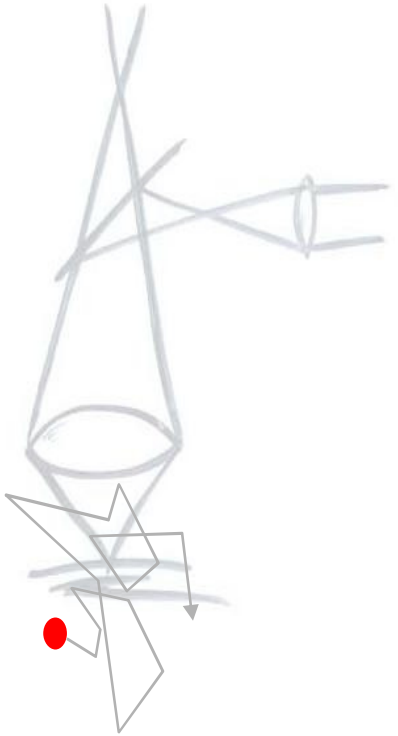
MICROSYSTEMS



Fluorescence correlation spectroscopy & related techniques

Tips&Tricks

Prepare experiment: Fluorescent labels

Prerequisite of FCS & FCCS: mobil, fluorescent molecules

- Covalent labeling of DNA, proteins, beads, … with fluorescent dyes (biochemical assays, loading of cells with microinjection or membrane permeation)
- Expression of fluorescent proteins fused to protein of interest in living cells
- Staining of certain structures (like membranes) with high affinity dyes
- Labeling with fluorescent antibodies

Prepare experiment: Choice of dyes for covalent labeling

- In binding assayes: The smaller reactant must be labeled with an FCS suitable dye.
- Criteria for suitable dyes:
  - High brightness.
  - High photostability.
  - Low triplet transition rate.
  - Amino- and/or thiol-reactive derivatives should be available.
  - Fluorescence lifetime within the lower ns-range (small against diffusion time)
- Excitation wavelength criterion: availability of laser line.
- Emission wavelength criterion: avoid range of autofluorescence.
- Avoid non-specific binding of the dye to buffer components (as BSA, detergents, ...), and the interaction partners, especially the unlabelled partner. Hydrophobic dyes (as rhodamine) tend to bind to chamber surfaces, membranes and proteins.
- Regard dependence of photochemical properties of some dyes on measurement conditions as pH, light intensity, …(like GFP, FITC depend on pH and light intensity).

Prepare experiment: Choice of dyes

FITC, a common label in fluorescence microscopy, is not an FCS suitable dye. It shows

- poor photostability, resulting in bleaching,
- pH dependence of fluorescence: below pH 7 the quantum yield deceases dramatically
- Quenching by neigbouring FITC molecules at labeling ratios of FITC/particle higher than one
- moderate molecular brightness, that means non-optimal signal to noise ratio

Prepare experiment: Choice of dyes

List of FCS suitable dyes:

Alexa dyes	Molecular Probes
Cy dyes	Amersham Pharmazia
Rhodamin Green, 6G, B, Lissamin	Sigma, …
EvoBlue	Evotec
DY dyes	Dyomics
TAMRA	
ROX	
TMR	
Resorufin	
Texas Red	

Prepare experiment: Covalent labeling of samples

- Label particle with appropriate dye (optimal 1 label per particle).
- Purify labeled particle to high purity (<10 kDa: via HPLC).
- Check sample for unspecific binding, autofluorescence, ...
- Characterize labeled sample and buffers

Prepare experiment: Choice of dyes

List of FCS suitable fluorescent proteins.

- GFP
- YFP
- RFP (from Roger Tsien)
- Cross correlation pair: GFP-RFP

Difficult:
- CFP

Not suitable:
- BFP
- DsRed, HsRed

Prepare experiment: Characterization of chemicals used

How to check for dye properties

1.) Start new types of experiments always by the characterization of the chosen free dye in the appropriate buffer and evaluate the following parameters:

- diffusion time (usually between 20 and 80 µs)
- triplet percentage (< 25 %)
- triplet decay time (1 .. 10 µs)
- count rate per particle (depending on the laser intensity and buffer conditions, maximum should be above 20 kHz)
- particle number (optimal range: N = 0.5 .. 2).
  You can calculate the concentration from this value using the actual size of the confocal volume (ca. 10 nM).

2.) Perform FCS measurements of mixtures containing the dye and the assay components.

3.) Compare the parameters determined from both measurements. Check for non-specific binding and alteration of dye properties.

Prepare experiment: Characterization of chemicals used

Avoid aggregation of fluorescent molecules

Some lipophilic dyes are commenly dissolved in organic solvents (as DMSO, DMF, tBuOH, ...). Depending on the solvent concentration some organic solvents cause aggregation of the dyes ore labeled substrates. Strong aggregation prevents reasonable FCS analysis and may even inhibit biological activity.

Freezing of the sample may cause aggregation as well. If possible, avoid it.

Aggregation might be reduced or prevented using detergents. Keep in mind, that detergents may show autofluorescence.

Sonication may destroy aggregates.

Prepare experiment: Characterization of chemicals used

Check for and minimize background fluorescence

- Check all chemicals used for autofluorescence, especially:
  - Detergents
  - non-specific DNA
- Buffer: Background fluorescence must be be checked prior to experiments. The background count rate must be below 10% of the total signal. A “good” buffer has a count rate of less than 2 kHz.
- Reduction of fluorescence by:
  - Treatment with UV-light for several hours
  - use HPLC-grade chemicals
  - filter the buffer through a filter with a pore diameter of 0.2 µm

Prepare experiment: Choice of the right objective

Necessary properties:

1. High numerical aperture
2. Water immersion
3. Correction ring

1. Why a high numerical aperture is necessary?

FCS is a single molecule detection technique. To get as much photons as possible a high numerical aperture is essential. The is essential for a good signal to noise ratio.

Prepare experiment: Choice of the right objective

2. Why one should use a water immersion objective?

- FCS samples are mostly in water-like conditions (either cells in cell culture medium or biochemical experiments in buffer). To avoid refractive index mismatch one needs to take an water immersion objective. A refractive index mismatch would lead to a distortion of the shape of the confocal volume. Furthermore, the degree of distortion would even depend on the focus point within the sample: the deeper one goes into the sample the worse this distortion.
- For FCS, the shape of the confocal volume is really crucial: In FCS data analysis biophysical models are applied that assume a certain shape of the confocal volume. If the shape does not meet this assumption the results of the analysis are not right.

Prepare experiment: Choice of the right objective

3. What is the correction ring good for?

- To obtain the correct shape of the confocal volume, on an inverted microscope the sample must be mounted on a cover slip #1.5 (=140-180 µm thickness). However, cover glass thickness slightly varies. To adapt to this a correction ring is necessary.
- Additionally, a refractive index of the sample solution differing from that of pure water can be compensated. Note that substances such as glycerol, PEG, and saccharides will change the viscosity and the refractive index of the solution.
- On an upright microscope an objective with integrated cover glass might be used alternatively.

System characterization: Theoretical calculation of diffusion coefficient

I. Spherical, globular molecules

For T=293 K (=20°C) and η_{water}=0.01 g/cm^3=10^{-3} Pa s:

$$D = \frac{k * T}{6\pi * \eta * r} = \frac{2.15 * 10^{-19}}{r}$$

$$r = \sqrt[3]{\frac{3m/N_A}{4\pi * \rho}} = 7.36 * 10^{-11} * \sqrt[3]{\frac{m}{\rho}}$$

D	- diffusion coefficient	[m^2/s]
T	- absolute temperature	[K]
k	- Bolzmann constant k=1.38*10^{-23} J/K	[J/K]
η	- viscosity of medium	[Pa s]
r	- hydrodynamic radius	[m]
m	- molecular mass	[g/mol]
N_A	- Avogadro's Number N_A=6.023*10^{23} mol^{-1}	[mol^{-1}]
ρ	- mean density of the molecule	[g/cm^3]

For proteins with ρ=1.2 g/cm^3:

$$D = \frac{3.10 * 10^{-9}}{\sqrt[3]{m}}$$

Molecule class	Mean density ρ
Proteins	1.2 g/cm^3
Nucleic acid	1.8 g/cm^3
Lipids	0.9 .. 1.1 g/cm^3

System characterization: Theoretical calculation of diffusion coefficient

II. Rod-like molecules

For T=293 K (=20°C) and η_{water}=0.01 g/cm^3=10^{-3} Pa s:

$$D = \frac{A * k * T}{3\pi * \eta * L} = \frac{A}{L} * 4.29 * 10^{-19}$$

$$A = \ln(L/d) + 0.312 + \frac{0.565}{L/d} - \frac{0.1}{(L/d)^2}$$

D	- diffusion coefficient	[m^2/s]
T	- absolute temperature	[K]
k	- Bolzmann constant k=1.38*10^{-23} J/K	[J/K]
η	- viscosity of medium	[Pa s]
A	- correction factor	
L	- length of the rod	[m]
d	- diameter of the rod	[m]
bp	- number of base pairs	
N_A	- Avogadro's Number N_A=6.023*10^{23} mol^{-1}	[mol^{-1}]

DNA:

$$D = \frac{A}{bp} * 1.26 * 10^{-9}$$

L= 3.4*10^{-10}m*bp
d=23.8* 10^{-10}m
L/d=0.142*bp

Data evaluation: Determination the of concentration using FCS

- Determine the confocal volume at a certain laser intensity. Set the intensity as low as possible.
- Measure number of particles of your sample at the same laser intensity.
- If possible, work in the recommended concentration range: 1 ..100 nM.

$$c = \frac{N}{N_A * V} = 1.66 * 10^{-24} \frac{N}{V}$$

c - concentration [mol/m^3]
1 mol/m^3 = 1 mM
N - number of molecules
N_A- Avogadro's number [mol^{-1}]
N_A= 6.023*10^{23} mol^{-1}
V - confocal volume [m^3]
1 m^3 = 10^3 l

- **Mind:** Number of molecules and confocal volume depend on laser intensity!
- **Mind:** Background fluorescence must not exceed 10% of the signal fluorescence!
- **Recommendation:** Set up calibration curves using dilution series.

Determination of concentration using FCS - Tips and tricks

- Non-specific adsorption to the walls of the sample carrier can lower the effective concentration of compounds in solution.
- Error in evaluation of the size of the confocal volume may lead to false concentration values. The confocal volume is effected by variations in laser intensity.
- Laser intensity should be as low as possible. To obtain comparable results it should be kept constant.
- Make a calibration dilution series using the laser intensity intended to use during measurement.
- If possible, prepare dilution series of your sample.
  (Optimal measurement range: 1 .. 100 nM)