Lecture 7
FCS, Autocorrelation, PCH, Cross-correlation

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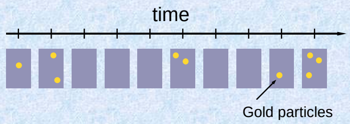
Principles of Fluorescence Techniques
Laboratory for Fluorescence Dynamics

Figure and slide acknowledgements:
Enrico Gratton

Historic Experiment: 1st Application of Correlation Spectroscopy

(Svedberg & Inouye, 1911) *Occupancy Fluctuation*



12000200132412310211113112511102331333221112242212261221423452411413114231001004211231232011110001 11_21100132000001001100010002322100211000020100 1_33312200023122102401 1102_12221122310001103311102101100101030113121210 10121111211_10003221012302012121321110110023312242110001203010100221734410101002112211444421211440132123314313011222123310121111222412231113322132110000410432012120011322231200_25321203323311110021002201301132113120010131432211221122323442223032142153220020214212323204311231200331422345213411041232222022 1

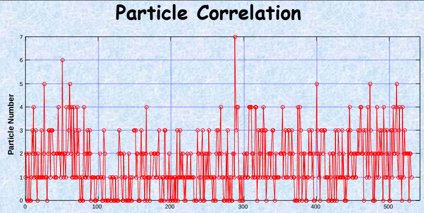
Svedberg and Inouye, *Zeitschr. F. physik. Chemie* 1911, **77**:145

Collected data by counting (by visual inspection) the number of particles in the observation volume as a function of time using a "ultra microscope"

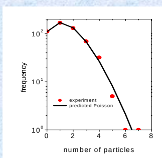
! Statistical analysis of raw data required

Particle Correlation

Graph of Raw Data:



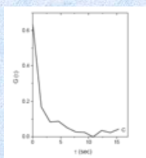
***Histogram of particle counts**



- **Poisson statistics**

$\langle N \rangle = 1.55$

***Autocorrelation**



- **Autocorrelation not available in the original paper. It can be easily calculated today.**

$\langle N \rangle = \frac{1}{G(0)} = 1.56$

What we learn from the correlation function?

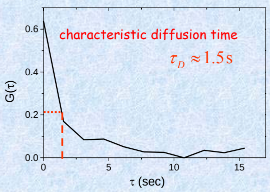
Svedberg claimed: Gold colloids with radius R = 3 nm $D_{\text{Expected}} = \frac{k_B T}{6\pi\eta R} \approx 70\,\frac{\mu\text{m}^2}{\text{s}}$ (Stokes-Einstein)

Experimental facts:

Slit

$2\,\mu\text{m}$

$\langle \Delta x^2 \rangle \approx 2D\tau_D$



$\Rightarrow D_{Exp} \approx 1\,\frac{\mu\text{m}^2}{\text{s}}$

$\Rightarrow R \approx 200\,\text{nm}$

Conclusion: Bad sample preparation

The ultramicroscope was invented in 1903 (*Siedentopf and Zsigmondy*). They already concluded that scattering will not be suitable to observe single molecules, but fluorescence could.

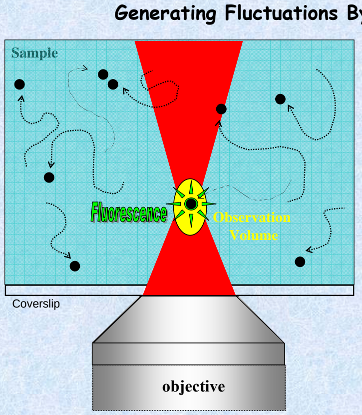
Fluorescence Correlation Spectroscopy (FCS)

In FCS
Fluctuations are in the Fluorescence Signal

Diffusion
Enzymatic Activity
Phase Fluctuations
Conformational Dynamics
Rotational Motion
Protein Folding

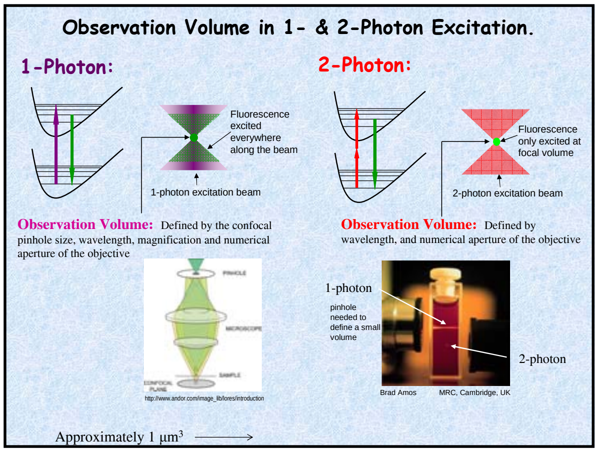
Example of processes that could generate fluctuations

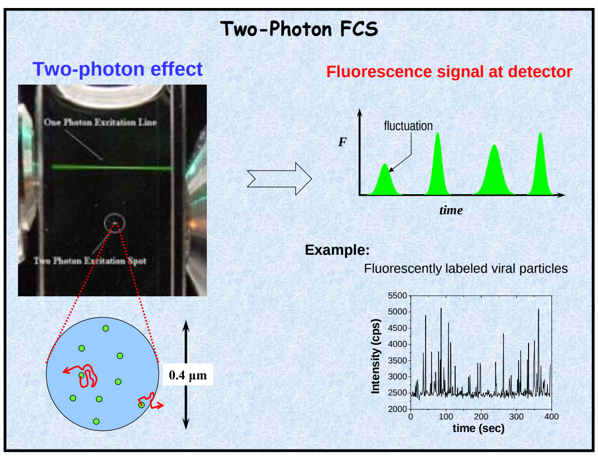
Generating Fluctuations By Motion

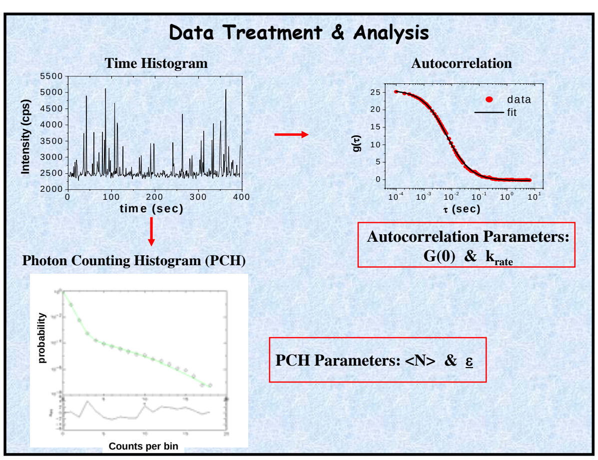


What is Observed?

1. **The Rate of Motion**
2. **The Concentration of Particles**
3. **Changes in the Particle Fluorescence while under Observation, for example conformational transitions**


Observation Volume in 1- & 2-Photon Excitation.
1-Photon:
Fluorescence excited everywhere along the beam
1-photon excitation beam
Observation Volume: Defined by the confocal pinhole size, wavelength, magnification and numerical aperture of the objective
2-Photon:
Fluorescence only excited at focal volume
2-photon excitation beam
Observation Volume: Defined by wavelength, and numerical aperture of the objective
1-photon
pinhole needed to define a small volume
2-photon
Brad Amos MRC, Cambridge, UK
Approximately 1 µm³


Two-Photon FCS
Two-photon effect
One Photon Excitation Line
Two Photon Excitation Spot
0.4 µm
Fluorescence signal at detector
fluctuation
F
time
Example:
Fluorescently labeled viral particles
Intensity (cps)
time (sec)


Data Treatment & Analysis
Time Histogram
Intensity (cps)
time (sec)
Autocorrelation
data
fit
g(τ)
τ (sec)
Autocorrelation Parameters:
G(0) & k_rate
Photon Counting Histogram (PCH)
probability
Counts per bin
PCH Parameters: <N> & ε

Autocorrelation Function

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

Factors influencing the fluorescence signal:

$$F(t) = \kappa Q \int d\mathbf{r}\, W(\mathbf{r}) C(\mathbf{r},t)$$

κQ = quantum yield and detector sensitivity (how bright is our probe). This term could contain the fluctuation of the fluorescence intensity due to internal processes

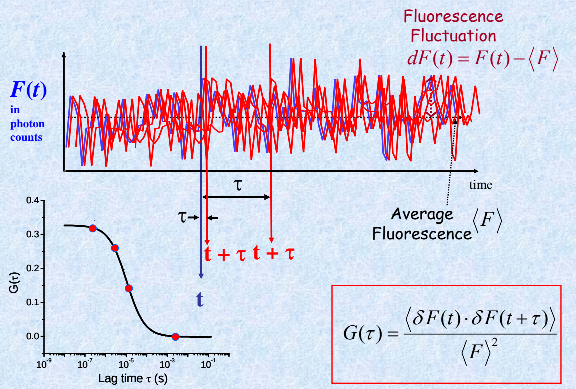
W(r) describes our observation volume

C(r,t) is a function of the fluorophore concentration over time. This is the term that contains the "physics" of the diffusion processes

Average fluorescence signal: $\langle F(t)\rangle$

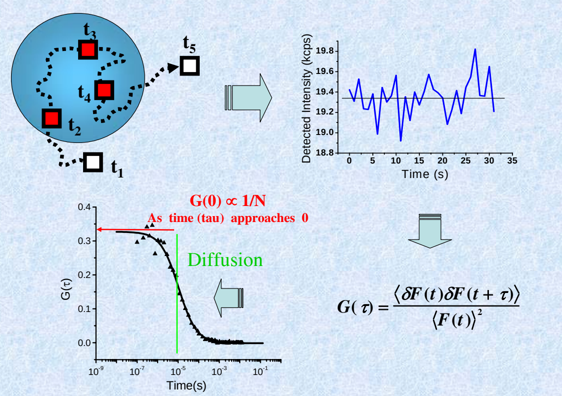
Fluorescence fluctuation: $\delta F(t) = F(t) - \langle F(t)\rangle$

Calculating the Autocorrelation Function



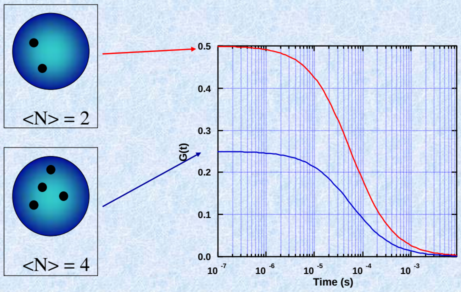
$$G(\tau) = \frac{\langle \delta F(t)\cdot \delta F(t+\tau)\rangle}{\langle F\rangle^2}$$

The Autocorrelation Function



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

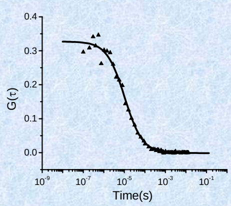
The Effects of Particle Concentration on the Autocorrelation Curve



Why Is G(0) Proportional to 1/Particle Number?

A Poisson distribution describes the statistics of particle occupancy fluctuations. In a Poissonian system the variance is proportional to the average number of fluctuating species:

$$\langle Particle\_Number \rangle = Variance$$

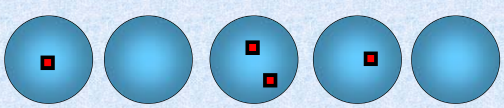


$$G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

$$G(0) = \frac{\langle \delta F(t)^2 \rangle}{\langle F(t) \rangle^2} = \frac{\langle (F(t) - \langle F(t) \rangle)^2 \rangle}{\langle F(t) \rangle^2}$$

$$G(0) = \frac{Variance}{\langle N \rangle^2} = \frac{1}{\langle N \rangle}$$

G(0), Particle Brightness and Poisson Statistics



1 0 0 0 0 0 0 0 0 2 0 1 1 1 0 0 0 0 0 0 1 0 0 0 0 0 0 0 1 0 1 0 0 0 1 0 0 1 0 0

Time

Average = 0.275 **Variance = 0.256**

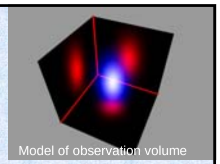
$$\langle N \rangle \propto Average^2 / Variance = \frac{0.275^2}{0.256} = 0.296$$

Lets increase the particle brightness by 4x:

4 0 0 0 0 0 0 0 0 8 0 4 4 4 0 0 0 0 0 0 4 0 0 0 0 0 0 0 4 0 4 0 0 0 4 0 0 4 0 0

Average = 1.1 Variance = 4.09 $\langle N \rangle \propto$ **0.296**

Effect of Shape on the (2-Photon) Autocorrelation Functions:
(for simple diffusion)


Model of observation volume

For a 3-dimensional Gaussian excitation volume:

$$G(\tau)=\frac{\gamma}{N}\left(1+\frac{8D\tau}{w_0^2}\right)^{-1}\left(1+\frac{8D\tau}{z_0^2}\right)^{-1/2}$$

For a 2-dimensional Gaussian excitation volume:

1-photon equation contains a 4, instead of 8

$$G(\tau)=\frac{\gamma}{N}\left(1+\frac{8D\tau}{w_0^2}\right)^{-1}$$

γ: shape factor (0.354 for 3DG, 0.5 for 2DG)
N: average number of particles inside volume
D: Diffusion coefficient
w_o: radial beam waist of two-photon laser spot
z_o: axial beam waist of two-photon laser spot

Additional Equations:

3D Gaussian Confocor analysis:

$$G(\tau)=1+\frac{1}{N}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\cdot\left(1+S^2\cdot\frac{\tau}{\tau_D}\right)^{-\frac{1}{2}}$$

... where N is the average particle number, τ_D is the diffusion time (related to D, $\tau_D=w^2/8D$, for two photon and $\tau_D=w^2/4D$ for 1-photon excitation), and S is a shape parameter, equivalent to w/z in the previous equations.

Note: The offset of one is caused by a different definition of G(τ) : $G(\tau)=\frac{\langle F(t+\tau)\cdot F(t)\rangle}{\langle F\rangle^2}$

Triplet state term:

$$(1+\frac{T}{1-T}e^{\frac{-\tau}{\tau_T}})$$

..where T is the triplet state amplitude and τ_T is the triplet lifetime.

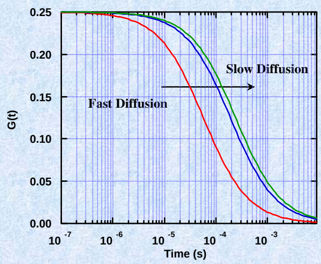
Orders of magnitude (for 10nM solution, small molecule, water)

Volume	Device	Size(μm)	Molecules	Diffusion Time (s)
milliliter	cuvette	10000	$6x10^{12}$	10^4
microliter	plate well	1000	$6x10^9$	10^2
nanoliter	microfabrication	100	$6x10^6$	1
picoliter	typical cell	10	$6x10^3$	10^{-2}
femtoliter	confocal volume	1	$6x10^0$	10^{-4}
attoliter	nanofabrication	0.1	$6x10^{-3}$	10^{-6}

The Effects of Particle Size on the Autocorrelation Curve

Diffusion Constants

300 um²/s
90 um²/s
71 um²/s



Stokes-Einstein Equation:

$$D = \frac{k \cdot T}{6 \cdot \pi \cdot \eta \cdot r}$$

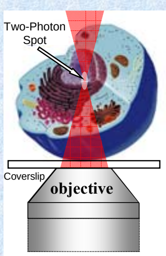
and

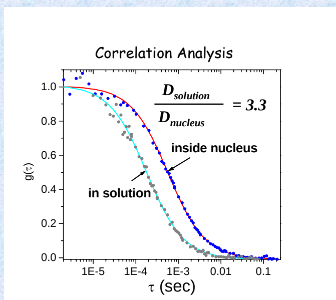
$$MW \propto Volume \propto r^3$$

Monomer --> Dimer

Only a change in D by a factor of $2^{1/3}$, or 1.26

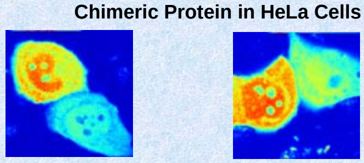
FCS inside living cells



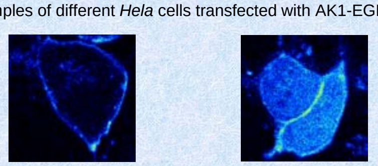


Measure the diffusion coefficient of Green Fluorescent Protein (GFP) in aqueous solution in inside the nucleus of a cell.

Autocorrelation Adenylate Kinase -EGFP Chimeric Protein in HeLa Cells



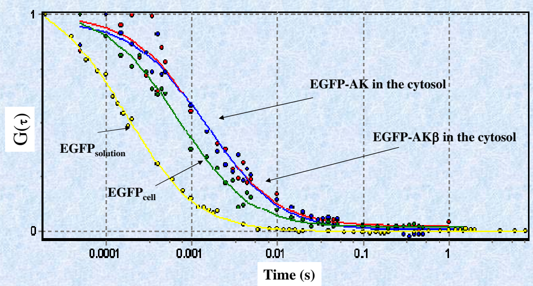
Examples of different *Hela* cells transfected with AK1-EGFP



Examples of different *Hela* cells transfected with AK1β -EGFP

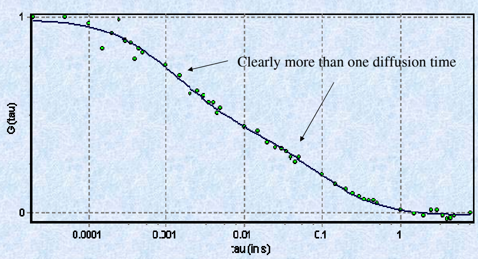
Qiao Qiao Ruan, Y. Chen, M. Glaser & W. Mantulin Dept. Biochem & Dept Physics- LFD Univ Il, USA

Autocorrelation of EGFP & Adenylate Kinase -EGFP



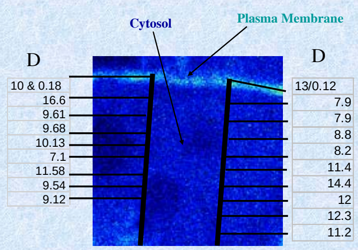
Normalized autocorrelation curve of EGFP in solution ( ), EGFP in the cell (•), AK1-EGFP in the cell(•), AK1β-EGFP in the cytoplasm of the cell(•).

Autocorrelation of Adenylate Kinase –EGFP on the Membrane



A mixture of AK1b-EGFP in the cytoplasm and membrane of the cell.

Autocorrelation Adenylate Kinaseβ -EGFP



Diffusion constants (um^2/s) of AK EGFP-AKβ in the cytosol -EGFP in the cell (HeLa). At the membrane, a dual diffusion rate is calculated from FCS data. Away from the plasma membrane, single diffusion constants are found.

Multiple Species

Case 1: Species differ in their diffusion constant D

Autocorrelation function can be used:

$$G(\tau)_{sample} = \sum_{i=1}^{M} f_i^2 \cdot G(0)_i \cdot \left(1 + \frac{8D\tau}{w_{2DG}^2}\right)^{-1}$$ (2D-Gaussian Shape)

! f_i is the fractional fluorescence intensity of species *i*.

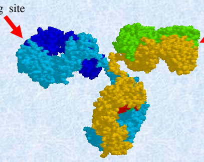
$$G(0)_{sample} = \sum f_i^2 \cdot G(0)_i$$

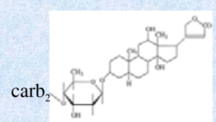
$G(0)_{sample}$ is no longer γ/N !

Antibody - Hapten Interactions

Binding site

Binding site



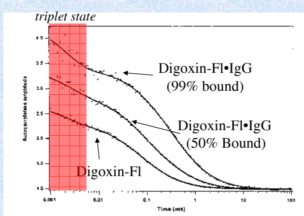


Mouse IgG: The two heavy chains are shown in yellow and light blue. The two light chains are shown in green and dark blue..*J.Harris, S.B.Larson, K.W.Hasel, A.McPherson, "Refined structure of an intact IgG2a monoclonal antibody", Biochemistry 36: 1581, (1997).*

Digoxin: a cardiac glycoside used to treat congestive heart failure. Digoxin competes with potassium for a binding site on an enzyme, referred to as potassium-ATPase. Digoxin inhibits the Na-K ATPase pump in the myocardial cell membrane.

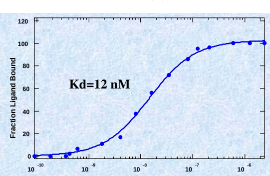
Anti-Digoxin Antibody (IgG) Binding to Digoxin-Fluorescein

Autocorrelation curves:



Binding titration from the autocorrelation analyses:

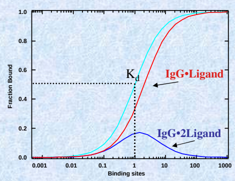
$$F_b = \frac{m \cdot S_{free}}{K_d + S_{free}} + c$$

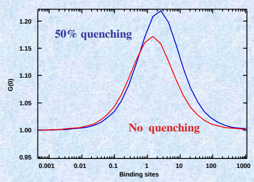


S. Tetin, K. Swift, & , E, Matayoshi , 2003

Two Binding Site Model

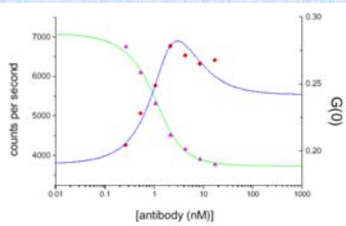
IgG + 2 Ligand-Fl ⟷ IgG•Ligand-Fl + Ligand-Fl ⟷ IgG•2Ligand-Fl





[Ligand]=1, G(0)=1/N, K_d=1.0

Digoxin-FL Binding to IgG: G(0) Profile



	Lifetime (nsec)	molecular fraction (lifetime)	cpsm	Molecular fraction (G(0))
Digoxin	4.01	100%	29000	100%
Ligated Digoxin(C_1)	4.03	53.6%	23600	52%
Ligated Digoxin(C_2)	1.25	46.4%	7100	48%

Y. Chen , Ph.D. Dissertation; Chen et. al., Biophys. J (2000) 79: 1074

Multiple Species

Case 2: Species vary by a difference in brightness

assuming that $D_1 \approx D_2$

The quantity G(0) becomes the only parameter to distinguish species, but we know that:

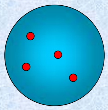
$$G(0)_{sample} = \sum f_i^2 \cdot G(0)_i$$

The autocorrelation function is not suitable for analysis of this kind of data without additional information.

We need a different type of analysis

Photon Counting Histogram (PCH)

Aim: **To resolve species from differences in their molecular brightness**



Poisson Distribution for particle number: $p(N) = \frac{\langle N \rangle^N \cdot e^{-\langle N \rangle}}{N!}$

But distribution of photon counts is Non-Poissonian: $p(k) = PCH(\varepsilon, \langle N \rangle)$

Single Species: p(k) is the probability of observing **k** photon counts

Sources of Non-Poissonian Noise

Detector Noise
Diffusing Particles in an Inhomogeneous Excitation Beam*
Particle Number Fluctuations*
Multiple Species*

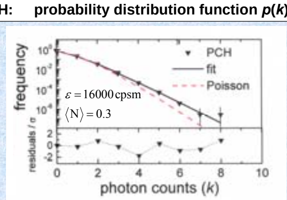
Photon Counting Histogram (PCH)

Aim: **To resolve species from differences in their molecular brightness**

Molecular brightness ε : The average photon count rate of a single fluorophore

PCH: probability distribution function *p*(*k*)

where *p*(***k***) is the probability of observing ***k*** photon counts



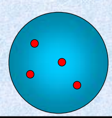
Single Species:

$$p(k) = PCH(\varepsilon, \langle N \rangle)$$

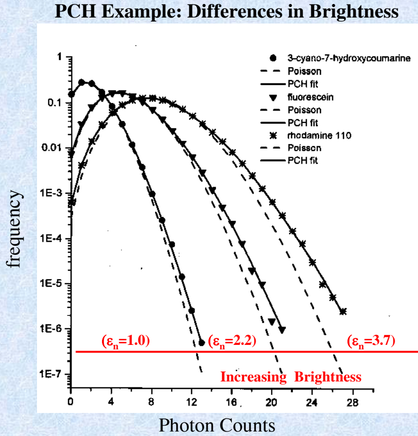
Note: PCH is Non-Poissonian!

Sources of Non-Poissonian Noise

- Detector Noise
- Diffusing Particles in an Inhomogeneous Excitation Beam*
- Particle Number Fluctuations*
- Multiple Species*



PCH Example: Differences in Brightness



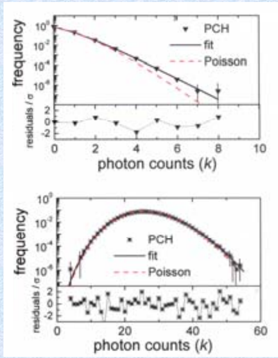
Single Species PCH: Concentration

5.5 nM Fluorescein

Fit:
ε = 16,000 cpsm
N = 0.3

550 nM Fluorescein

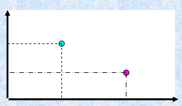
Fit:
ε = 16,000 cpsm
N = 33

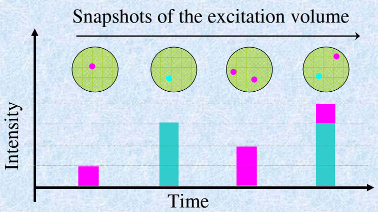


As particle concentration increases the PCH approaches a Poisson distribution

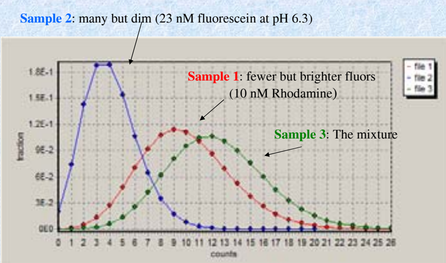
Photon Counting Histogram: Multiple Species

Binary Mixture: $p(k) = PCH(\varepsilon_1, \langle N_1 \rangle) \otimes PCH(\varepsilon_2, \langle N_2 \rangle)$





Photon Counting Histogram: Multiple Species

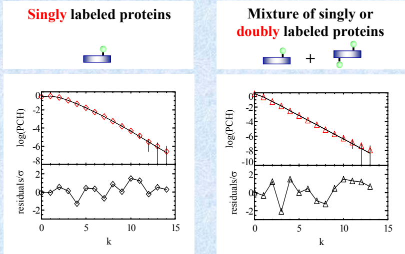


The occupancy fluctuations for each specie in the mixture becomes a convolution of the individual specie histograms. The resulting histogram is then broader than expected for a single species.

$$p(k) = PCH(\varepsilon_1, \langle N_1 \rangle) \otimes PCH(\varepsilon_2, \langle N_2 \rangle)$$

Resolve a protein mixture with a brightness ratio of two

Alcohol dehydrogenase labeling experiments

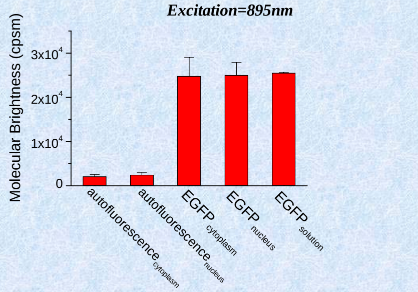


c	ε_1 kcpsm	$\overline{N}_1$	ε_2 kcpsm	$\overline{N}_2$
Sample A	$26.2^{+0.19}_{-0.18}$	$0.540^{+0.004}_{-0.004}$	-----	-----
Sample B	$25.1^{+0.6}_{-1.2}$	$0.155^{+0.007}_{-0.002}$	56^{+10}_{-10}	$0.006^{+0.008}_{-0.003}$

Both species have same

- color
- fluorescence lifetime
- diffusion coefficient
- polarization

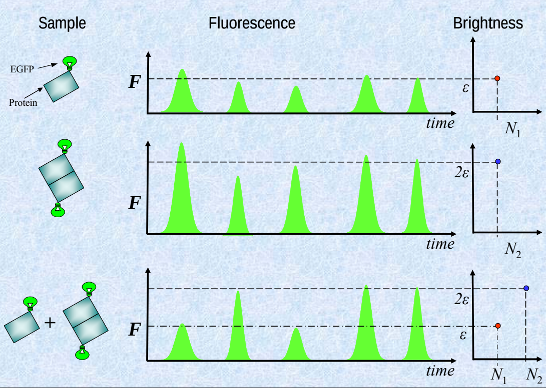
PCH in cells: Brightness of EGFP



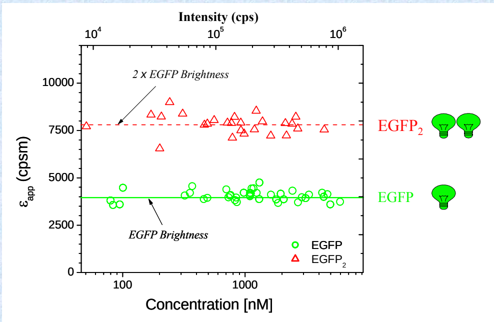
The molecular brightness of EGFP is a factor ten higher than that of the autofluorescence in HeLa cells

Chen Y, Mueller JD, Ruan Q, Gratton E (2002) Biophysical Journal, 82, 133 .

Brightness Encodes Stoichiometry



Brightness and Stoichiometry



Brightness of $EGFP_2$ is twice the brightness of EGFP

Chen Y, Wei LN, Mueller JD, PNAS (2003) 100, 15492-15497

Distinguish Homo- and Hetero-interactions in living cells

ECFP: EYFP:

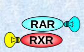
	Apparent Brightness	A	B	A B	A + B	A A
$\lambda_{2\gamma} = 905\,\text{nm}$	A, B	ε	ε	2ε	ε	2ε
$\lambda_{2\gamma} = 965\,\text{nm}$	A, B	ε	0	ε	ε	2ε

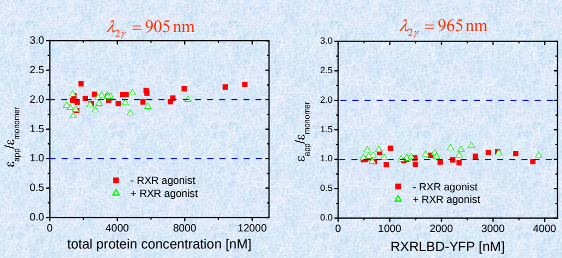
- single detection channel experiment
- distinguish between CFP and YFP by excitation (not by emission)!
- brightness of CFP and YFP is identical at 905nm (with the appropriate filters)
- you can choose conditions so that the brightness is not changed by FRET between CFP and YFP
- determine the expressed protein concentrations of each cell!

PCH analysis of a heterodimer in living cells

The nuclear receptors RAR and RXR form a tight heterodimer in vitro. We investigate their stoichiometry in the nucleus of COS cells.

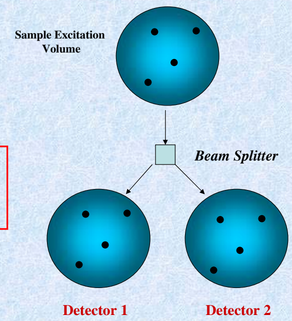
We expect:





Chen Y, Li-Na Wei, Mueller JD, Biophys. J., (2005) 88, 4366-4377

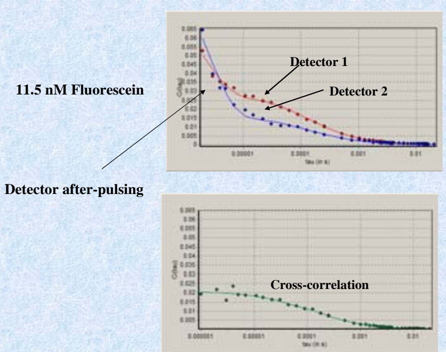
Two Channel Detection: Cross-correlation



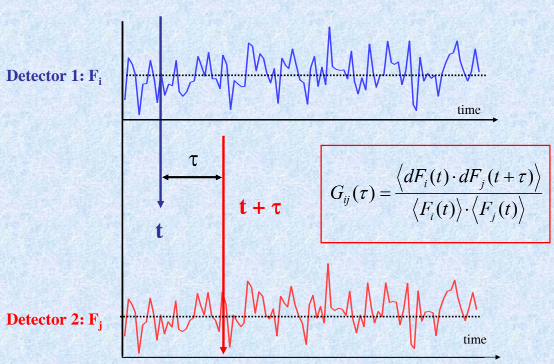
1. Isolate correlated signals.
2. Corrects for PMT noise

Each detector observes the same particles

Removal of Detector Noise by Cross-correlation



Calculating the Cross-correlation Function



$$G_{ij}(\tau) = \frac{\langle dF_i(t) \cdot dF_j(t+\tau) \rangle}{\langle F_i(t) \rangle \cdot \langle F_j(t) \rangle}$$

Cross-correlation Calculations

One uses the same fitting functions you would use for the standard autocorrelation curves.

Thus, for a 3-dimensional Gaussian excitation volume one uses:

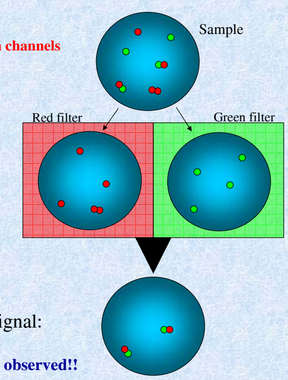
$$G_{12}(\tau) = \frac{\gamma}{N_{12}}\left(1+\frac{8D_{12}\tau}{w^2}\right)^{-1}\left(1+\frac{8D_{12}\tau}{z^2}\right)^{-1/2}$$

G_{12} is commonly used to denote the cross-correlation and G_1 and G_2 for the autocorrelation of the individual detectors. Sometimes you will see $G_x(0)$ or $C(0)$ used for the cross-correlation.

Two-Color Cross-correlation

The cross-correlation
ONLY if particles are observed in both channels

Each detector observes particles with a particular color



The cross-correlation signal:

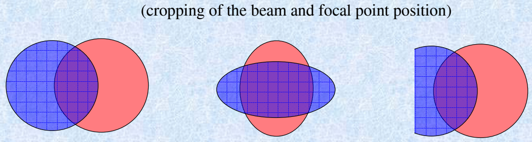
Only the green-red molecules are observed!!

Experimental Concerns: Excitation Focusing & Emission Collection

We assume exact match of the observation volumes in our calculations which is difficult to obtain experimentally.

Excitation side:
- (1) Laser alignment
- (2) Chromatic aberration
- (3) Spherical aberration

Emission side:
- (1) Chromatic aberrations
- (2) Spherical aberrations
- (3) Improper alignment of detectors or pinhole (cropping of the beam and focal point position)



Two-color Cross-correlation

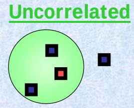
Equations are similar to those for the cross correlation using a simple beam splitter:

$$G_{ij}(\tau) = \frac{\langle dF_i(t) \cdot dF_j(t+\tau)\rangle}{\langle F_i(t)\rangle \cdot \langle F_j(t)\rangle}$$

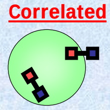
Information Content	Signal
Correlated signal from particles having **both colors**.	$G_{12}(\tau)$
Autocorrelation from channel 1 on the **green particles**.	$G_1(\tau)$
Autocorrelation from channel 2 on the **red particles**.	$G_2(\tau)$

Spectral Crosstalk

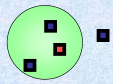
Uncorrelated

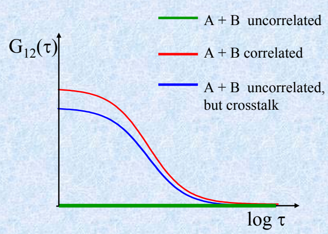


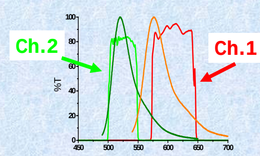
Correlated



Uncorrelated+Crosstalk

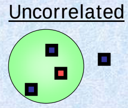


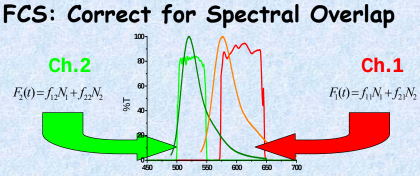




Two-Color FCS: Correct for Spectral Overlap

Uncorrelated

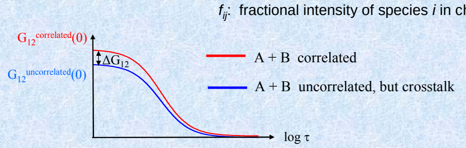


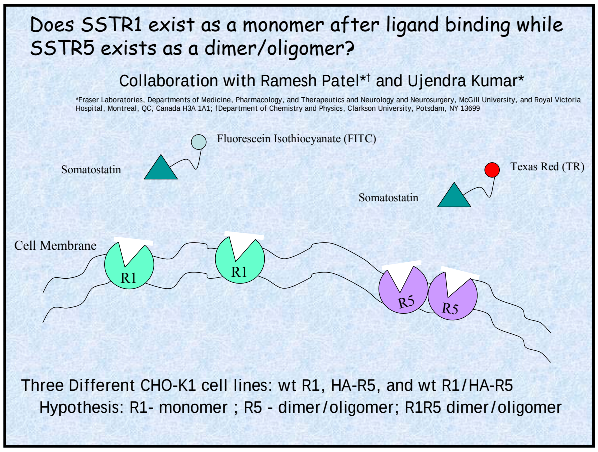


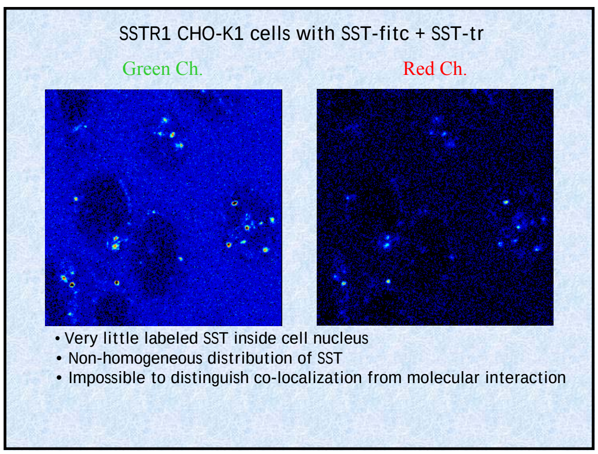
For two ***uncorrelated*** species, the amplitude of the cross-correlation is proportional to:

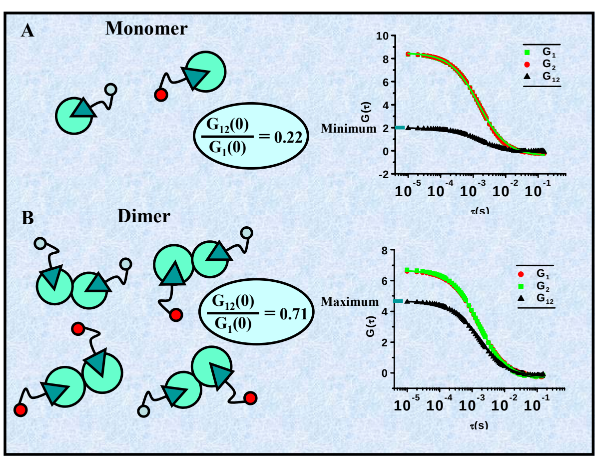
$$G_{12}^{uncorrelated}(0) \propto \left[\frac{f_{11}f_{12}\langle N_1\rangle + f_{21}f_{22}\langle N_2\rangle}{f_{11}f_{12}\langle N_1\rangle^2 + (f_{11}f_{22} + f_{21}f_{12})\langle N_1\rangle\langle N_2\rangle + f_{21}f_{22}\langle N_2\rangle^2}\right]$$

f_{ij}: fractional intensity of species *i* in channel *j*

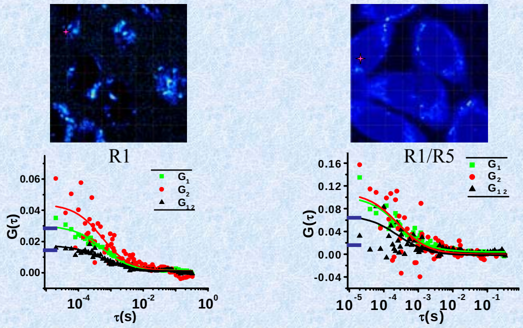



Does SSTR1 exist as a monomer after ligand binding while SSTR5 exists as a dimer/oligomer?
Collaboration with Ramesh Patel*† and Ujendra Kumar*
*Fraser Laboratories, Departments of Medicine, Pharmacology, and Therapeutics and Neurology and Neurosurgery, McGill University, and Royal Victoria Hospital, Montreal, QC, Canada H3A 1A1; †Department of Chemistry and Physics, Clarkson University, Potsdam, NY 13699
Fluorescein Isothiocyanate (FITC)
Somatostatin
Texas Red (TR)
Somatostatin
Cell Membrane
R1
R1
R5
R5
Three Different CHO-K1 cell lines: wt R1, HA-R5, and wt R1/HA-R5
Hypothesis: R1- monomer ; R5 - dimer/oligomer; R1R5 dimer/oligomer


SSTR1 CHO-K1 cells with SST-fitc + SST-tr
Green Ch.
Red Ch.
• Very little labeled SST inside cell nucleus
• Non-homogeneous distribution of SST
• Impossible to distinguish co-localization from molecular interaction


A
Monomer
G12(0) / G1(0) = 0.22
Minimum
G1
G2
G12
G(τ)
τ(s)
B
Dimer
G12(0) / G1(0) = 0.71
Maximum
G1
G2
G12
G(τ)
τ(s)

Experimentally derived auto- and cross-correlation curves from live R1 and R5/R1 expressing CHO-K1 cells using dual-color two-photon FCS.



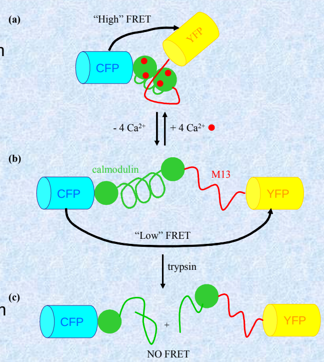
The R5/R1 expressing cells have a greater cross-correlation relative to the simulated boundaries than the R1 expressing cells, indicating a higher level of dimer/oligomer formation.

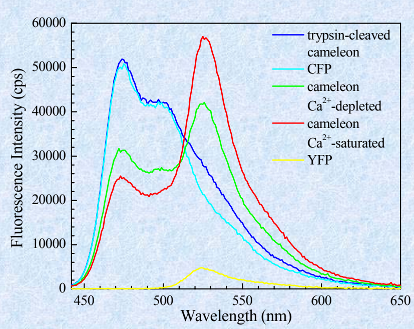
Patel, R.C., *et al.*, *Ligand binding to somatostatin receptors induces receptor-specific oligomer formation in live cells.* PNAS, 2002. **99**(5): p. 3294-3299

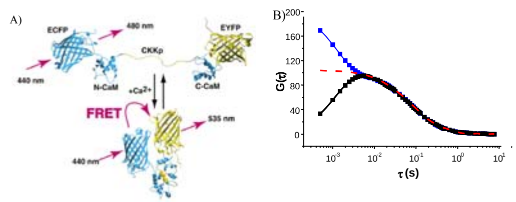
Molecular Dynamics

What if the distance/orientation is not constant?

- Fluorescence fluctuation can result from FRET or Quenching
- FCS can determine the rate at which this occurs
- This will yield hard to get information (in the μs to ms range) on the internal motion of biomolecules

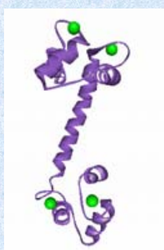


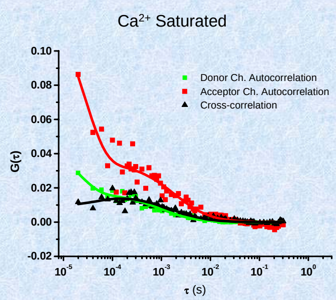




. A) Cameleon fusion protein consisting of ECFP, calmodulin, and EYFP. [Truong, 2001 #1293] Calmodulin undergoes a conformational change that allows the ECFP/EYFP FRET pair to get cl ose enough for efficient energy transfer. Fluctuations between the folded and unfolded states will yield a measurable kinetic component for the cross-correlation. B) Simulation of how such a fluctuation would show up in the autocorrelation and cross-correlation. Red dashed line indicates pure diffusion.

In vitro Cameleon Data

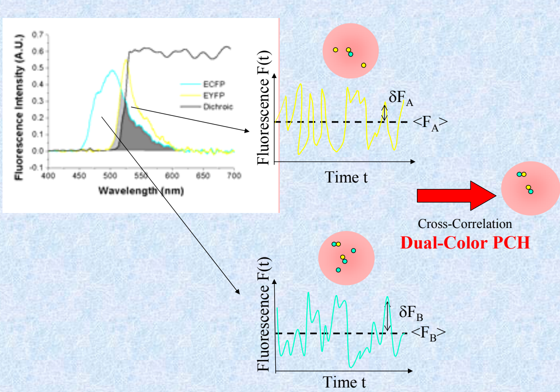


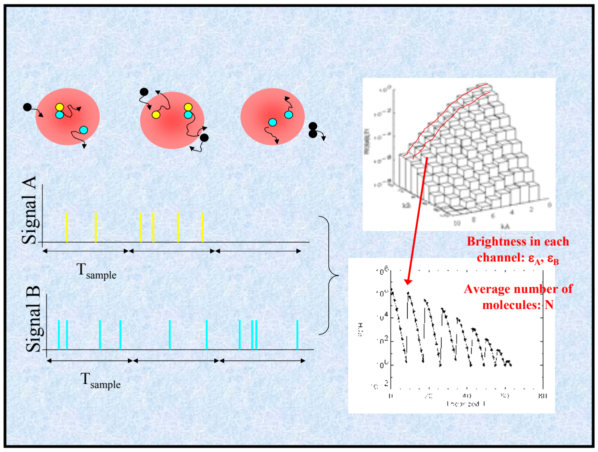


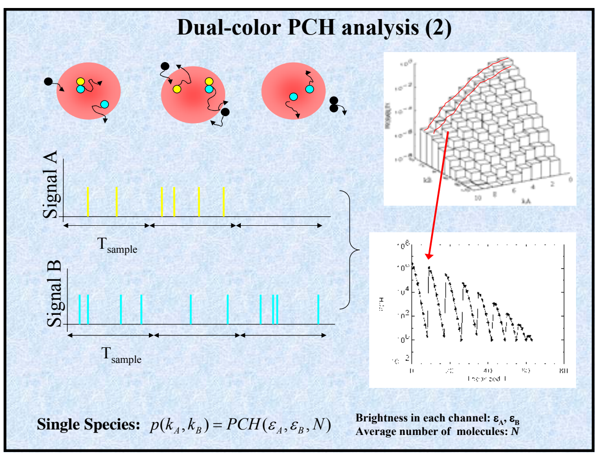
Crystallization And Preliminary X-Ray Analysis Of Two New Crystal Forms Of Calmodulin, B.Rupp, D.Marshak and S.Parkin, Acta Crystallogr. D 52, 411 (1996)

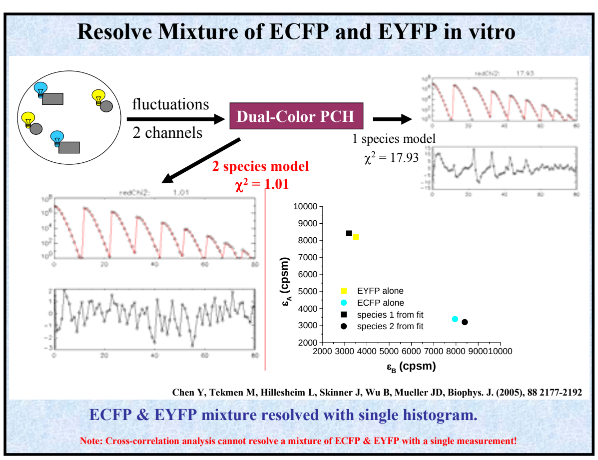
Are the fast kinetics (~20 μs) due to conformational changes or to fluorophore blinking?

Dual-color PCH analysis (1)




Signal A
Signal B
T_{sample}
Brightness in each channel: ε_A, ε_B
Average number of molecules: N


Dual-color PCH analysis (2)
Signal A
Signal B
T_{sample}
Single Species: $p(k_A, k_B) = PCH(\varepsilon_A, \varepsilon_B, N)$
Brightness in each channel: ε_A, ε_B
Average number of molecules: *N*


Resolve Mixture of ECFP and EYFP in vitro
fluctuations
2 channels
Dual-Color PCH
1 species model
$\chi^2 = 17.93$
2 species model
$\chi^2 = 1.01$
ε_A (cpsm)
ε_B (cpsm)
EYFP alone
ECFP alone
species 1 from fit
species 2 from fit
Chen Y, Tekmen M, Hillesheim L, Skinner J, Wu B, Mueller JD, Biophys. J. (2005), 88 2177-2192
ECFP & EYFP mixture resolved with single histogram.
Note: Cross-correlation analysis cannot resolve a mixture of ECFP & EYFP with a single measurement!