Fundamentals of Fluctuation Spectroscopy II:

Multiple Species
Reactions
Scanning FCS

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Autocorrelation Function, Multiple Species

The fluorescence intensity for multiple *non-interacting* particles is given by:

$$F(t) = \sum_{i=1}^{M} \kappa_i Q_i \int d\mathbf{r}\, W(\mathbf{r}) C_i(\mathbf{r},t)$$

The ACF is given by:

$$G(\tau) = \frac{\sum_{i=1}^{M} \sum_{j=1}^{M} \kappa_i Q_i \kappa_j Q_j \iint d\mathbf{r} d\mathbf{r}'\, W(\mathbf{r}) W(\mathbf{r}') \left\langle \delta C_i(\mathbf{r},\tau) \delta C_j(\mathbf{r}',0) \right\rangle}{\left[\sum_{i=1}^{M} \kappa_i Q_i \left\langle C_i \right\rangle \int d\mathbf{r}\, W(\mathbf{r}) \right]^2}$$

$$\left\langle \delta C_i(\mathbf{r},\tau) \delta C_j(\mathbf{r}',0) \right\rangle = \delta_{ij} \left\langle \delta C_i(\mathbf{r},\tau) \delta C_i(\mathbf{r}',0) \right\rangle$$

$$G(\tau) = \frac{\sum_{i=1}^{M} \kappa_i^2 Q_i^2 \iint d\mathbf{r} d\mathbf{r}'\, W(\mathbf{r}) W(\mathbf{r}') \left\langle \delta C_i(\mathbf{r},\tau) \delta C_i(\mathbf{r}',0) \right\rangle}{\left[\sum_{i=1}^{M} \kappa_i Q_i \left\langle C_i \right\rangle \int d\mathbf{r}\, W(\mathbf{r}) \right]^2}$$

$$G(\tau) = \sum_{i=1}^{M} \Im_i^2\, G_{Di}\left(\tau, N_i, \tau_{D_i}\right)$$

where

$$G_{D_i}(N_i, D_i, \tau) = \frac{\gamma}{\left\langle N_i \right\rangle} \left(\frac{1}{1+\tau/\tau_{D_i}} \right) \left(\frac{1}{1+\left(w_r/w_z\right)^2 \tau/\tau_{D_i}} \right)^{1/2}$$

$$\Im_i = \frac{\kappa_i Q_i \left\langle C_i \right\rangle}{\sum_{k=1}^{M} \kappa_k Q_k \left\langle C_k \right\rangle} = \frac{\varepsilon_i \left\langle N_i \right\rangle}{\sum_{k=1}^{M} \varepsilon_k \left\langle N_k \right\rangle} \quad \text{and} \quad \tau_{D_i} = \frac{{w_r}^2}{4D_i}$$

Measurements in a Fluorescence Background

Situation: Large number of weakly fluorescencing particles:

i.e. Although $Q_S >> Q_B$, $N_S << N_B \Rightarrow$

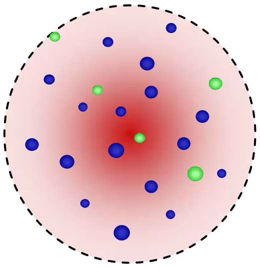
$$G_B(0)=\left(\frac{\gamma}{\langle N_B\rangle}\right) << \left(\frac{\gamma}{\langle N_S\rangle}\right)=G_S(0)$$

$$G(\tau)_{eff}=\Im_S^2 G_{Diff}\left(\tau, N_S, \tau_{D_S}\right)+\cancelto{0}{\Im_B^2 G_{Diff}\left(\tau, N_B, \tau_{D_B}\right)}$$

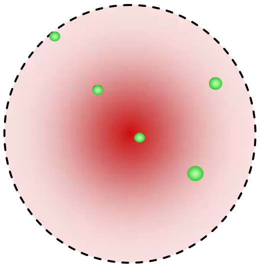
$$G(\tau)_{eff}=\Im_S^2 G_{Diff}\left(\tau, N_S, \tau_{D_S}\right)$$

The amplitude of the fluctuations are diminished, hence the amplitude of the autocorrelation function is reduced by the square of the fractional intensity

With Background



Without Background



Multi-background Components

A background made of multiple components can be treated as a single component.

$$G_B(\tau) = \left(\frac{I_{B1}}{I_{B1}+I_{B2}}\right)^2 G_{B1}(\tau) + \left(\frac{I_{B2}}{I_{B1}+I_{B2}}\right)^2 G_{B2}(\tau)$$

$$G_{Meas}(\tau) = \left(\frac{I_S}{I_S+I_{B1}+I_{B2}}\right)^2 G_S(\tau) + \left(\frac{I_{B1}}{I_S+I_{B1}+I_{B2}}\right)^2 G_{B1}(\tau) + \left(\frac{I_{B2}}{I_S+I_{B1}+I_{B2}}\right)^2 G_{B2}(\tau)$$

$$G_{Meas}(\tau) = \left(\frac{I_S}{I_S+I_{B1}+I_{B2}}\right)^2 G_S(\tau) + \left(\frac{I_{B1}+I_{B2}}{I_S+I_{B1}+I_{B2}}\right)^2 \left[\left(\frac{I_{B1}}{I_{B1}+I_{B2}}\right)^2 G_{B1}(\tau) + \left(\frac{I_{B2}}{I_{B1}+I_{B2}}\right)^2 G_{B2}(\tau)\right]$$

$$G_{Meas}(\tau) = \left(\frac{I_S}{I_S+I_B}\right)^2 G_S(\tau) + \left(\frac{I_B}{I_S+I_B}\right)^2 G_B(\tau)$$

where $I_B = I_{B1} + I_{B2}$

High-Order Autocorrelation Functions

Definition of high order ACF:

$$G_{m,n}(\tau) = \frac{\left\langle \delta A^{m}(t+\tau)\,\delta A^{n}(t)\right\rangle - \left\langle \delta A^{m}(t)\right\rangle\left\langle \delta A^{n}(t)\right\rangle}{\left\langle A(t)\right\rangle^{m+n}}$$

For equilibrium measurements, $G_{m,n}$ is an even function,

$$G_{m,n} = G_{n,m}$$

The first, non-trivial correlation function is

$$G_{1,1}(\tau) = \frac{\left\langle \delta A(t+\tau)\,\delta A(t)\right\rangle}{\left\langle A(t)\right\rangle^{2}}$$

$$G_{1,1}(0) = 2h_2B_2$$

$$G_{1,2}(0) = 4h_3B_3$$

$$G_{2,2}(0) = 8h_4B_4 + 8h_2^2B_2^2$$

$$G_{1,3}(0) = 8h_4B_4 + 12\,h_2^2B_2^2$$

where

$$B_n = \frac{\sum_{i=1}^{M}\varepsilon_i^n\left\langle N_i\right\rangle}{\left[\sum_{i=1}^{M}\varepsilon_i\left\langle N_i\right\rangle\right]^k} \quad ; \quad h_n = \frac{\int d\mathbf{r}\left(W(\mathbf{r})/W(0)\right)^n}{\left[\int d\mathbf{r}\left(W(\mathbf{r})/W(0)\right)\right]}$$

and

$$\varepsilon_i = \kappa_i Q_i W(0)$$

High-Order Autocorrelation Functions

The high-order autocorrelation amplitudes provide:

- number densities
- molecular brightness

for measurements with multiple species

$$\varepsilon_2 = \varepsilon_1 \frac{z^2 B_3 - z B_2}{z B_2 - 1}$$

$$\langle N_2 \rangle = \frac{z\left(z B_2 - 1\right)\varepsilon_1^2}{\left[\varepsilon_2\left(\varepsilon_2 - \varepsilon_1\right)\right]}$$

$$\langle N_1 \rangle = z - \frac{\varepsilon_2}{\varepsilon_1}\langle N_2 \rangle$$

$$\varepsilon_1 = \frac{z\varepsilon_2 \langle N_2 \rangle}{\left(1 - z\right)\langle N_1 \rangle}$$

where

$$z = \frac{\left\{\left(B_4 - B_2 B_3\right) \pm \left[\left(B_2 B_3 - B_4\right)^2 - 4\left(B_2^2 - B_3\right)\left(B_3^2 - B_2 B_4\right)\right]^{\frac{1}{2}}\right\}}{2\left(B_3^2 - B_2 B_4\right)}$$

The same parameters,

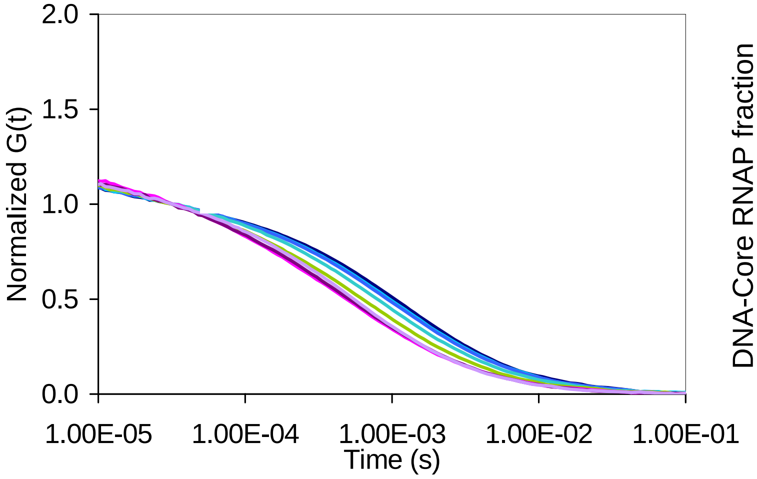
$\langle N_1 \rangle$, ε_1, $\langle N_2 \rangle$, ε_2

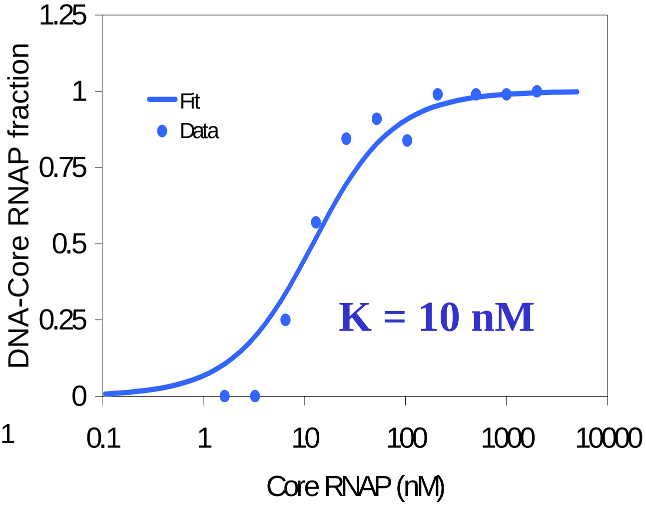
can also be determined using PCH analysis

Molecular Interactions

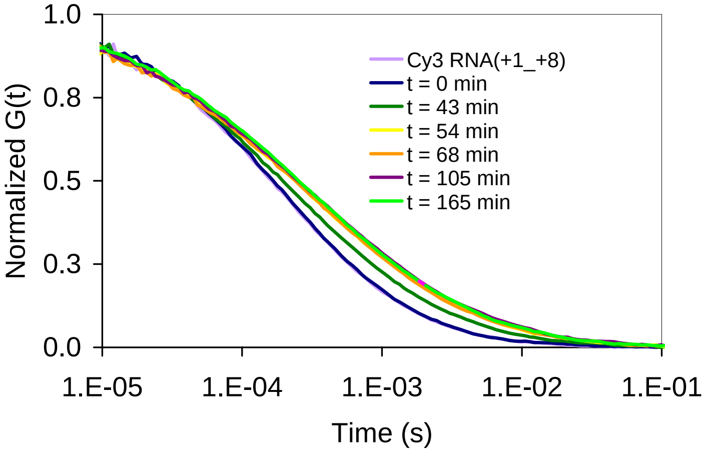
Titrations and slow reactions resulting in a change in the diffusion constant changes can be followed using FCS

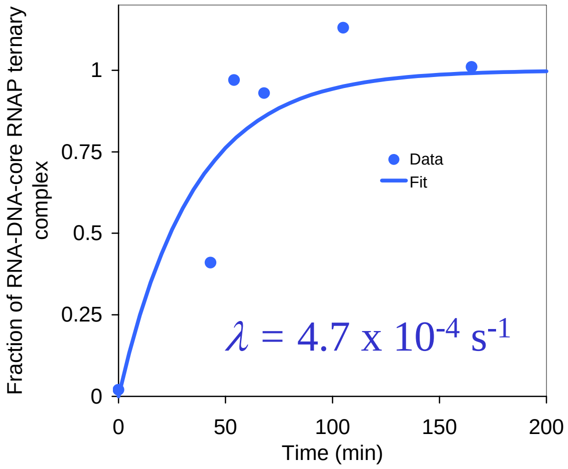
Titration of RNA Polymerase and DNA w/ 9 bp Artificial Bubble





Ternary Complex Formation (RNA Polymerase, DNA, RNA)



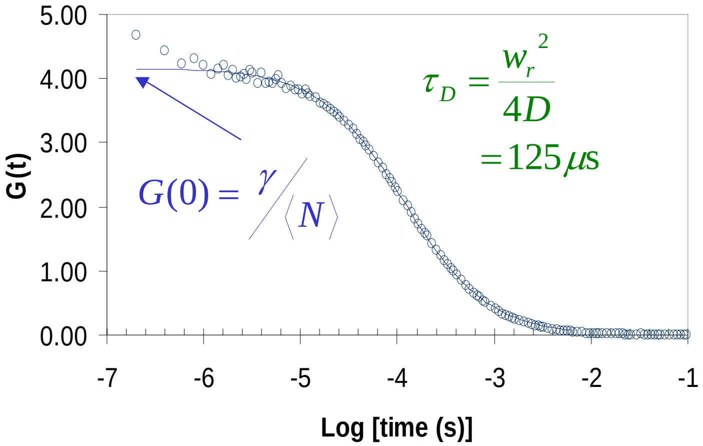


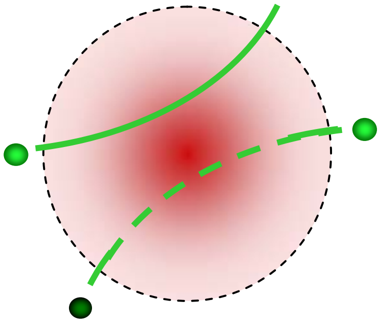
Reaction Kinetics

From freely diffusing particles,
we can determine:

Average number of molecules or concentration

Diffusion coefficients or PSF





Fluctuations *also* arise from:

Triplet states

Conformational Motions

Chemical reactions

Unimolecular

$$A_1 \underset{k_-}{\overset{k_+}{\Leftrightarrow}} A_2$$

Bimolecular

$$M + L \underset{k_-}{\overset{k_+}{\Leftrightarrow}} ML$$

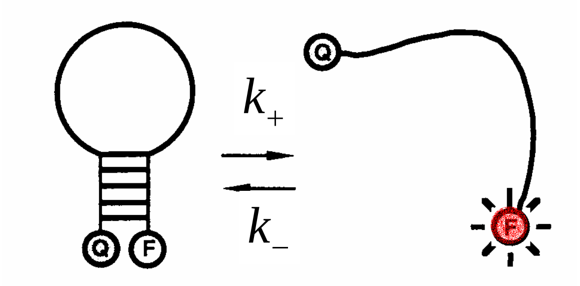
We can determine:

The equilibrium coefficient, $K = k_+ / k_-$, and reaction rate $\lambda = k_+ + k_-$.

Hence, the microscopic rate coefficients,

➡ k_+ and k_-

Unimolecular Reaction



$$A \overset{k_+}{\underset{k_-}{\Leftrightarrow}} B$$

$$\left\langle \delta C_i(\mathbf{r},\tau)\delta C_j(\mathbf{r}',0)\right\rangle \neq \delta_{ij}\left\langle \delta C_i(\mathbf{r},\tau)\delta C_i(\mathbf{r}',0)\right\rangle$$

$$\frac{\partial}{\partial t}\begin{pmatrix} C_A(\mathbf{r},t) \\ C_B(\mathbf{r},t)\end{pmatrix} = \begin{pmatrix} D_A\nabla^2 - k_+ & k_- \\ k_+ & D_B\nabla^2 - k_- \end{pmatrix}\begin{pmatrix} C_A(\mathbf{r},t) \\ C_B(\mathbf{r},t)\end{pmatrix}$$

Solve the equation for the Eigenvalues

For $D_A = D_B = D$, the ACF has a closed form.

$$\left\langle \delta C_k(\mathbf{r},\tau)\delta C_\ell(\mathbf{r}',0)\right\rangle = F_{\mathbf{r}'}^{-1}\left\{\sum_s \mathbf{X}_\ell^{(s)}\, e^{-\nu^2 D+\lambda^{(s)}\tau} \sum_j \mathbf{X}_j^{-1(s)} F_\nu\left\{\left\langle \delta C_k(\mathbf{r},0)\delta C_j(\mathbf{r}'',0)\right\rangle\right\}\right\}$$

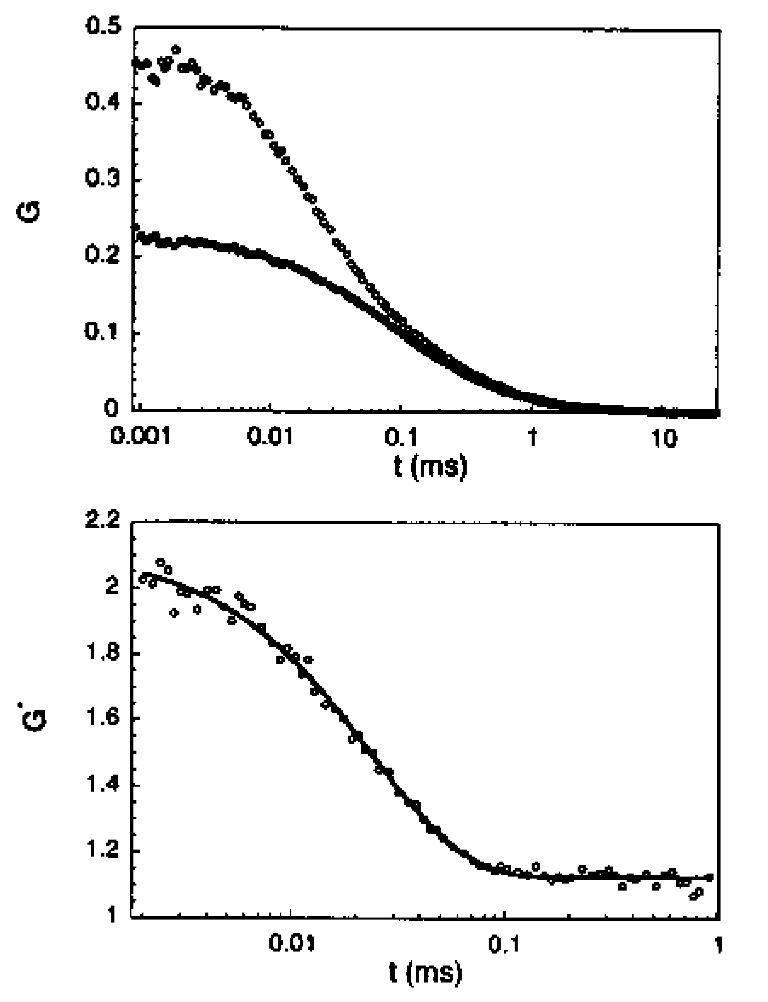
$$= \frac{\left\langle C_\ell\right\rangle e^{-\frac{(\mathbf{r}-\mathbf{r}')^2}{4D\tau}}}{(4\pi D\tau)^{\frac{3}{2}}}\left\{\sum_s \mathbf{X}_\ell^{(s)}\, e^{\lambda^{(s)}\tau}\ \mathbf{X}_k^{-1(s)}\right\}$$

where $-\nu^2 D+\lambda^{(s)}$ are the Eigenvalues

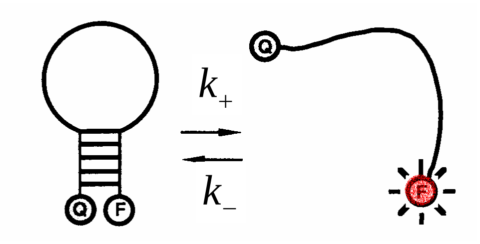
$$G(\tau) = G_D\left(N_A + N_B, D, \tau\right)\left[1 + K\left(\Im_A - \frac{\Im_B}{K}\right)^2 e^{-\lambda\tau}\right]$$

where $K = k_+ / k_-$ and $\lambda = k_+ + k_-$

Conformational Fluctuations of DNA



Bonnet, Krichevsky,
Libchaber *PNAS* (1998)
95:8602



$$G_b(\tau) = G_{b,Diffusion}(\tau)\left(1 + \frac{1}{K}\exp(-\lambda\tau)\right)$$

$$G_c(\tau) = G_{c,Diffusion}(\tau)$$

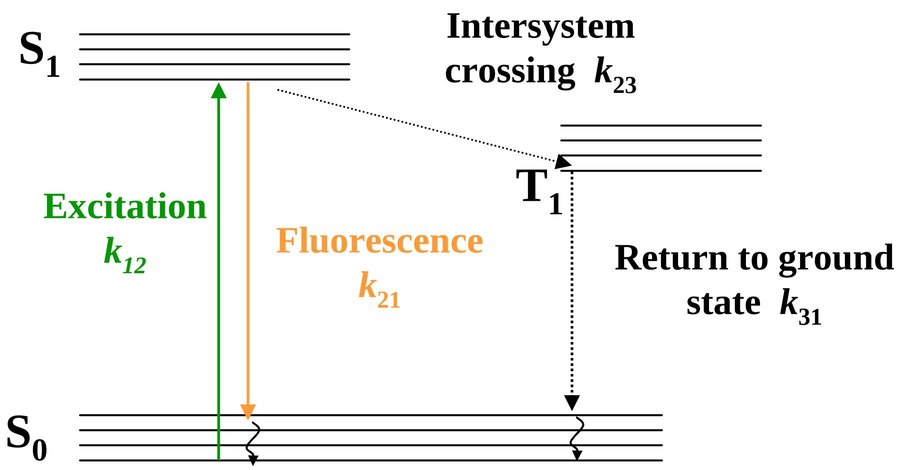
Diffusion term drops out of the ratio

$$\frac{G_b(\tau)}{G_c(\tau)} = \frac{G_b(0)}{G_c(0)}\left(1 + \frac{1}{K}\exp(-\lambda\tau)\right)$$

$$\lambda = k_+ + k_-$$

$$1/\lambda = 24.2\ \mu s$$

Triplet State



If we ignore the spatial distribution of k_{12}

$$\frac{d}{dt}\begin{pmatrix} S_0(t) \\ S_1(t) \\ T_1(t) \end{pmatrix} = \begin{pmatrix} -k_{12} & k_{21} & k_{31} \\ k_{12} & -(k_{23}+k_{21}) & 0 \\ 0 & k_{23} & -k_{31} \end{pmatrix}\begin{pmatrix} S_0(t) \\ S_1(t) \\ T_1(t) \end{pmatrix}$$

$$g(\tau) = 1 + \frac{1}{N^*}\left(\frac{1}{1+4D\tau/w_1^2}\right)\left(\frac{1}{1+4D\tau/w_2^2}\right)^{1/2}\left[1 - T_{eq} + T_{eq}\, e^{-\lambda\tau}\right]$$

from Widengren, Rigler, Mets, *J. Fluorescence* 1994 **4**:255

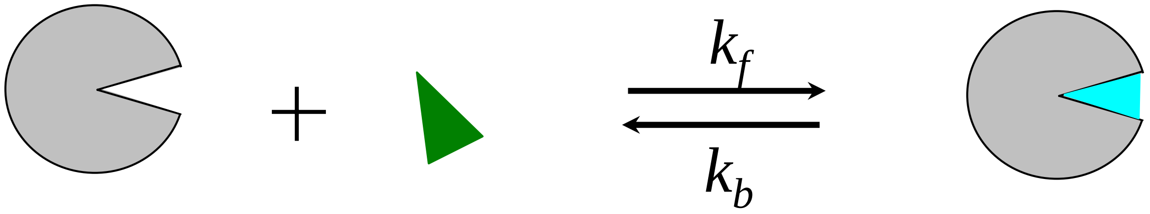
$$\lambda = -k_{31} - \frac{k_{23}k_{12}}{k_{12}+k_{21}}$$

N* is the number of molecules in the singlet states

Normalizing to the total number of molecules, N, and switching to familiar notation

$$G(\tau) = G_D(N, D, \tau)\left[1 + \frac{T_{eq}}{1-T_{eq}}e^{-\lambda\tau}\right]$$

Bimolecular Processes



$$M + L \underset{k_b}{\overset{k_f}{\Leftrightarrow}} ML$$

$$\frac{\partial}{\partial t}\begin{pmatrix} C_M(\mathbf{r},t) \\ C_L(\mathbf{r},t) \\ C_{ML}(\mathbf{r},t) \end{pmatrix} = \begin{pmatrix} D_M\nabla^2 - k_f\langle C_L\rangle & -k_f\langle C_M\rangle & k_b \\ -k_f\langle C_L\rangle & D_L\nabla^2 - k_f\langle C_M\rangle & k_b \\ k_f\langle C_L\rangle & k_f\langle C_M\rangle & D_{ML}\nabla^2 - k_b \end{pmatrix}\begin{pmatrix} C_M(\mathbf{r},t) \\ C_L(\mathbf{r},t) \\ C_{ML}(\mathbf{r},t) \end{pmatrix}$$

For $D_M = D_{ML} >> D_L$ and $\langle C_L\rangle >> \langle C_M\rangle$, it is possible to solve the ACF in a closed form

$$G(\tau) = G_D\left(N_M + N_{ML}, D, \tau\right)\left[\left(\mathfrak{I}_M + \mathfrak{I}_{ML}\right)^2 + K\langle C_L\rangle\left(\mathfrak{I}_M - \frac{\mathfrak{I}_{ML}}{K\langle C_L\rangle}\right)^2 e^{-\lambda\tau}\right]$$

$$+ G_D\left(N_L, D_L, \tau\right)\mathfrak{I}_L^2.$$

where $K = k_f / k_b$ and $\lambda = k_f\left(\langle C_M\rangle + \langle C_L\rangle\right) + k_b$

Bimolecular Autocorrelation Function

If $\langle C_L \rangle \sim \langle C_M \rangle$, then it is necessary to assume

$$D_M = D_{ML} << D_L$$

$$\lambda >> 1/\tau_D$$

Then, the ACF can be approximated in closed form.

$$\begin{aligned}
G(\tau) = {} & G_D\left(N_M + N_{ML}, D, \tau\right)\left(\Im_M + \Im_{ML}\right)^2 \\
& + \frac{\langle C_{ML} \rangle k_f}{\lambda} G_D\left(N_M + N_{ML}, D^{(+)}, \tau\right)\left[\Im_M - \frac{\langle C_M \rangle}{\langle C_{ML} \rangle}\Im_{ML} - \frac{\langle C_M \rangle + \langle C_{ML} \rangle}{\langle C_{ML} \rangle}\Im_L\right]^2 \\
& + \frac{\langle C_{ML} \rangle\left(k_f \langle C_B \rangle + k_b\right)}{\langle C_M \rangle \lambda} G_D\left(N_M + N_{ML}, D^{(-)}, \tau\right)\left[\Im_M - \frac{\langle C_M \rangle}{\langle C_{ML} \rangle}\Im_{ML} + \frac{\langle C_M \rangle + \langle C_{ML} \rangle}{\langle C_{ML} \rangle}\Im_L\right]^2 e^{-\lambda\tau}
\end{aligned}$$

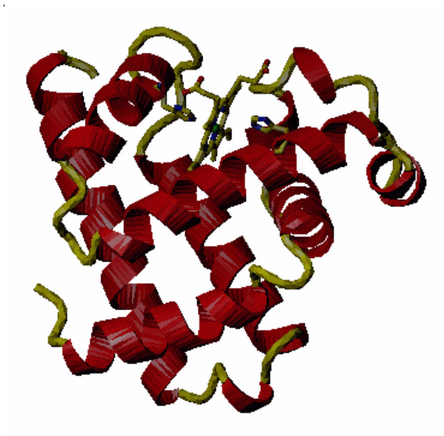
where $$D^{(+)} = \left[D_L \frac{\langle C_L \rangle}{\langle C_L \rangle + \langle C_{ML} \rangle f} + D_M \frac{\langle C_{ML} \rangle}{\langle C_L \rangle + \langle C_{ML} \rangle f}\right],$$

$$D^{(-)} = \left[D_L \frac{\langle C_{ML} \rangle}{\langle C_L \rangle + \langle C_{ML} \rangle f} + D_M \frac{\langle C_L \rangle}{\langle C_L \rangle + \langle C_{ML} \rangle f}\right],$$

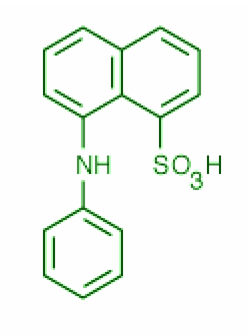
and $$f = \frac{\langle C_M \rangle}{\langle C_M \rangle + \langle C_{ML} \rangle}.$$

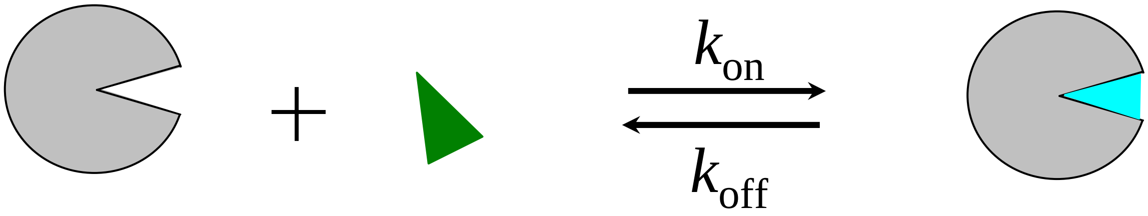
ANS Binding to Apomyoglobin

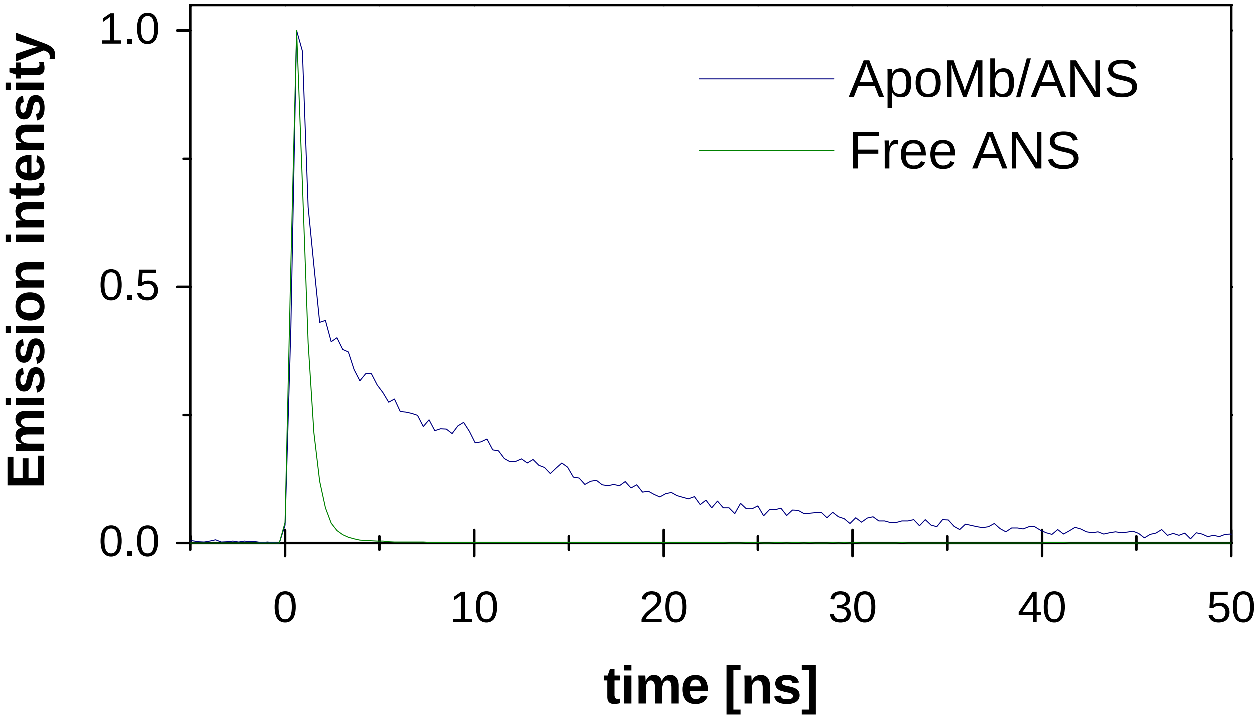
Myoglobin



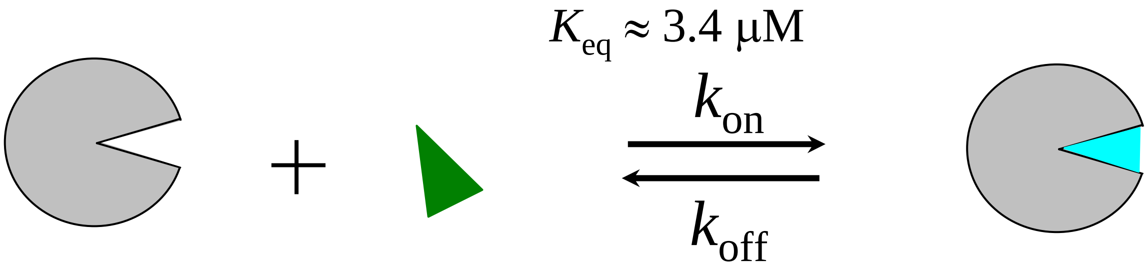
ANS







ANS Binding to Apomyoglobin



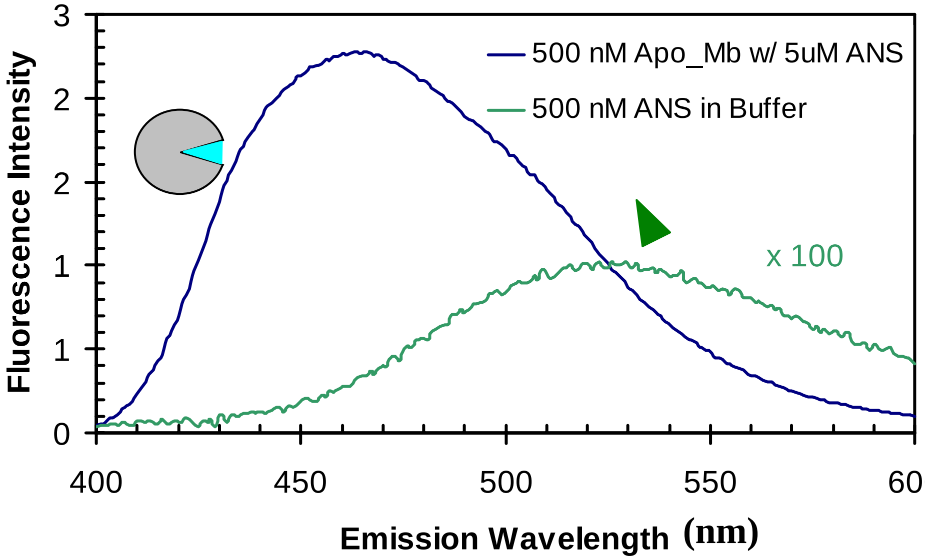
$K_{eq} \approx 3.4\ \mu\text{M}$

$$\text{} + \text{} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}}$$

Weakly Fluorescent

Strongly Fluorescent

1 Photon Fluorescence of ANS and Apo_Mb-ANS



$$k_r = k_{on}(< C_{Mb} > + < C_{ANS} >) + k_{off}$$

$k_{on} \approx 2 \times 10^9\ \text{M}^{-1}\text{s}^{-1}$ Diffusion Limited
or Protein Controlled

$k'_{on} = 2100\ \text{s}^{-1}$ for 50 nM ANS, 1 μM Apo Mb

$k_{off} = \text{K}_{eq}k_{on} = 6800\ \text{s}^{-1}$

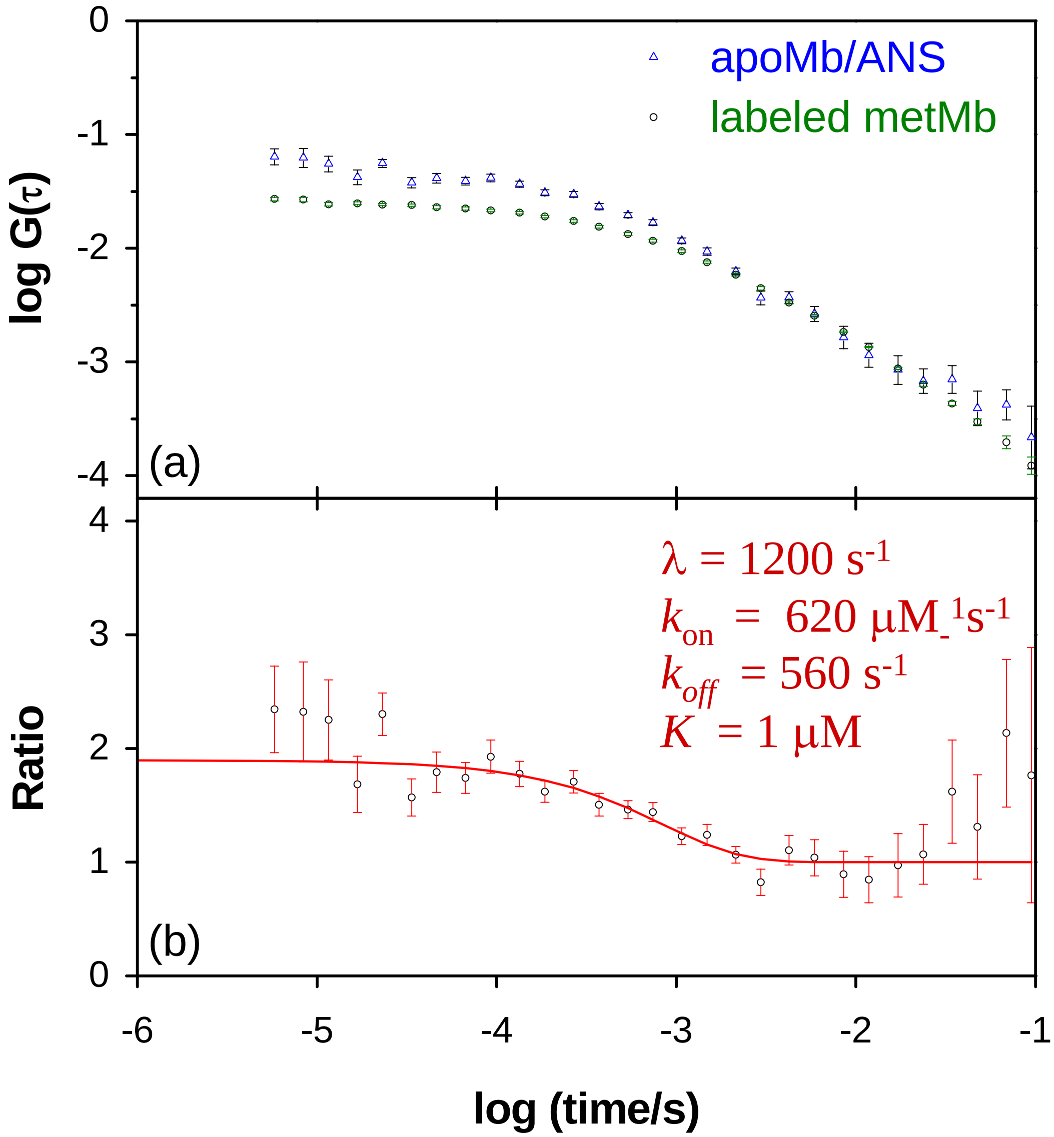
$k_r = k'_{on} + k_{off} = 8900$ s-1 or 112 μs

ANS Reaction Rate to Horse ApoMb

Ratio autocorrelation functions,
diffusion term cancels

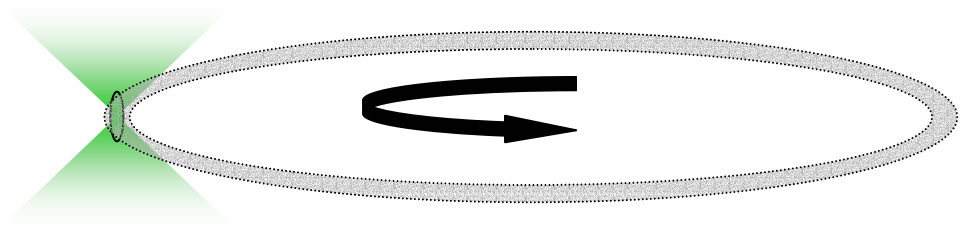
$$\frac{G_{apoMbANS}(\tau)}{G_{MbOG}(\tau)} = \frac{N_{MbOG}}{N_{apoMbANS}}\left[1+\frac{\exp(-k_r t)}{K\langle C_{ANS}\rangle}\right]$$

‘300’ nM Apo Mb, 1 mM ANS in Buffer (pH 7)



Scanning FCS

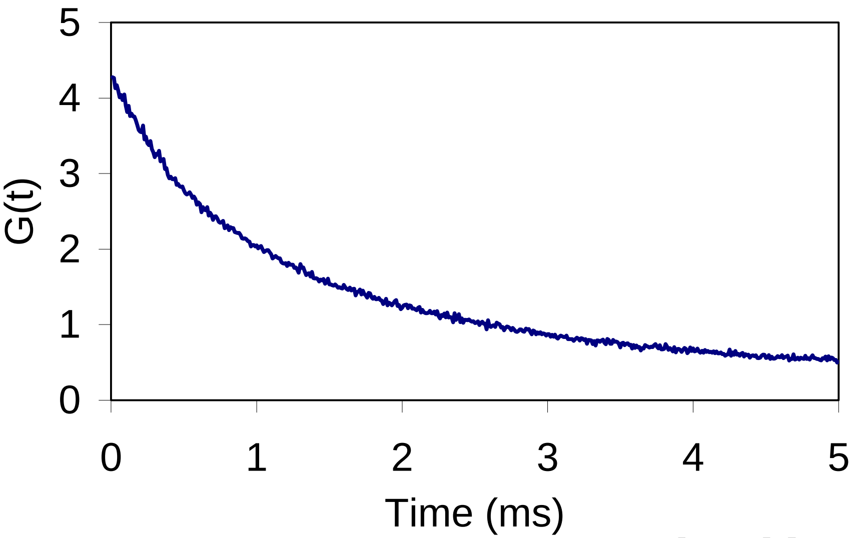
Periodic scanning of the sample or laser beam

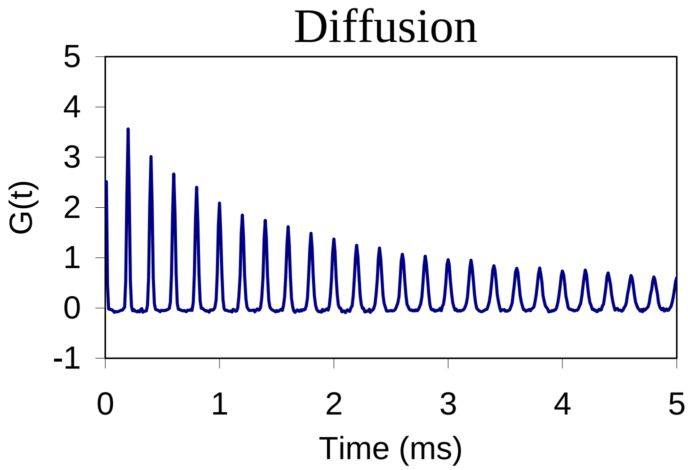


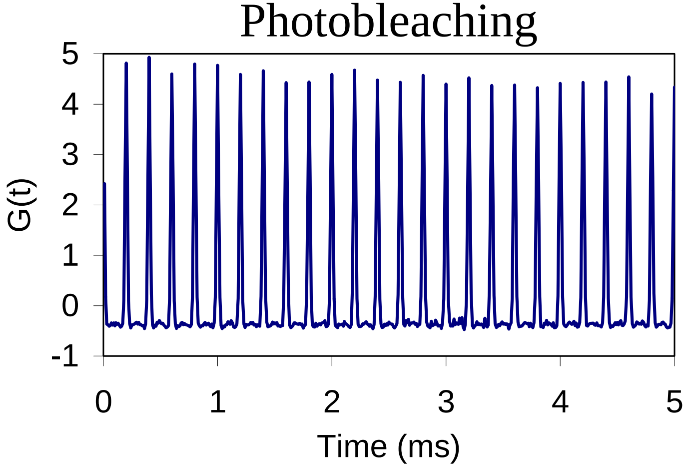
$$\mathbf{r}_s = A_x \cos(\omega t)\hat{e}_x + A_y \sin(\omega t)\hat{e}_y$$

Advantageous for:
- low concentrations
- slow diffusing molecules
- photosensitive molecules

e.g. Is the ACF decay due to diffusion or photobleaching?




Diffusion


Photobleaching

Scanning FCS

$$\mathbf{r}_s(t) = A_x \cos(\omega t)\hat{e}_x + A_y \sin(\omega t)\hat{e}_y$$

Laser Scanning

$$G(\tau) = \frac{\left\langle \iint d\mathbf{r}\, d\mathbf{r}'\, W(\mathbf{r} + \mathbf{r}_s(t+\tau)) W(\mathbf{r} + \mathbf{r}_s(t)) \delta C(\mathbf{r}, t+\tau) \delta C(\mathbf{r}', t) \right\rangle}{\left[\langle C \rangle \int d\mathbf{r}\, W(\mathbf{r})\right]^2}$$

Sample Scanning or change of variables: $\tilde{\mathbf{r}} = \mathbf{r} + \mathbf{r}_s(t); \quad \tilde{\mathbf{r}}' = \mathbf{r}' + \mathbf{r}_s(t)$

$$G(\tau) = \frac{\left\langle \iint d\tilde{\mathbf{r}}\, d\tilde{\mathbf{r}}'\, W(\tilde{\mathbf{r}}) W(\tilde{\mathbf{r}}') \delta C(\tilde{\mathbf{r}} - \mathbf{r}_s(t+\tau), t+\tau) \delta C(\tilde{\mathbf{r}}' - \mathbf{r}_s(t), t) \right\rangle}{\left[\langle C \rangle \int d\mathbf{r}\, W(\mathbf{r})\right]^2}$$

Laser scanning and sample scanning are theoretically equivalent

For circular scanning in the plane of the radial component of the PSF

$$\mathbf{r}_s(t) = A\left(\cos(\omega t)\hat{e}_x + \sin(\omega t)\hat{e}_y\right)$$

$$G(\tau) = G_D(N, D, \tau)\ \exp\left(- \frac{4A^2(1 - \cos(\omega\tau))}{w_r^2 \left(1 + \frac{2\tau}{\tau_D}\right)} \right)$$

The envelope of the ACF from S-FCS is that of pure diffusion!