Fluorescence techniques
- Fluorescence correlation (FCS)
  - Principle
  - Applications

Superresolution techniques
- Structured illumination
- 4Pi
- STED
- PALM
- STORM
**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 -> $F(t)$. 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: different diffusion models**

The relative fluorescence fluctuations decrease for increasing 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

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

3D brownian diffusion

$$G(\tau) = \frac{1}{N(1 + \tau/\tau_D)} \frac{1}{\sqrt{1 + \tau/S^2 \tau_D}} + 1$$

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

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

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

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 $v$

$$G_{flow}(\tau) = \frac{1}{V_{eff} \cdot \langle C \rangle} \cdot \frac{1}{1 + \frac{\tau}{\tau_D}} \cdot \frac{1}{\sqrt{1 + \frac{r_0^2}{z_0^2} \cdot \frac{\tau}{\tau_D}}}$$

- $\left( \frac{v_x^2 + v_y^2}{r_0^2} \cdot \tau^2 \right) \cdot \left( \frac{1}{1 + \frac{\tau}{\tau_D}} \right)$
- $\left( \frac{v_z^2}{z_0^2} \cdot \tau \right) \cdot \left( \frac{1}{1 + \frac{r_0^2}{z_0^2} \cdot \frac{\tau}{\tau_D}} \right)$

Directed flow

FCS: fluorophore photophysics complicates the interpretation

If the photophysics does not influence the diffusion dynamics:

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

**BUT:** \( G_{\text{photoph}}(\tau) \) is not always known

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

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

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, ...

Remarks: 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)

**Change of diffusion coefficient upon unfolding**

Guanidinium hydrochloride induces unfolding

Beware of modifications of the refractive index mismatch

**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>Condition</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 $V_{\text{eff}}$ is identical for the two excitation wavelengths and there is negligible overlap between emission and absorption spectra:

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

$M_{ij}$ 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)

Red channel autocorr. + Green channel autocorr. = Cross-correlation

Reaction kinetics: $k = 8 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$

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  \( \rightarrow \) flow measurements in microstructured channels
Use EM-CCD for multiple spot detection

Scanning FCS

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## Comparison FRAP, FCS, SPT

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle and observable(s)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRAP</td>
<td>Level and rate of fluorescence recovery with time following the photobleaching of a limited area of observation</td>
<td>• Well established for FRAP in spot mode</td>
</tr>
<tr>
<td></td>
<td>• Translational diffusion</td>
<td>• Ensemble average method</td>
</tr>
<tr>
<td></td>
<td>• Mobile fraction</td>
<td>• Weakly sensitive to separate subpopulations with different diffusion characteristics</td>
</tr>
<tr>
<td>FCS and ICS</td>
<td>Fluorescence fluctuations induced by low numbers of diffusing fluorescent molecules into a limited area of observation</td>
<td>• Observation area usually larger than the diffraction limit</td>
</tr>
<tr>
<td></td>
<td>• Molecular concentration</td>
<td>• Averaging of thousands of single-molecule diffusion events within short acquisition times</td>
</tr>
<tr>
<td></td>
<td>• Translational diffusion of multiple subpopulations</td>
<td>• Low probe concentration (femto range)</td>
</tr>
<tr>
<td></td>
<td>• Molecular clustering</td>
<td>• Highly sensitive to clustering (photon counting histogram analysis)</td>
</tr>
<tr>
<td></td>
<td>• Count rate per molecule</td>
<td>• Single &amp; multiple colors</td>
</tr>
<tr>
<td>SPT and SDT</td>
<td>Scattered light from a single bead particle Or fluorescence signal from a single emitter</td>
<td>• In standard approach, it is difficult to determine the diffusion of slow-diffusing molecules</td>
</tr>
<tr>
<td></td>
<td>• Trajectories of individual particles or fluorescent molecules</td>
<td>• Observation area usually close to the diffraction limit</td>
</tr>
<tr>
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<td>• Translational diffusion in different cell area</td>
<td>• Excellent spatial precision (dependent on the signal/noise ratio)</td>
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<tr>
<td></td>
<td>• Access to different mode of motion</td>
<td>• Temporal resolution limited by the frequency of image acquisition</td>
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<tr>
<td></td>
<td></td>
<td>• Spatio-temporal identification of the diffusion heterogeneities</td>
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<tr>
<td></td>
<td></td>
<td>• Experimental bias favoring slow-diffusing particles/molecules</td>
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<tr>
<td></td>
<td></td>
<td>• Statistics quality strongly related to the length of the trajectories</td>
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<td></td>
<td></td>
<td>• Valence of the tagged molecules often weakly determined</td>
</tr>
</tbody>
</table>

Superresolution techniques
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

\[
\delta_d := \frac{1}{\sqrt{4 \pi \Lambda_0 \cdot (t - t_0) \cdot \Gamma_0 (d)}} \cdot \frac{\lambda}{n_a} \cdot \lambda \cdot \gamma
\]

Resolution as a function of the distance \( d \)

\[
\Gamma_0 (d) := \int \frac{1}{\sqrt{2 \pi}} \frac{J_1^2 (\alpha r_{01}) + J_1^2 (\alpha r_{02})}{r_{01}^2 + r_{02}^2} \left( \frac{d}{2} \right) \frac{J_1 (\alpha r_{01}) J_2 (\alpha r_{01})}{r_{01}^3} \right) \cdot dx \cdot dy,
\]

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.

Structured illumination to increase the axial resolution by a factor of 2

The reconstruction procedure in reciprocal space

Actin cytoskeleton at the edge of a HeLa cell

conventional  
Structured illumination

M. G. L. Gustafsson, J. Microscopy 198, 82 (2000)
Saturated structured illumination

Principle

Field of 50-nm fluorescent beads

Taking into account 3 harmonics

M. G. L. Gustafsson, PNAS 102, 13081 (2005)
**Cellular imaging with 3D structured illumination**

Principle: 3 interfering beams to generate patterns along z as well as x and y

Invaginations of the nuclear envelope in mitotic prophase in fixed mouse myoblast cells (C2C12)

4Pi confocal microscopy

Use two microscope objectives with a common focus to increase the aperture of the microscope.

Point spread functions

**Confocal**

Axial FWHM: 540 nm

**4Pi illumination**

Axial FWHM: 138 nm

Axial side peaks: 0.4

Due to the non-linearity of the process, the edges can be extremely steep.

STimulated Emission Depletion (STED) microscopy

Fluorescence lifetime: \(\sim 3\) ns, STED pulse duration: 100 ps

\[
\eta(r) = \exp\left[-\sigma \tau I_{\text{STED}}(r)\right]
\]

\[
h(r) = h_{\text{exc}}(r) \eta(r)
\]

Fluorescence probability

\[
h_{\text{exc}}(r) = C \cos^2(\pi r n \sin \alpha / \lambda_{\text{exc}})
\]

Normalized excitation probability (lower limit)

\[
h(r) = h_{\text{exc}}(r) \eta(r)
\]

Probability to detect a photon at \(r\)

\[
I_{\text{STED}}(r) = s I_{\text{sat}} \sin^2(\pi r n \sin \alpha / \lambda_{\text{STED}})
\]

Fluorescence drop to 1/e

For: \(\lambda_{\text{exc}} \approx \lambda_{\text{STED}} \equiv \lambda\) and \(r < \lambda / 2n\) the FWHM of \(h(r)\) will be (approx. by a Taylor series to the second order)

\[
\Delta r = \frac{\sqrt{8}}{\pi} \frac{\lambda}{n \sin \alpha \sqrt{1 + s}} \approx 0.45 \frac{\lambda}{n \sin \alpha \sqrt{1 + s}}
\]

STimulated Emission Depletion (STED) microscopy

Synaptotagmin I molecules (transmembrane synaptic vesicle proteins) form distinct spots on endosomes.

protein-heavy subunit of neurofilaments in human neuroblastoma.

FIXED CELLS !!

Two-Color STED

Mitochondria labeled with antibodies specific for the $F_1 F_0$ ATP synthase (red) and the TOM complex (green)

G. Donnert et al., Biophys. J. 92, L67 (2007)
Prevent xy and z patterns from interfering using two different lasers
Constructive interference of the excitation and xy STED beam, destructive for the z STED beam

S. W. Hell group, Göttingen
R. Schmidt et al., Nature Meth. 5 539 (2008)
STED with a spherical focal spot

Mitochondrion inside a mammalian (Vero) cell

21-nm fluorescent beads

S. W. Hell group, Göttingen
R. Schmidt et al., Nature Meth. 5 539 (2008)
**STED on live cells detects synaptic vesicle movement**

Observation of a 2.5-µm by 1.8-µm field of view

Single synaptic vesicles in neurons observed via labeling of synaptotagmin molecules at their surface

I_{STED} = 400 MW/cm²

**S. W. Hell group, Göttingen**

Superresolution microscopy based on single-molecule microscopy

Principle

Does not require high intensities like STED but requires long acquisition times.

**Photoactivation Localization Microscopy (PALM)**

Photoactivatable fluorescent proteins
Switching between neutral and ionized form


Total internal reflection setup
Photoactivation: 405 nm
Imaging: 561 nm

Observation of up to $10^5$ molecules/µm$^2$
Exposure time: 0.5-1 s
For a full stack: 2-12h
-> FIXED CELLS

Important: High contrast between activated and inactive state


Labeled actin in fox lung fibroblasts
**3D Biplane PALM (BP-PALM)**

**Object plane 500 nm closer to the objective**


Fit with the experimentally determined 3D PSF instead of a 2D Gaussian fit

- z-localization in a range of 1 µm without scanning
- Imaging of 4 µm fluorescently labeled beads with scanning
- **Localization precision in x,y: 20-40 nm in z: 75 nm**

M. F. Juette et al., Nature Meth. 5, 527 (2008)
Stochastic optical reconstruction microscopy (STORM)

Photoactivation of Cy5: 532 nm, 1 W/cm²
Observation of Cy5: 633 nm, 30 W/cm²

Imaging resolution: 20 nm

Positions after correction for sample drift

STORM: applications

Protein RecA bound to DNA and labeled by Cy5-Cy3 switches

Multi-color (STORM)

Red, clathrin: Cy3-Alexa 647  
Activation at 457 nm

Green, microtubules: Cy2-Alexa 647  
Activation at 532 nm

Multi-color imaging resolution: 30 nm

3D STORM microtubules

Wide field

Standard deviation: 9 nm in x, 11 nm in y, 22 nm in z

Limitations: oxygen scavenging necessary measurements only in fixed samples

B. Huang et al., Science 319, 810 (2008)
Bibliography (course 4)

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

Superresolution
Cited publications

**Biophotonics seminars**
*(10h45-12h15)*
February 2: Beatrice David, Caliper Life Sciences
February 16: B. Querleux, L'Oreal
February 23: F. Lacombe, Mauna Kea Technologies

**Exam: Monday February 23 14h-17h**
Written exam (1h30 on the first 3 ½ courses, 1h30 on the second part)
All documents authorized.