptor dye. The thrombin inhibitor inhibits the thrombin enzyme activity.

QD-dye conjugates as energy-transfer-based pH sensors

Spectral shift of the acceptor dye upon protonation at low pH
-> better overlap, higher FRET efficiency

Bawendi group, MIT
M. Bawendi and coll., JACS 128, 13320 (2006)
Detection of ERK receptor phosphorylation using FRET

Stimulation by epidermal growth factor (EGF) induces Receptor phosphorylation

Donor lifetime changes due to FRET

Single-pair FRET (sp-FRET)

- Results in agreement with the dye distance
- Detection of different subpopulations
- Measurement of enzymatic cleavage activity

P. G. Schultz and S. Weiss groups
Folded vs. unfolded proteins using FRET

Addition of a denaturant

Limit on reconfiguration time imposes limit on free-energy barrier

Exploiting long lifetime donors: the case of rare-earth ions


Fluorescence techniques
 Fluorescence lifetime imaging
Fluorescence lifetime imaging (FLIM)

The fluorescence quantum yield \( q \) is determined by the radiative and nonradiative transition rates \( k_{rad} \) and \( k_{nrad} \) and is sensitive to the local environment.

\[
q = \frac{k_{rad}}{k_{rad} + k_{nrad}}
\]

It is easier to measure the lifetime \( \tau \).

\[
\tau = \frac{1}{k_{rad} + k_{nrad}}
\]

In biological samples, fluorophore lifetime can depend on \([\text{Ca}^{2+}],[\text{pO}_2], \text{pH}\)

Example: low pH in cancer tissues

Time-domain lifetime measurements
2D-Fluorescence lifetime imaging (2D-FLIM)
Detection using a gated image intensifier

Acceleration voltage
Can be switched on and off
-> gating

75 ps-2 ns

- Wide-field technique
- Only the photons arriving during the acceleration gate are detected.


P. French website

Figure 1. (a) Schematic of experimental FLIM system and (b) the acquisition process.
**Time-domain lifetime measurements**

**Principle:**  
*Time-correlated single-photon counting*

- Periodic light signal (e.g. excited by a pulsed laser)
- Fast electronics (TAC: Time to amplitude converter) to detect the arrival time of individual photons
- Reconstruction of the waveform from the individual arrival time measurements.

**Requirement:** Not more than one photon in one period

**Detectors:**  
- Avalanche photodiodes (APD)  
- Photomultipliers

- Point-by-point detection (single detector).
- No photons are lost. All photons are detected.  
  -> optimum signal-to-noise ratio for a given number of detected photons

**BUT:** the signal has to be reduced to remain in the single-photon counting regime.

Combination with scanning: confocal  
or non-linear microscopy  
Also used for fluorescence correlation

Becker & Hickl GmbH website
## Gated image intensifiers vs. time-correlated single-photon counting

<table>
<thead>
<tr>
<th>Gated image intensifiers</th>
<th>Time-correlated single-photon counting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highest accuracy per second</td>
<td>Highest accuracy per photon</td>
</tr>
<tr>
<td>2D signal output</td>
<td>Requires scanning</td>
</tr>
<tr>
<td>Best for applications like endoscopy</td>
<td>Best for FLIM with a confocal microscope</td>
</tr>
</tbody>
</table>
Time-domain lifetime measurements

Streak camera

Advantages:
High temporal resolution (~1 ps), adequate for short lifetimes (ns or sub-ns)

Disadvantages:
Expensive
Low lifetime dynamic range
Frequency-domain lifetime measurements

Adequate for long lifetimes (µs-ms).

Excitation intensity:
\[ I_e(t) = I_{e0} + I_{e\omega} \cos(\omega t + \varphi_e) \]

Emission intensity:
\[ I_f(t) = I_{f0} + I_{f\omega} \cos(\omega t + \varphi_f) \]

- Phase shift \( \Delta \phi \)
- Demodulation factor:
\[ M = \frac{I_{f\omega}/I_{f0}}{I_{e\omega}/I_{e0}} \]

For a monoexponential decay:
\[ \omega \tau_f = \tan(\Delta \phi), \]
\[ \omega \tau_f = \sqrt{\frac{1}{M^2} - 1} \]

For multiexponential decays, measurements at multiple frequencies \( \omega \) are necessary. Inexpensive technique, but complicated analysis for non monoexponential decays.

A. Deniset-Besseau, thesis Université d’Orsay, 2008

**FLIM variants**

**FLIM**
FLIM (2D-gated detection) + optical sectioning

\[ z = z_0 \]

**FLIM+structured illumination**

Fig. 2. Microscope images (x, y plane) of cotton wool stained with Coumarin 314 and DASPI for two different \( z \) positions separated by 315 \( \mu \)m. (a), (e) Conventional fluorescence intensity images. (b), (f) Corresponding conventional FLIM maps. (c), (g) Sectioned fluorescence intensity images. (d), (h) Sectioned FLIM maps. All lifetime false-color scales span from 700 ps (blue) to 2.6 ns (pink).

**FLIM**

\[ z = z_0 + 315 \mu \text{m} \]

**FLIM+structured illumination**

P. French + T. Wilson groups
FLIM variants

FLIM (2D-gated detection) + spectral measurements + optical sectioning

P. French + T. Wilson groups
FLIM to study ordered lipid domains

**Ordered lipid phase**

**Disordered lipid phase**

Spectral blueshift in the ordered lipid phase


LUV: Large Unilamellar Vesicles
DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine
DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
PSM: n-palmitoyl-sphingomyelin

Lifetime increase in the ordered lipid phase

P. French + A. Magee groups
FLIM to study ordered lipid domains

Enhanced contrast with lifetime imaging

Effect of temperature and cholesterol depletion

P. French + A. Magee groups
Fluorescence techniques
Fluorescence recovery after photobleaching
Fluorescence recovery after photobleaching (FRAP)

A. Fluorescence recovery after photobleaching – FRAP

- Ensemble method
- Difficult to differentiate between different subpopulations

Fluorescence techniques
Fluorescence correlation spectroscopy
**Fluorescence correlation spectroscopy (FCS)**

Developed in the 1970s as a miniaturization of dynamic light scattering

**Principle:** Excited molecules in the focal volume give rise to a fluorescence signal. The fluorescence signal fluctuates in time $\rightarrow F(t)$. These fluctuations can be quantified by calculating the normalized autocorrelation function $G(\tau)$.

$$G(\tau) = \frac{\langle F(t) \cdot F(t + \tau) \rangle}{\langle F(t) \rangle^2} = \frac{\langle \delta F(t) \cdot \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} + 1$$

$$\delta F(t) = F(t) - \langle F(t) \rangle$$

$$\langle F(t) \rangle = \frac{1}{T} \int_0^T F(t) dt.$$
**Fluorescence correlation spectroscopy (FCS)**

The fluorescence **fluctuations** may result from:
- a change in the number of fluorophores in the observation volume due to **diffusion**
- a change in fluorescence properties of the molecule as a consequence of
  - a **chemical reaction**
  - a **conformational fluctuation**.

**Optimize signal-to-noise ratio:**
Here, the fluctuations are the signal.
The relative fluorescence fluctuations increase for decreasing numbers of emitting molecules
- **minimize the average number of emitting molecules/particles in the focal volume**
  (0.1 to 1000) -> concentrations $10^{-10}$-$10^{-6}$ M for a focal volume of ~1fL
- minimize the **focal volume**, reduce the emitter concentration

$$G(\tau = 0) = \frac{\left\langle \delta F(t)^2 \right\rangle}{\left\langle F(t) \right\rangle^2} + 1 = \left( \frac{\sqrt{N}}{N} \right)^2 + 1 = \frac{1}{N} + 1$$
Fluorescence correlation spectroscopy
Signal due to diffusion

2D brownian diffusion (e.g. in a membrane)

\[ G_{2D}(\tau) = \frac{1}{N} \cdot \frac{1}{1 + \frac{\tau}{\tau_D}} + 1 \]

3D brownian diffusion

For a depth/diameter \((1/e^2)\) ratio \(S\) of the gaussian 3D excitation volume:

\[ G(\tau) = \frac{1}{N(1 + \tau/\tau_D)} \cdot \frac{1}{1 + \tau/S^2\tau_D} + 1 \]

Amplitude at \(\tau=0\) gives the number of molecules in the focal volume \(V_{\text{eff}}\)

Correction term due to the fact that the focal volume is not spherical.


Lateral diffusion time \(\tau_D\):
Time during which the molecule stays in the focal volume

\[ \tau_D = \frac{r_0^2}{4D} \]

\(r_0\): lateral \(1/e^2\) diameter
Fluorescence correlation spectroscopy: different diffusion models

Brownian diffusion + directed flow with velocity $v$

$$G_{flow}(\tau) = \frac{1}{V_{eff} \cdot \langle C \rangle} \cdot \frac{1}{1+S^2} \cdot \frac{1}{\sqrt{1 + \frac{r_0^2}{z_0^2} \cdot \frac{\tau}{\tau_D}}} \cdot e^{-\left(\frac{(v_x^2+v_y^2)}{r_0^2} \cdot \tau^2\right) \cdot \left(\frac{1}{1+\frac{\tau}{\tau_D}}\right)} \cdot e^{-\left(\frac{(v_z^2)}{z_0^2} \cdot \tau^2\right) \cdot \left(\frac{1}{1+\frac{r_0^2}{z_0^2} \cdot \frac{\tau}{\tau_D}}\right)}$$

FCS: fluorophore photophysics complicates the interpretation

If the photophysics does not influence the diffusion dynamics:

\[ G(\tau) = G_{\text{photoph}}(\tau) \cdot G_{\text{diff}}(\tau) \]

\textbf{BUT: } \[ G_{\text{photoph}}(\tau) \text{ is not always known} \]

More generally:
For a chemical reaction or conformational change of the diffusing species with a characteristic time \( \tau_R \):

\[ G(\tau) = \frac{1 - A + A \exp(-\tau/\tau_R)}{N(1 - A)} \cdot \frac{1}{1 + \frac{\tau}{\tau_D}} \cdot \frac{1}{\sqrt{1 + \frac{\tau}{S^2\tau_D}}} + 1 \]

\textbf{A: } fraction of the molecules that undergoes the change
FCS applications

- Determine concentrations, esp. concentration changes (S. Charier et al, J. Am. Chem. Soc. 127, 15491 (2005))
- Determine the diffusion coefficient
- Determine the particle size
  
  Stokes-Einstein equation
  \[ D_i = \frac{kT}{6\pi \eta V R_{h,i}} \]
  \( \eta V \): viscosity
  \( R_h \): hydrodynamic radius (size+solvation shell)

- Detect binding processes in vitro and in vivo: mass change leads to a change in diffusion coefficient
  
  for a spherical particle in solution: \( D \propto \sqrt[3]{m} \), \( m \) molecular mass

- Detect events on very short time scales: rotational diffusion, isomerization, ...

Comparison to single-molecule tracking

**Advantages:** can measure much faster dynamic processes

**Drawbacks:** accurate calibration of the focal volume is important
  
  requires fitting of the data with a model

FCS applications: measure diffusion

2D and 3D images with scanning

Giant Unilamellar Vesicle
Green: dye in the liquid-ordered phase (Alexa488-labeled cholera toxin B-subunit)
Red: dye in the liquid-disordered phase (dye dil)

**Slower diffusion in the liquid-ordered phase.**

P. Schwille group
FCS applications: detect conformational changes

IFABP (intestinal fatty acid binding protein, 15 kDa)

Beware of modifications of the refractive index mismatch

Change of diffusion coefficient upon unfolding

Guanidinium hydrochloride induces unfolding

TABLE 2 Diffusion coefficient and the hydrodynamic radius ($R_{h}$) of IFABP determined from the diffusion time measurements under different unfolding conditions

<table>
<thead>
<tr>
<th></th>
<th>$D^*$, cm$^2$/s</th>
<th>$R_{h}$, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>V60Alexa in the native state</td>
<td>$1.4 \times 10^{-6}$</td>
<td>17</td>
</tr>
<tr>
<td>V60Alexa unfolded (in presence of Gdn)</td>
<td>$0.8 \times 10^{-6}$</td>
<td>31</td>
</tr>
</tbody>
</table>

C. Frieden group
Dual-color Fluorescence Cross-Correlation (FCCS)

Cross-correlation between the signals $F_i$ and $F_j$:

$$G_x(\tau) = \frac{\langle \delta F_i(t) \cdot \delta F_j(t + \tau) \rangle}{\langle F_i(t) \rangle \cdot \langle F_j(t) \rangle}$$

If the excitation volume $V_{eff}$ is identical for the two excitation wavelengths and there is negligible overlap between emission and absorption spectra:

$$G_x(\tau) = \frac{\langle C_{ij} \rangle M_{ij}(\tau)}{V_{eff} \langle \langle C_i \rangle + \langle C_{ij} \rangle \rangle \langle \langle C_j \rangle + \langle C_{ij} \rangle \rangle}$$

$M_{ij}$ is the motion-related part of the correlation function.

The amplitude of $G_x(\tau)$ is proportional to the concentration of doubly labeled molecules.

P. Schwille et al., Biophys. J. 72, 1878 (1997)
Dual-color Fluorescence Cross-Correlation (FCCS)

P. Schwille et al., Biophys. J. 72, 1878 (1997)
FCS variants

More generally: 
\[ G(\Delta p) = \frac{\langle F(p)F(p + \Delta p) \rangle}{\langle F(p) \rangle^2} \]

Where \( p \) can be any combination of \( x, y, z, t \)

Image correlation FCS

Spatiotemporal image correlation FCS

Dual-spot FCS -> flow measurements in microstructured channels
Use EM-CCD for multiple spot detection

Scanning FCS

### Comparison FRAP, FCS, SPT

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle and observable(s)</th>
<th>Comments</th>
</tr>
</thead>
</table>
| FRAP         | Level and rate of fluorescence recovery with time following the photobleaching of a limited area of observation  

- Translational diffusion  
- Mobile fraction                                                                                                                                                                                                                                                                                                                                                                                            | Well established for FRAP in spot mode  

- Ensemble average method  
- Weakly sensitive to separate subpopulations with different diffusing characteristics  
- Observation area usually larger than the diffraction limit                                                                                                                                                                                                                                                                                                                                                           |
| FCS and ICS  | Fluorescence fluctuations induced by low numbers of diffusing fluorescent molecules into a limited area of observation  

- Molecular concentration  
- Translational diffusion of multiple subpopulations  
- Molecular clustering  
- Count rate per molecule                                                                                                                                                                                                                                                                                                                                                                                                  | Averaging of thousands of single-molecule diffusion events within short acquisition times  

- Time resolution: ~70 ns (APD)  

- Low probe concentration (nM range)  
- Highly sensitive to clustering (photon counting histogram analysis)  
- Single & multiple colors  

- In standard approach, weakly sensitive to determine the diffusion of slow-diffusing molecules  
- Observation area usually close to the diffraction limit |
| SPT and SDT  | Scattered light from a single bead particle or fluorescence signal from a single emitter  

- Trajectories of individual particles or fluorescent molecules  
- Translational diffusion in different cell area  
- Access to different mode of motion                                                                                                                                                                                                                                                                                                                                                                                  | Excellent spatial precision (dependent on the signal/noise ratio)  

- Temporal resolution limited by the frequency of image acquisition  

- Spatio-temporal identification of the diffusion heterogeneities  
- Experimental bias favoring slow-diffusing particles/molecules  
- Statistics quality strongly related to the length of the trajectories  

- Valence of the tagged molecules often weakly determined |

Bibliography (course 3)


Cited publications, in particular:

D. Marguet et al., EMBO J. 25, 3446 (2006) fluorescence correlation

Biophotonics seminars
Monday morning February 6: 10h45-12h15 Ana-Maria Pena, L’Oréal

Exam: Wednesday February 15 14h-17h
Written exam (1h30 on the 3 ½ courses by A. Alexandrou, 1h30 on the rest of the courses)
**All documents authorized.**

Bibliography projects upon request.