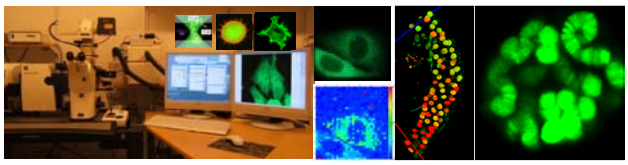

Fluorescentna mikroskopija. Tehnike oslikavanja, poboljšanje rezolucije zaobilaženjem difrakcione barijere i izučavanje dinamičkih procesa

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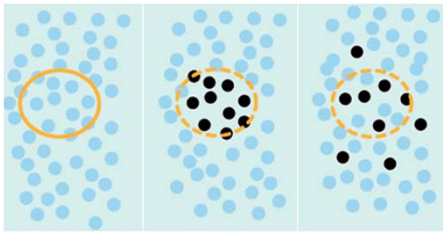
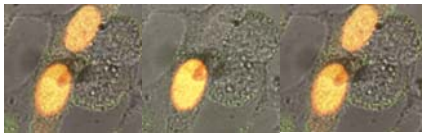
29. juni, Beograd, Srbija

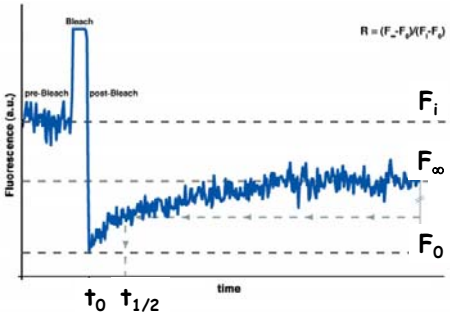
Outline

- **Quantitative study of molecular interactions and mobility in live cells**
  - **Fluorescence Recovery After Photobleaching (FRAP)**
  - **Főrster Resonance Energy Transfer (FRET)**
  - **Fluorescence Lifetime Imaging Microscopy (FLIM)**
  - **Fluorescence Correlation Spectroscopy (FCS)**
  - **Raster Image Correlation Spectroscopy (RICS)**
  - **Quantitative Confocal Laser Scanning Microscopy via massively parallel FCS (mpFCS)**

Fluorescence Recovery After Photobleaching (FRAP)







Percentage of recovery

$R = (F_\infty - F_0)/(F_i - F_0)$

Recovery half time ($t_{1/2}$)

is the time required for 50 % of the fluorescence to revert.

http://www.science.mcmaster.ca/biochem/faculty/truant/videos.htm; http://www.embl.de/eamnet/frap/FRAP6.html
http://www.biophysj.org/cgi/content/full/86/6/3473; http://www.youtube.com/watch?v=bhjP9PqfJRE

FRAP is governed by diffusion and chemical kinetics

$$A^* + B \underset{k_{off}}{\overset{k_{on}}{\Longleftrightarrow}} C^*$$

$$\frac{\partial[A^*]}{\partial t} = D_A \nabla^2 [A^*] - k_{on}[A^*][B] + k_{off}[C^*]$$

$$\frac{\partial[B]}{\partial t} = D_B \nabla^2 [B] - k_{on}[A^*][B] + k_{off}[C^*]$$

$$\frac{\partial[C^*]}{\partial t} = D_C \nabla^2 [C^*] + k_{on}[A^*][B] - k_{off}[C^*]$$

$$[A^*] + [A] = [A^*]_{eq}$$

$$[C^*] + [C] = [C^*]_{eq}$$

$$\frac{d[B]}{dt} = 0$$

the bound complex C is immobile

$$\frac{\partial[A^*]}{\partial t} = D_A \nabla^2 [A^*] - k_{on}[A^*][B] + k_{off}[C^*]$$

$$\frac{\partial[C^*]}{\partial t} = k_{on}[A^*][B] - k_{off}[C^*]$$

$$\nabla^2 = \frac{\partial^2}{\partial x^2} + \frac{\partial^2}{\partial y^2} + \frac{\partial^2}{\partial z^2}$$ - the Laplace operator (Laplacian)

D - diffusion coefficient for each of the three species
k_{on} – kinetic rate constant of the forward reaction
k_{off} – kinetic rate constant of the backward reaction

Sprague BL, Pego RL, Stavreva DA, McNally JG. Analysis of Binding Reactions by Fluorescence Recovery after Photobleaching Biophys J (2004) **86**: 3473-3495

Diffusion dominant FRAP

$$D = \frac{\varpi^2}{4 \cdot t_{1/2}}$$

ω - width of the beam

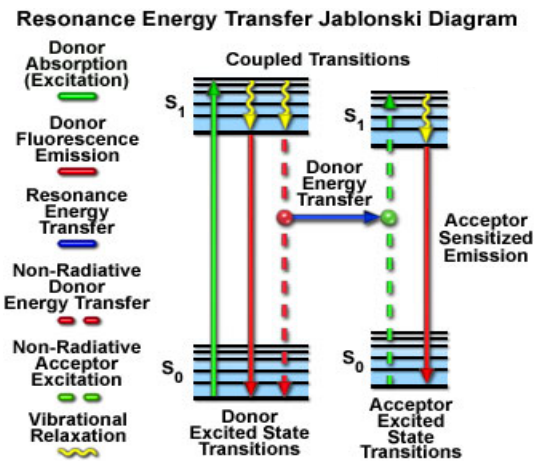
Percentage of FRAP recovery (R) reflects the abundance of the immobile fraction.

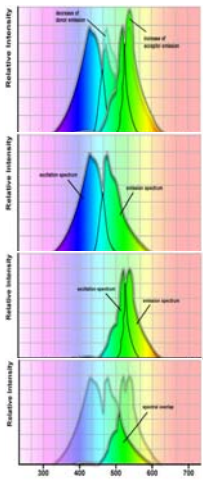
Reaction dominant FRAP

$$FRAP(t) = 1 - [C^*]_{eq} \cdot e^{k_{off} t}$$

$$[A^*]_{eq} + [C^*]_{eq} = 1$$

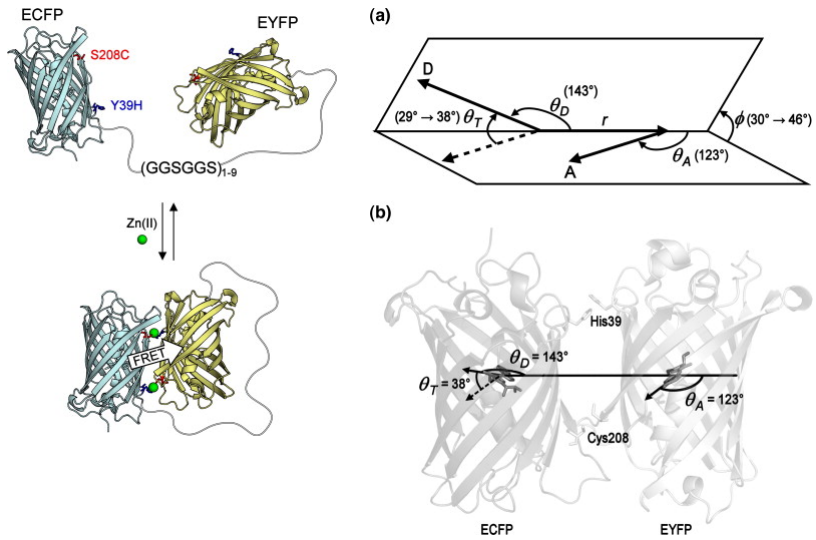
Förster Resonance Energy ransfer (FRET)





In biological studies, the most often applied genetic probes pair is the CFP-YFP. The R_0 distance for the CFP-YFP pair reported in various studies is 4.5 – 7.0 nm.

Molecular orientation



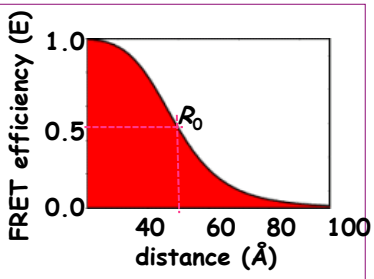
Evers et al. J Mol Biol 2007

Relative orientation of ECFP and EYFP transition dipole moments.

FRET efficiency

$$E = \frac{R_0^6}{R_0^6 + r_{DA}^6}$$

R_0 - Förster radius (at which 50% energy is transferred)
r_{DA} - distance between donor and acceptor



$$R_0^6 = C \cdot n^{-4} \cdot Q_D \cdot \kappa^2 \cdot J$$

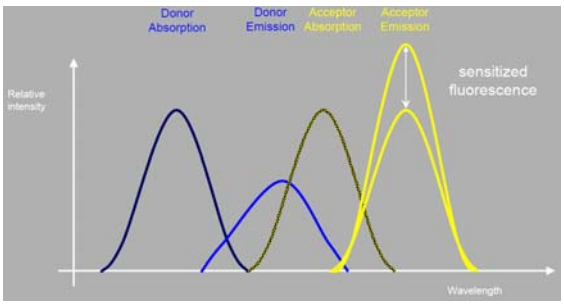
$$J = \frac{\int F_D(\lambda) \cdot \varepsilon_A(\lambda) \cdot \lambda^4 \cdot d\lambda}{\int F_D(\lambda) d\lambda}$$

Q_D - fluorescence quantum yield of the donor
ε_A - the absorption coefficient of the acceptor ($M^{-1}cm^{-1}$)
J - the overlap integral ($M^{-1}cm^{-1}nm^4$)
n - the refractive index
κ^2 - the relative angular dispositions of the donor emission and the acceptor absorption dipole moments
$F_D(\lambda)$ - donor fluorescence emission (arbitrary units)
C = 8.79×10^{-11} (M cm nm^2)

Pietraszewska-Bogiel A, Gadella TWJ. FRET microscopy: from principle to routine technology in cell biology. (2011) Journal of Microscopy, **241**:111–118

FRET effect on donor and acceptor emission spectra

Donor (Em.)	Acceptor (Exc.)
FITC (520 nm)	TRITC (550 nm)
Cy3 (566 nm)	Cy5 (649 nm)
EGFP(508 nm)	Cy3 (554 nm)
CFP (477 nm)	YFP (514 nm)
EGFP (508 nm)	YFP (514 nm)



FRET measuring techniques

- ***Intensity-based***
- ***Acceptor photobleaching (irreversible photodestruction of acceptor absorption)***
- ***Donor photobleaching***
- ***FRET via fluorescence lifetime measurements***

$$E = 1 - \frac{Q_{DA}}{Q_D} = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{\tau_{DA}}{\tau_D} = 1 - \frac{\tau_{bl,D}}{\tau_{bl,DA}} =$$

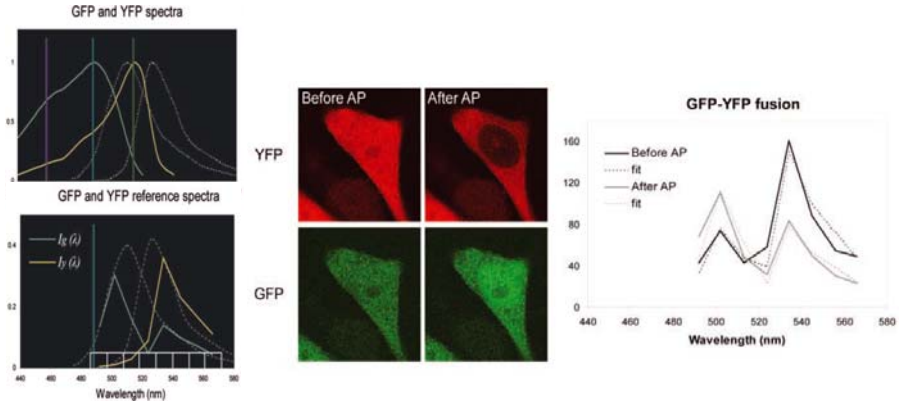
$$= \frac{\varepsilon_A}{\varepsilon_D} \cdot \left(\frac{SE}{I_A} \right) = \frac{\varepsilon_A}{\varepsilon_D} \cdot \left(\frac{I_{AD}}{I_A} - 1 \right)$$

Q_{DA} – *quantum yield of the donor in the presence of the acceptor*
Q_D – *quantum yield of the donor in the absence of the acceptor*
I_{DA} – *fluorescence emission intensity of the donor in the presence of the acceptor*
I_D – *fluorescence emission intensity of the donor in the absence of the acceptor*
τ_{DA} – *fluorescence lifetime of the donor in the presence of the acceptor*
τ_D – *fluorescence lifetime of the donor in the absence of the acceptor*
$\tau_{bl,DA}$ – *donor photobleaching time in the presence of the acceptor*
$\tau_{bl,D}$ – *donor photobleaching time in the absence of the acceptor*
SE – *sensitized emission, increased acceptor fluorescence emission intensity (I)*
ε – *molar absorption coefficient*

FRET by aceptor photobleaching

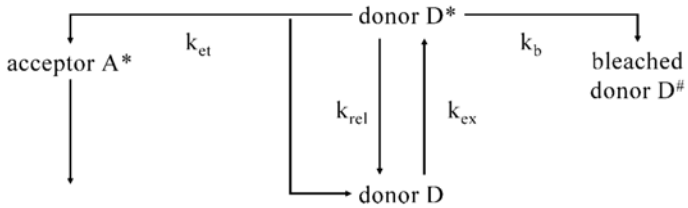
FRET by acceptor photobleaching measures the difference in donor fluorescence intensity in the sample before and after destroying the acceptor by photobleaching:

$$FRET_{eff} = (D_{post} - D_{pre})/D_{post}$$



Dinant et al_J Microscopy 2008; http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2818.2008.02020.x/pdf
http://bti.cornell.edu/pdfs/PCIC_Manuals/AppNote_FRET_AB.pdf

FRET by donor photobleaching

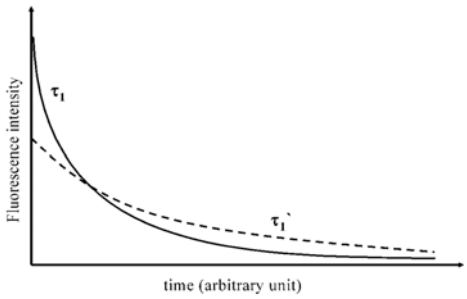


$$E = k_{et}/(k_{rel} + k_{et}) = 1 - \frac{\tau_{da}}{\tau_d}$$

$$\tau_1 \approx (k_{rel})/(k_{ex}k_b)$$

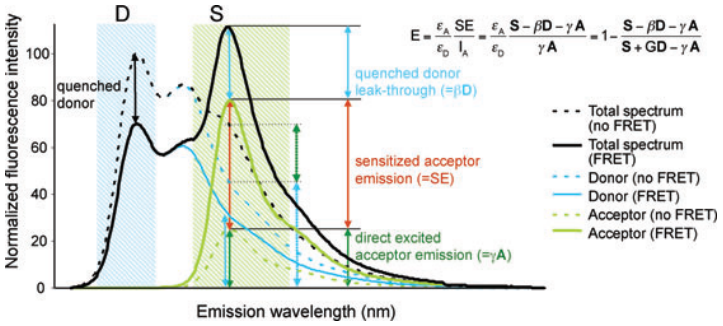
$$\tau_1' \approx (k_{et} + k_{rel})/(k_{ex}k_b)$$

$$E = 1 - \tau_1/\tau_1'$$

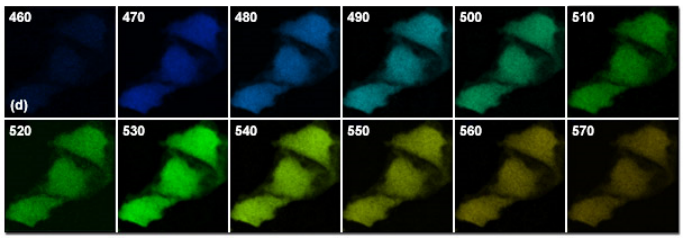


Szentesi et al. Cytometry A 2005
http://onlinelibrary.wiley.com/doi/10.1002/cyto.a.20175/pdf

Spectral FRET

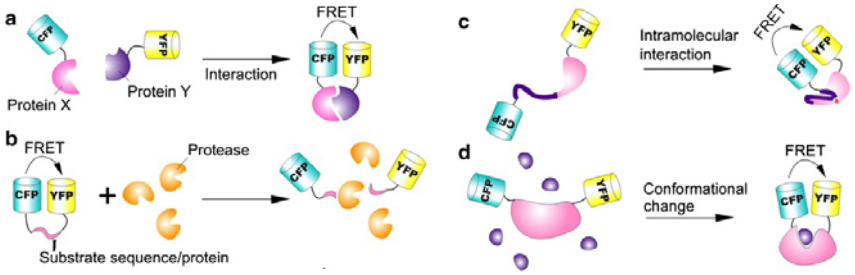


$$E = \frac{\varepsilon_A}{\varepsilon_D}\frac{SE}{I_A} = \frac{\varepsilon_A}{\varepsilon_D}\frac{S-\beta D-\gamma A}{\gamma A} = 1-\frac{S-\beta D-\gamma A}{S+GD-\gamma A}$$



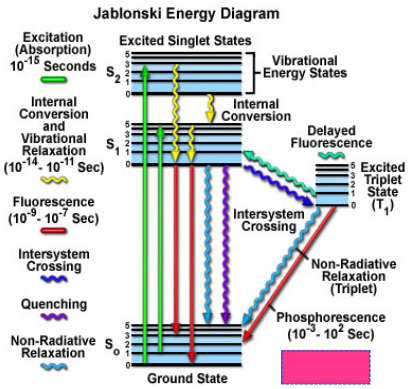
http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/images/spectralfretfigure7.jpg
http://www.ctschina.com.hk/literature/microscope/micpaper005.pdf

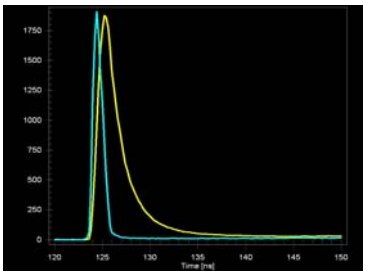
Which biological problems can be studied by FRET



Du W. Wang Y, Luo Q. Liu B-F. Optical molecular imaging for systems biology: from molecule to organism. Anal Bioanal Chem (2006) **386:** 444-457

Fluorescence Lifetime Imaging Microscopy (FLIM)





Fluorescence lifetime, τ, *is defined as the time required for the fluorescence intensity* to decay by the factor 1/e.

The experimentally measured lifetime (τ) contains contributions from internal conversion (IC) and intersystem crossing (ISC):

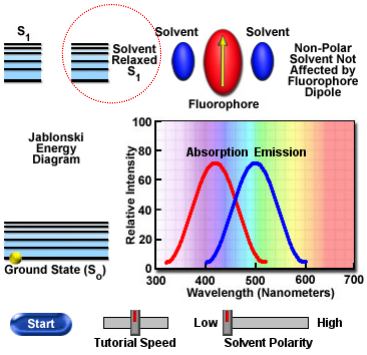
$$\frac{1}{\tau} = k_F + k_{IC} + k_{ISC}$$

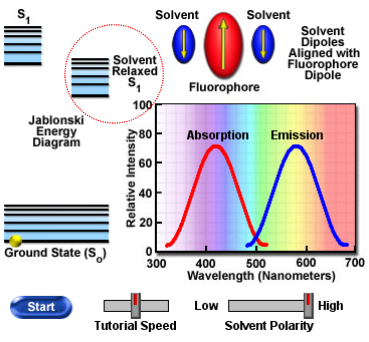
$$\tau_0 = \frac{1}{k_F} = \frac{\tau}{Q}$$

The natural lifetime (τ_0) describes the contribution from fluorescence emission only.
Q is the fluorescence quantum yield.

http://www.olympusconfocal.com/applications/flimintro.html

Why is fluorescence lifetime changing?





http://www.olympusmicro.com/primer/java/jablonski/solventeffects/index.html
http://pubs.acs.org/doi/pdf/10.1021/ac50036a030

How can we measure fluorescence lifetime?

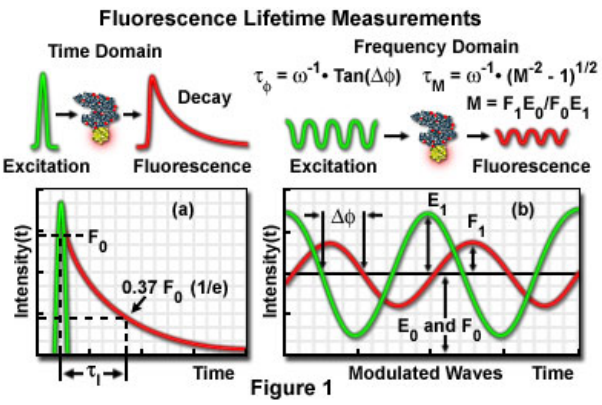
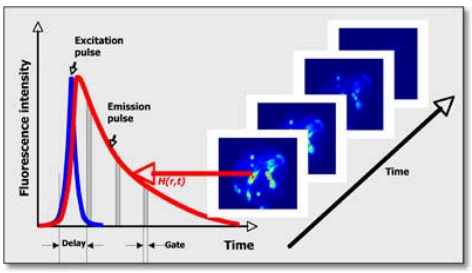
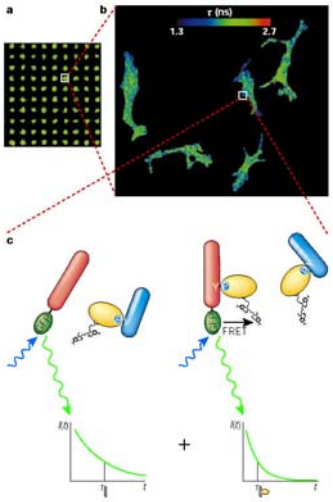


Figure 1

http://www.olympusconfocal.com/applications/flimintro.html

Examples of FLIM application





Phizicky E, Bastiaens PIH, Zhu H, Snyder M, Fields S. Protein analysis on a proteomic scale. Nature 2003, **422**, 208-215

Fluorescence Correlation Spectroscopy (FCS)

Milestones in FCS development

1905/1906 Einstein and von Smoluchowski
Fluctuation theory of light scattering

1911 Svedberg observed fluctuations in the number of colloidal gold particles under a microscope

1913 Perrin anticipated fluorescence fluctuation studies

1957 Laser development

1957 Confocal microscope

1961/1964 Solid state single photon detectors

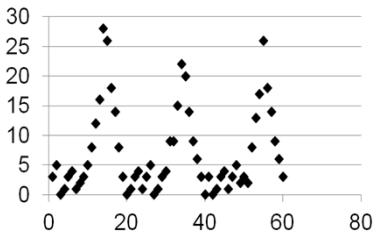
1967 Autocorrelator

Moder  work of

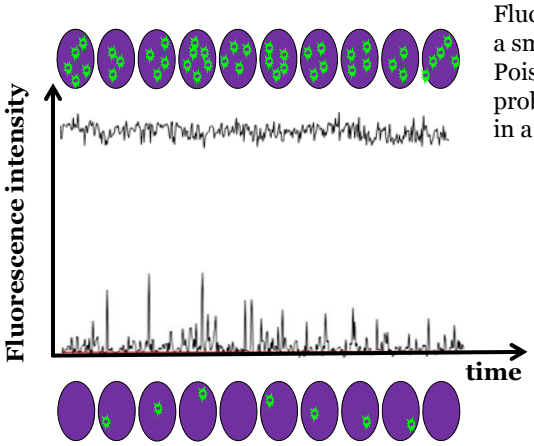
Magde D,
Elson EL,
Ehrenber

Fluctuation analysis

3 5 0 1 3 4 1 2 3 5 8 12 16 28 26 18 14
8 3 0 1 3 4 1 3 5 0 1 3 4 9 9 15 22 20
14 9 6 3 0 3 0 1 3 4 1 3 5 2 3 2 8 13 17
26 18 14 9 6 3



Fluctuation analysis

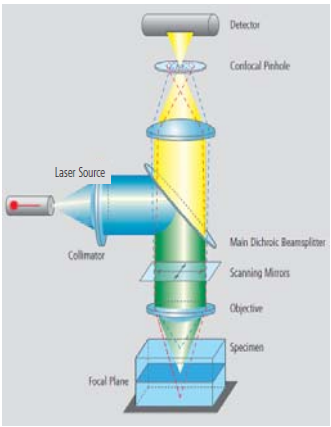


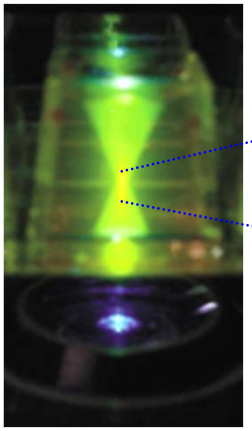
Fluctuations in molecular number in a small volume can be described by a Poisson process, which states that the probability of observing N molecules in a given interval of time is:

$$P(N) = \frac{\langle N \rangle^N e^{-\langle N \rangle}}{N!}$$

$$\frac{1}{\bar{N}} = \frac{\langle (N - \bar{N})^2 \rangle}{(\bar{N})^2} = \frac{Var}{(\bar{N})^2}$$

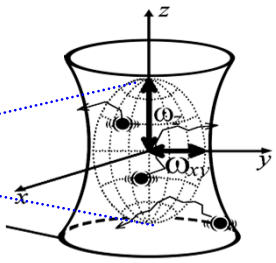
FCS coupling with confocal microscopy





http://www.es.hokudai.ac.jp/publish/outline/2003/ries14_1.png

0.5 μm × 0.5 μm × 1.5 μm



Single-molecule sensitivity

Real time analysis

Live cells

Fluorescence intensity distribution in the OVE

$$F(t) = \int_V I_{exc}(\vec{r}) \cdot Q(\vec{r}) \cdot S(\vec{r}) \cdot c(\vec{r},t) dV$$

I_{exc} – laser intensity profile
Q – quantum yield of the fluorophore
S – photon detection sensitivity of the instrument
c – concentration of the fluorophore

By combining all terms that characterize fluorescence emission and detection, and by assuming that the spatial distribution of the emitted light can be approximated by a three-dimensional Gaussian:

$$W(r) = e^{-2\frac{x^2+y^2}{r_0^2}} \cdot e^{-2\frac{z^2}{z_0^2}}$$

which decays to $1/e^2$ at r_0 in the lateral direction and for $z = z_0$ in the axial direction, fluorescence intensity distribution across the observation volume element can be described as:

$$F(t) = \int_V W(\vec{r}) \cdot c(\vec{r},t) dV$$

By substituting F(t) in the autocorrelation function and considering only free 3D diffusion:

$$\langle \delta c(\vec{r},0) \cdot \delta c(\vec{r}',\tau) \rangle = \langle c \rangle \cdot \frac{1}{(4\pi D\tau)^{-\frac{3}{2}}} \cdot e^{-\frac{(\vec{r}-\vec{r}')^2}{4D\tau}}$$

the autocorrelation function for a single, freely diffusing species of molecules:

$$G(\tau) = \frac{1}{V_{eff}\langle c \rangle} \cdot \frac{1}{1+\frac{\tau}{\tau_D}} \cdot \frac{1}{\sqrt{1+\left(\frac{r_0}{z_0}\right)^2 \cdot \frac{\tau}{\tau_D}}}$$

$$G(0) = \frac{1}{V_{eff}\langle c \rangle} = \frac{1}{\langle N \rangle} \qquad \tau_D = \frac{r_0^2}{4D}$$

$$D = \frac{k \cdot T}{6\pi\eta_v R_h} \qquad \frac{1}{D} \propto \frac{6\pi\eta}{kT} \cdot \sqrt[3]{M}$$

Schwille P, Haustein E. Fluorescence Correlation Spectroscopy. An Introduction to its Concepts and Applications. Biophysics Textbook Online 2004

$$\frac{\partial \delta C(\mathbf{r},t)}{\partial t} = D\nabla^2 \delta C(\mathbf{r},t)$$

$$\delta C(\mathbf{r},t) = \int d\boldsymbol{\rho}\, e^{i\boldsymbol{\rho}\cdot\mathbf{r}} \delta C_f(\boldsymbol{\rho},t)$$

$$\frac{\partial \delta C(\mathbf{r},t)}{\partial t} = \int d\boldsymbol{\rho}\, e^{i\boldsymbol{\rho}\cdot\mathbf{r}} \left\{ -D\rho^2 \delta C_f(\boldsymbol{\rho},t) \right\}$$

$$\delta C(\mathbf{r},t) = \int d\boldsymbol{\rho}\, e^{i\boldsymbol{\rho}\cdot\mathbf{r}} \left\{ \delta C_f(\boldsymbol{\rho},0) e^{-D\rho^2 t} \right\}$$

$$\langle \delta C(\mathbf{r},t)\delta C(\mathbf{r}',0)\rangle = \int d\boldsymbol{\rho}\, e^{i\boldsymbol{\rho}\cdot\mathbf{r}} \left\{ \langle \delta C_f(\boldsymbol{\rho},0)\delta C(\mathbf{r}',0)\rangle e^{-D\rho^2 t} \right\}$$

$$= \int d\boldsymbol{\rho}\, e^{i\boldsymbol{\rho}\cdot\mathbf{r}} \left\{ \frac{1}{2\pi} \int d\mathbf{r}''\, e^{-i\boldsymbol{\rho}\cdot\mathbf{r}''} \langle \delta C(\mathbf{r}'',0)\delta C(\mathbf{r}',0)\rangle e^{-} \right.$$

$$= \frac{\langle C\rangle}{2\pi} \int d\boldsymbol{\rho}\, e^{i\boldsymbol{\rho}\cdot|\mathbf{r}-\mathbf{r}'|} - D\rho^2 t$$

$$\langle \delta C(\mathbf{r},t)\delta C(\mathbf{r}',0)\rangle = \frac{\langle C\rangle}{(4\pi Dt)^{\frac{3}{2}}} e^{-\left(\frac{|\mathbf{r}-\mathbf{r}'|^2}{4Dt}\right)}$$

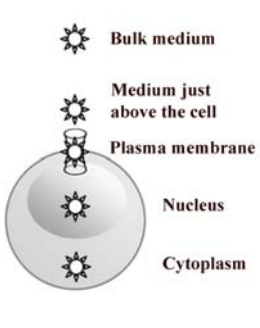
$$G(\tau) = \frac{\iint d\mathbf{r}d\mathbf{r}'\, W(\mathbf{r})W(\mathbf{r}')\langle \delta C(\mathbf{r},\tau)\delta C(\mathbf{r}',0)\rangle}{\left[\langle C\rangle \int d\mathbf{r}\, W(\mathbf{r})\right]^2}$$

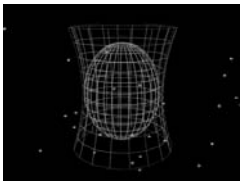
$$G_D(\tau,N,\tau_D) = \frac{\gamma}{\langle N\rangle}\left(\frac{1}{1+\tau/\tau_D}\right)\left(\frac{1}{1+(w_r/w_z)^2\tau/\tau_D}\right)^{1/2}$$

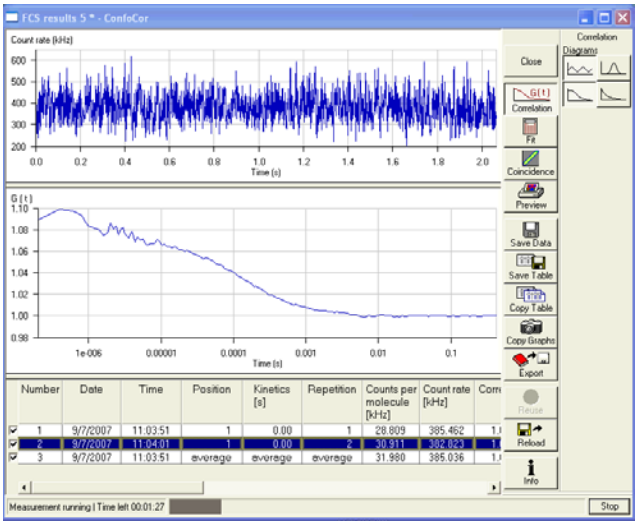
w^2

http://www.cup.uni-muenchen.de/pc/lamb/FCS_Notes/FCS_1.pdf

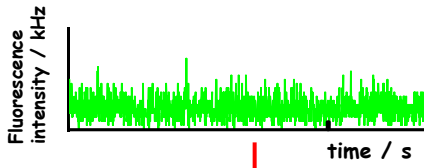
FCS measurement

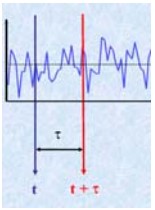






Temporal auto-correlation analysis

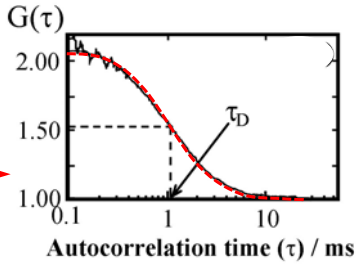




$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

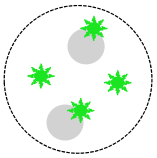
$\delta F(t) = F(t) - \langle F(t) \rangle$

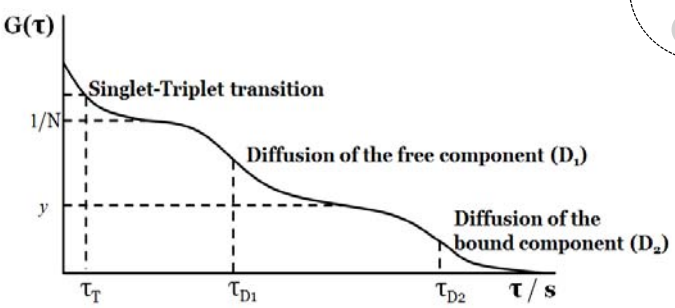
$\delta F(t + \tau) = F(t + \tau) - \langle F(t) \rangle$



$$G(\tau) = 1 + \frac{1}{N} \cdot \frac{1}{\left(1 + \frac{\tau}{\tau_D}\right)\sqrt{1 + \frac{w_{xy}^2}{w_z^2}\frac{\tau}{\tau_D}}}$$

FCS in chemical reaction systems





$$G(\tau) = 1 + \frac{1}{N} \cdot \left(\frac{1-y}{\left(1+\frac{\tau}{\tau_{D1}}\right)\sqrt{1+\frac{w_{xy}^2}{w_z^2}\frac{\tau}{\tau_{D1}}}} + \frac{y}{\left(1+\frac{\tau}{\tau_{D2}}\right)} \right) \cdot \left[1 - F + F \exp\left(\frac{-\tau}{\tau_C}\right) \right]$$

Vukojević et al. Cell Mol Life Sci. 2005 62:535.

FCS in chemical reaction systems

$$A + B^{*} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB^{*}$$

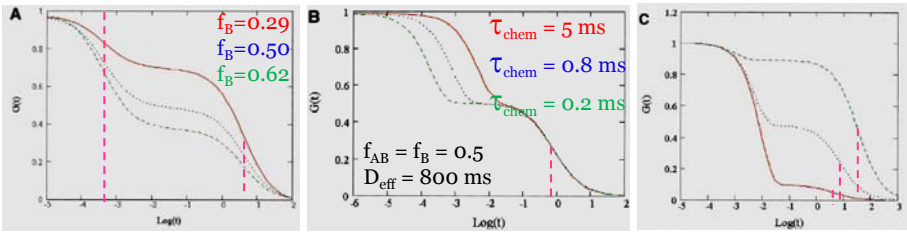
$\tau_{D,B} << \tau_{D,A} \approx \tau_{D,AB}$

$\tau_{chem} = k_{on}(C_A + C_B) + k_{off}$

$R_{chem} = 1/\tau_{chem}$

$f_{AB} = 1 - f_B$

$K = k_{on}/k_{off}$



$\tau_{D,B} << \tau_{chem}$

$\tau_{D,B}$ = 6 ms; τ_{chem} = 30 s

$\omega = 5\times10^{-6}$ m

$D_B = 1.5\times10^{-8}$ m²/s

$D_{AB} = 1.5\times10^{-12}$ m²/s

Elson EL. Traffic 2001 **2:** 789-796

$\tau_{D,B} >> \tau_{chem}$

$D_{eff} = f_B D_B + f_{AB} D_{AB}$

$f_{AB} = KC_A/(1 + KC_A)$

$f_B = 1 - f_{AB}$

Brightness increases 10× upon binding!

τ_{chem} = 1.1 ms f_B = 0.11

τ_{chem} = 5.3 ms f_B = 0.47

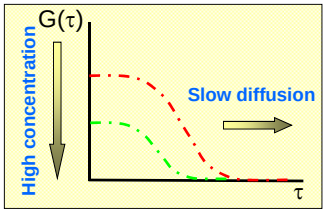
τ_{chem} = 9.3 ms f_B = 0.93

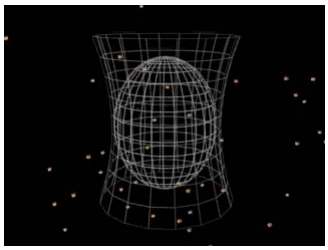
D_{AB} = 35

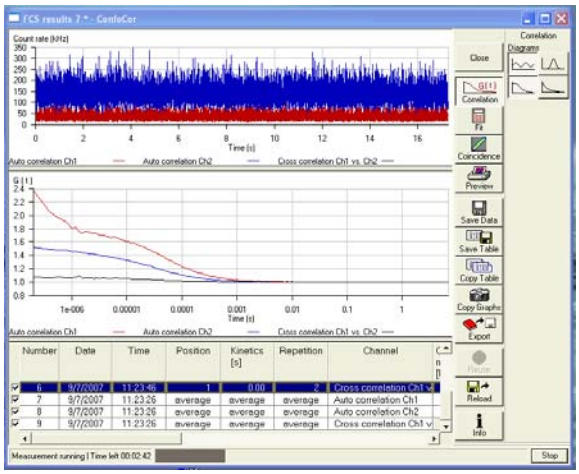
D_{AB} = 7.8

D_{AB} = 4.5

Dual-color FCS, non-interacting molecules







$1/N_{red} \approx 0.6$

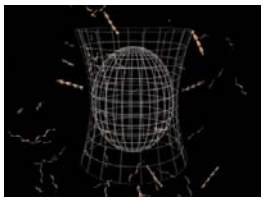
$N_{red} \approx 1.7$

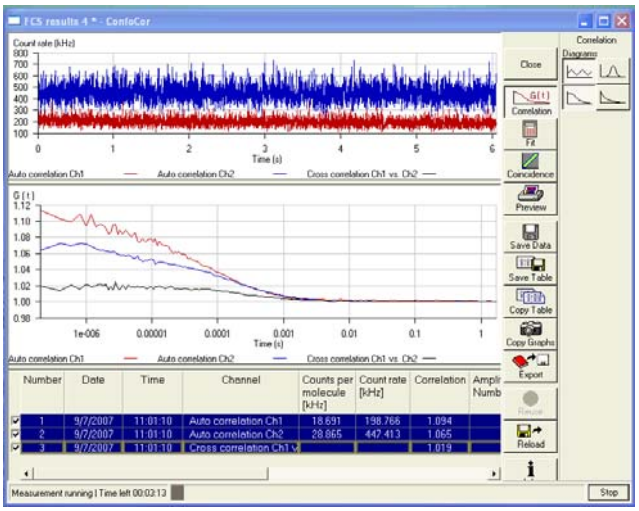
$1/N_{green} \approx 0.3$

$N_{green} \approx 3.3$

$\tau_{D,red} \approx \tau_{D,green}$

Fluorescence Cross-Correlation Spectroscopy





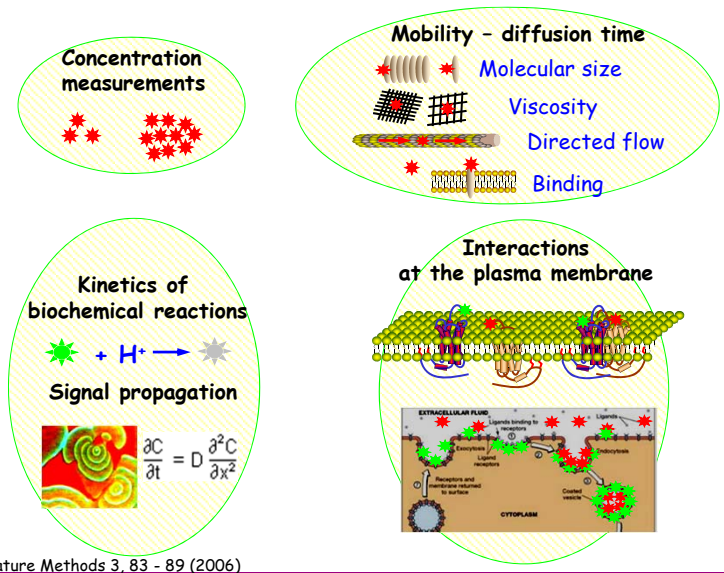
$A_{red+greenred}$ = 0.08
$A_{green+greenred}$ = 0.05
A_{cc} = 0.02

$$\frac{1}{N_{cc}} = \frac{N_{gr}}{(N_{gr}+N_g)(N_{gr}+N_r)}$$

A_{red} = 12.5 – 5 = 7.5
A_{green} = 20 - 5 = 15

$N_{green,red}$ = 5
$\tau_{Dgreen,red}$ = 0.5 ms
K_D = 2.2x10^{-8} M

What can we study by FCS/FCCS?



Bacia et al. Nature Methods 3, 83 - 89 (2006)

Examples of autocorrelation functions

AF1 Two-dimensional diffusion of a single fluorescent species

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

AF2 Free diffusion of a single species and triplet formation

$$G(\tau)=1+\frac{1}{N}\cdot\frac{1}{\left(1+\frac{\tau}{\tau_D}\right)\sqrt{1+\frac{w_{xy}^2}{w_z^2}\frac{\tau}{\tau_D}}}\cdot\left[1+\frac{T}{1-T}\exp-\left(\frac{\tau}{\tau_T}\right)\right]$$

T, average equilibrium fraction of molecules in triplet state
τ_T, triplet correlation time, related to rate constants for intersystem crossing and t

AF3 Free diffusion of unbound ligand and the ligand-receptor complex

$$G(\tau)=1+\frac{1}{N}\cdot\left(\frac{1-y}{\left(1+\frac{\tau}{\tau_{D_1}}\right)\sqrt{1+\frac{w_{xy}^2}{w_z^2}\frac{\tau}{\tau_{D_1}}}}+\frac{y}{\left(1+\frac{\tau}{\tau_{D_2}}\right)\sqrt{1+\frac{w_{xy}^2}{w_z^2}\frac{\tau}{\tau_{D_2}}}}\right)$$

y, fraction of ligand bound to the receptor
τ_{D_1}, diffusion time of the unbound ligand
τ_{D_2}, diffusion time of the bound ligand

AF4 Free diffusion of unbound ligand and diffusion of a ligand bound to a surface

$$G(\tau)=1+\frac{1}{N}\cdot\left(\frac{1-y}{\left(1+\frac{\tau}{\tau_{D_1}}\right)\sqrt{1+\frac{w_{xy}^2}{w_z^2}\frac{\tau}{\tau_{D_1}}}}+\frac{y}{\left(1+\frac{\tau}{\tau_{D_2}}\right)}\right)$$

y, fraction of bound ligand
τ_{D_1}, diffusion time of the unbound ligand
τ_{D_2}, diffusion time of the bound ligand

AF5 Concentration fluctuations around a chemical equilibrium coupled with diffusion

$$G(\tau)=1+\frac{1}{N(1-F)}\cdot\frac{1}{\left(1+\frac{\tau}{\tau_D}\right)\sqrt{1+\frac{w_{xy}^2}{w_z^2}\frac{\tau}{\tau_D}}}\cdot\left[1-F+F\exp\left(\frac{-\tau}{\tau_C}\right)\right]$$

The chemical reaction analyzed is AB ⇔ A* + B; A* is the only fluorescent species
F, average fraction of molecules in the non-fluorescent state AB
τ_C, chemical relaxation time from which rate constants are derived

AF6 Restricted (anomalous) diffusion due to interactions with fixed or mobile structures

$$G(\tau)=1+\frac{1}{N}\cdot\frac{1}{\left(1+\left(\frac{\tau}{\tau_D}\right)^{\alpha}\right)\sqrt{1+\frac{w_{xy}^2}{w_z^2}\left(\frac{\tau}{\tau_D}\right)^{\alpha}}}$$

α, restriction coefficient; for free (Brownian) diffusion $\alpha = 1$, and for restricted diffusion $\alpha < 1$

AF7 Active transport

$$G(\tau)=1+\frac{1}{N}\cdot\exp\left(-\left(\tau\cdot\frac{v}{w_{xy}}\right)^2\right)$$

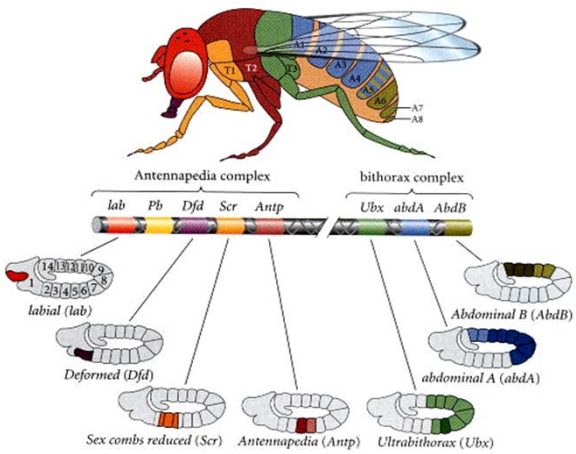
v, velocity of active radial transport

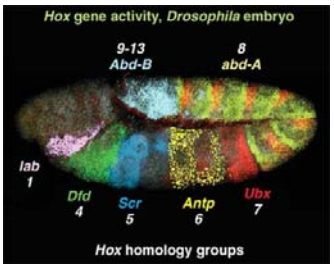
AF8 Mixed mode diffusion – active transport coupled with diffusion

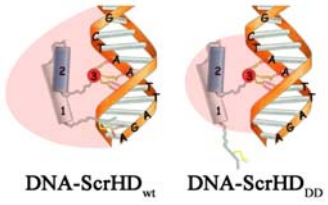
$$G(\tau)=1+\frac{1}{N}\cdot\frac{1}{\left(1+\frac{\tau}{\tau_D}\right)\sqrt{1+\frac{w_{xy}^2}{w_z^2}\frac{\tau}{\tau_D}}}\cdot\exp\left(-\left(\tau\cdot\frac{v}{w_{xy}}\right)^2\cdot\frac{1}{\left(1+\frac{\tau}{\tau_D}\right)}\right)$$

Vukojević et al. Cell Mol Life Sci. 2005 62:535.

Scr transcription factor

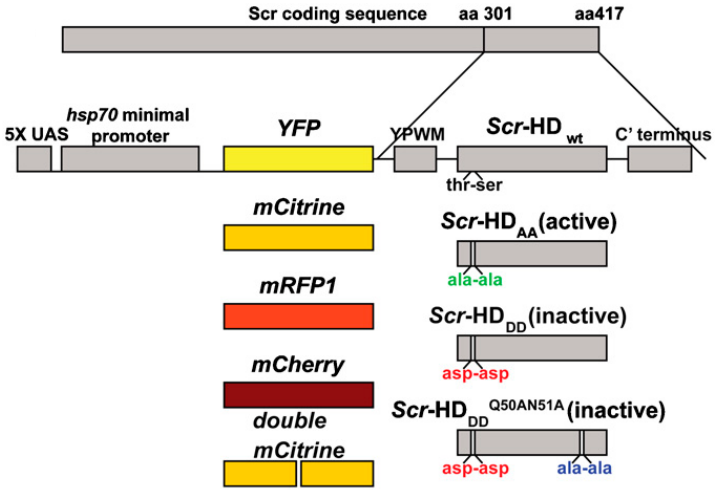






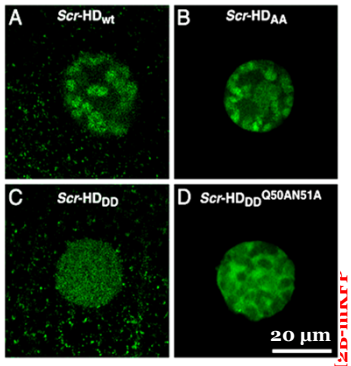
http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=dbio&part=A1971
http://scienceblogs.com/pharyngula/upload/2006/09/septuple_hox.jpg

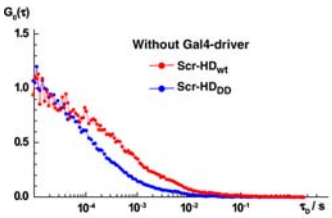
Model development

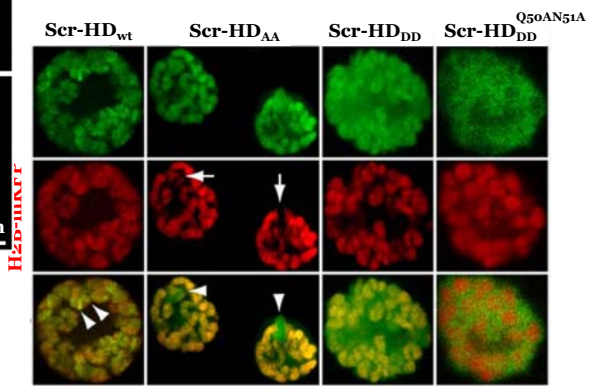


Vukojević et al. PNAS 2010 107:4093-4098.
Papadopoulos et al. PNAS 2010 107:4087-4092.

Model validation by fluorescence imaging and FCS

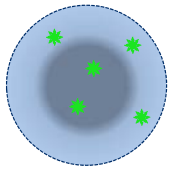


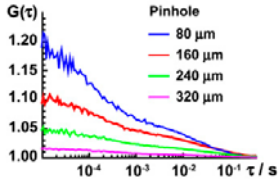


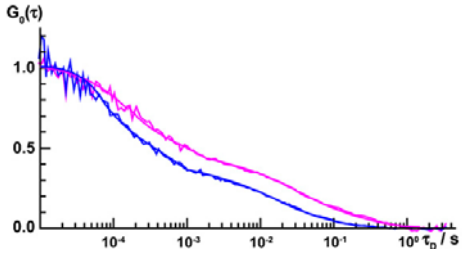


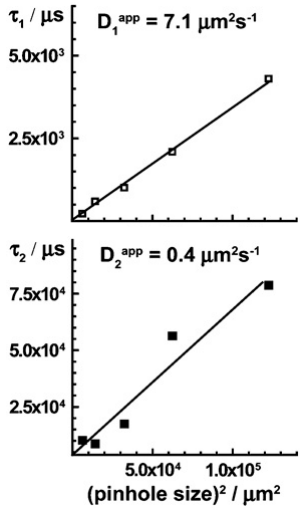
Vukojević et al. PNAS 2010 107:4093-4098. Papadopoulos et al. PNAS 2010 107:4087-4092.

Pinhole size scan – distinguishing diffusion from chemical kinetics





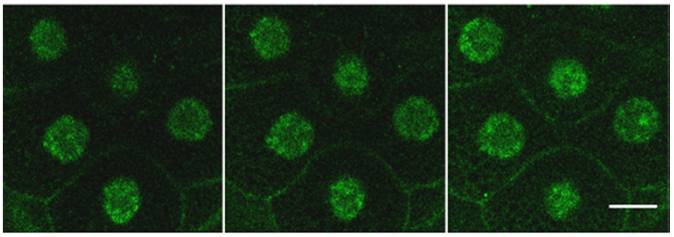


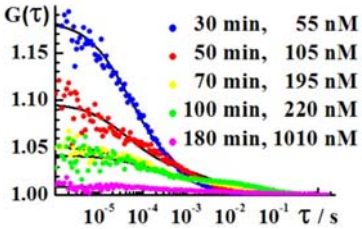


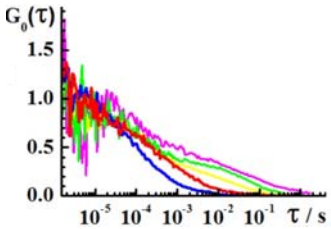
Vukojević et al. PNAS 2010 **107:**4093-4098.
Elson E. Traffic 2001 **2:** 789-796

Heat shock-induced increase in *Scr* expression

30 min **70 min** **140 min**

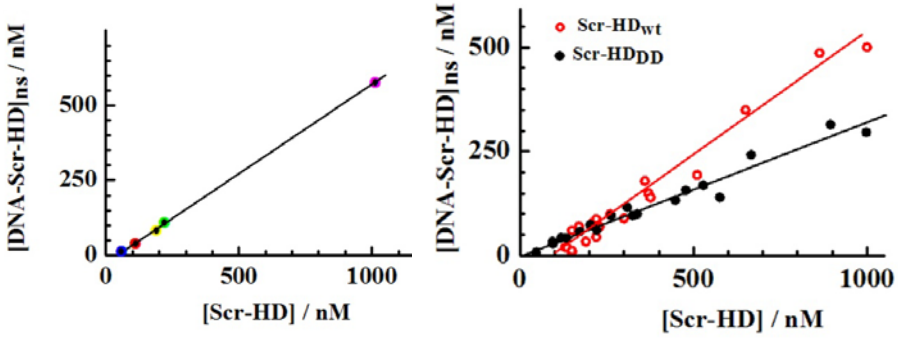






Vukojević et al. PNAS 2010 107:4093-4098.

Scr-HD$_{wt}$ and Scr-HD$_{DD}$ "binding curves"



Vukojević et al. PNAS 2010 **107:**4093-4098.

Kinetics of nonspecific and specific binding

$$DNA + SCR_{DD} \underset{k_{-ns}}{\overset{k_{ns}}{\Longleftrightarrow}} DNA - SCR_{DD}$$

$$[DNA - ScrHD_{DD}] = \frac{k_{ns} \cdot [DNA]_0}{(k_{-ns} + k_{ns} \cdot [DNA]_0)} \cdot [ScrHD_{DD}]_0$$

$$\frac{k_{ns} \cdot [DNA]_0}{(k_{-ns} + k_{ns} \cdot [DNA]_0)} = (0.35 \pm 0.15)$$

$$DNA + SCR_{wt} \underset{k_{-ns}}{\overset{k_{ns}}{\Longleftrightarrow}} (DNA - SCR_{wt})_{ns} + DNA_s \underset{k_{-s}}{\overset{k_s}{\Longleftrightarrow}} (DNA - SCR_{wt})_s$$

$$[(DNA - SCR_{wt})_{ns}] = \frac{k_{ns} \cdot [DNA]_0}{k_{-ns} + k_s + k_{ns} \cdot [DNA]_0} \cdot [SCR]_0 - \frac{k_{ns} \cdot [DNA]_0 - k_{-s}}{k_{-ns} + k_s + k_{ns} \cdot [DNA]_0} \cdot [(DNA - SCR_{wt})_s]$$

$$\frac{k_{ns} \cdot [DNA]_0}{k_{-ns} + k_s \cdot [DNA_s] + k_{ns} \cdot [DNA]_0} = (0.62 \pm 0.08) \qquad \frac{k_{ns} \cdot [DNA]_0 - k_{-s}}{k_{-ns} + k_s \cdot [DNA_s] + k_{ns} \cdot [DNA]_0} \cdot [(DNA - ScrHD_{wt})_s] = (60 \pm 40)\ nM$$

Estimation of the number of binding sites and dissociation constants

TAATCG or TAATGG

$$C_{\text{sites}}^{\text{non-specific}} = \frac{n}{V_{\text{nucleus}}} = \frac{1.94 \times 10^{-16}}{4.187 \times 10^{-12}} = 46\ \mu\text{M}$$

$$K_d^{ScrHD_{DD},ns} = (80 \pm 50)\ \mu M$$

$$K_d^{wt,ns} = (25 \pm 15)\ \mu M$$

AGATTAATCG

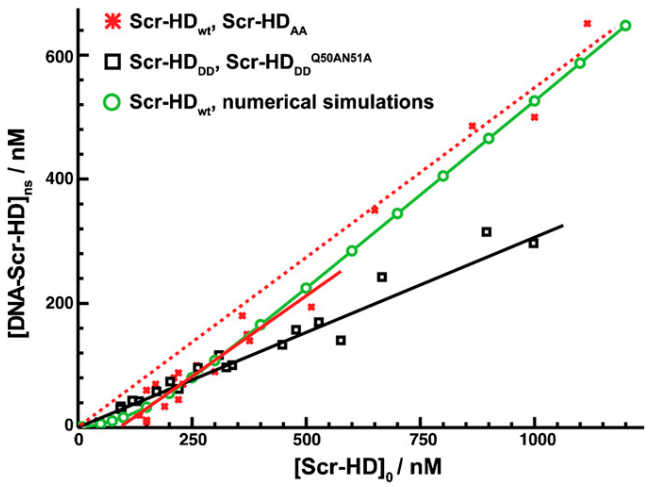
$$C_{\text{sites}}^{\text{specific}} = \frac{n}{V_{\text{nucleus}}} = \frac{0.578 \times 10^{-18} \times 0.665}{4.187 \times 10^{-12}} = 92\ \text{nM}$$

$$K_d^{wt,s} = (7 \pm 5)\ nM$$

$$\left[\left(DNA - ScrHD_{wt}\right)_s\right] = (80 \pm 50)\ nM$$

Vukojević et al. PNAS 2010 **107:**4093-4098.

Simulation of Scr-HD binding to nuclear DNA



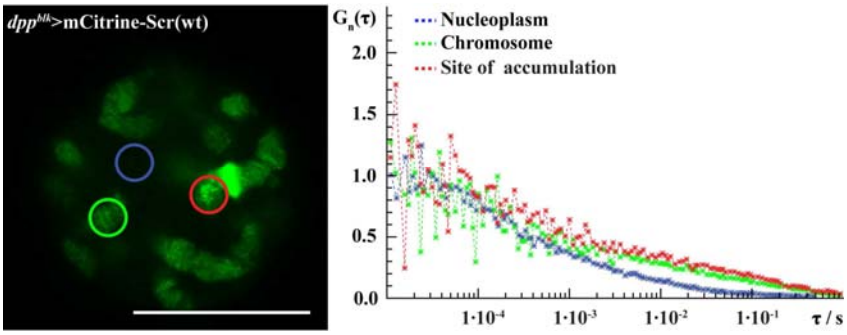
Vukojević et al. PNAS 2010 107:4093-4098.

Iso-electric points for Scr-HD variants at different pH

pH	Charge			
	mCitrine-Scr-HD_{wt}	mCitrine-Scr-HD_{AA}	mCitrine-Scr-HD_{DD}	mCitrine-Scr-$HD_{DD}^{Q50AN51A}$
7.00	7.2	7.2	5.2	5.2
7.10	6.4	6.4	4.4	4.4
7.20*	**5.7**	**5.7**	**3.7**	**3.7**
7.30*	**5.1**	**5.1**	**3.1**	**3.1**
7.40*	**4.5**	**4.5**	**2.5**	**2.5**
7.50*	**4.0**	**4.0**	**2.0**	**2.0**
7.60*	**3.5**	**3.5**	**1.5**	**1.5**
7.70*	**3.1**	**3.1**	**1.1**	**1.1**
7.80*	**2.7**	**2.7**	**0.7**	**0.7**
7.90*	**2.3**	**2.3**	**0.3**	**0.3**
8.00*	**1.9**	**1.9**	**-0.1**	**-0.1**
8.10*	**1.5**	**1.5**	**-0.5**	**-0.5**
8.20*	**1.0**	**1.0**	**-1.0**	**-1.0**
8.30	0.5	0.5	-1.5	-1.5
8.40	0.0	0.0	-2.0	-2.0
8.50	-0.5	-0.5	-2.5	-2.5
8.60	-1.1	-1.1	-3.1	-3.1
8.70	-1.8	-1.8	-3.8	-3.8
8.80	-2.6	-2.6	-4.6	-4.6
8.90	-3.4	-3.4	-5.4	-5.4

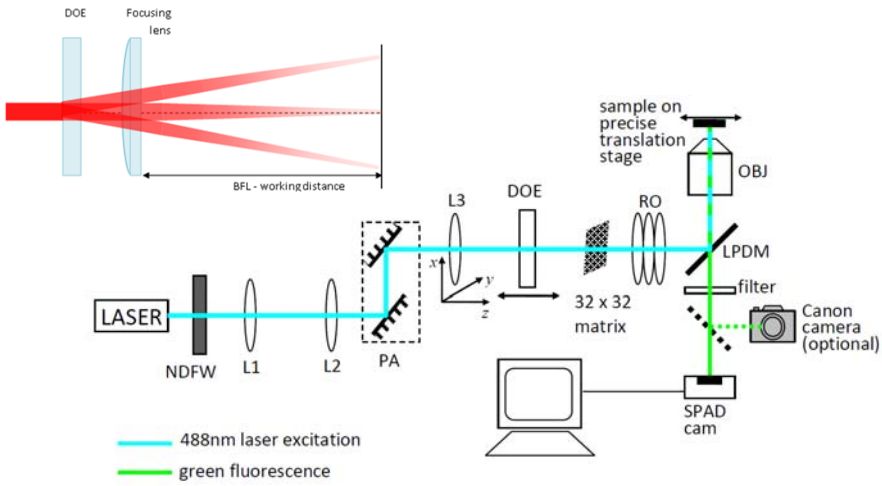
Iso-electric points estimated using the Protein Calculator v3.3
(http://www.scripps.edu/cgi-bin/cdputnam/protcalc3)
*Experimentally measured pH in salivary gland cells isolated from third instar larvae
(Schneider S., Wiinsch S., Schwab A., and Oberleithney H. (1996) Mol Cell Endocrinol **116**, 73-79)

Limitations of single-point FCS



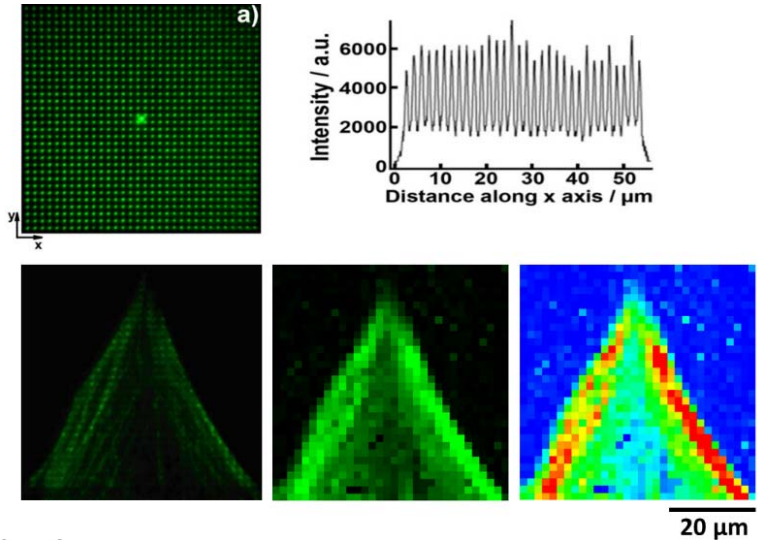
Vukojević et al. PNAS 2010 107:4093-4098.
Papadopoulos et al. Mech Dev. 2015 pii: S0925-4773(15)30029-0.

Overcoming the limited overview of classical FCS by massively parallel FCS



Vitali *et al.* IEEE J. Select. Topics Quantum Electron. 2014, *in press*
Krmpot et al., submitted

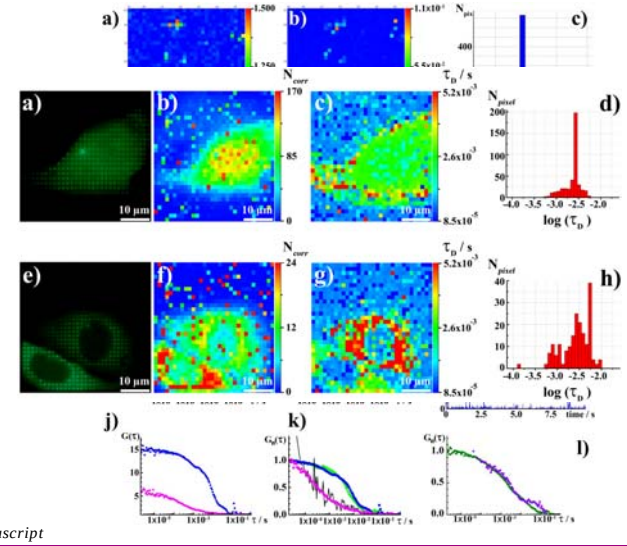
Fast confocal imaging without scanning



Krmpot et al. *manuscript*

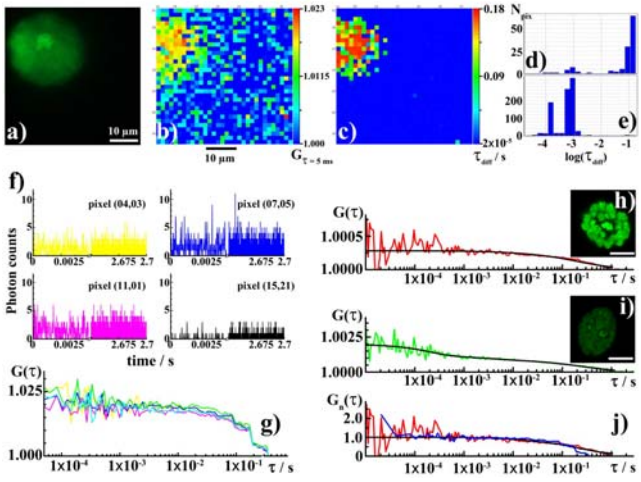
Quantitative confocal fluorescence microscopy imaging of quantum dots in aqueous suspension

7×10^{-10} M



Krmpot et al. *manuscript*

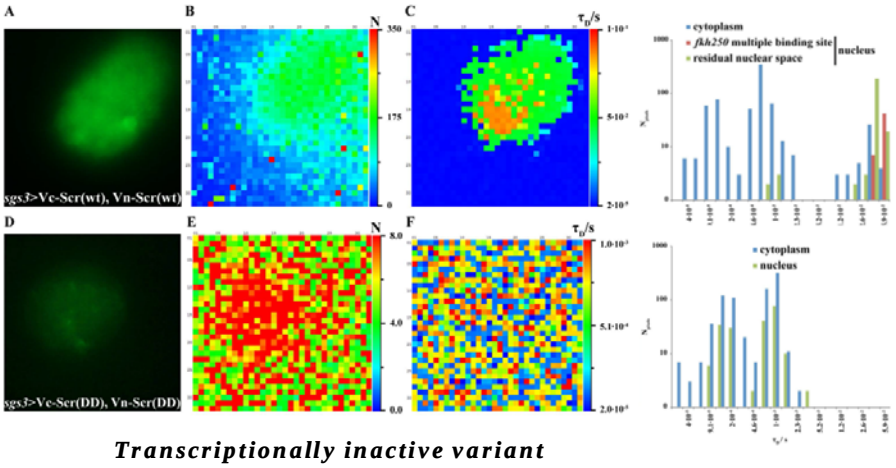
Quantitative fluorescence microscopy imaging *via* massively parallel FCS



Krmpot et al. submitted

Quantitative fluorescence microscopy imaging of transcription factor activity by mpFCS

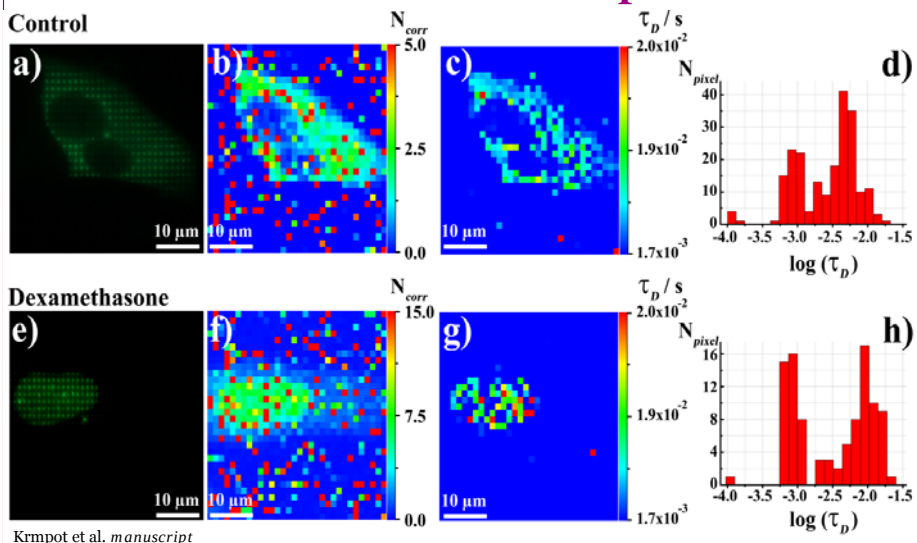
Transcriptionally active variant



Transcriptionally inactive variant

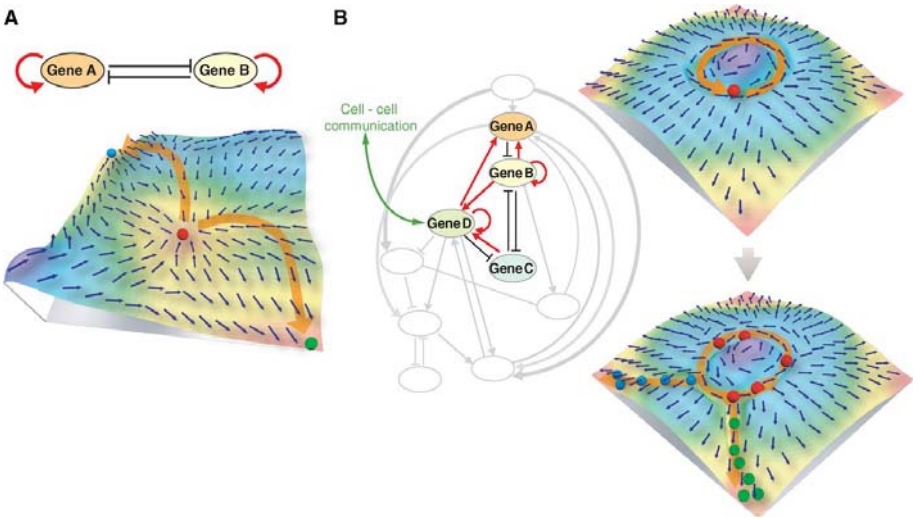
Papadopoulos et al. Mech Dev. 2015 pii: S0925-4773(15)30029-0.

Ligand-induced glucocorticoid receptor (GR) internalization *via* mpFCS



Krmpot et al. *manuscript*

Dynamical gene transcription landscapes



Chikara Furusawa, Kunihiko Kaneko, A Dynamical-Systems View of Stem Cell Biology. Science 2012, 338:6104 pp. 215-217 , DOI: 10.1126/science.1224311

Your imagination sets the limits!