~~TOC~~

2fFCS

2fFCS is an abbreviation **2 F**ocus **F**luorescence **C**orrelation **S**pectroscopy. Dual focus FCS might also be used.

Correlation analysis that is applied to the fluctuations of the fluorescence intensity. The cross-correlation between the occurrence of events in two different detection volumes is used to introduce an absolute diffusion length into the analysis.

Experimental Setup

Pinhole Selection

The conjugated pinhole size should be chosen slightly larger than the excitation spot diameter. The conjugated pinhole size is the pinhole diameter divided by the magnification of the objective.

e.g:

- 60x 1.2N.A. Objective, 640nm excitation
- excitation FWHM = 350 nm (typical for 4x out-coupler!, the 10x out-coupler will have a larger excitation spot)

Usually the size of the confocal volume or the diameter of the excitation spot is given as its FWHM. However the airy disc diameter is bigger and the 1/e2 diameter must be used.

Single Focus

$2ln(2)=\frac{FWHM^2}{w_0^2}$

$w_0=0.849 \cdot FWHM$

The diameter of the excitation spot therefore is:

$ d_{exc}=2 \cdot w_0 = 594 nm $

Together with the 60x magnification of the objective the airy disc at the pinhole location is

$ d_{PH}=60\cdot 594nm = 35.6\mu m $

This equals 1 airy unit (AU)

Occasionally, pinhole size can be used to adjust amount of photons to change the signal intensity and increase SNR. In addition to the “optimal” 1 AU, Pinhole 1-3 AU is the range of choice. Bigger pinhole give you stronger signal but with the compromised confocal effects.

PicoQuant usually uses a 50 micron pinhole (1.4 AU) to get maximum detection efficiency, somewhat sacrificing background rejection.

Two Foci

The distance between the foci in two-focus FCS is determined by the Nomarski prism. Usually the distance is around 400 nm. Therefore the 2 foci (separated by 400 nm) will occupy:

$ d_{exc, 2fFCS} = 2 \cdot w_0 + 400 nm = 994 nm $

Together with the 60x magnification of the objective both airy discs together, at the the pinhole location, have a diameter

$ d_{PH, 2fFCS}=60\cdot 994nm =59.6\mu m $

Adjusting the Pinhole

1. use a suitable dye solution sample to get an approx. count-rate of 105 cps. e.g. $10^{-9}M$ aqueous Atto655
2. make sure both lasers have the same power
3. using the time trace oscilloscope optimize the pinhole position **with** Nomarski Prism and **both** lasers.
4. switch to TCSPC oscilloscope
5. select linear scale
6. optimize with horizontal knob, this knob affects both lasers equally
7. optimize with vertical knob until both lasers show same intensity. When turning, you should see the decreasing of one laser while the other stays at the same level for a bit then, also the second decreases.
8. if you can't get them both to their **respective** maximum value at the same time, the pinhole is too small. (This means that you are either cutting one or the other or both)

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