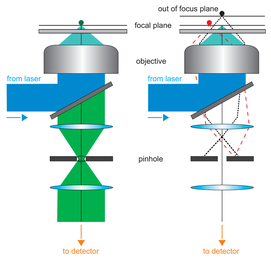
If you are interested in receiving a printed copy of the poster, please send an email to info@picoquant.com.

Quick Reference for Confocal Time-Resolved Microscopy (FLIM, FRET, FCS)

PicoQuant

Confocal Principle

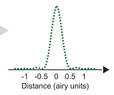


Point Spread Function (PSF) - Confocal Fluorescence Microscopy

Point Spread Function (PSF)
(Ideal imaging system)

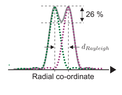
Point-like object

Diffraction pattern

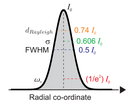


3D Gaussian approximation

Rayleigh criterion



Typical values for the FWHM of the PSF depending on the type of objective and the wavelength: ~ 250 to 450 nm



Confocal volume

λ_{exc}	excitation wavelength
λ_{em}	emission wavelength
σ	standard deviation
NA	numerical aperture
PH	pinhole size
I_0	amplitude of the Gaussian
K	shape parameter
z_0	1/e² radius along the axial direction

$$d_{Rayleigh} \approx 0.68 FWHM \approx 0.8\omega_0 \approx 1.6\sigma$$

$$d_{Rayleigh} \approx \frac{\lambda_{av}}{2 \cdot NA} \quad \text{, for } PH \approx 1A.U.$$

$$\lambda_{av} = \sqrt{2}\frac{\lambda_{exc}\lambda_{em}}{\sqrt{\lambda_{exc}^2 + \lambda_{em}^2}}$$

$$\kappa = \frac{z_0}{\omega_0}$$

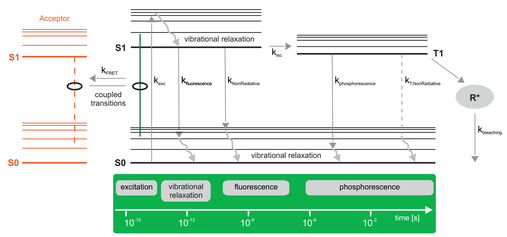
$$V_{confocal} = \left(\frac{\pi}{2}\right)^{3/2} \omega_0^2 z_0$$

$$w(x,y,z) = e^{-2\frac{x^2+y^2}{\omega_0^2}} \cdot e^{-2\frac{z^2}{z_0^2}}$$

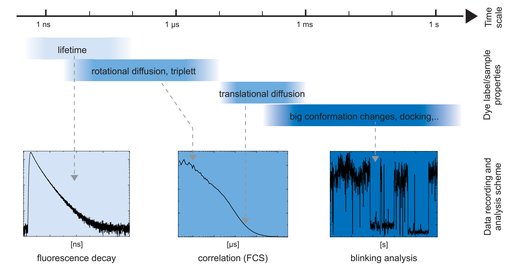
Reference:
Fluorescence Microscopy: From Principles to Biological Applications, Edited by Ulrich Kubitschek

Simplified Perrin - Jablonski Energy Diagram

$$\tau = \frac{1}{k_{fluorescence} + k_{FRET} + k_{NonRadiative}} \qquad Q.Y. = \frac{k_{fluorescence}}{k_{fluorescence} + k_{FRET} + k_{NonRadiative}}$$

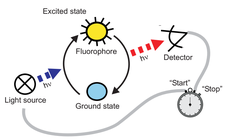


Time Scales and Techniques

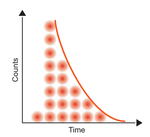


Time-Correlated Single Photon Counting (TCSPC)

Measurement principle



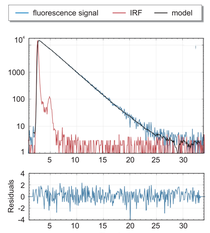
Fluorescence decay histogram



For TCSPC we need:
- A defined "start" of the experiment: pulsed excitation
- A defined "stop" of the experiment: photon counting detector
- A fast "stopwatch" to measure time difference between "start" and "stop"

- Repeat this time measurement many times and count "how many photons have arrived after what time"
- Sort the photons into a histogram according to their arrival times
- Exponential fit of fluorescence decay to obtain fluorescence lifetime

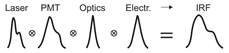
Fitting Fluorescence Decays



Exponential decay fit:

$$Dec(t) = \int_0^{\infty} IRF(\hat{t}Fluor(t-\hat{t}))d\hat{t}$$

Instrument Response Function (IRF):



Examples of fitting models:

N-exponential decay

$$Fluor(t) = \sum_{i=1}^{n} A_i e^{-t/\tau_i}$$

Decay distribution models

$$Fluor(t) = \int_0^{\infty} \alpha(\tau) e^{-t/\tau} d\tau$$

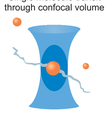
Average lifetime:

$$\tau_{av,amplitude} = \frac{\sum A_i \tau_i}{\sum A_i} \qquad \tau_{av,intensity} = \frac{\sum I_i \tau_i}{\sum I_i}$$

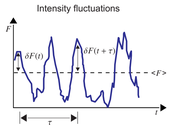
$\alpha(\tau)$	lifetime distribution i.e. ⎍⎍⎍
I_i	the total counts corresponding to lifetime component i

Fluorescence Correlation Spectroscopy (FCS) and Diffusion

Single molecule transit through confocal volume

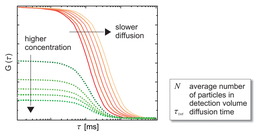


Intensity fluctuations



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

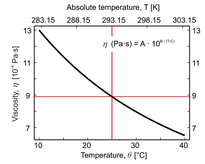
Autocorrelation curve



N	average number of particles in detection volume
τ_{Dif}	diffusion time

$$G(\tau) = \frac{1}{N} \cdot \left(1 + \frac{\tau}{\tau_{Dif}}\right)^{-1} \cdot \left(1 + \frac{\tau}{\kappa^2 \cdot \tau_{Dif}}\right)^{-\frac{1}{2}}$$

Absolute temperature, T [K]



A	= 2.414·10⁻⁵ Pa·s
B	= 247.8 K
C	= 140 K
T	temperature in Kelvin
θ	temperature in °C

Stokes-Einstein (Spherical particles)

$$D(T) = D(25°C) \cdot \frac{T}{298.15\ \text{K}} \cdot \frac{8.9 \cdot 10^{-4}\ \text{Pa} \cdot \text{s}}{\eta(T)}$$

$$D(\theta) = D(25°C) \cdot \frac{\theta + 273.15}{298.15\ \text{K}} \cdot \frac{8.9 \cdot 10^{-4}\ \text{Pa} \cdot \text{s}}{\eta(\theta)}$$

$$D = \frac{k_B T}{6\pi\eta R_H} \qquad R_H \propto M^{-1/3}$$

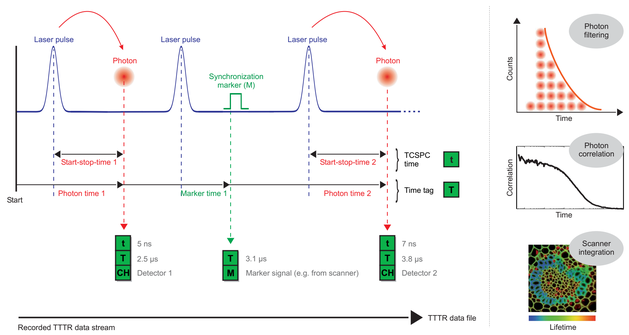
$$V_{eff} = \pi^{3/2} \omega_0^2 z_0 = 2^{3/2} V_{confocal}$$

$$\omega_0 = 2\sqrt{D \cdot \tau_{Dif}}$$

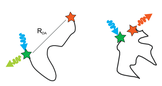
k_B	Boltzmann constant
T	temperature
R_H	hydration radius of diffusing particle
η	viscosity of solvent
M	mass of diffusing particle

Typical values for the effective FCS volume are 0.5-2 fl depending on the objective and the wavelength and the pinhole.

Time Tagged Time-Resolved (TTTR) Data Acquisition

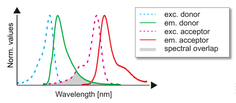


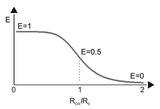
Förster Resonance Energy Transfer (FRET)



FRET efficiency

$$E = \frac{1}{1 + \left(\frac{R_{DA}}{R_0}\right)^6} = \frac{k_{FRET}}{k_{fluorescence} + k_{NonRadiative} + k_{FRET}}$$





Förster radius

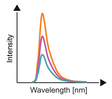
$$R_0 = \left[\frac{9000 \cdot ln10 \cdot J\kappa^2 \Phi_D}{128\pi^5 N_A \cdot n^4}\right]^{\frac{1}{6}}$$

Spectral overlap integral

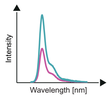
$$J(\lambda) = \int_0^{\infty} F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$$

R_0	Förster radius ~ 4-7 nm
E	FRET efficiency
J	spectral overlap integral
n	refractive index of the medium between the donor and acceptor
Φ_D	quantum yield of the donor
κ^2	orientation factor
N_A	Avogadro number
$F_D(\lambda)$	donor emission
$\varepsilon_A(\lambda)$	normalised acceptor absorption
λ	wavelength

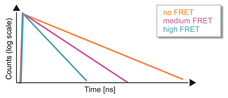
Donor emission



Acceptor emission



Donor lifetime

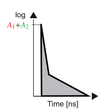


$$E = \frac{F_A / \Phi_A}{F_D / \Phi_D + F_A / \Phi_A}$$

Φ_D, Φ_A	quantum yields
F_D, F_A	fluorescence counts corrected for background and detection efficiencies
τ_{DA} / $\tau_{D\,(no\ FRET)}$	lifetime of the donor in the presence of the acceptor / lifetime of the donor in the absence of the acceptor

$$E = 1 - \frac{\tau_{DA}}{\tau_{D\,(no\ FRET)}}$$

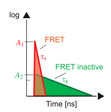
Determination of donor species



Fit of n-exponential decay

$$Fluor(t) = \sum_{i=1}^{n} A_i e^{-t/\tau_i}$$

in this case n = 2



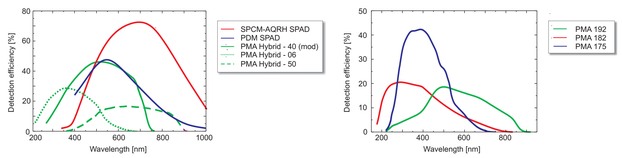
Fraction of FRET-active donors

$$A_{DA} = \frac{A_1}{A_1 + A_2}$$

Fraction of free (FRET-inactive) donors

$$A_D = \frac{A_2}{A_1 + A_2}$$

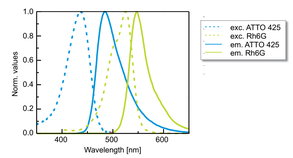
Single Photon Sensitive Detectors

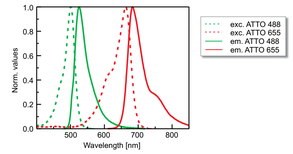


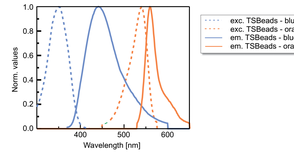
	PMA Series	PMA Hybrid Series	SPCM-AQRH	PDM Series
Type	PMT	Hybrid PMT	SPAD	SPAD
Timing resolution (detector FWHM)	< 180 ps	< 50 to < 140 ps	< 200 to < 600 ps	< 50 to < 250 ps
Max. detection efficiency	40 % at 400 nm*	45 % at 500 nm*	70 % at 700 nm	49 % at 550 nm
Spectral range	185 - 920 nm	220 - 890 nm	400 - 1100 nm	400 - 1100 nm
Diameter of active area	8 mm	3, 5 or 6 mm	180 µm	20, 50 or 100 µm

* model dependent, see detection efficiency curves above

Properties of Standard Samples







Fluorophore	Max. excitation [nm]	Max. emission [nm]	Molar absorption coefficient [cm M]⁻¹	Quantum yield	Lifetime [ns]
ATTO 425	436	484	45000	0.9	3.6
ATTO 485	501	523	90000	0.8	4.1
ATTO 655	663	684	125000	0.3	1.8
Rh6G	530	555	116000	0.95	4.1

Fluorophore	Max. emission [nm]	Diffusion coefficient in water at 25°C (298.15 K) [10⁻⁶ cm²s⁻¹]	Lifetime in water [ns]
ATTO 425 - carboxylic acid	484	4.07	3.6
ATTO 655 - maleimid	686	4.26	1.8
ATTO 655 - carboxylic acid	685	4.25	1.8
ATTO 655 - NHS esther	685	3.6	1.8
Cy5	670	3.3	0.9
Alexa 647	665	3.4	1.0
Alexa 633	647	4.14	3.2
Rhodamine 6G	550	4.5	3.9
Rhodamine B	560	4.6	1.7
Rhodamine 123	530	4.7	4.0 (in PBS)
Rhodamine 110	535	4.25	4.0
Fluorescein	520	4.11	4.3
Oregon Green 488	550	4.10	4.1
Atto488-carboxylic acid	523	4.0	4.1
TetraSpeck Beads, 0.1 µm diameter	430, 515, 580, 680	0.044	--

For more details please see our Technical Note on "Absolute Diffusion Coefficients: Compilation of Reference Data for FCS Calibration" available for download on our website.

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