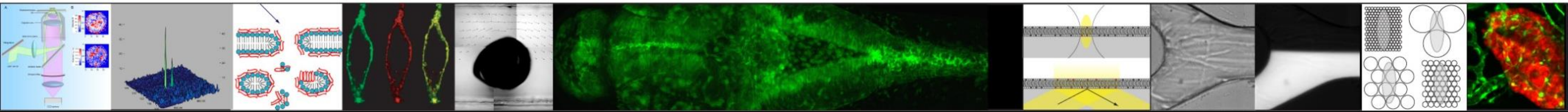
Biophysical Fluorescence Laboratory

National University of Singapore

Department of Chemistry



Fluorescence Correlation and Cross-Correlation Spectroscopy for the measurement of molecular dynamics and interactions

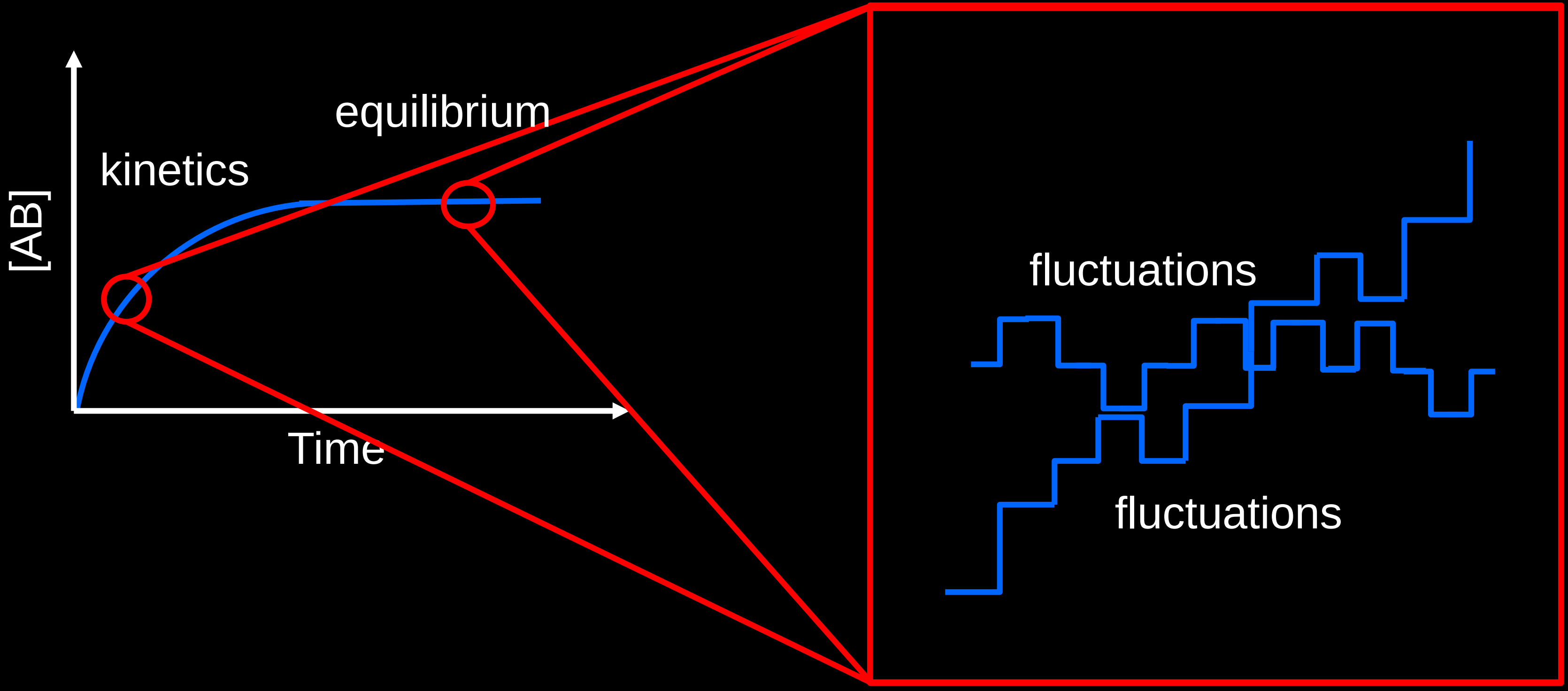
Thorsten Wohland

Outline

- Fluorescence Correlation Spectroscopy (FCS)
  - Introduction to basics of FCS
  - How to use amplitude, width and shape to obtain quantitative information
- Fluorescence Cross-Correlation Spectroscopy (FCCS)
  - Measurement of interactions and affinity constants (K_ds)
- FCS limitations and workarounds
- Imaging FCS
  - Motivation and principles
  - Example: Organization of Wnt3 in zebrafish membranes

Fluctuations

$$A + B \rightleftharpoons AB$$



Correlations

$$\langle a \cdot b \rangle \neq \langle a \rangle \langle b \rangle$$

$$g = \frac{\langle a \cdot b \rangle}{\langle a \rangle \langle b \rangle}$$

Anti-correlation $g < 1$

No correlation $g = 1$

Correlation $g > 1$

X. Shi and T. Wohland, "*Fluorescence Correlation Spectroscopy*", in Nanoscopy, CRC Press, 2010

Autocorrelations

$$\langle a(t)\cdot a(t)\rangle \geq \langle a(t)\rangle\langle a(t)\rangle$$

$$\langle a(t)\cdot a(t+\tau)\rangle \geq \langle a(t)\rangle\langle a(t+\tau)\rangle$$

$$G(\tau)=\frac{\langle a(t)\cdot a(t+\tau)\rangle}{\langle a(t)\rangle\langle a(t+\tau)\rangle}$$

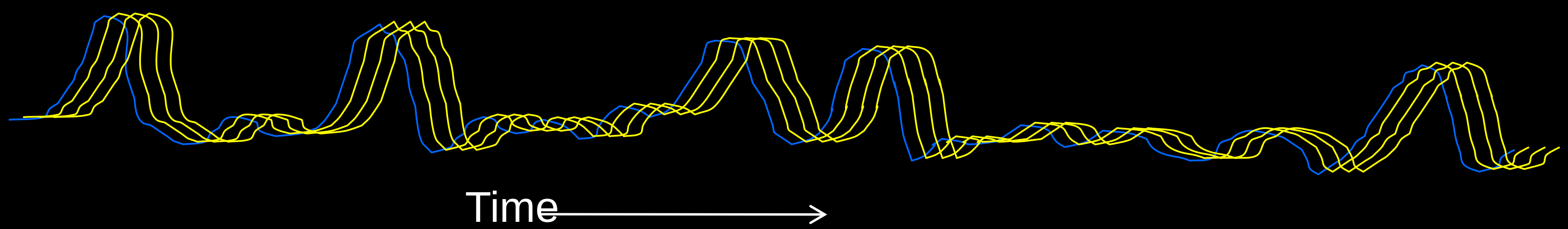
$$G(\tau)=\frac{\langle F(t+\tau)F(t)\rangle}{\langle F(t+\tau)\rangle\langle F(t)\rangle}=\frac{\langle F(t+\tau)F(t)\rangle}{\langle F(t)\rangle^2}$$

Stationary Processes

Short time shifts τ

$$\langle F(t)\cdot F(t+\tau)\rangle \,?\, \langle F(t)\rangle\langle F(t+\tau)\rangle$$

Blue: *F(t)*
Yellow: *F(t+τ)*



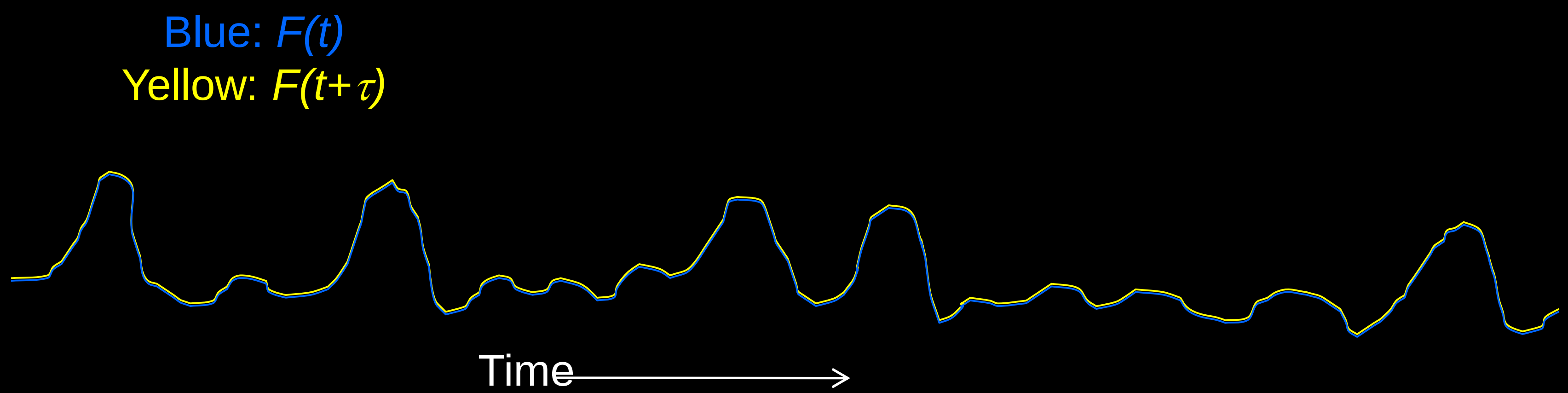
$$\langle F(t)\cdot F(t+\tau_3)\rangle$$

The intensity peaks always overlap to some extent and thus

$$\langle F(t)\cdot F(t+\tau)\rangle \geq \langle F(t)\rangle\langle F(t+\tau)\rangle$$

Long time shifts τ

$$\langle F(t)\cdot F(t+\tau)\rangle ? \langle F(t)\rangle\langle F(t+\tau)\rangle$$



$$\langle F(t)\cdot F(t+\tau_3)\rangle$$

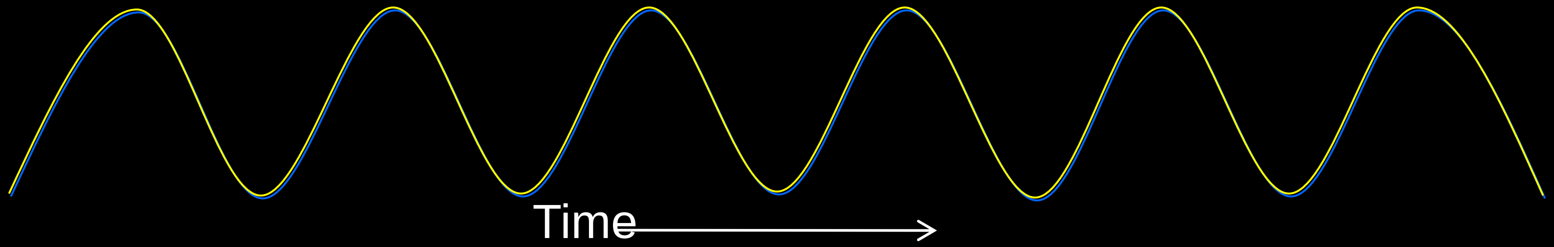
The intensity trace contains a random pattern of intensity peaks. Therefore an overlap of all/many peaks is only achievable at short times.

$$\langle F(t)\cdot F(t+\tau)\rangle = \langle F(t)\rangle\langle F(t+\tau)\rangle$$

Periodic signals

$$\langle F(t)\cdot F(t+\tau)\rangle ? \langle F(t)\rangle\langle F(t+\tau)\rangle$$

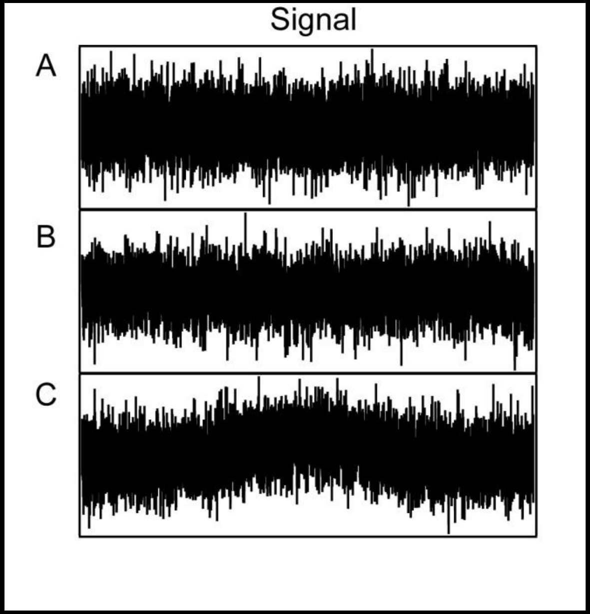
Blue: *F(t)*
Yellow: *F(t+τ)*



$$\langle F(t)\cdot F(t+\tau_3)\rangle$$

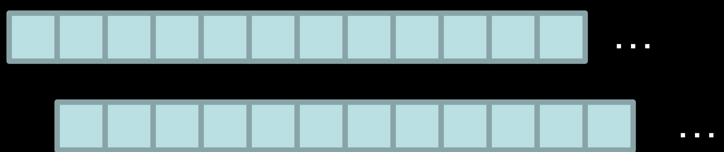
The intensity trace contains a regular pattern of intensity peaks (i.e. it is repeated). Therefore an overlap of all/many peaks is achievable periodically and the correlation function will show that periodicity.

ACF: Autocorrelation Function (the correlation of a variable with itself)

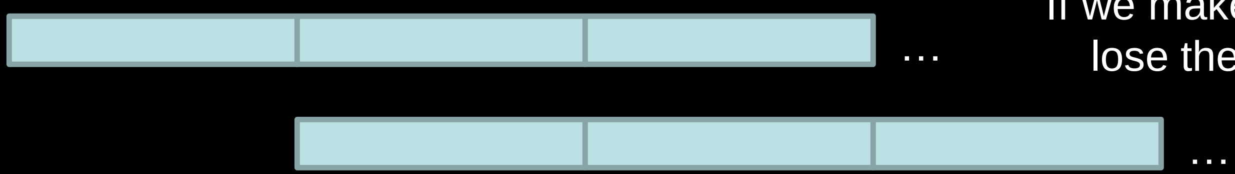


How is an ACF calculated practically?

Intensity values recorded every nanosecond



To calculate the correlation for the range of seconds you would need 1 billion values …



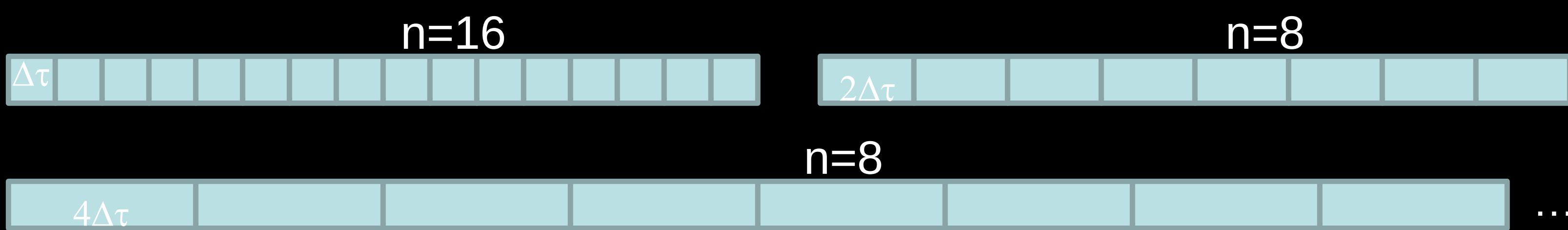
If we make the time bins larger then we lose the information at short times.

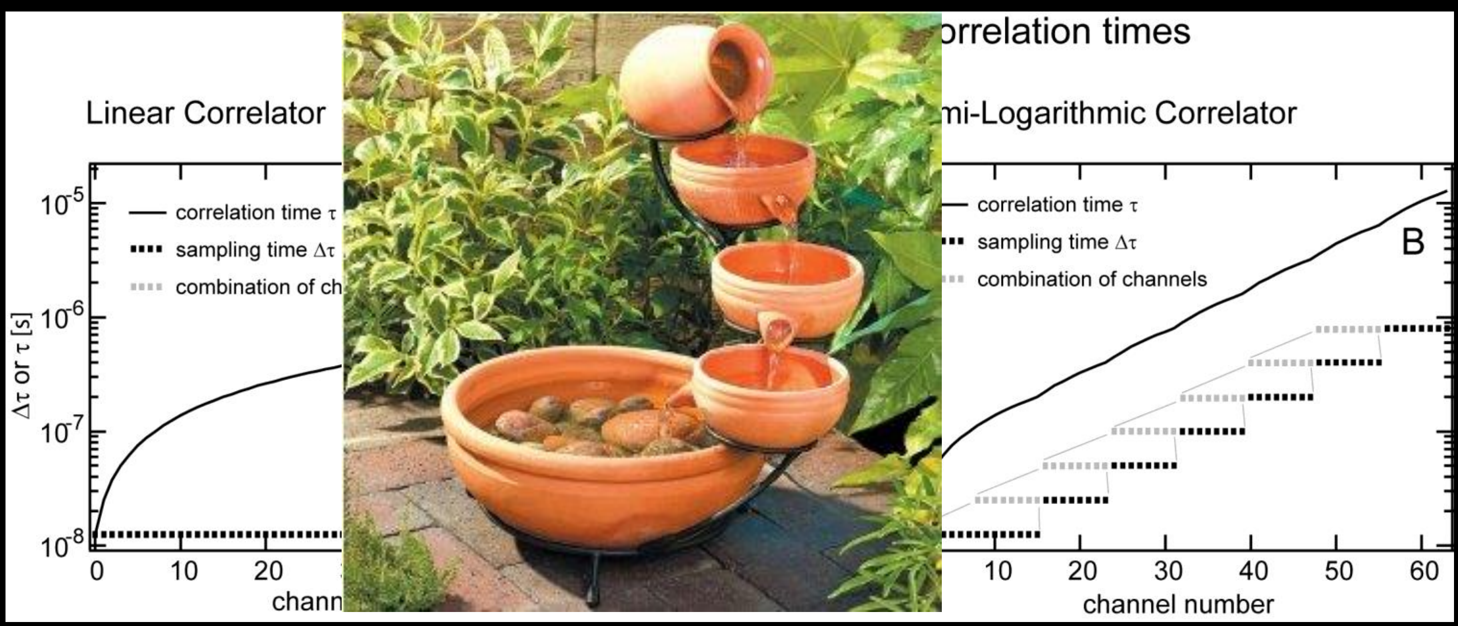
So best would be to use a varying time scheme.

T. Wohland, R.Rigler, H. Vogel, Biophys. J. 2001, 80(6), 2987-2999.

Correlation Time Schemes

The tyipical scheme used is called the semi-logarithmic time scale. The first n channels have a time $\Delta\tau$. The second group contains n/2 channels with 2 $\Delta\tau$. The next group n/2 channels with 4 $\Delta\tau$.





Correlation Time Schemes

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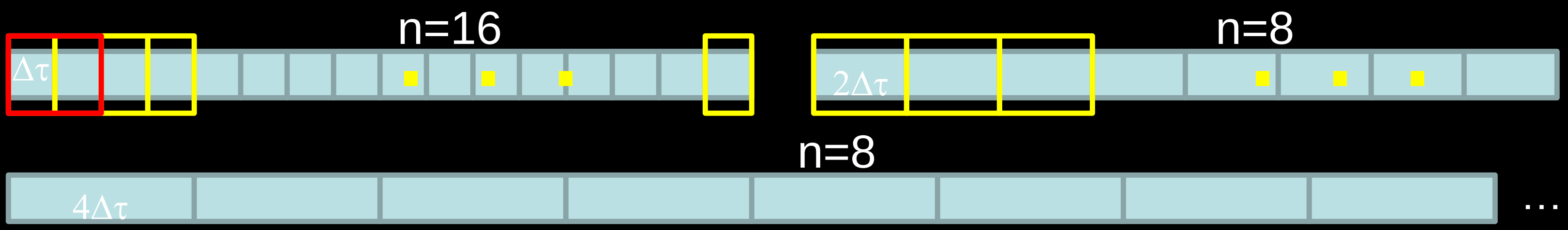
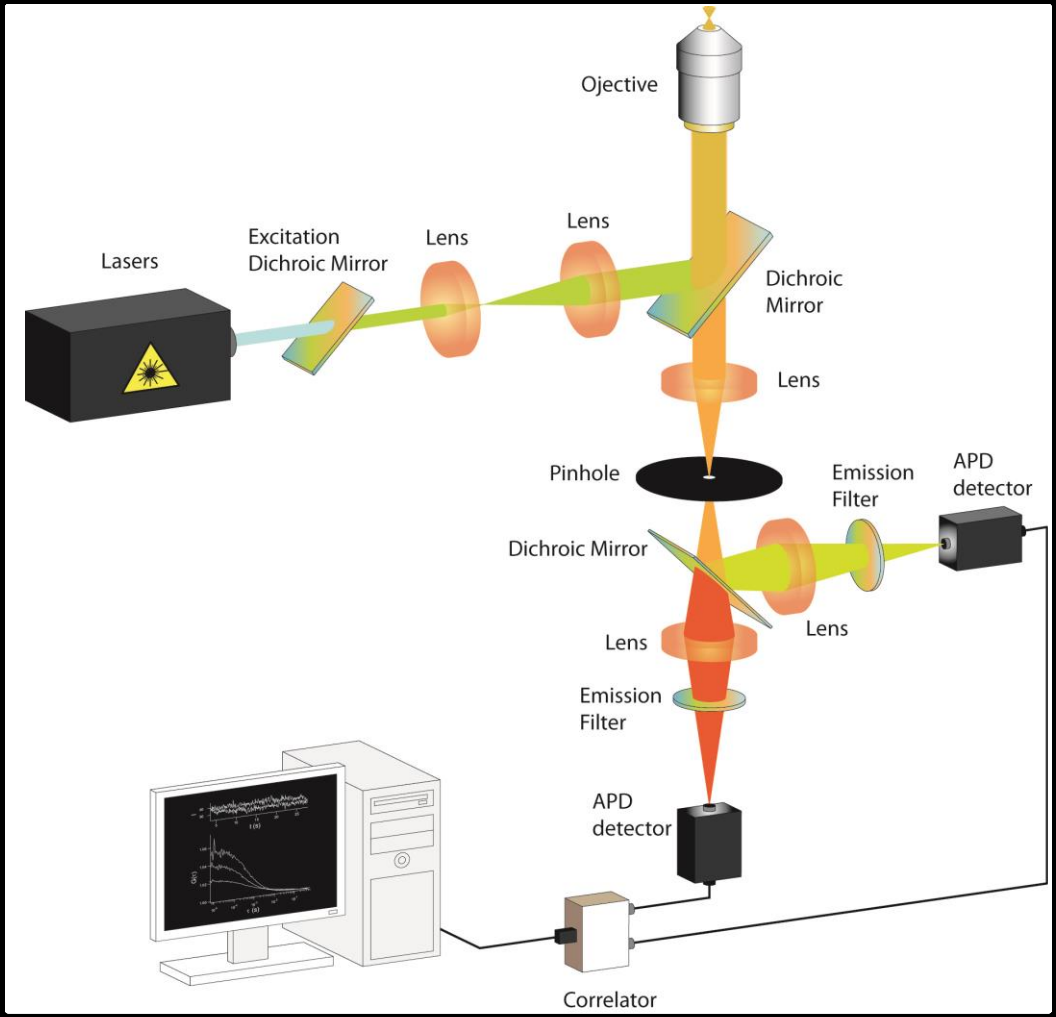
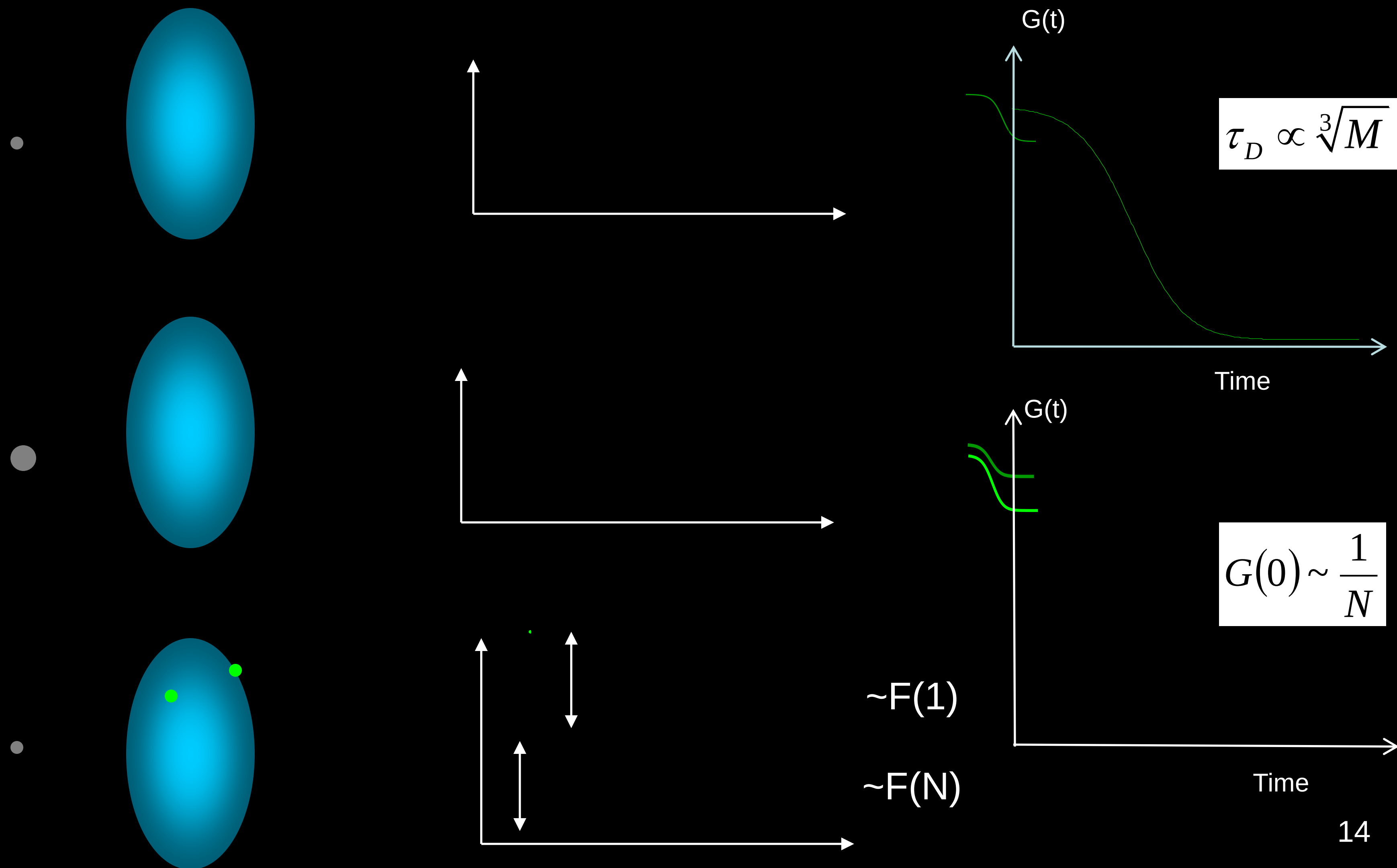


1) Each time a new measurement of length $\Delta\tau$ comes in, calculate all ACF values for lag times 0 to $16\Delta\tau$.
2) After 2 measurements of $\Delta\tau$, correlate the last two newest measurements with all channels in group 2. Then take the last two channels of group 1 and combine them into one channel with width $2\Delta\tau$ of group 2 and shift.

Confocal FCS setup

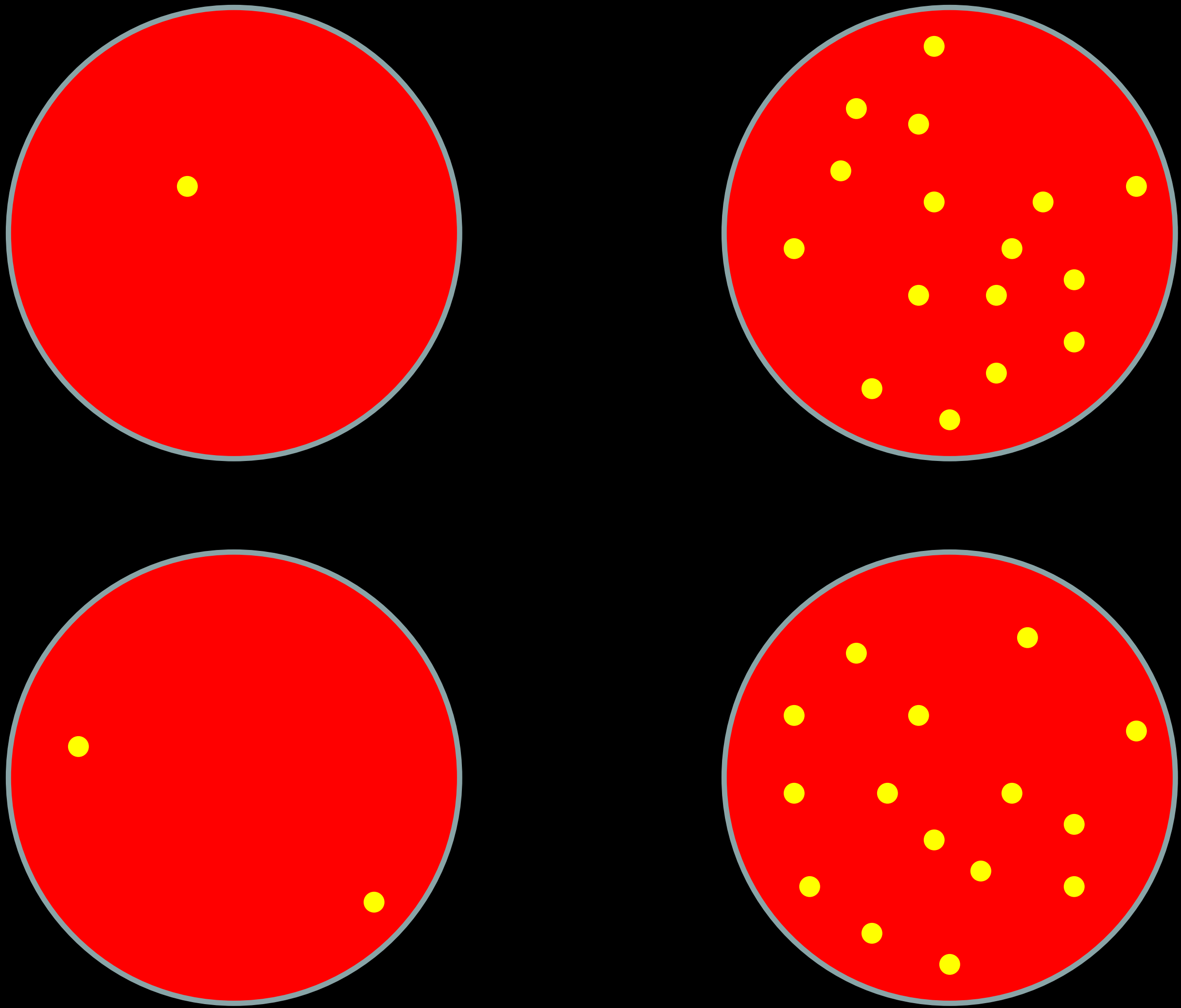


FCS: Characteristic Parameters



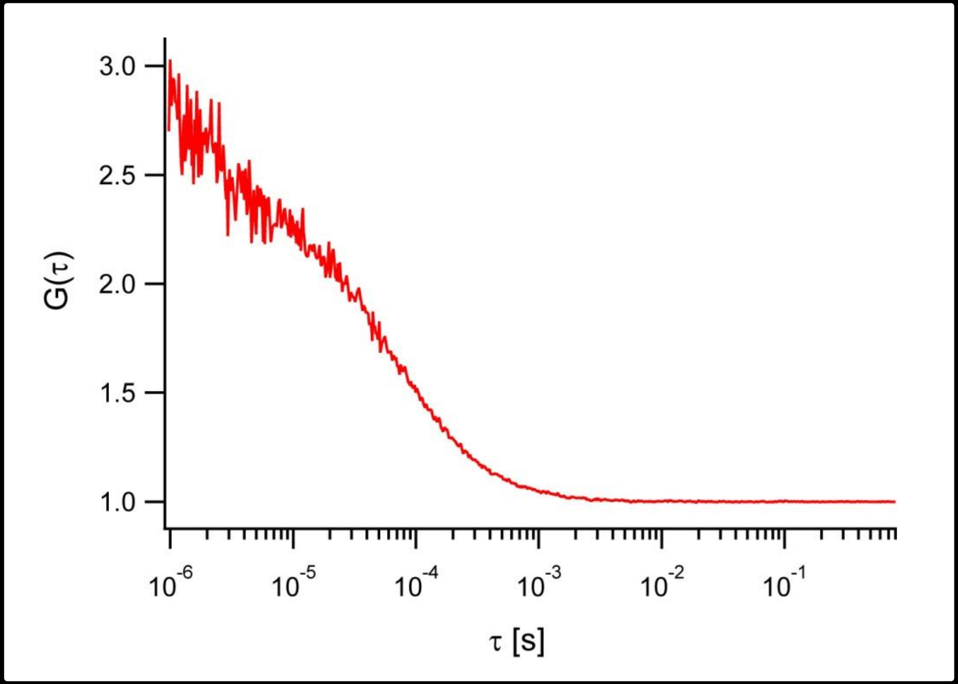
$$\tau_D \propto \sqrt[3]{M}$$

$$G(0) \sim \frac{1}{N}$$



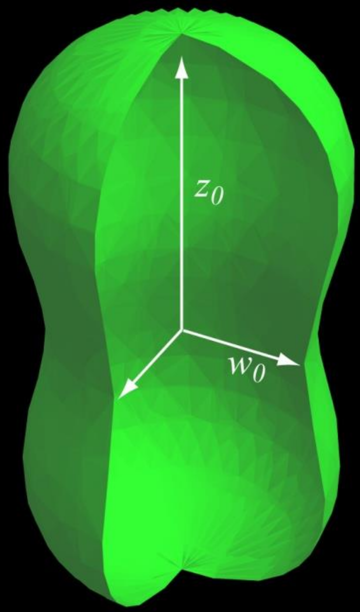
Correlation Functions

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



Correlation Functions

$$G(\tau) = \frac{1}{\langle C \rangle \pi^{3/2} w_0^2 z_0} \left(1 + \frac{4D\tau}{w_0^2}\right)^{-1/2} \left(1 + \frac{4D\tau}{w_0^2}\right)^{-1/2} \left(1 + \frac{4D\tau}{z_0^2}\right)^{-1/2} + 1$$

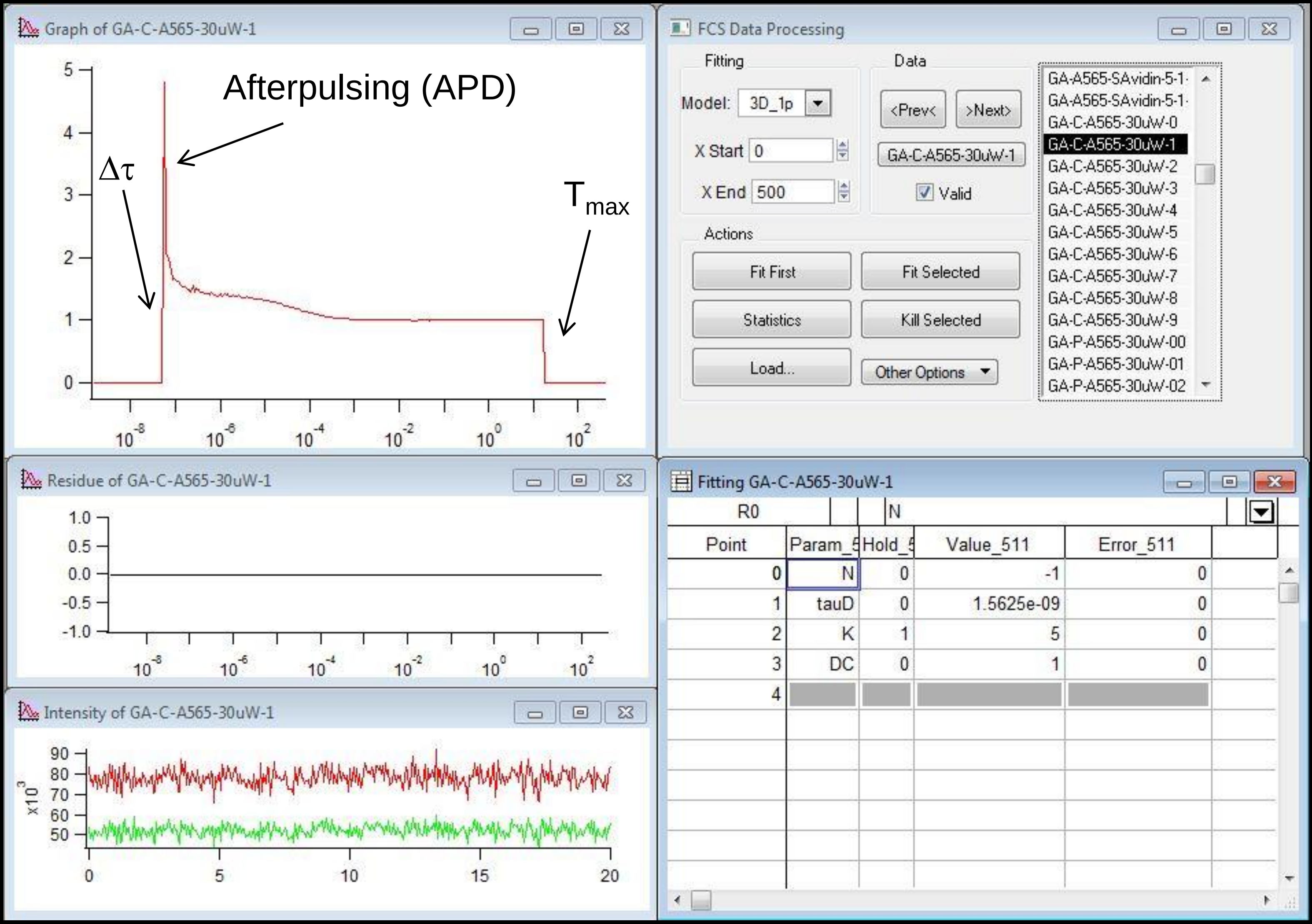


$z_0 = K w_0$

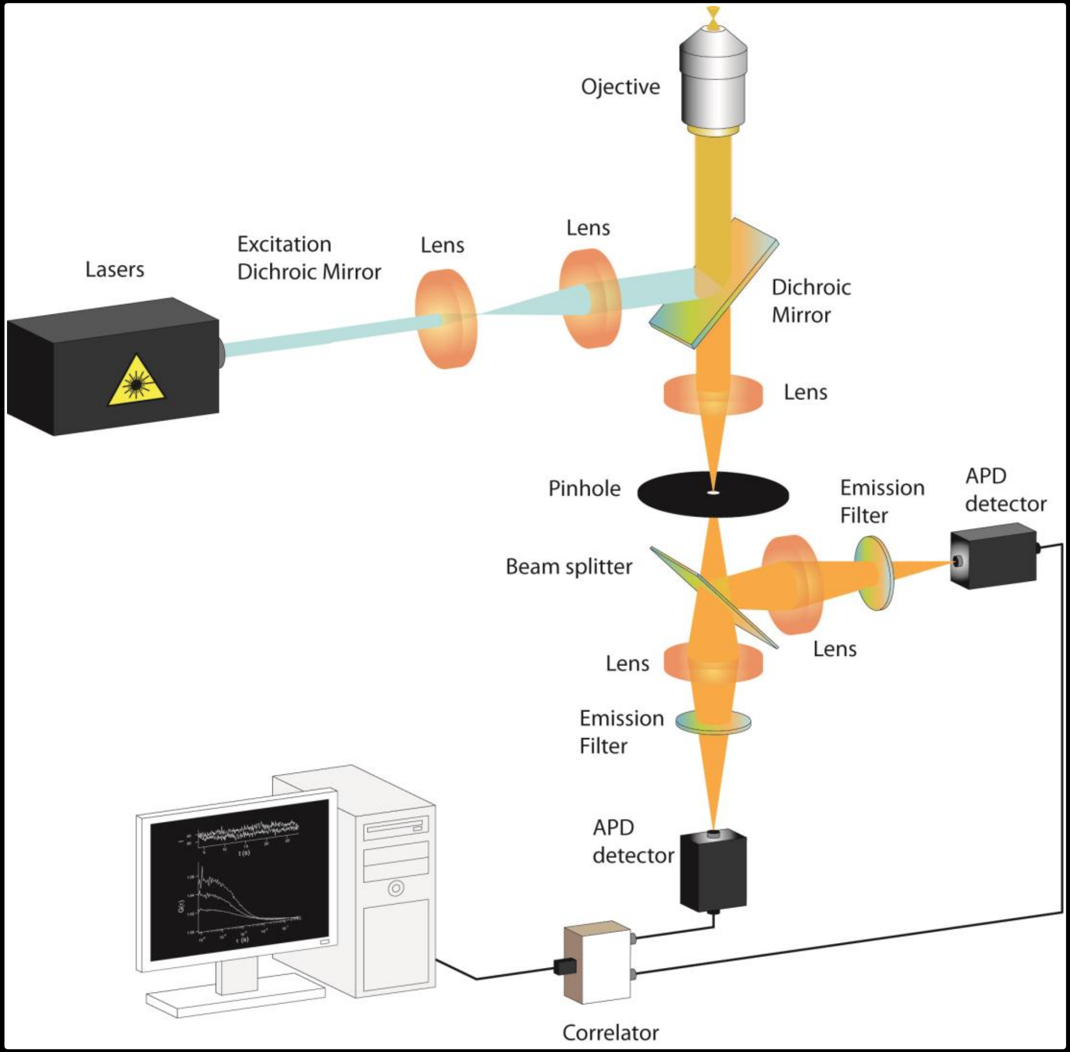
Number of particles	$N = \langle C \rangle V_{eff} = \langle C \rangle \pi^{3/2} w_0^2 z_0$
Correlation time	$\tau_D = \frac{w_0^2}{4D}$
Structure factor	$K = \frac{z_0}{w_0}$

$$G(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{K^2 \tau_D}\right)^{-1/2} + G_\infty$$

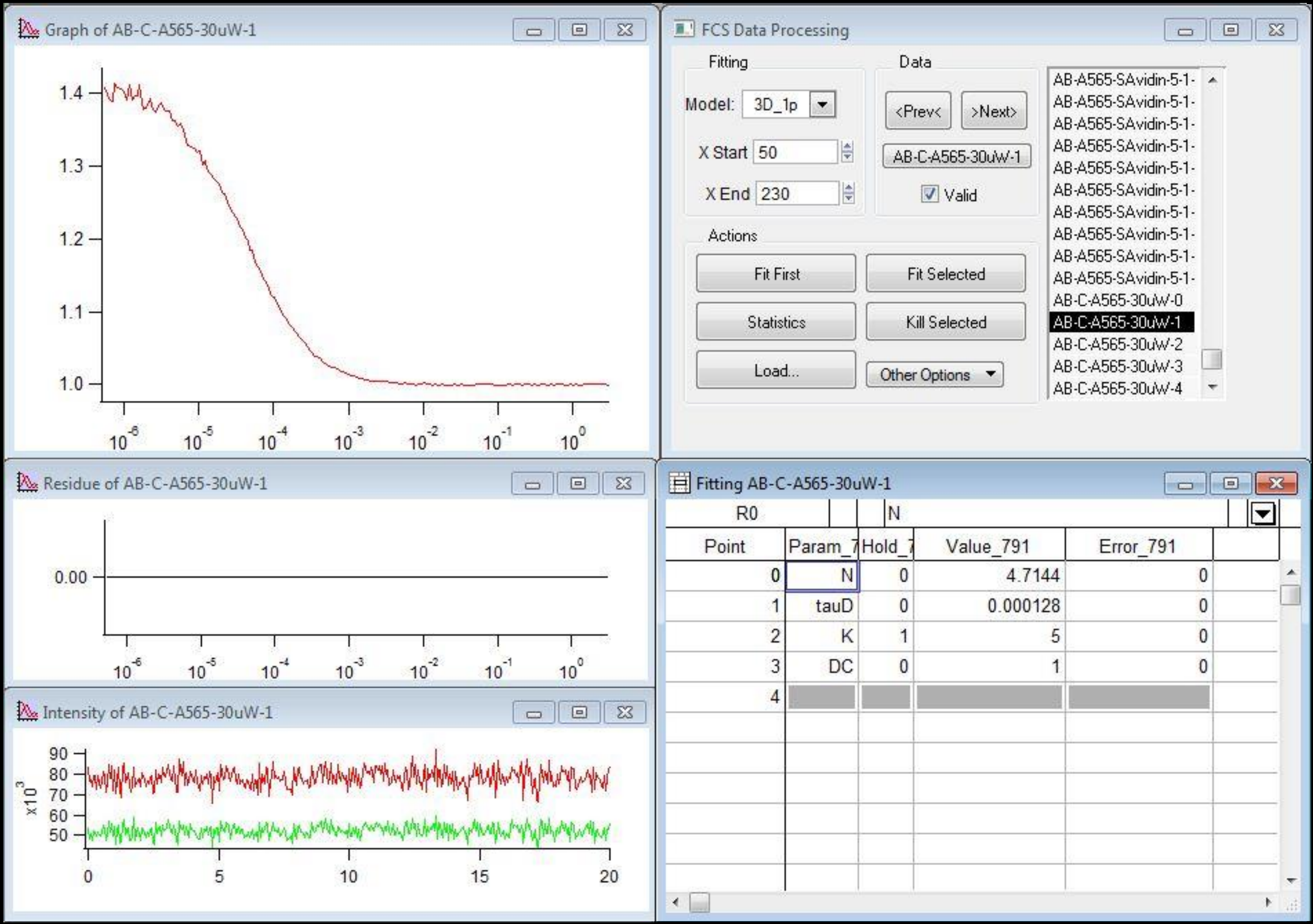
Data Fitting: Raw data



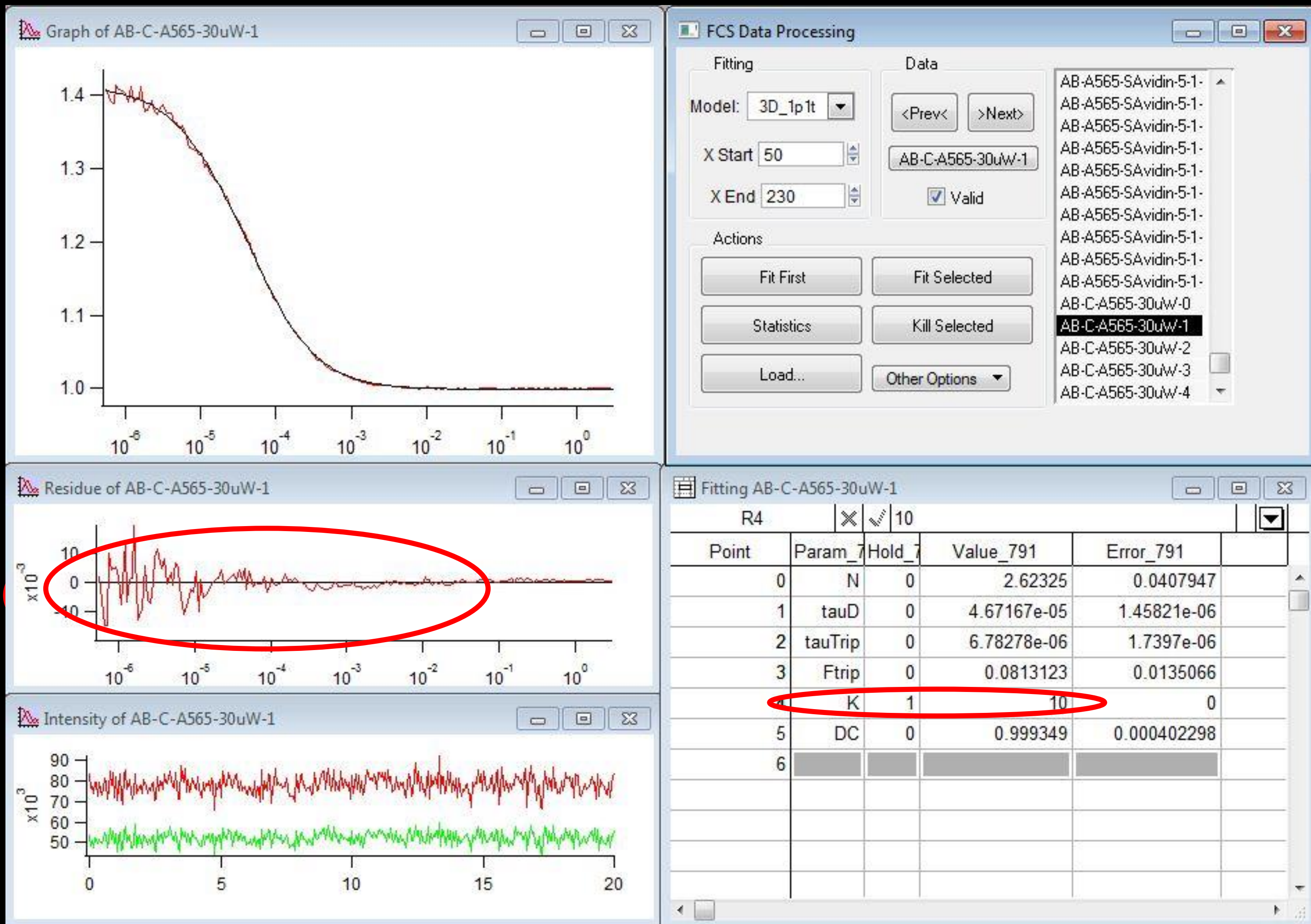
Removal of afterpulsing by cross-correlation



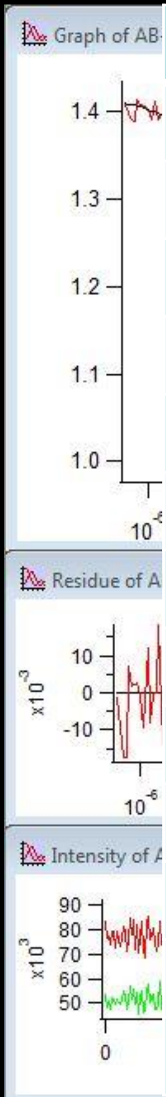
Data Fitting: Raw data



Data Fitting



Weighted data fits



	non-weighted fit	weighted fit
N	2.601	2.591
τ_D	4.781e-05	4.739e-05
τ_{Trip}	4.992e-06	4.406e-06
F_{Trip}	1.010e-01	8.187e-02
K	6.089	5.843
G_∞	1.000	1.000
	Standard deviation	
N	9.377e-02 (3.6 %)	6.294e-02 (2.4 %)
τ_D	4.254e-06 (8.9 %)	2.699e-06 (5.7 %)
τ_{Trip}	4.128e-06 (83 %)	2.439e-06 (55 %)
F_{Trip}	3.780e-02 (37 %)	1.853e-02 (23 %)
K	1.940 (32 %)	1.219 (21 %)
G_∞	8.698e-05	5.586e-05

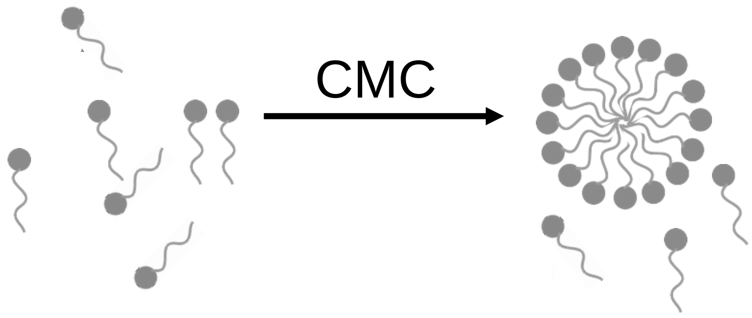
Measured over 10 experiments

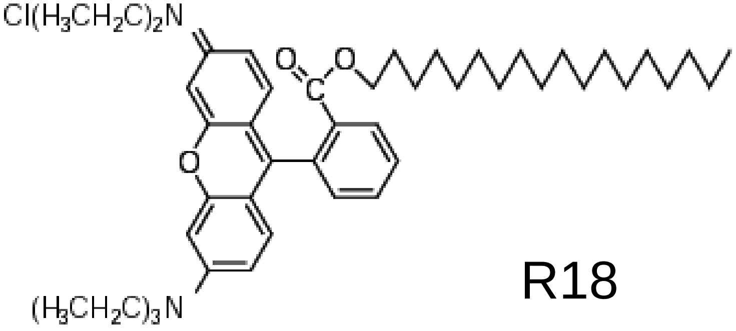
How to use the FCS amplitude

Aggregation numbers of detergent/lipid micelles

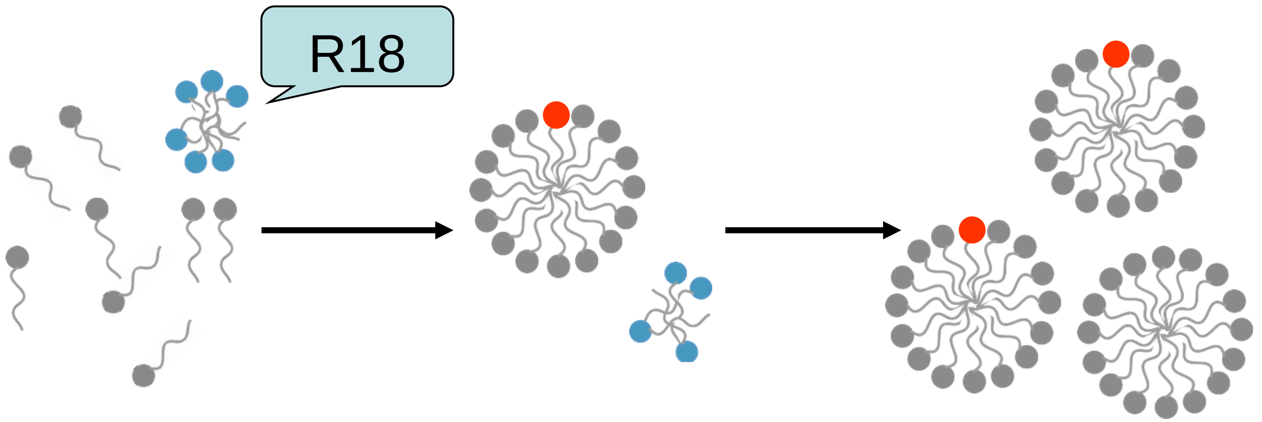
Determination of the aggregation of detergent and LPS

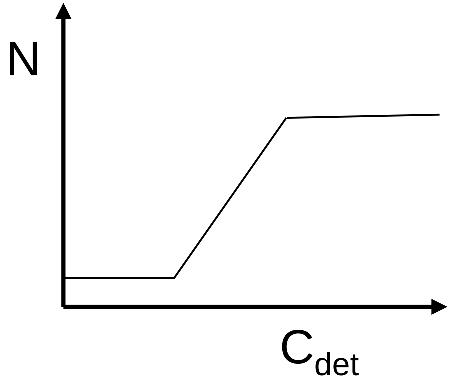
Micelle Formation



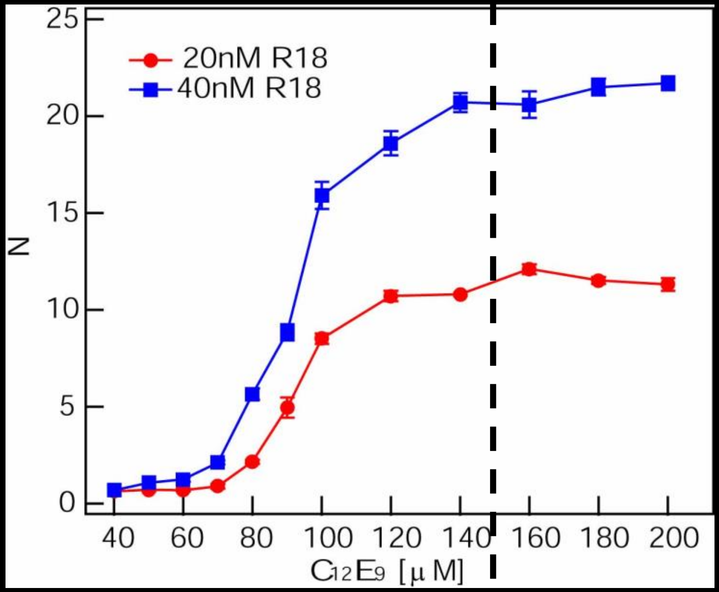


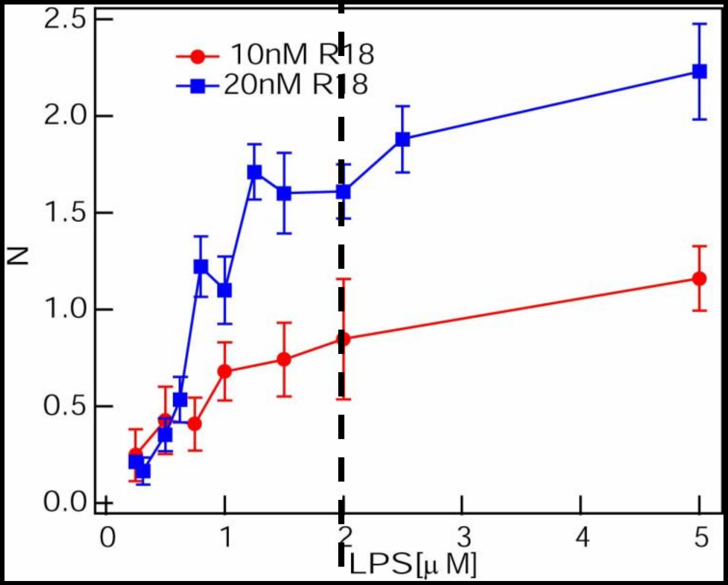
R18 non-flu oligomers -> Micelle formation->dissolution of R18 oligomers and incorporation into micelles with fluorescence increase





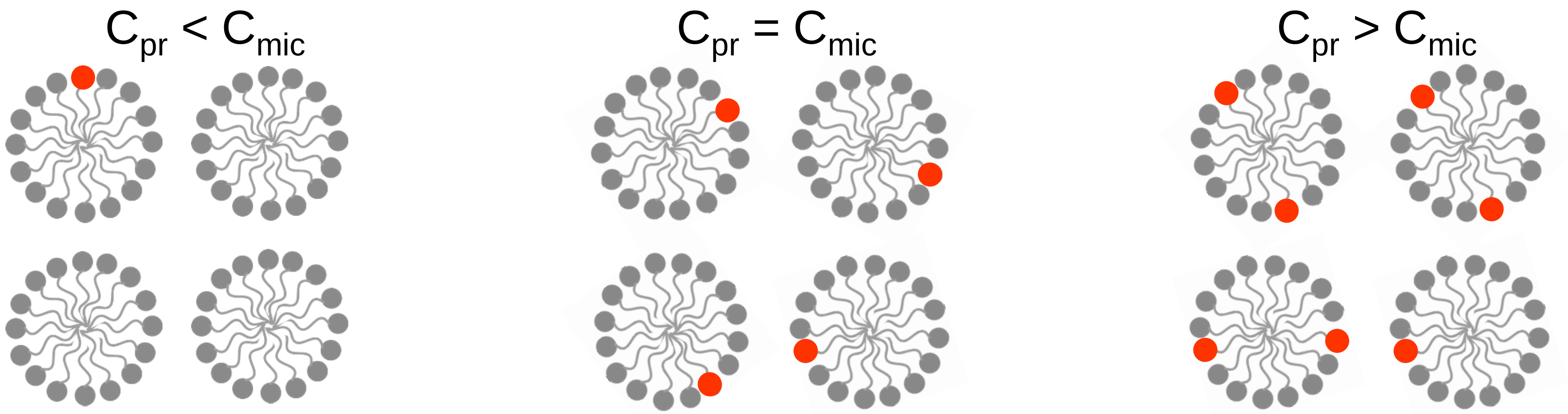
Determination of the aggregation of detergent and LPS

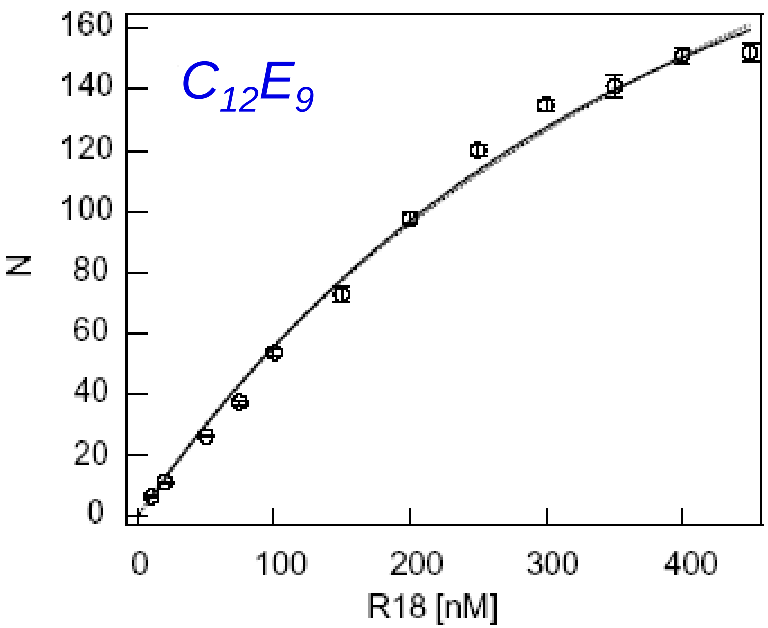


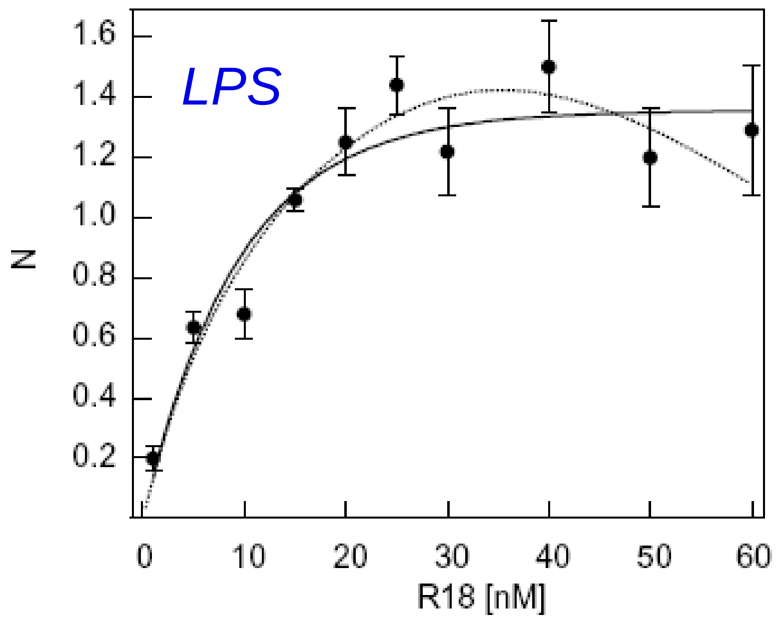


Yu et al., Analytica Chimica Acta 556 (2006) 216–225

Determination of the aggregation of detergent and LPS







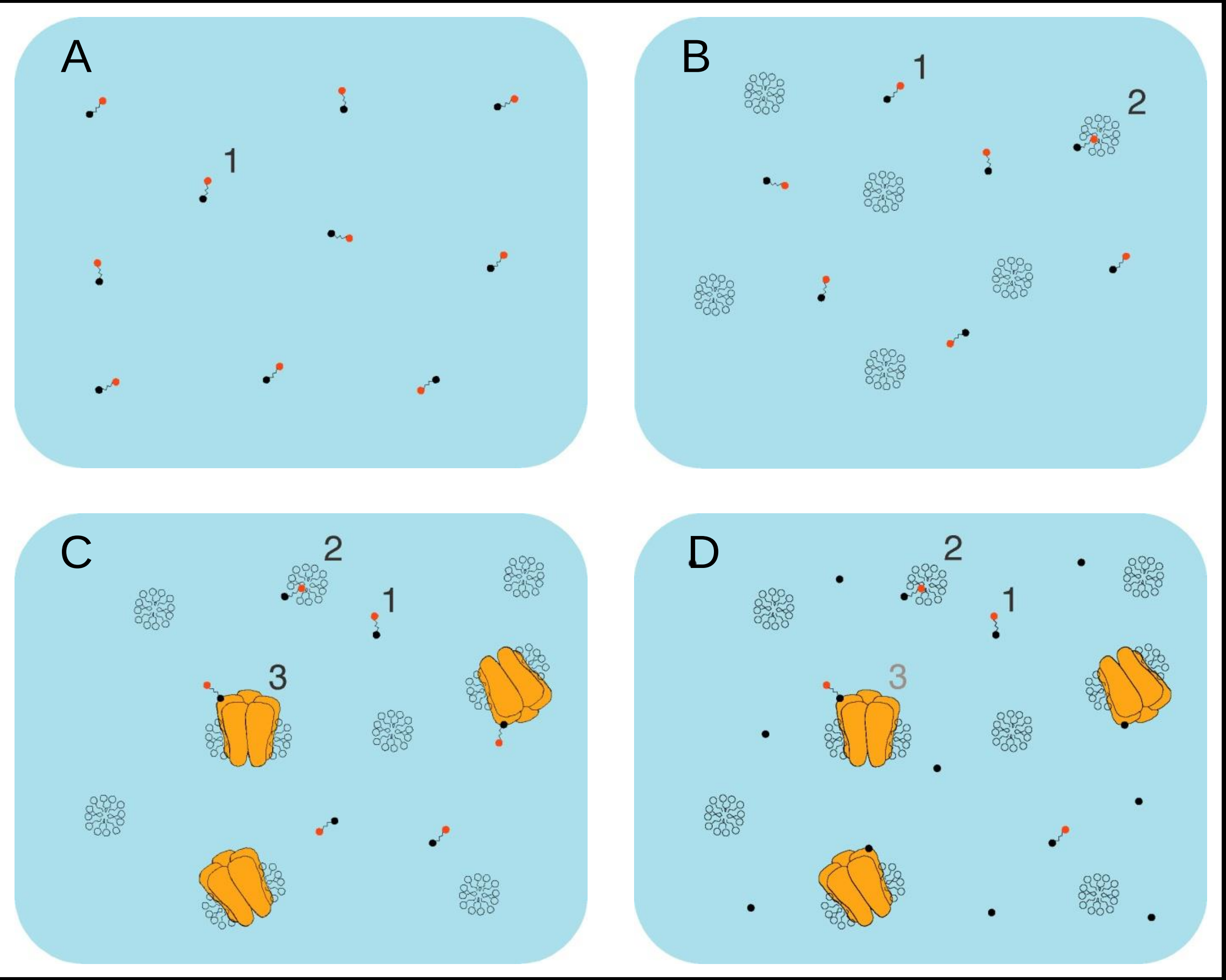
c_{cmc}=93-105 μM; N_{agg}=112-132
(c_{cmc}=80 μM; N_{agg}=120)

c_{cmc}=1.3-1.6 μM; N_{agg}=43-49

How to use the FCS width

Ligand affinities for the $5HT_3$ receptor

Measurements in Solution



Parameters: correlation times (τ_1, τ_2, τ_3),

fraction of particles (Y_1, Y_2, Y_3)

A: Ligand in Buffer solution

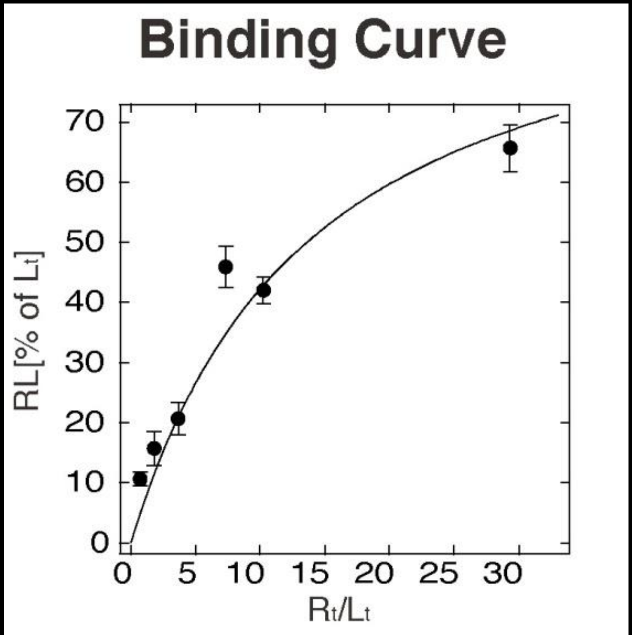
B: Ligand + Detergent

C: Ligand+Detergent +Receptor

D: Ligand+Detergent+ Receptor +Competitor

Ligand-Receptor Interactions

Ligands: 0.5 – 1.1 kDa	425 $\mu m^2/s$
$C_{12}E_9$ micelle: 60 - 70 kDa	73 $\mu m^2/s$
$5HT_{3As}$-R + micelle: ~320 kDa	35 $\mu m^2/s$



$$K_d^{FCS} = 15.7 \pm 8.0 nM$$

$$K_d^{RBA} = 18.0 \pm 2.0 nM$$

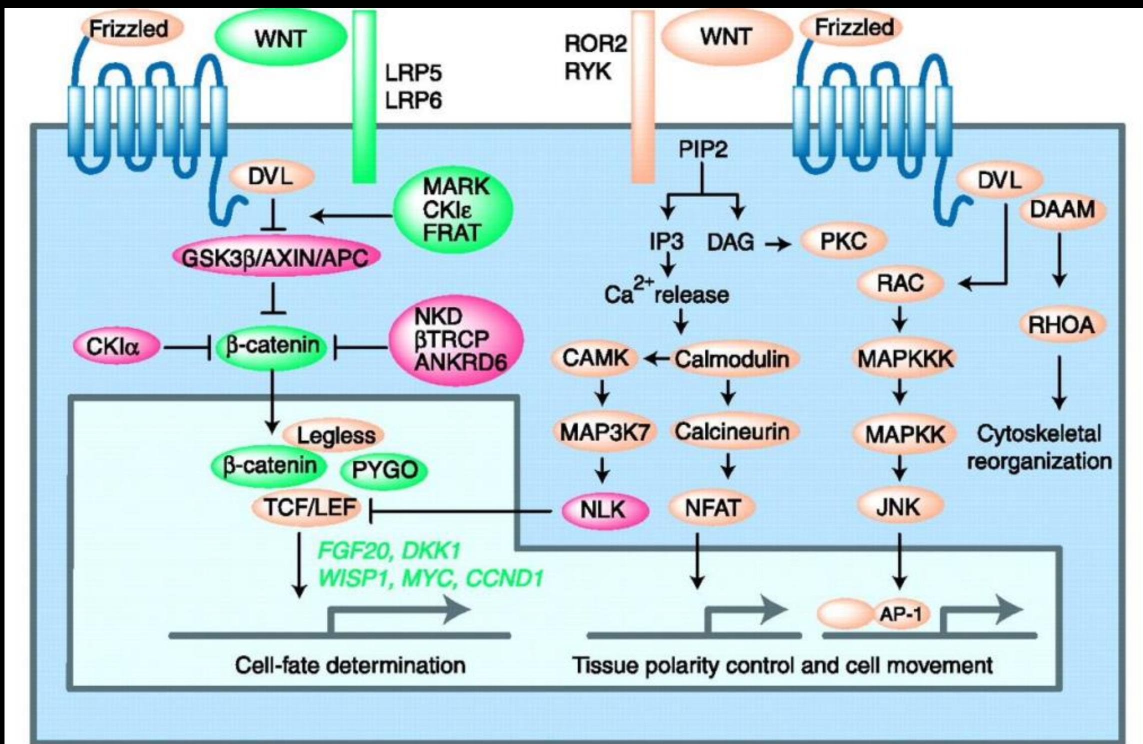
How to use the FCS shape

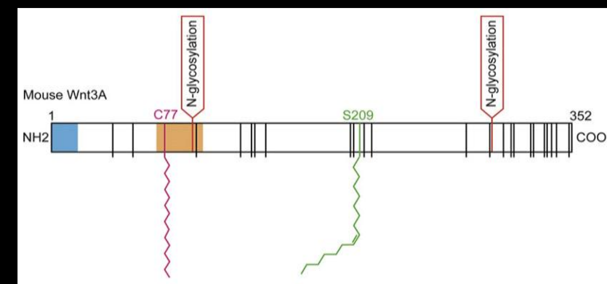
Determination of morphogen secretion in live zebrafish

Wnt Signaling

Canonical Wnt Pathway
Wnt/β-catenin signaling

Non-Canonical Wnt Pathway
β-catenin independent Wnt signaling

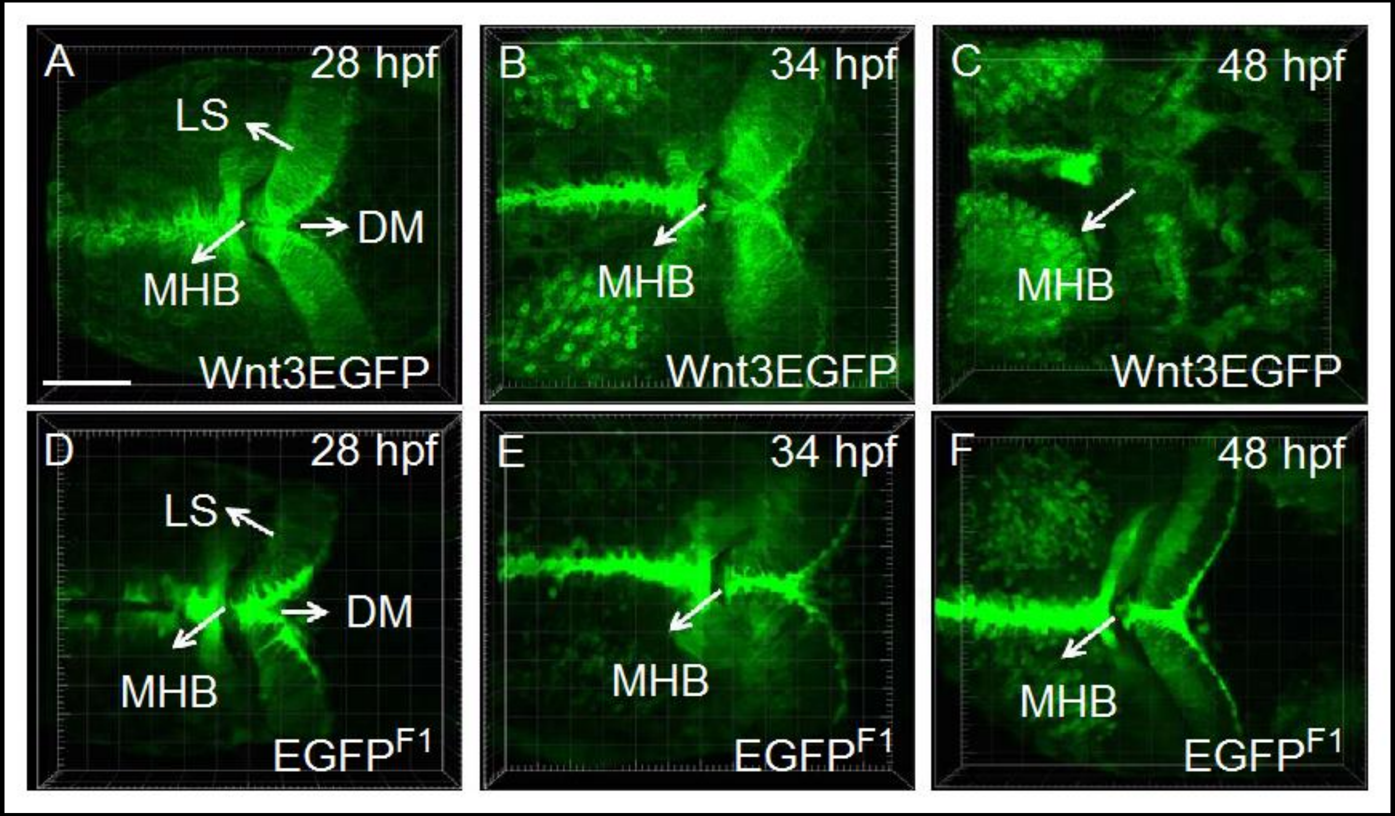




1. Is Wnt3 secreted?
2. Where in the membrane does Wnt3 reside?

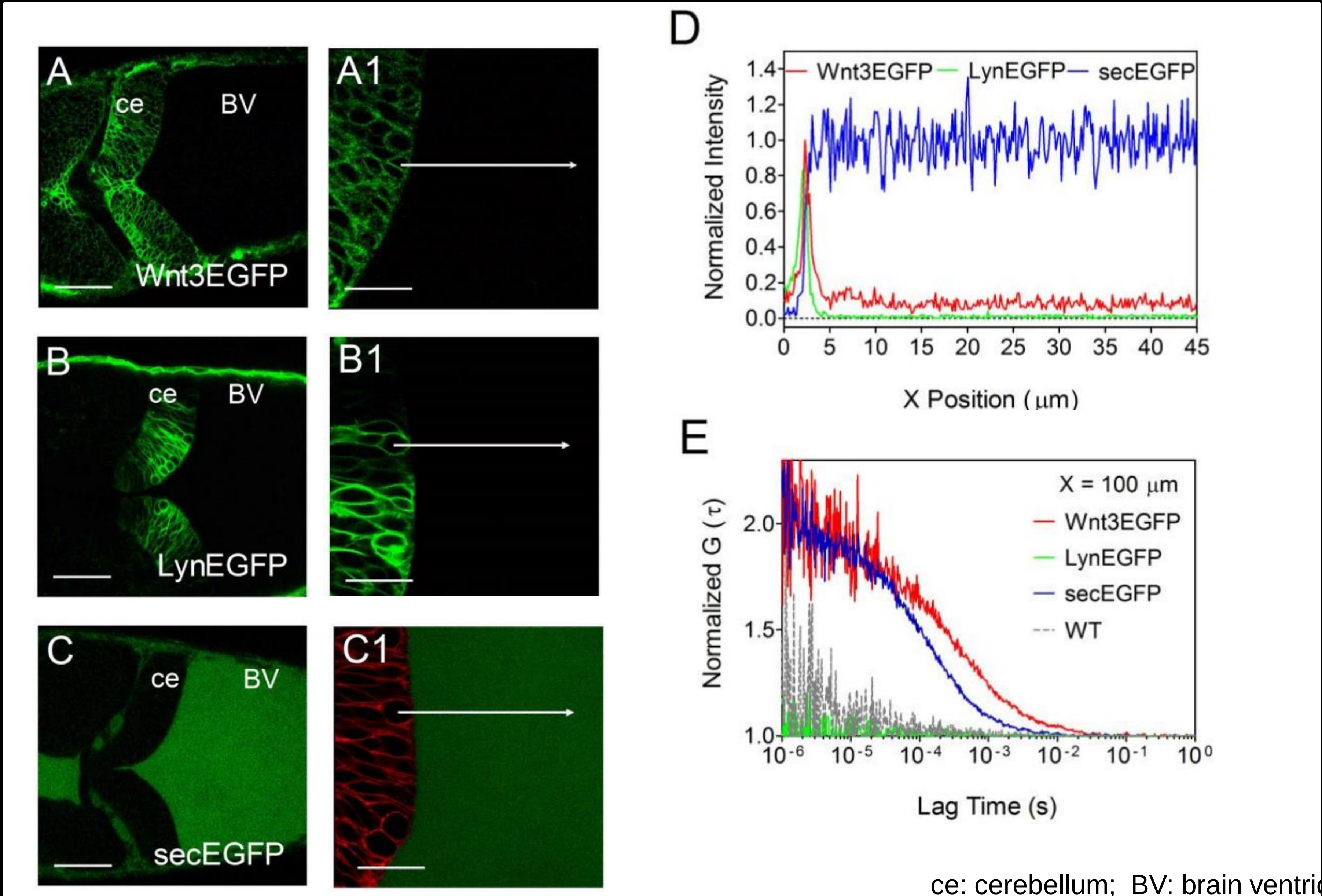
Katoh, M. *Clinical Cancer Research* **2007,** *13*, 4042-4045.

Wnt3EGFP Expression in the Cerebellum



ce: cerebellum; ot: optic tectum; MHB: midbrain hindbrain boundary; BV: brain ventricle; DM: dorsal midline; LS: lateral side.

Wnt3EGFP Secretion to the Brain Ventricle



ce: cerebellum; BV: brain ventric

Bayesian Model Selection

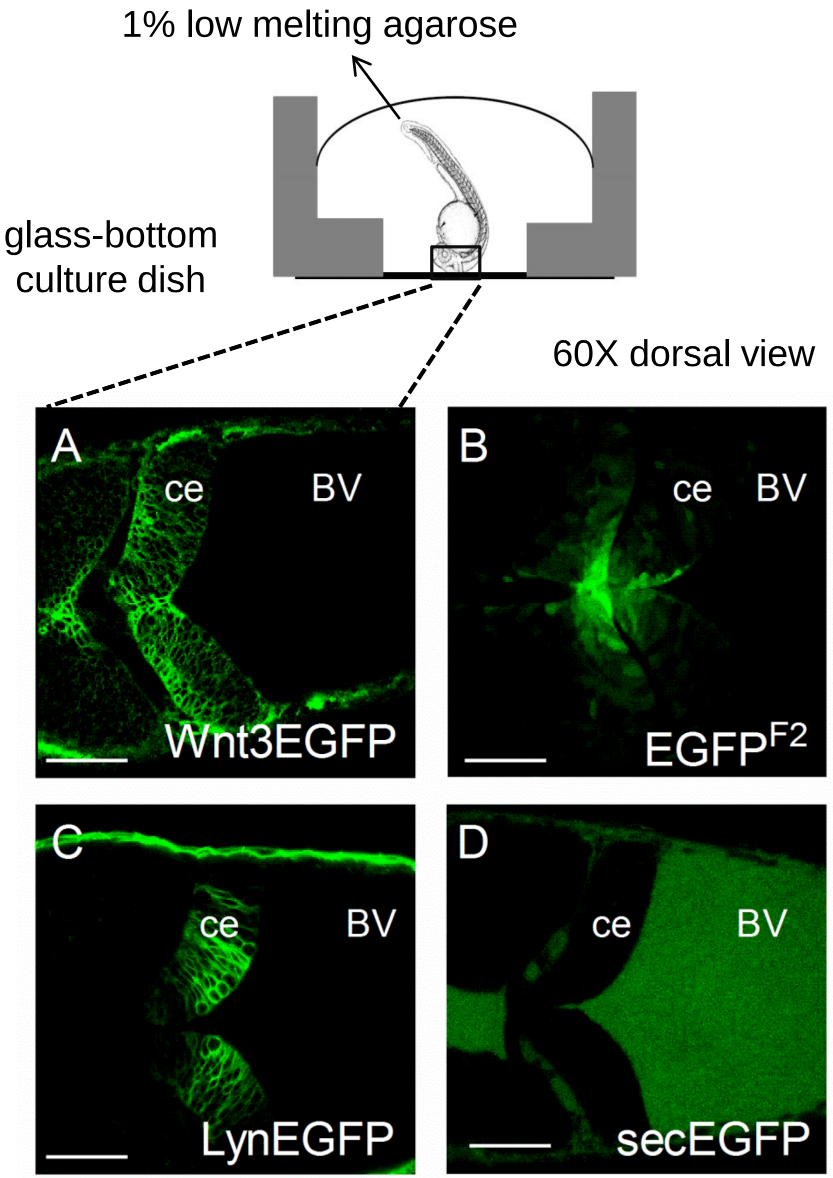
- Bayes' Theorem

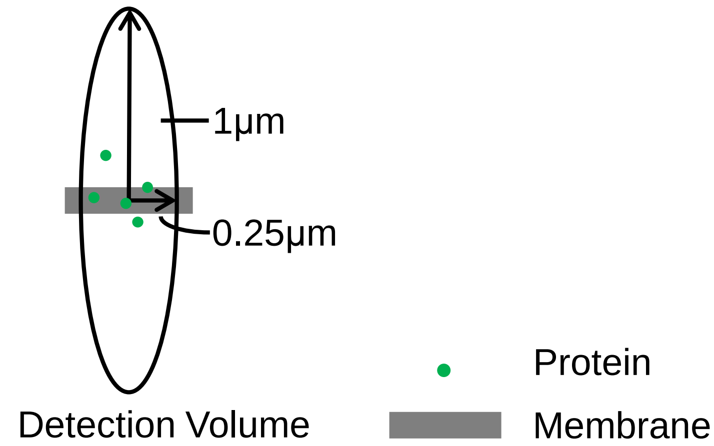
$$P(M_k|\mathbf{y}) = \frac{P(\mathbf{y}|M_k)P(M_k)}{P(\mathbf{y})}$$

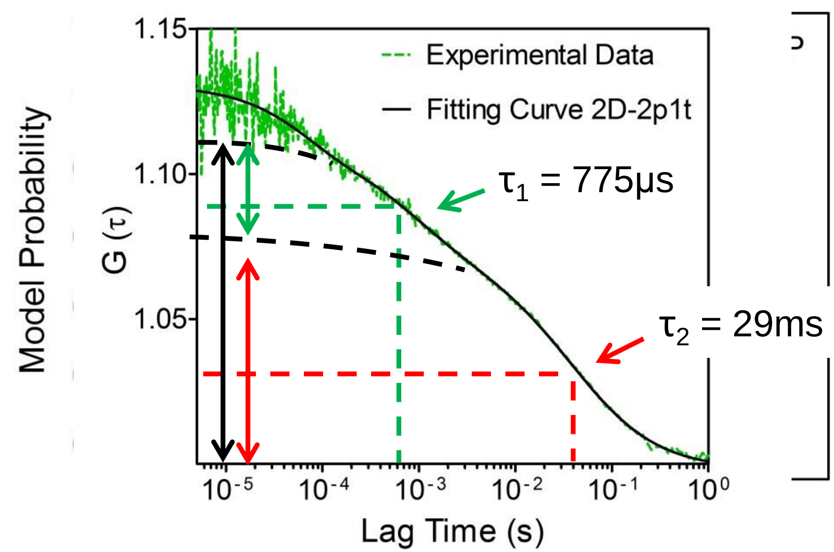
$$P(\mathbf{y}|M_k) = \int_{\beta} P(\mathbf{y}|\boldsymbol{\beta}, M_k)P(\boldsymbol{\beta}|M_k)\mathrm{d}\boldsymbol{\beta}$$

$$P(\mathbf{y}|\boldsymbol{\beta}) = \frac{1}{(2\pi)^{n/2}\sqrt{\det(\mathbf{C})}}\exp\left\{-\frac{1}{2}[\mathbf{y} - \mathbf{f}(\mathbf{x}, \boldsymbol{\beta})]^{\mathrm{T}}\mathbf{C}^{-1} \times [\mathbf{y} - \mathbf{f}(\mathbf{x}, \boldsymbol{\beta})]\right\}$$

Zebrafish FCS measurements



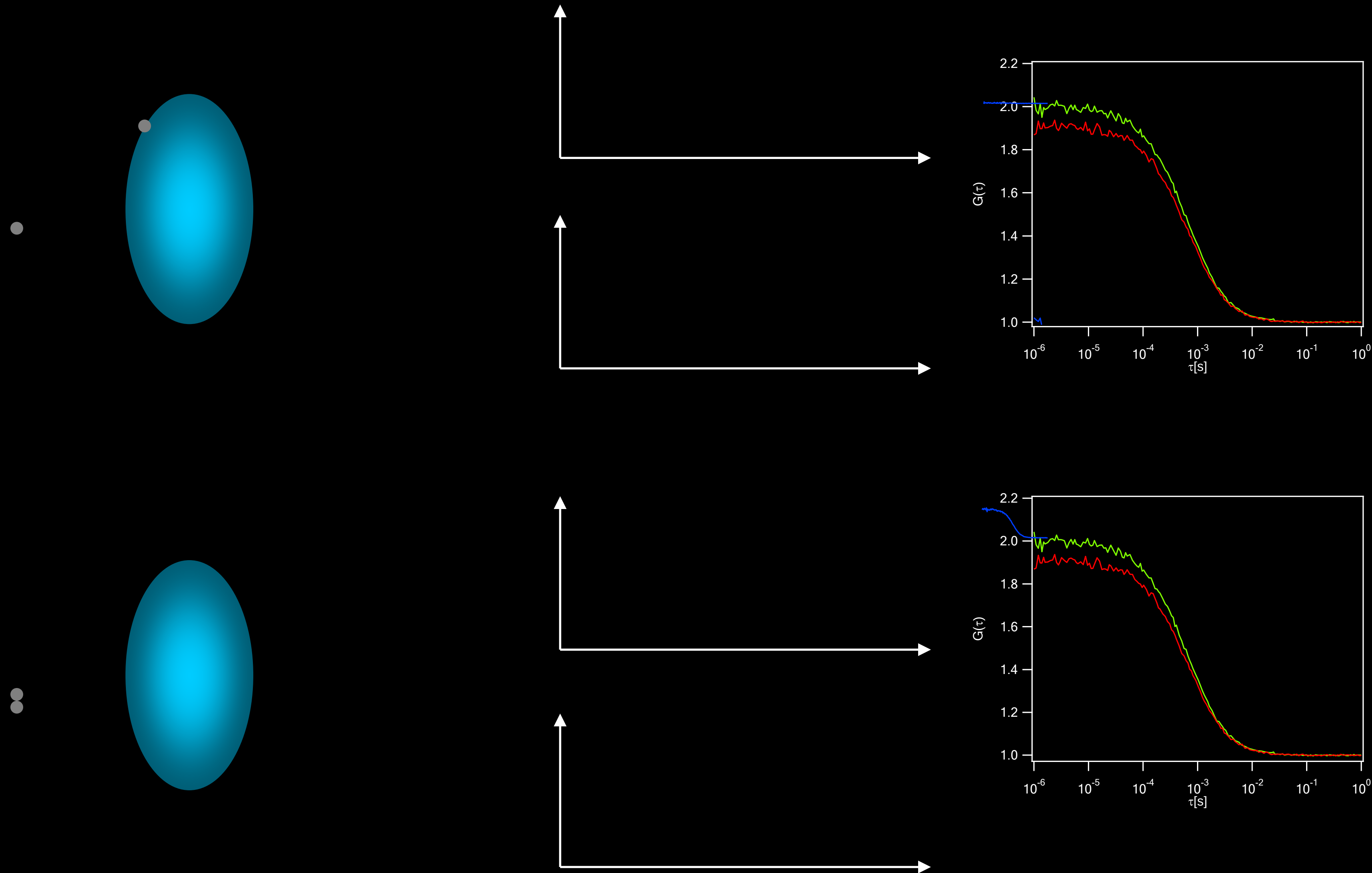




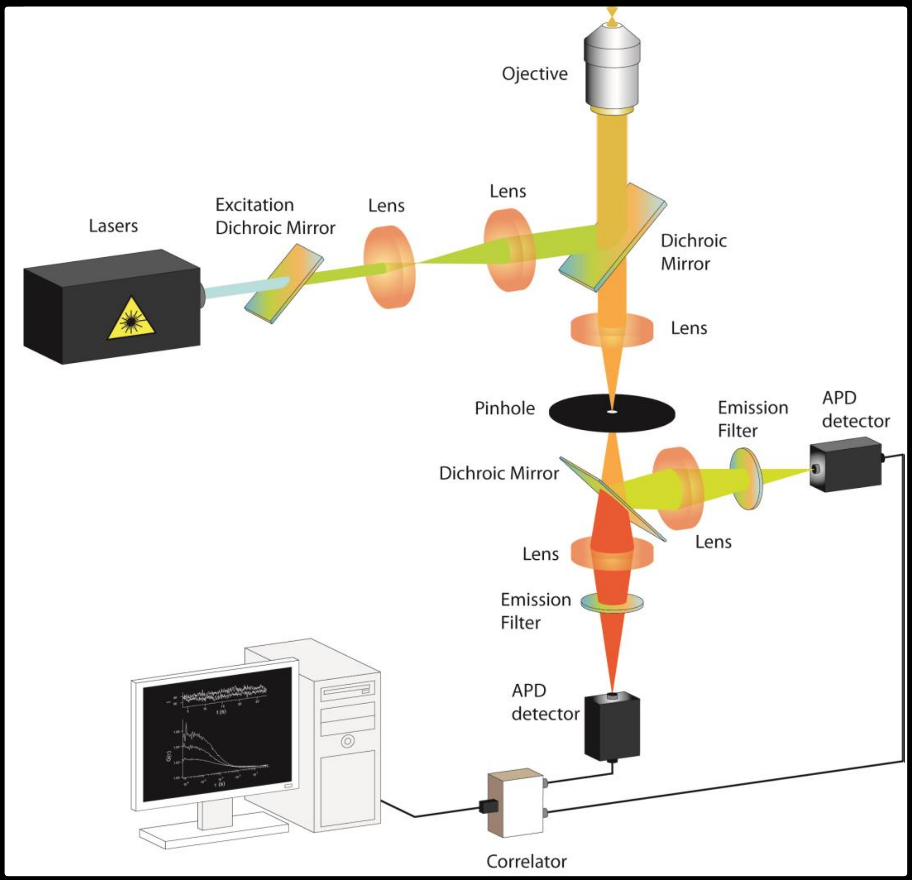
ce: cerebellum; BV: brain ventricle

Fluorescence Cross-Correlation Spectroscopy

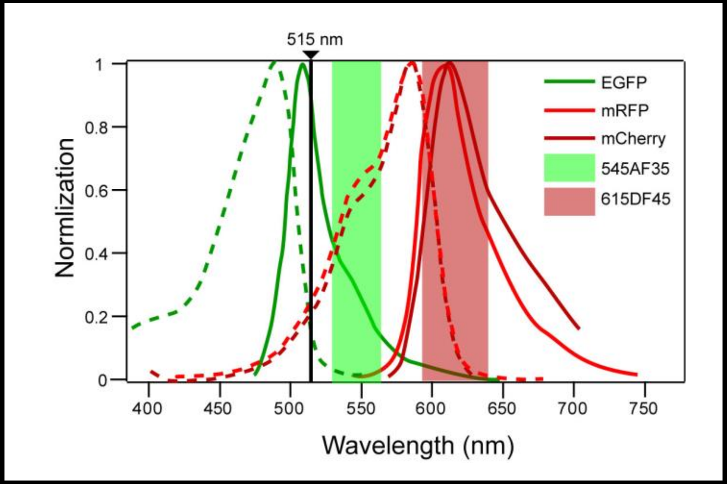
Fluorescence Cross-correlation Spectroscopy (FCCS)



SW-FCCS



Fluorophores:
Quantum dots
Tandem dyes (energy transfer dyes)
Organic dyes
Fluorescent proteins



~2000 counts per second and particle

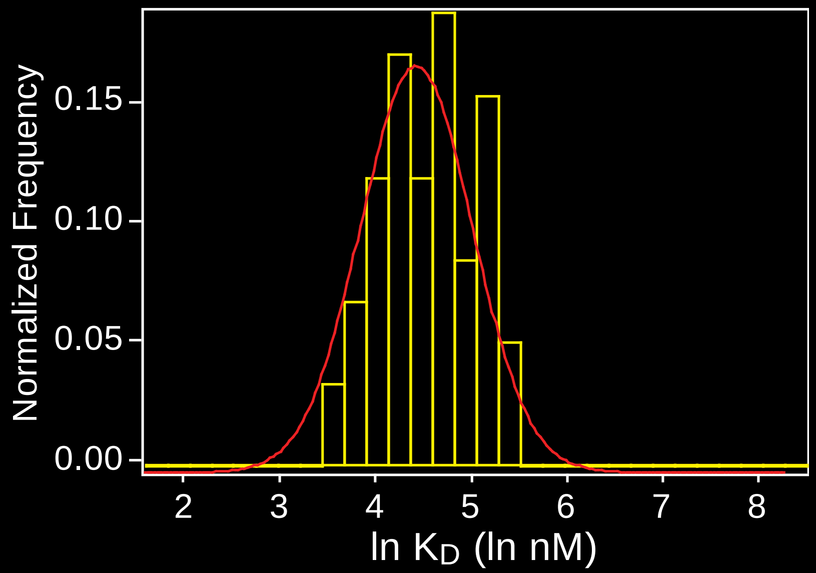
Ricka and Binkert, *Phys Rev A*, 39(5) :2646-52 (1989)
Hwang and Wohland, *ChemPhysChem* 5, 549-551 (2004)
Hwang and Wohland, *JCP*, 122, 114708 (2005)

How to determine the K_d

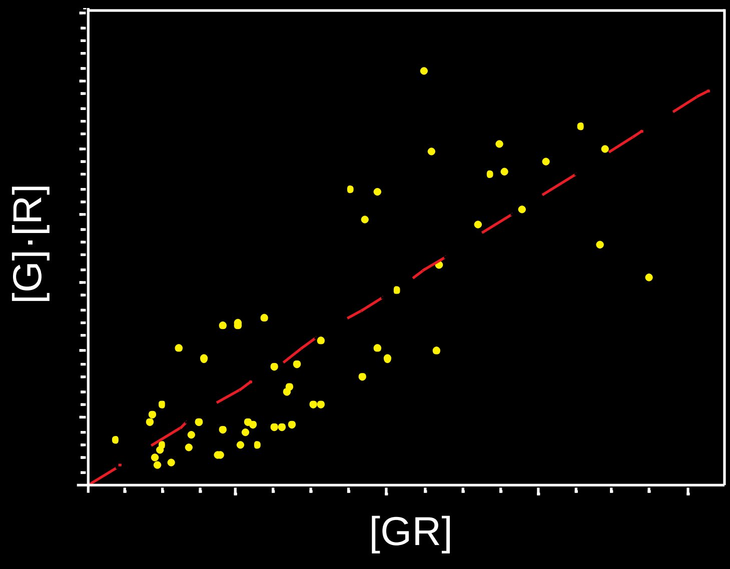
$$[G]+[R] \leftrightarrow [GR]$$

$$K_d = \frac{[G][R]}{[GR]} \longrightarrow [G][R] = K_d\,[GR]$$

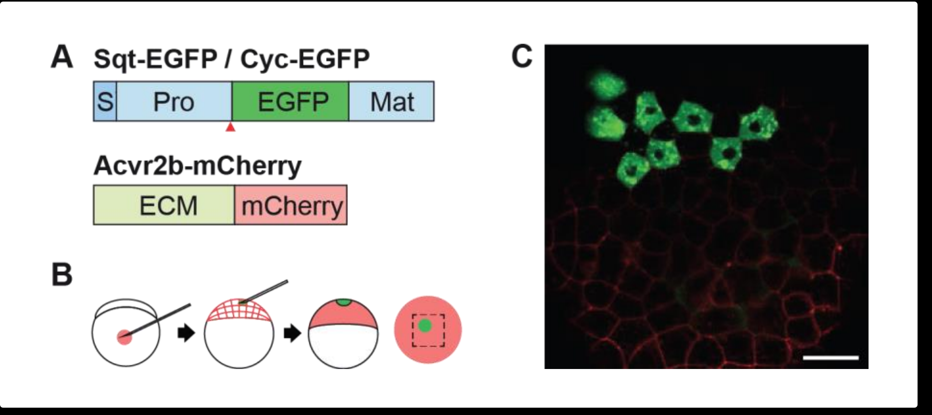
K_d distribution

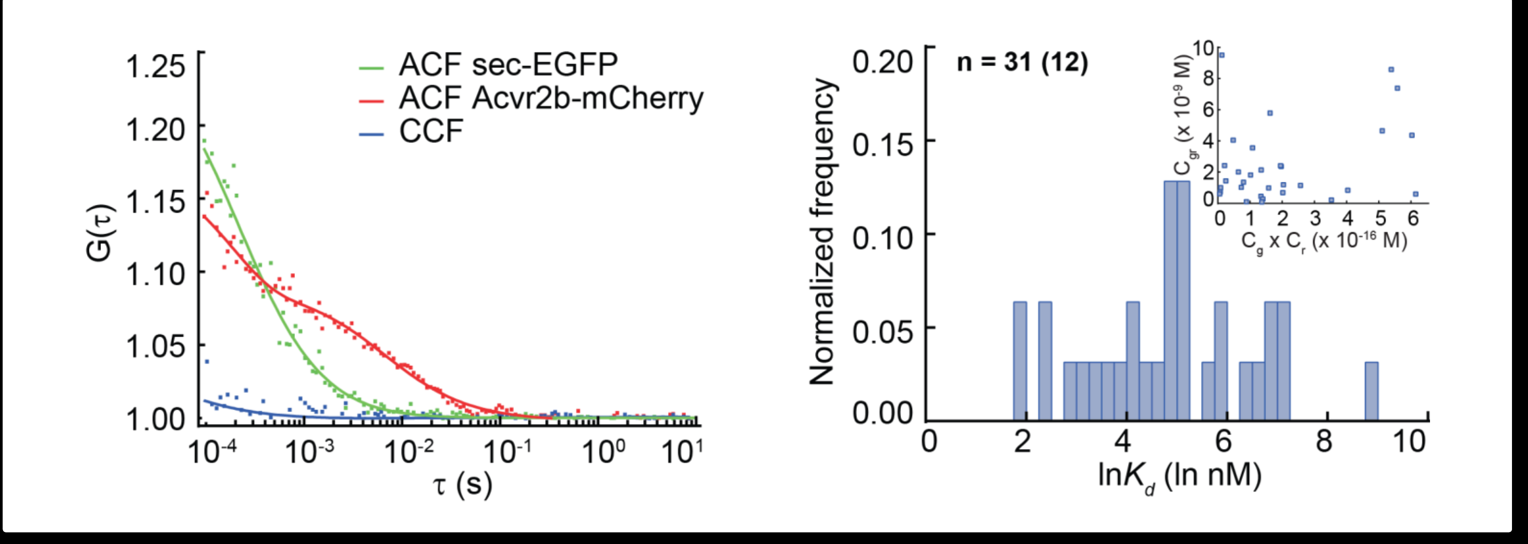


Line through origin with a slope of K_d



Nodal/Acvr2b interactions in live zebrafish





Wang et al., eLife 2016, 5:e13879

Examples of Applications

Membrane proteins: EGFR dimerization

Liu et al., *Biophys. J.* (93): 684-698 (2007).

Yavas et al., *Biophys. J.* 111(10) - 2241-2254 (2016)

Membrane and cytosolic proteins: EGFR activation

Ma et *al Front. Biosci*. Jan 1;3:22-32 (2011).

Cytosolic protein (cdc42 and effectors: IQGAP1, N-WASP etc.)

Shi et al., *Biophys. J.* (97)2:678-686 (2009).

Sudhaharan et al., *JBC* 284: 13602-13609 (2009).

Protein - DNA: Sox2/Oct4 DNA motif binding and cooperativity

EMBO Reports 2015 16(9)p1177

bioarxiv 052530

Nuclear proteins: K_ds of kinetochore protein interactions (CENPs)

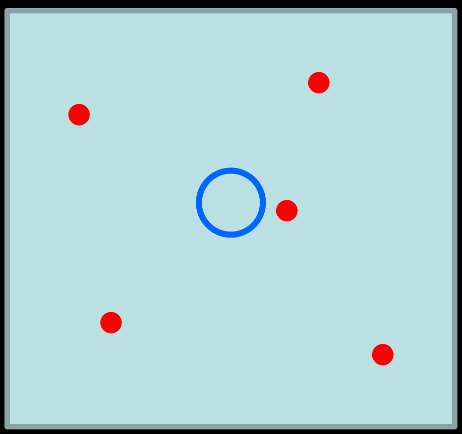
Hoischen et al. 2018 PLoS ONE. 13: e0192572–26.

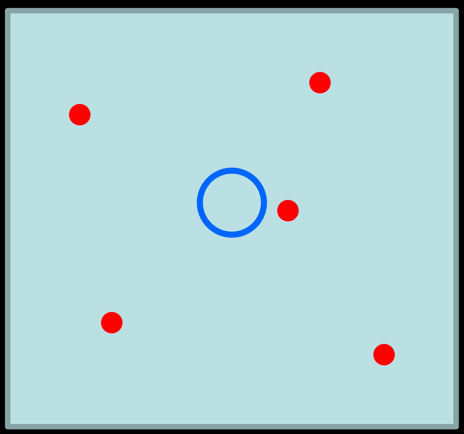
Some FCS limitations and solutions

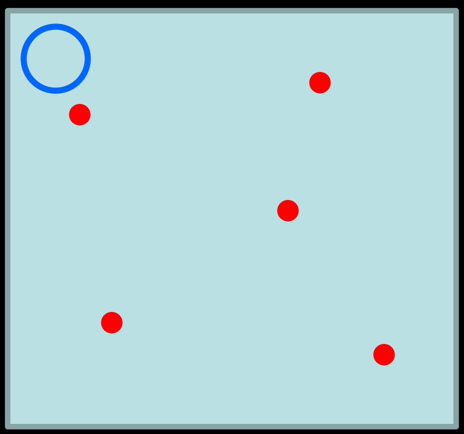
Scanning FCS:

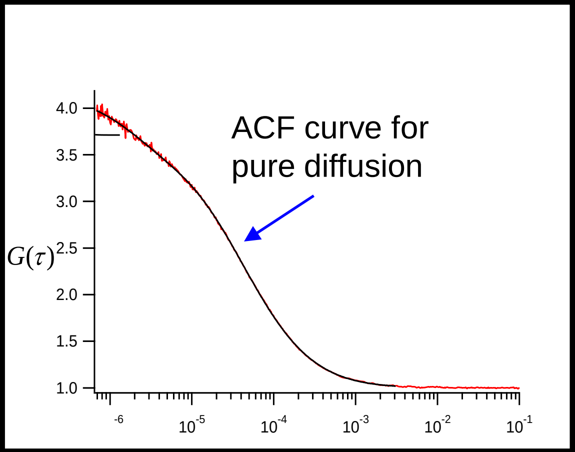
The problem of immobile particles

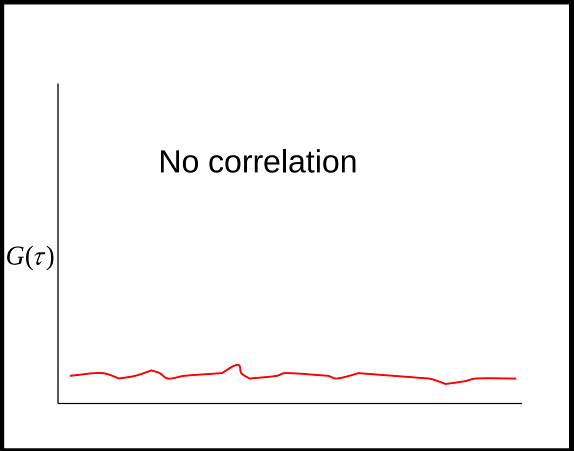
Moving particles	Immobile particles	Scanning Beam

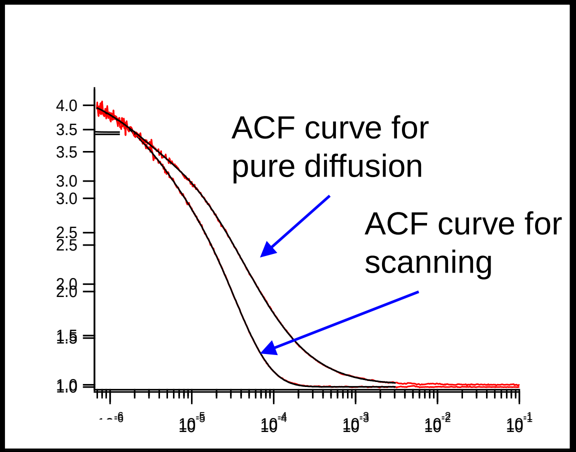






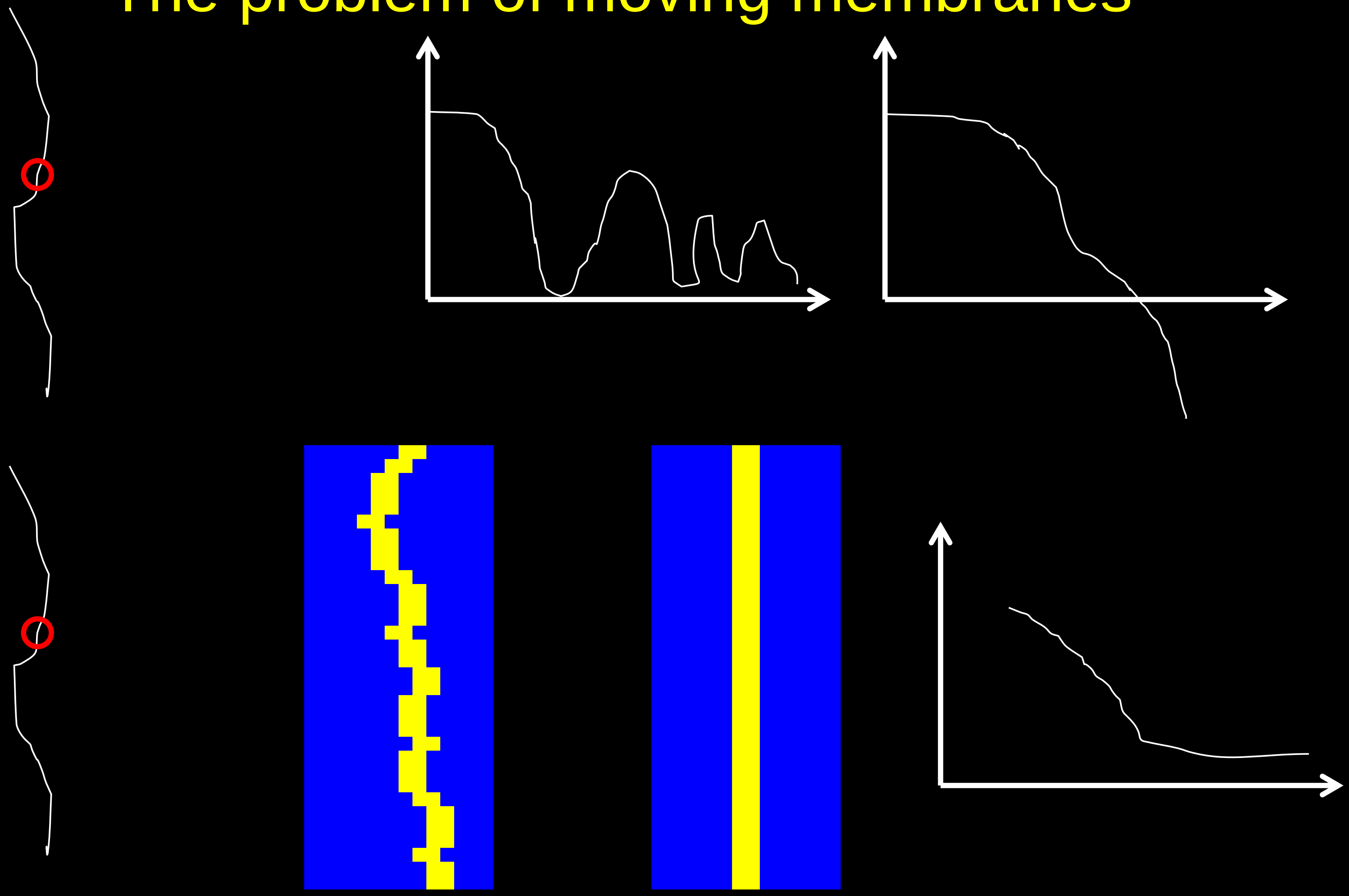






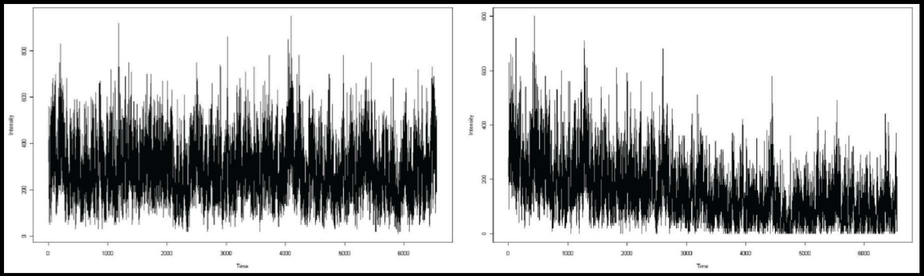
Scanning FCS:

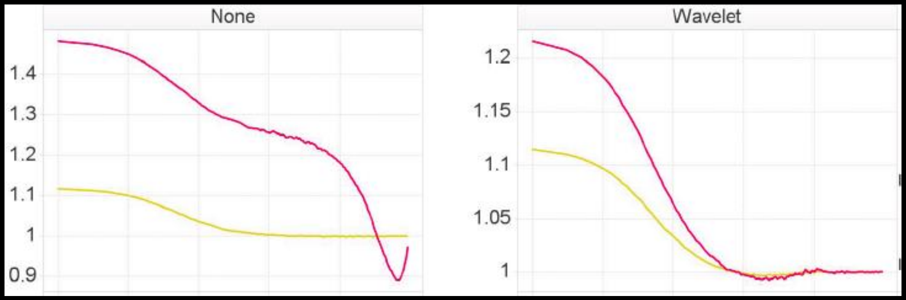
The problem of moving membranes



Bleach Correction

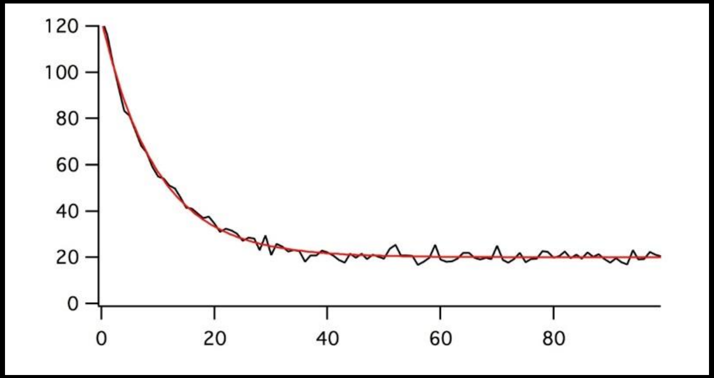
Wavelet Shrinking

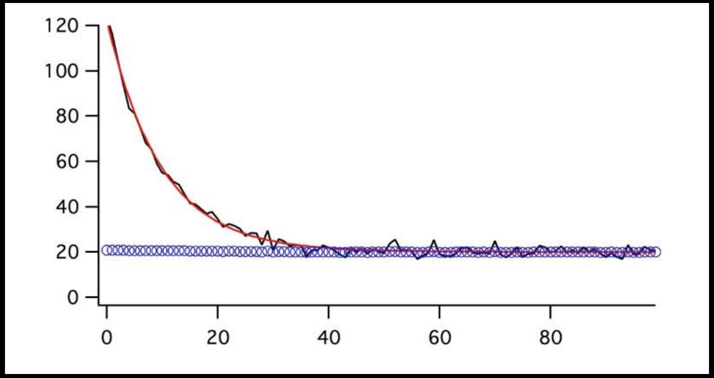




Lange, J.J. et al. 2013. In: Enderlein J, et al. editors. SPIE. pp. 859006–859006–15.

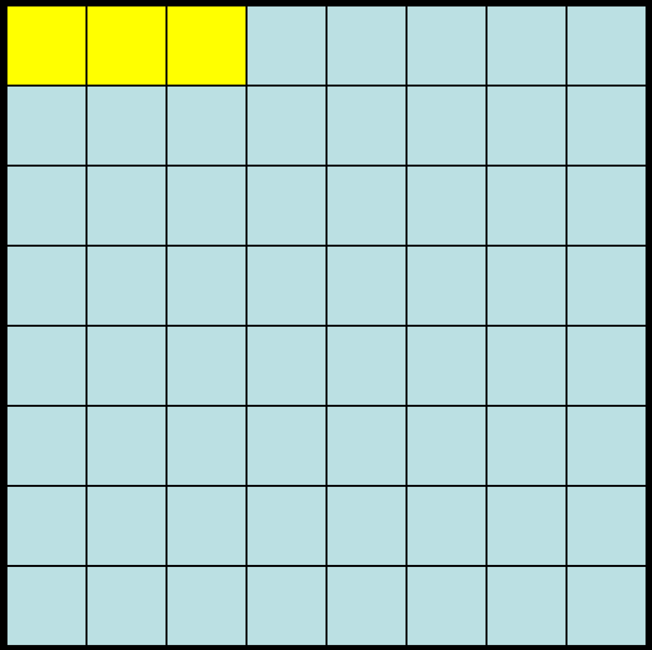
Fitting of a multi-exponential decay

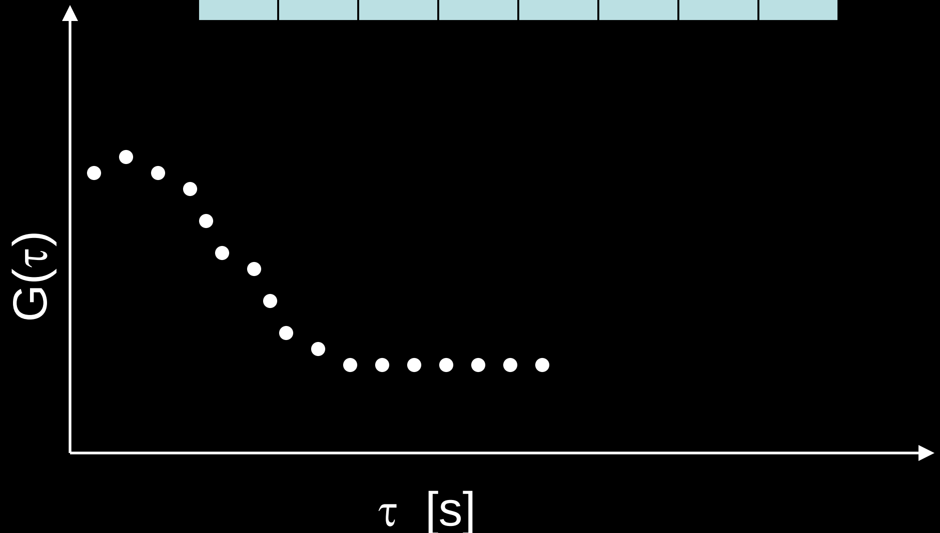


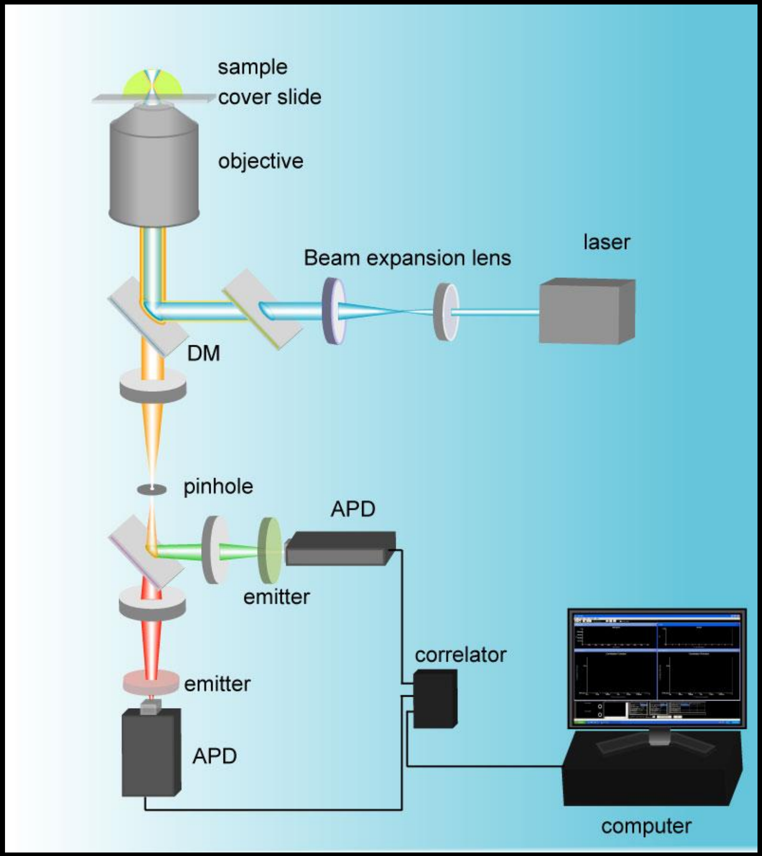


Imaging FCS

FCS in a confocal system

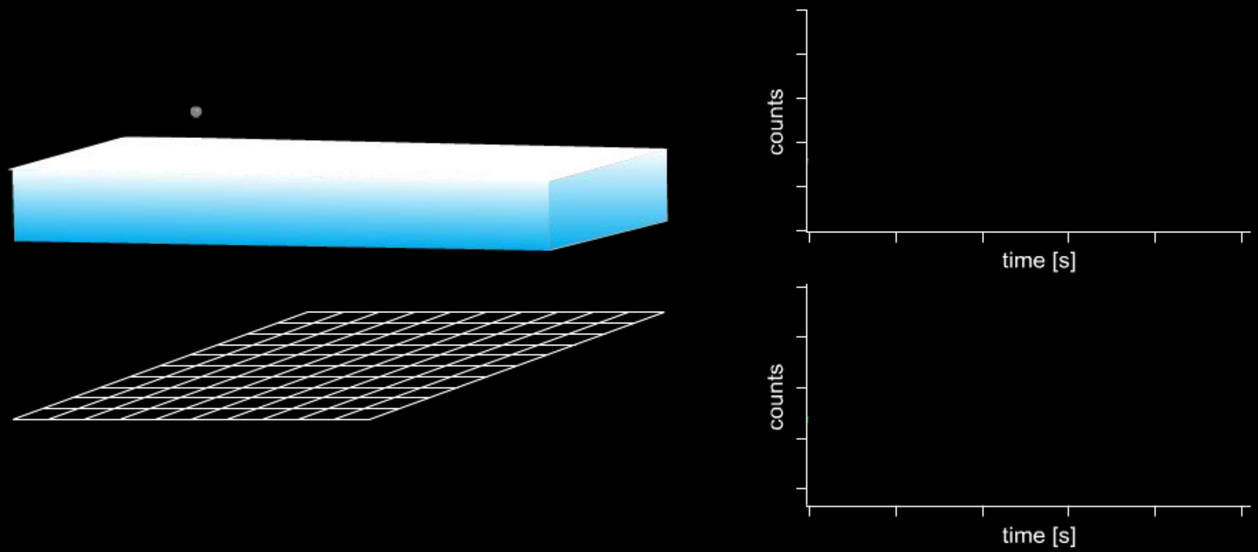


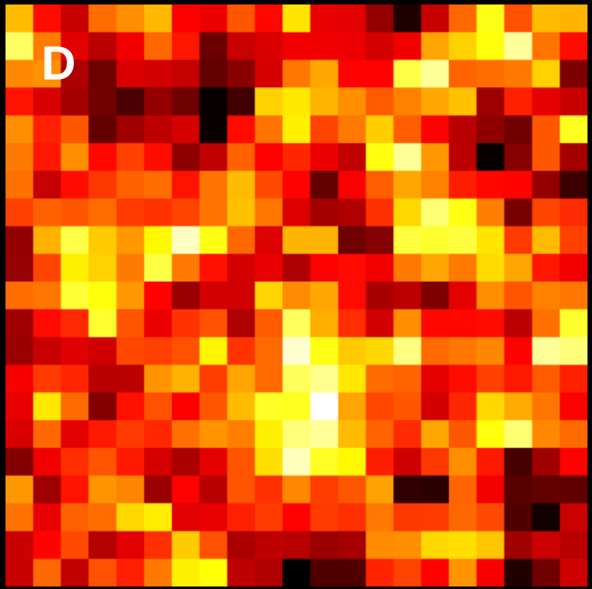


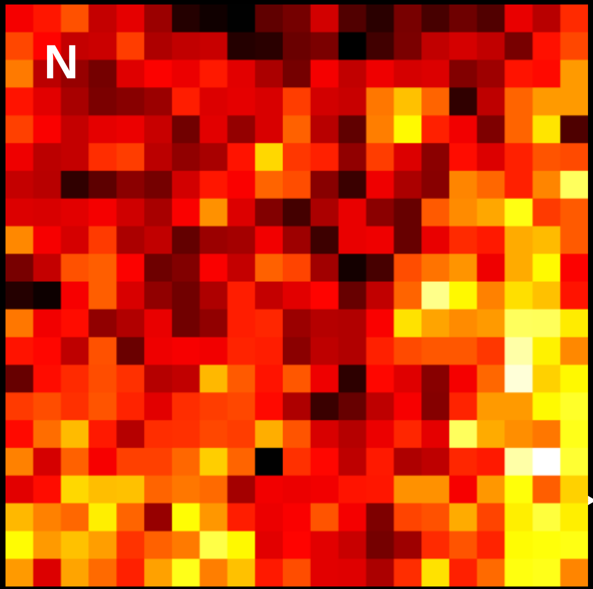


1) **Measurements are not simultaneous**
2) **Cell damage by long illumination times**

Imaging FCS

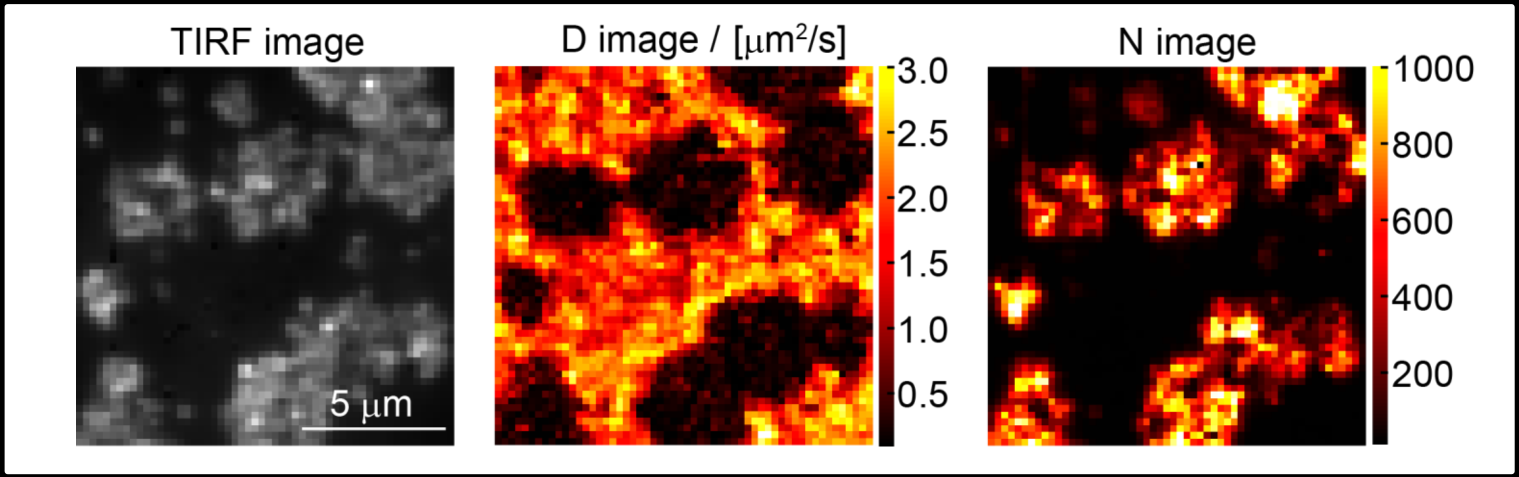




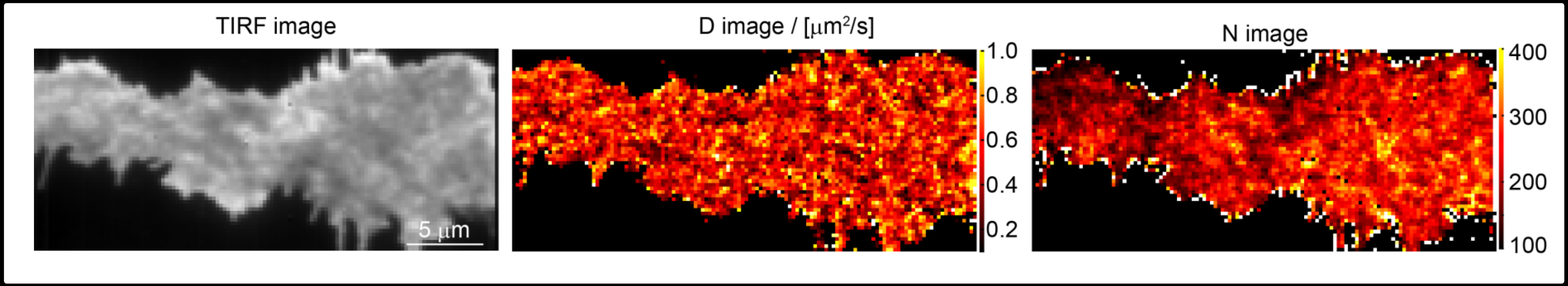


Examples

DLPC/DSPC bilayer on glass



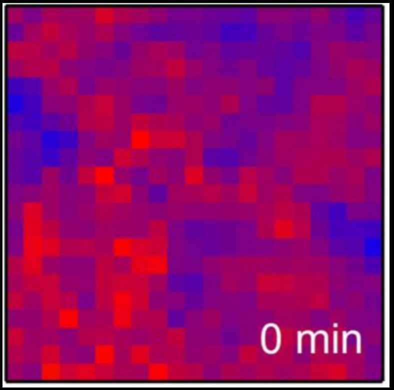
GFP-GPI on SH-SY5Y cells



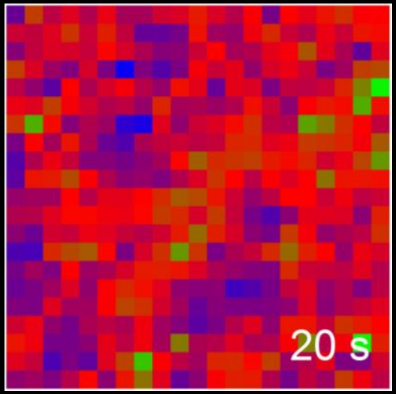
Bag et al. *Methods Appl. Fluoresc.* **4** (2016) 034003

FCS videos

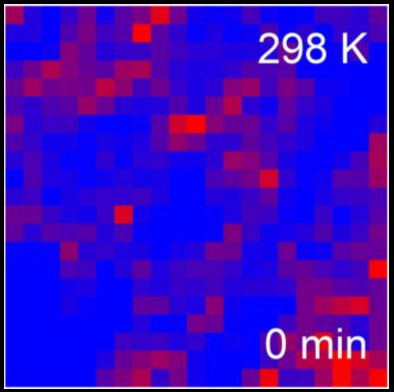
hIAPP treated DiI-C18 labelled SH-SY5Y cell



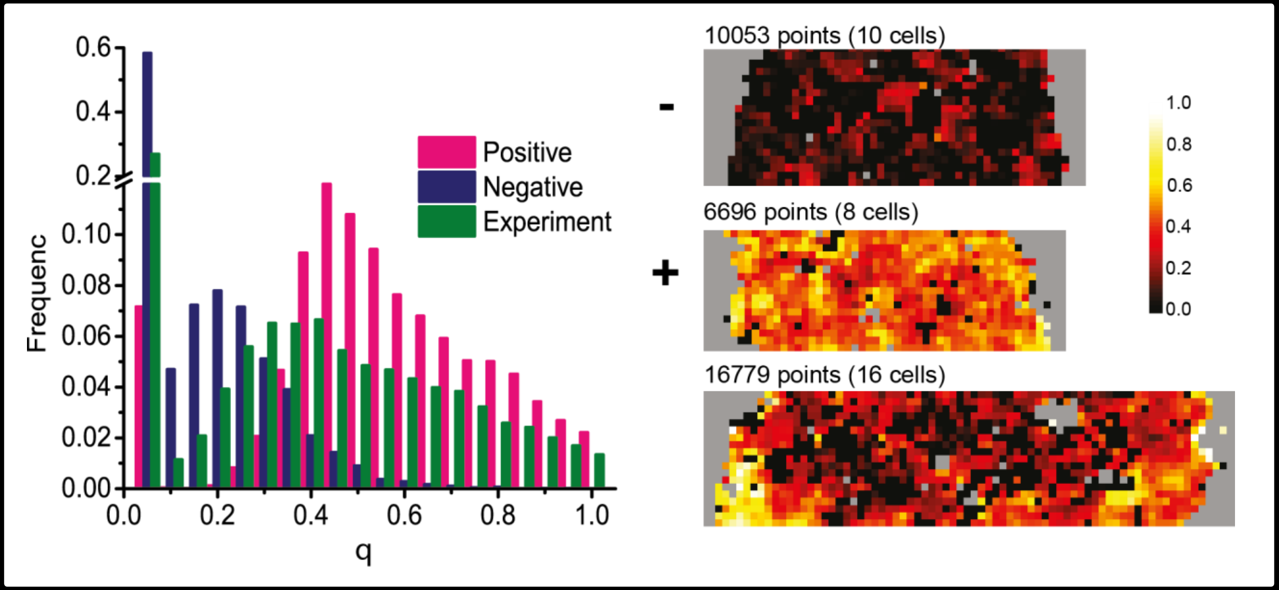
RhoPE labelled DOPC bilayer



GFP-GPI transfected SH-SY5Y cell at different temperature



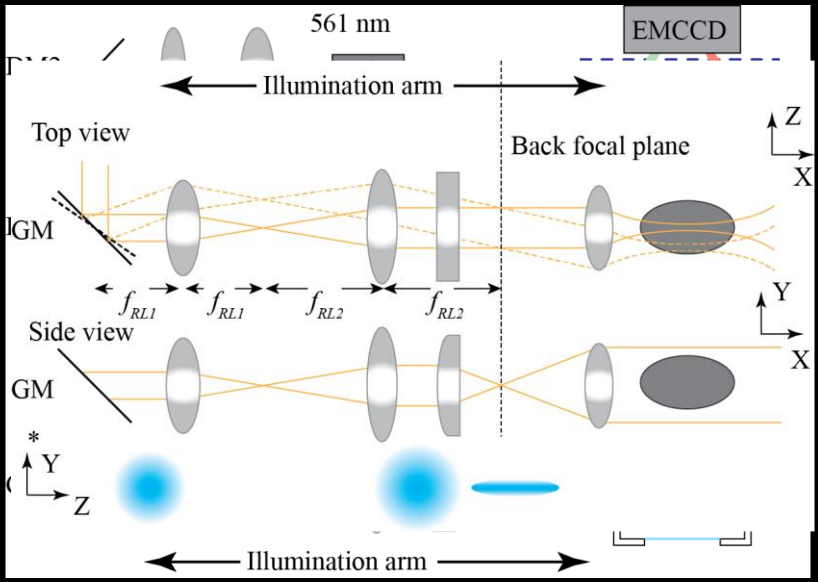
Imaging FCCS on EGFR

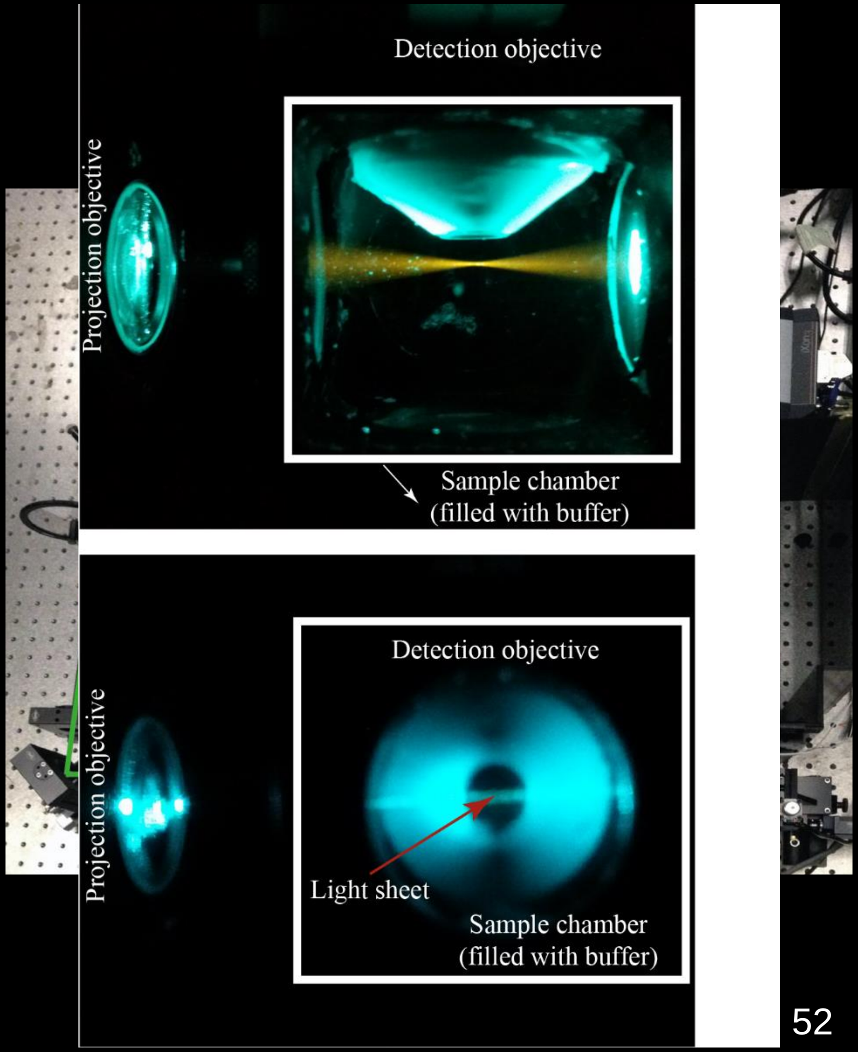


Degree of dimerization

$$q = G_{GR}(0)/\mathrm{Min}\{G_G(0), G_R(0)\}$$

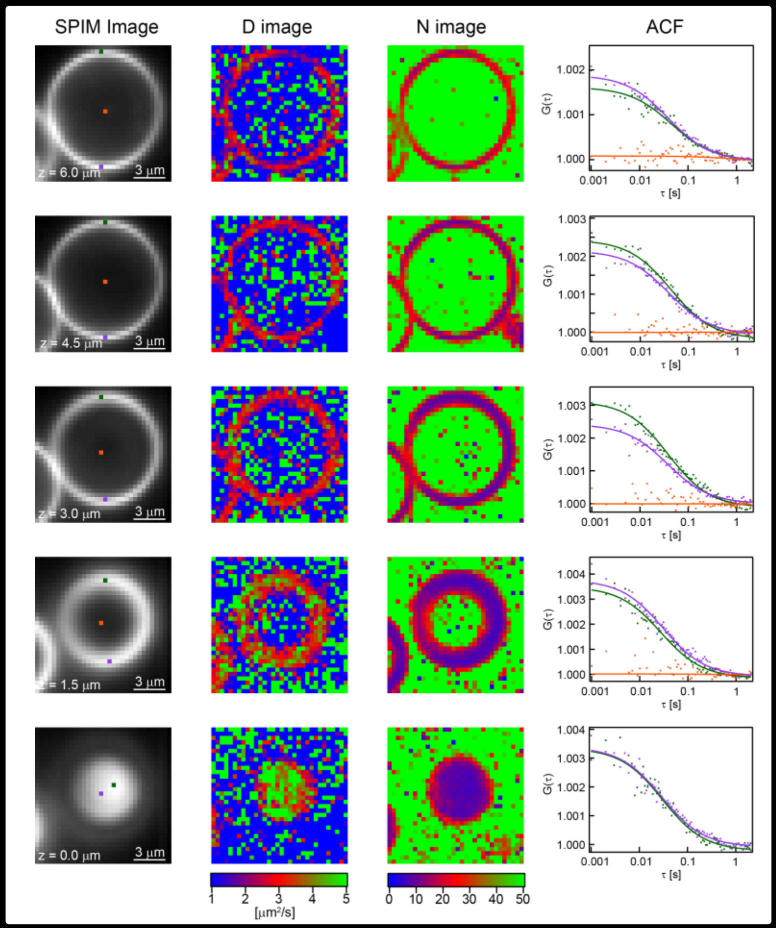
Single Plane Illumination Microscopy (SPIM)





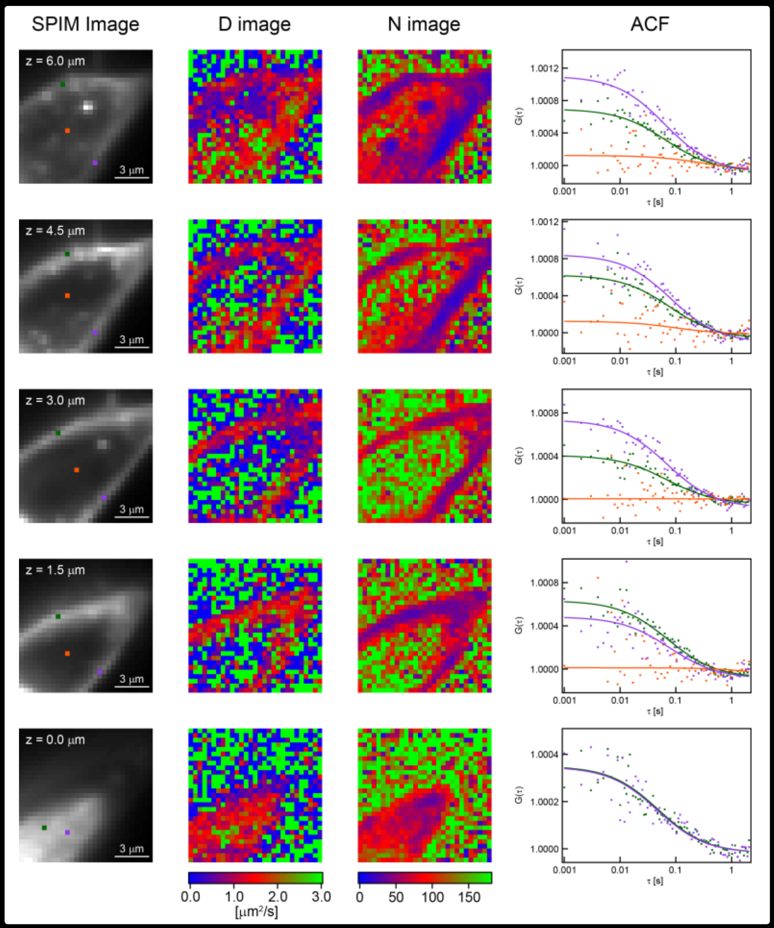
SPIM-FCS in 3D

Giant Unilamellar Vesicles (GUV)



RhoPE-labelled DOPC:DOPG (10:1)

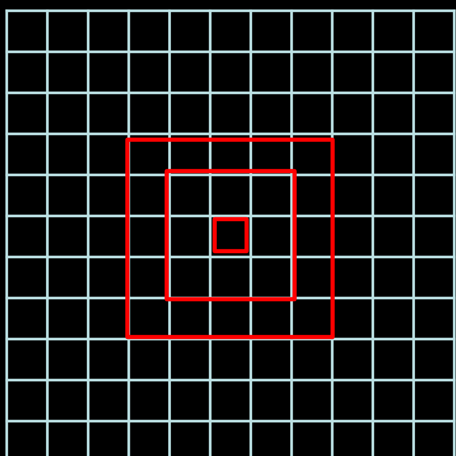
DiI-C_{18} labelled live SH-SY5Y cell



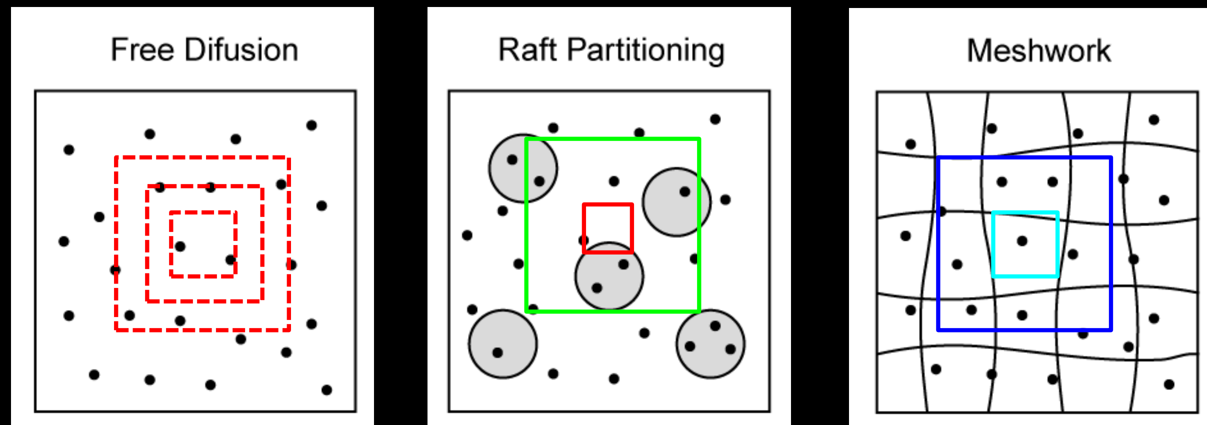
Ng et al., Biophys. J., (2016) accepted

Imaging FCS diffusion law

Pixel binning

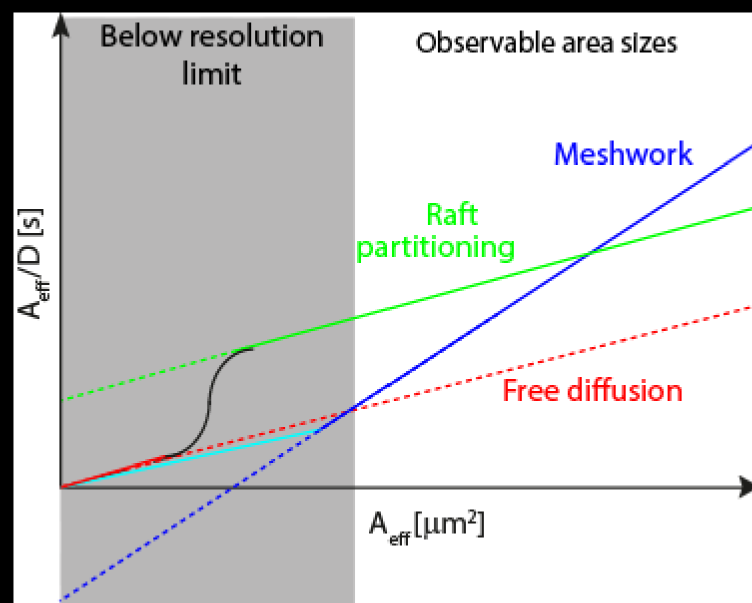


EMCCD chip



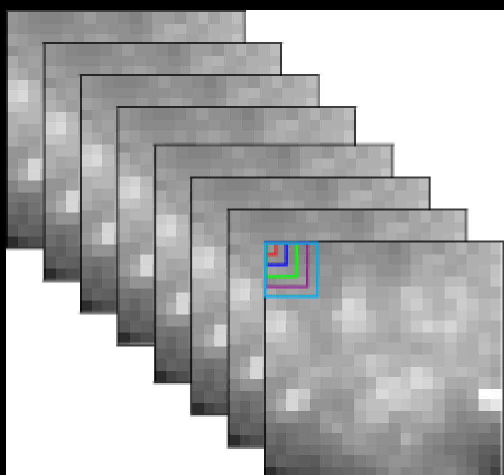
$D_{micro} = D_{macro}$ $D_{micro} < D_{macro}$ $D_{micro} > D_{macro}$

$$\tau_D(A_{eff}) = \tau_0 + \frac{A_{eff}}{D}$$

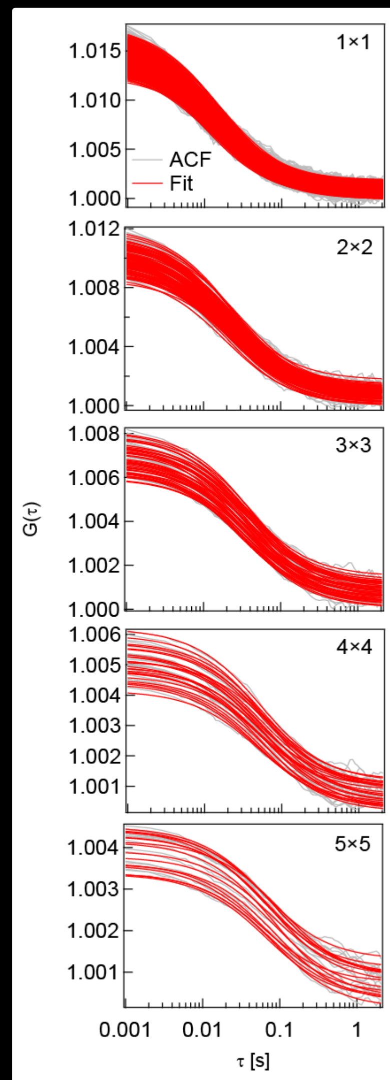


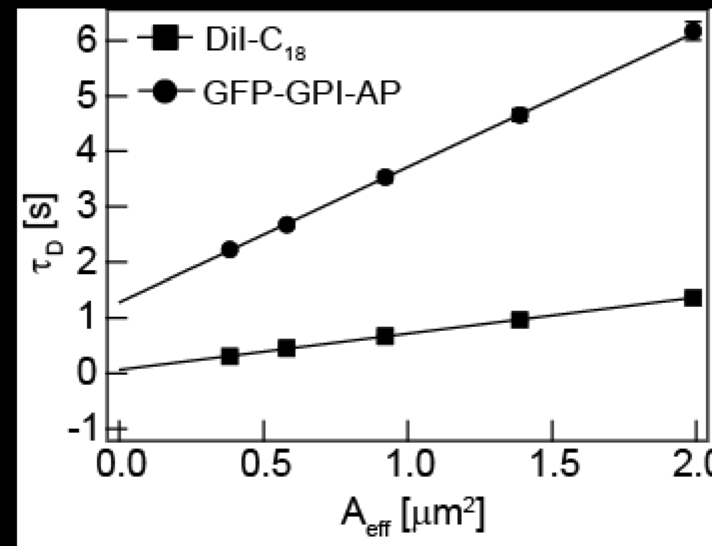
Wawrezinieck *et al. Biophysical Journal*, 2005
Huang and Pralle, *arXiv:1101.5087*

Imaging FCS diffusion law



$$\tau_D(A_{eff}) = \tau_0 + \frac{A_{eff}}{D_{eff}}$$

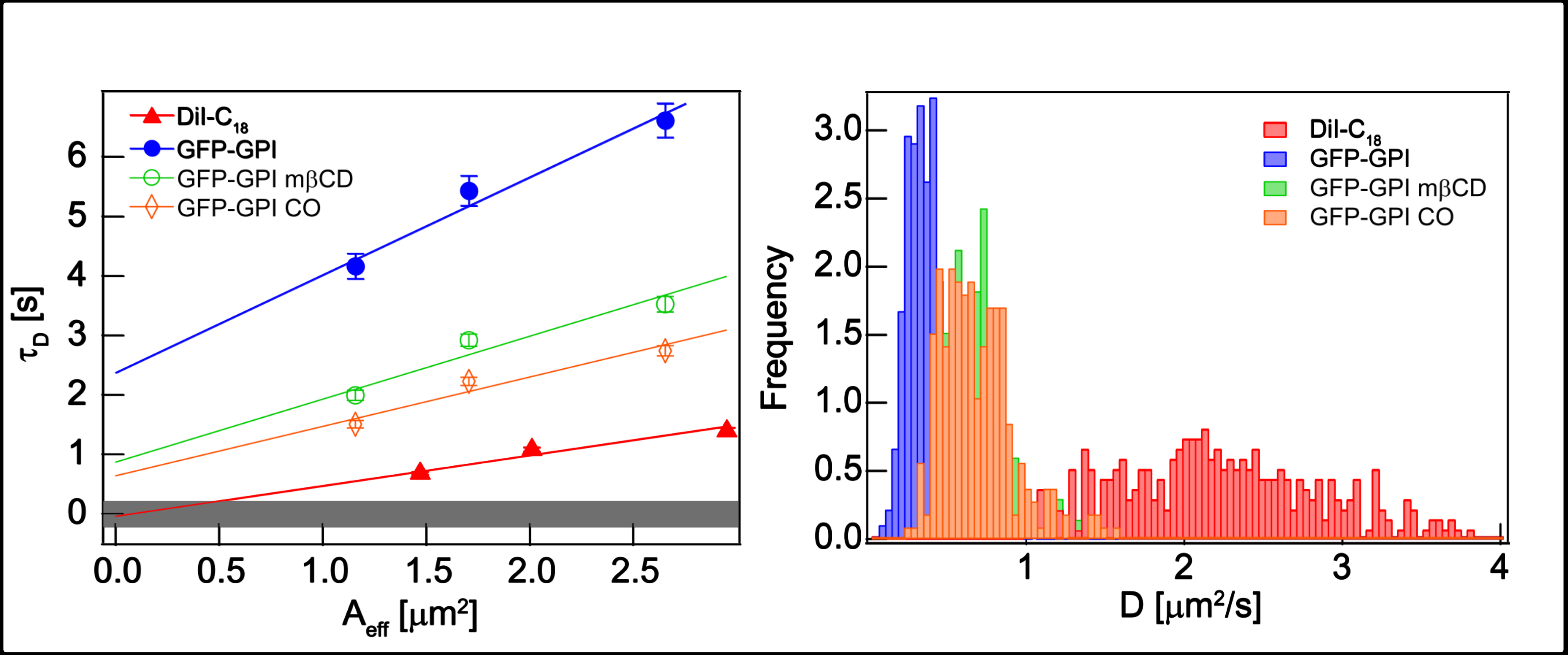




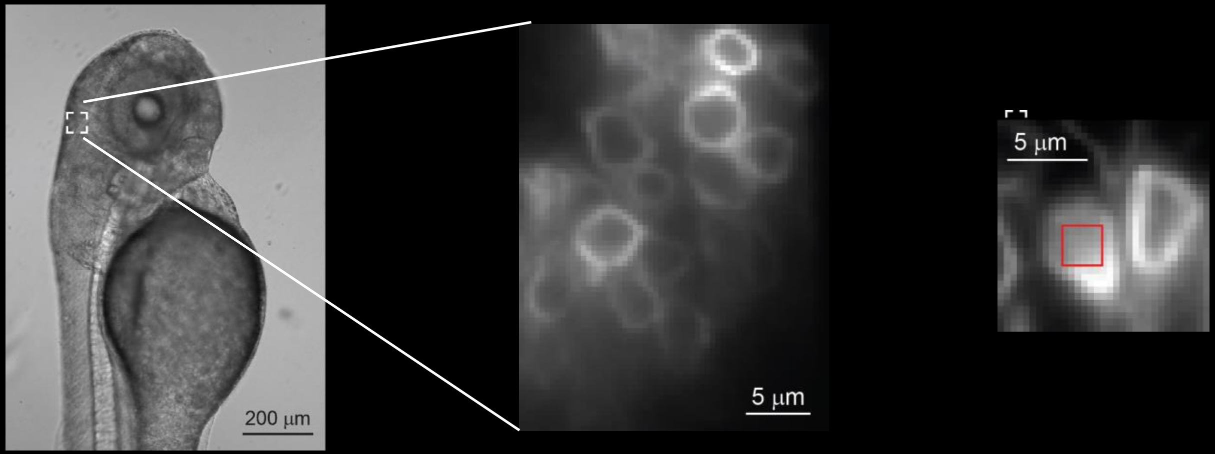
Diffusion mode	τ_0 [s]	D_{eff}/D_{ACF}
Free	0	1
Domain confined	> 0	> 1

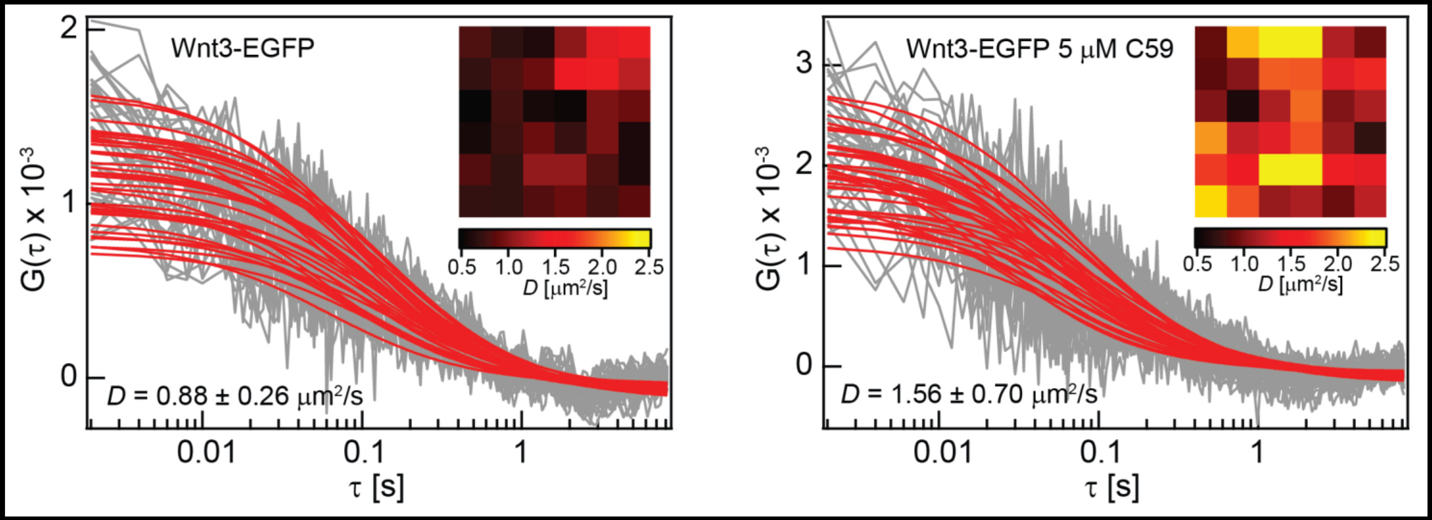
Bag et al., *Biophysical Journal*, 2015

SH-SY5Y membrane organization

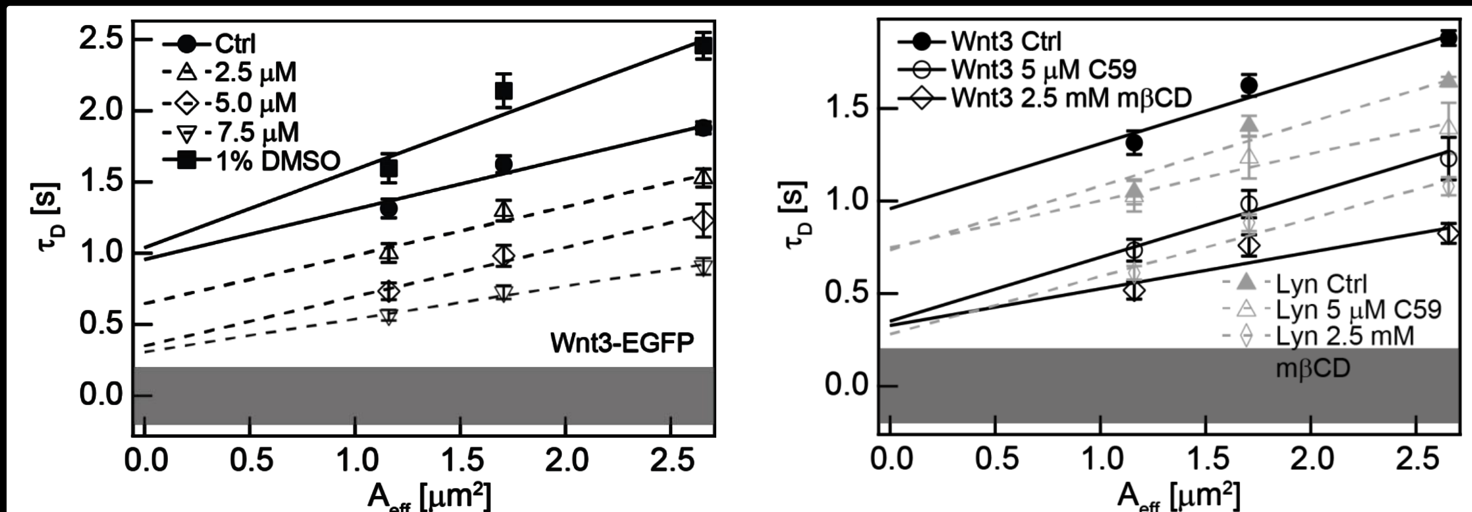


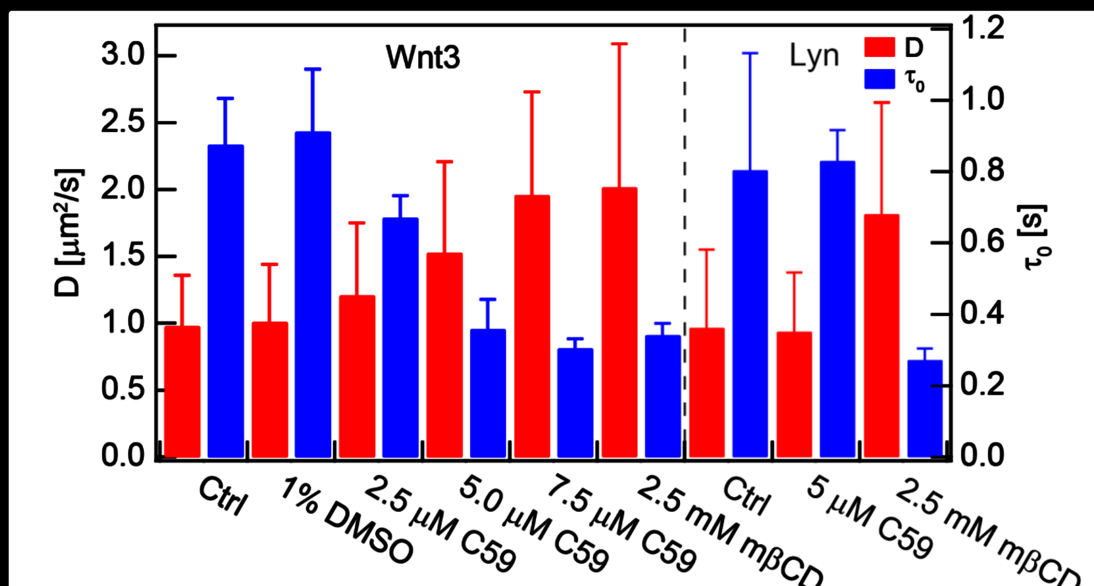
Wnt3 membrane localization in zebrafish





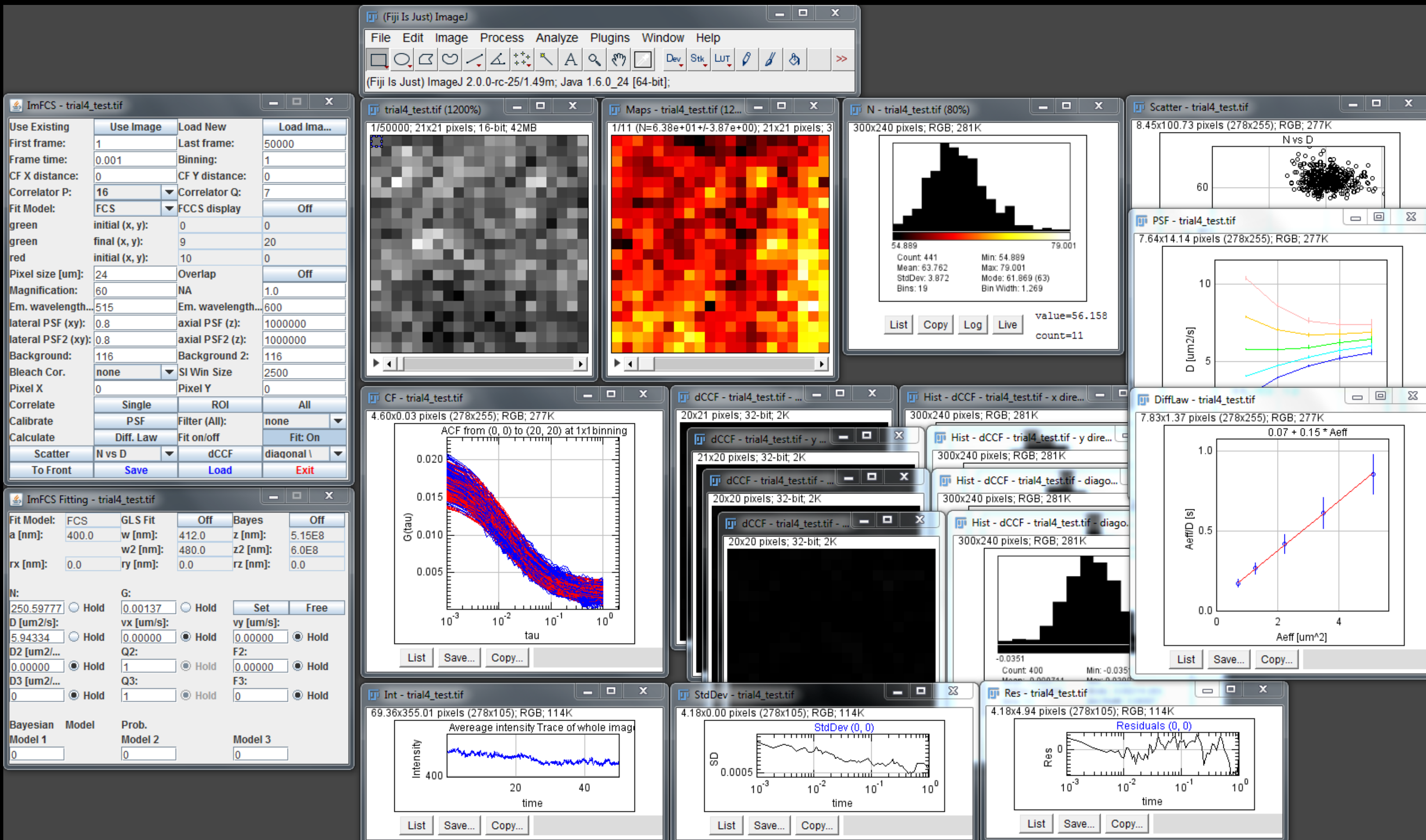
Inhibition of palmitoylation





ImageJ Plugin for Imaging FCS

http://dbs.nus.edu.sg/lab/BFL



Sankaran et al. *Opt. Exp.* 2010, 18 (24): 25468-25481
Bag and Wohland, *Ann. Rev. Phys. Chem.* 2014, 65: 225–48
Krieger et al. *Nat. Prot.* 2015, 10 (12) 1948-1972

Summary

- FCS provides measures for concentrations and diffusion coefficients
- These parameters can be quantified and can be used to derive secondary parameters (affinity, stoichiometry etc.)
- (SW-) FCCS provides an easy readout for interactions via ACF and CCF amplitudes
- Imaging FCS multiplexes FCS and FCCS measurements and can be used to make time lapse FCS videos
- TIRF and SPIM modes provide high S/N 2D and 3D measurements, respectively
- The spatiotemporal information in imaging FCS provides information beyond the diffraction limit via the diffusion laws

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Singapore Stem Cell Consortium (SSCC)

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SMA

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