The Fundamentals of Fluorescence Spectroscopy III:

Multidimensional Analysis

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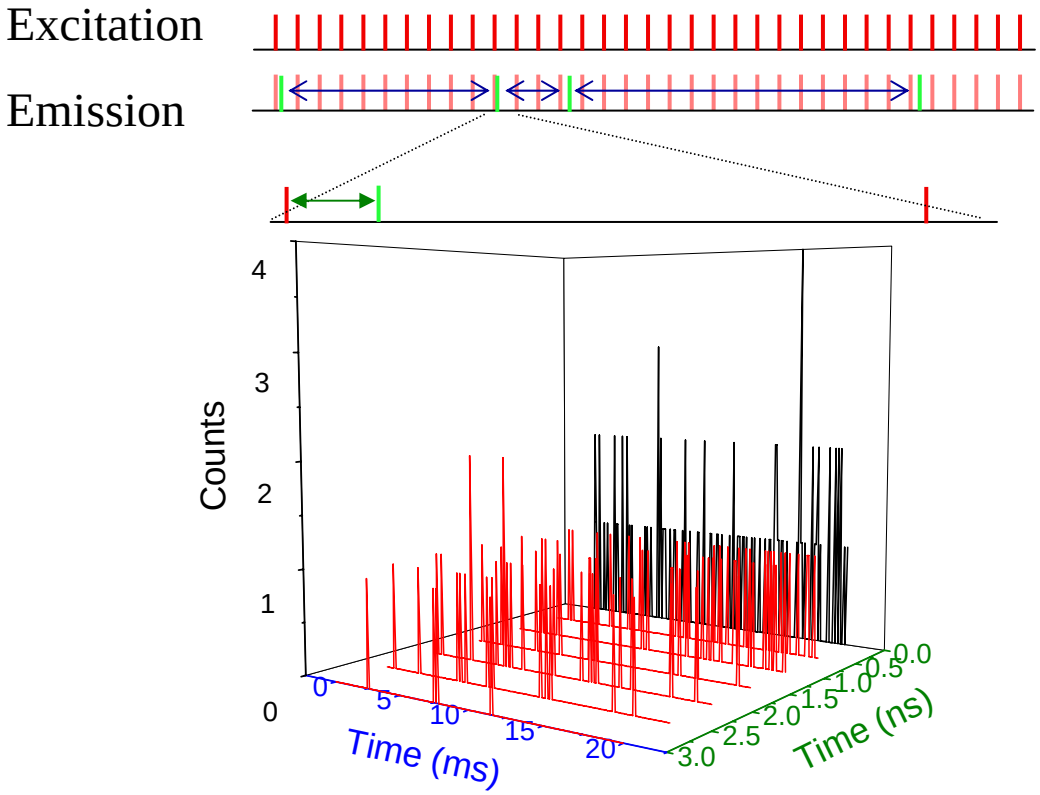
Time-Gated FCS

How can we enhance the capabilities of FCS?

Incorporate the additional information of the photon in the analysis

- Lifetime of the Excited State
- Wavelength
- Polarization



Use Multidimensional Analysis

Select photons for analysis based upon photon properties

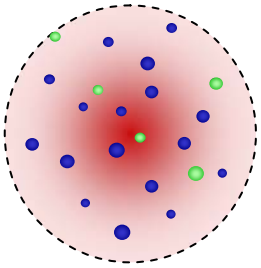
Time-Gated FCS

Use delay between the excitation pulse and fluorescence emission to discriminate between photons based upon their duration in the excited state

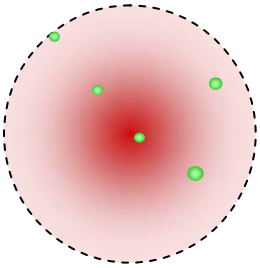
Removal of Fluorescent Background

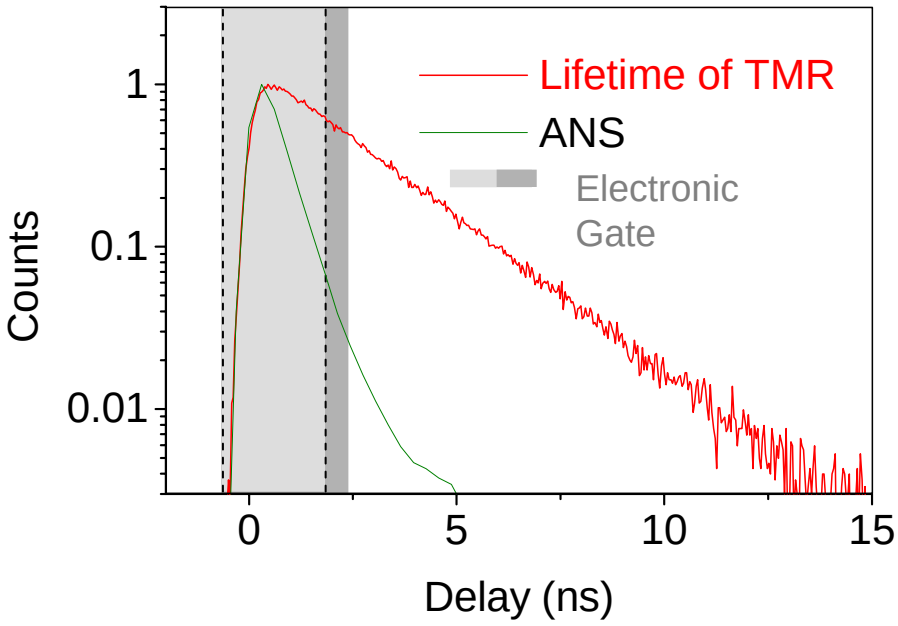
Quantitative measurements in the presence of a fluorescence background

Without Gate

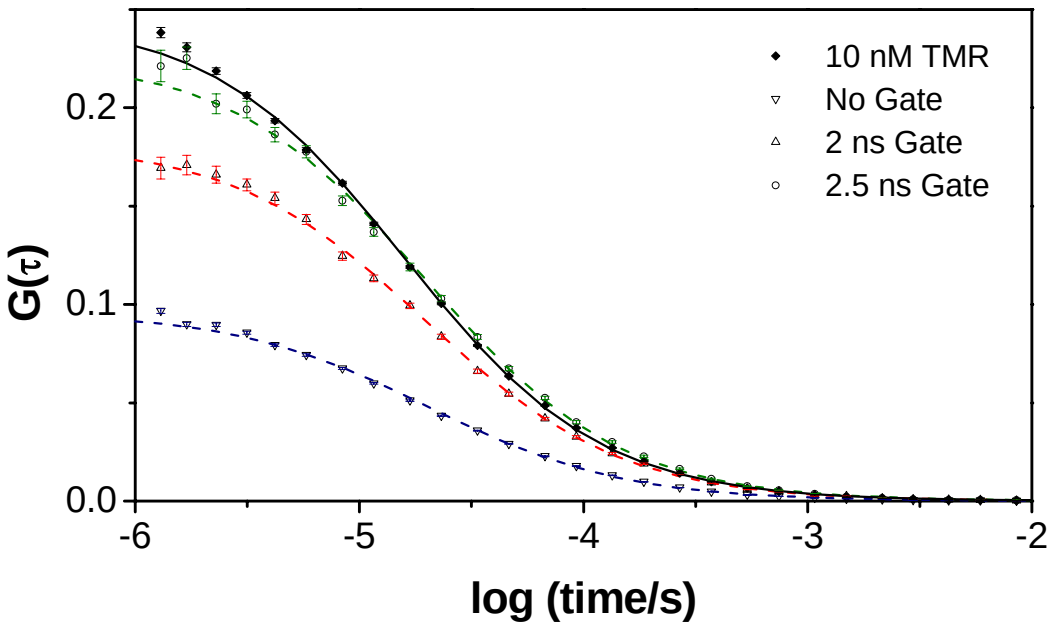


With Gate





10 nM TMR 9 μM ANS Mixture



Multiple Species Discrimination

Check sample inhomogeneity

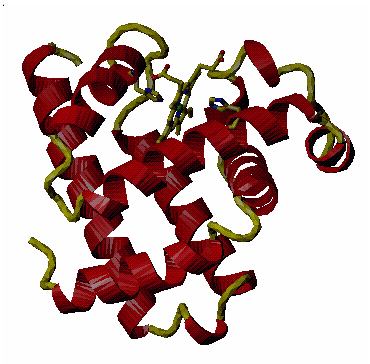
$$G(0)_{eff} = \mathfrak{I}_1^2 G_1(0) + \mathfrak{I}_2^2 G_2(0)$$

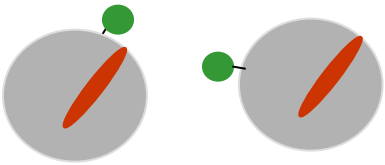
where $\mathfrak{I}_i$ is the relative intensity of the i^{th} species

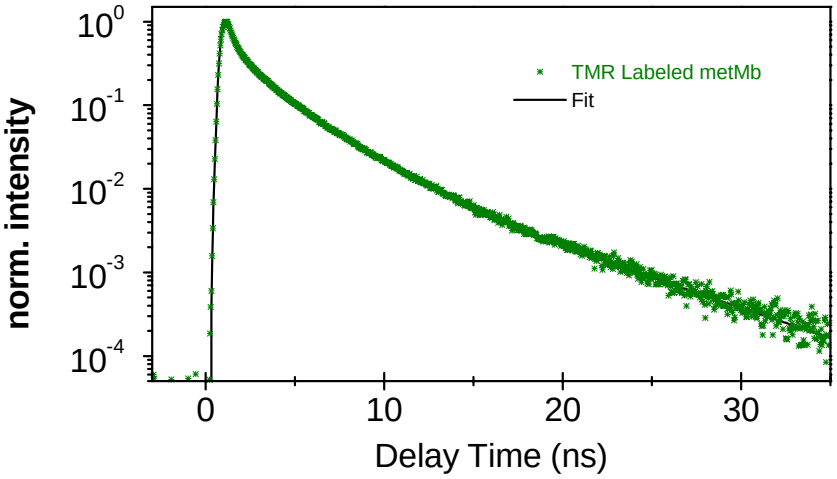
If sample contains multiple lifetime species,
G(0) will depend upon gate duration

MetMb stochastically labeled with TMR

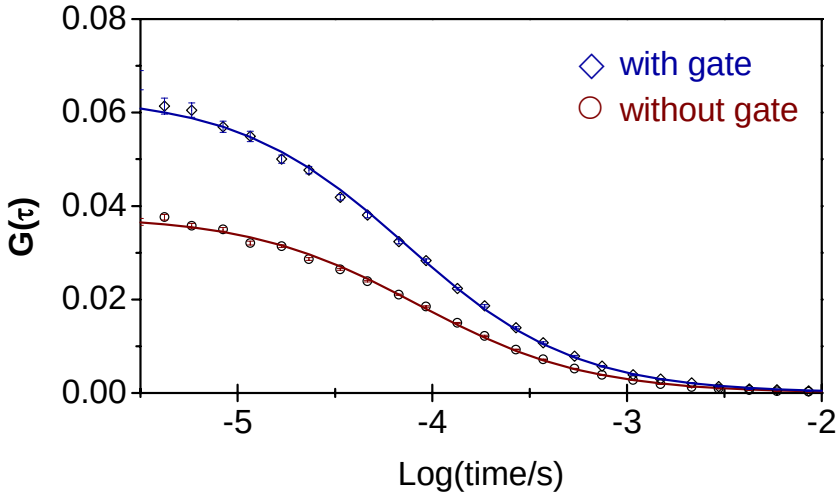
TMR labeled close to the heme is quenched







Autocorrelation Function



Static versus Dynamic Heterogeneity

Is the heterogeneity static or dynamic?

For non-interacting species:

Amplitude changes with gate duration

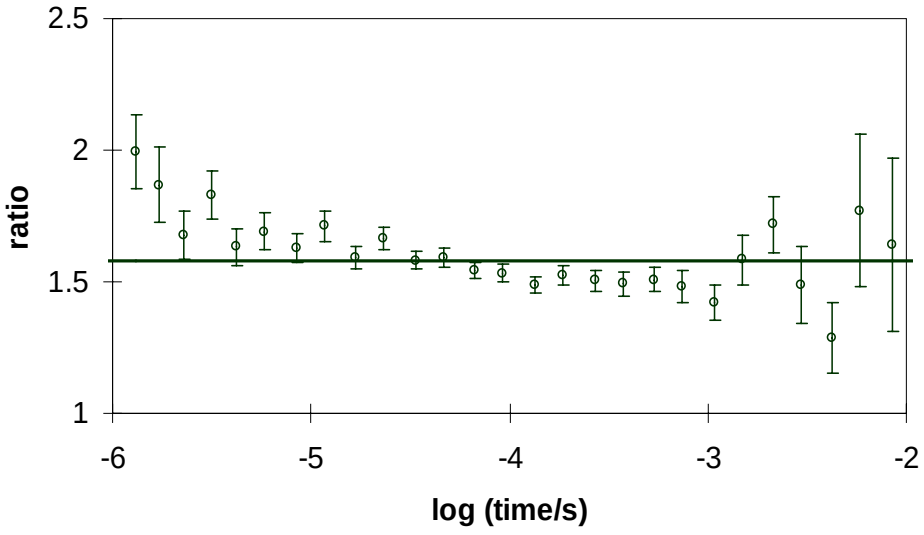
$$G(\tau) = \left(\frac{\mathfrak{I}_1^2}{N_1} + \frac{\mathfrak{I}_2^2}{N_2} \right) \times G_{Diff}(1, D, \tau)$$

For unimolecular reaction:

Amplitude of the relaxation will vary with gate duration

$$G(\tau) = \left\{ 1 + K\left(\mathfrak{I}_1 - \frac{\mathfrak{I}_2}{K} \right)^2 e^{-k_r \tau} \right\} \times G_{Diff}(N_1 + N_2, D, \tau)$$

Ratio measurements with and without gate!



Note*: Ratio analysis is sensitive to offsets in the ACF due to laser instabilities, vibrations, bleaching . . .

Enhancement of FCS

Are FCS measurements *Enhanced* by Time-Gating?

Signal-to-Noise Considerations:

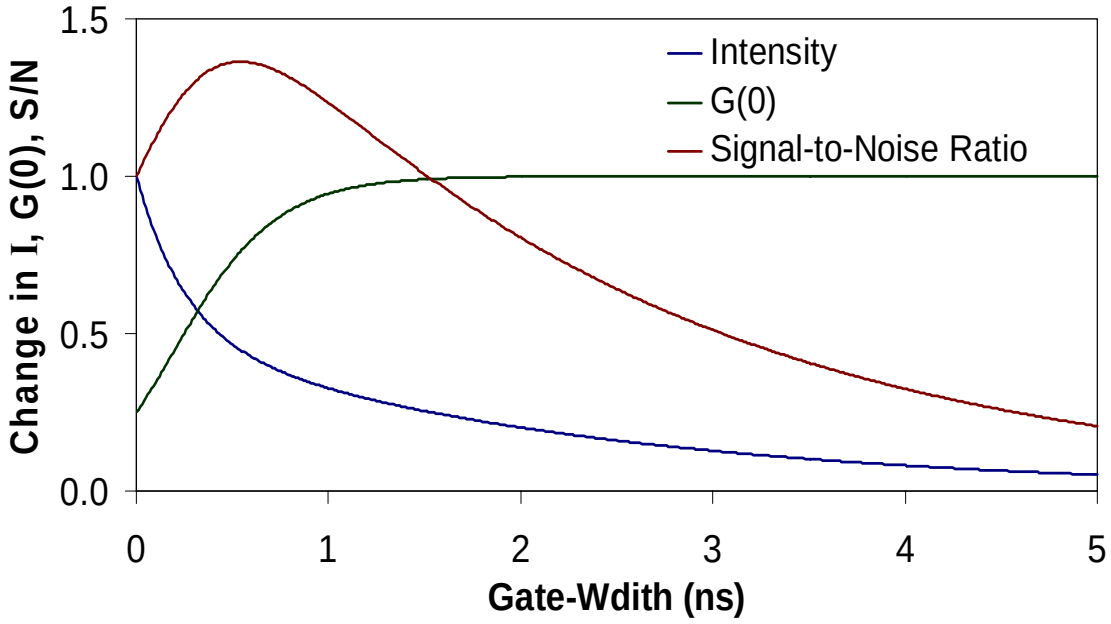
High Intensity Limit:

Uncertainty dominated by number of fluctuations:
Loss of photons by gating unimportant

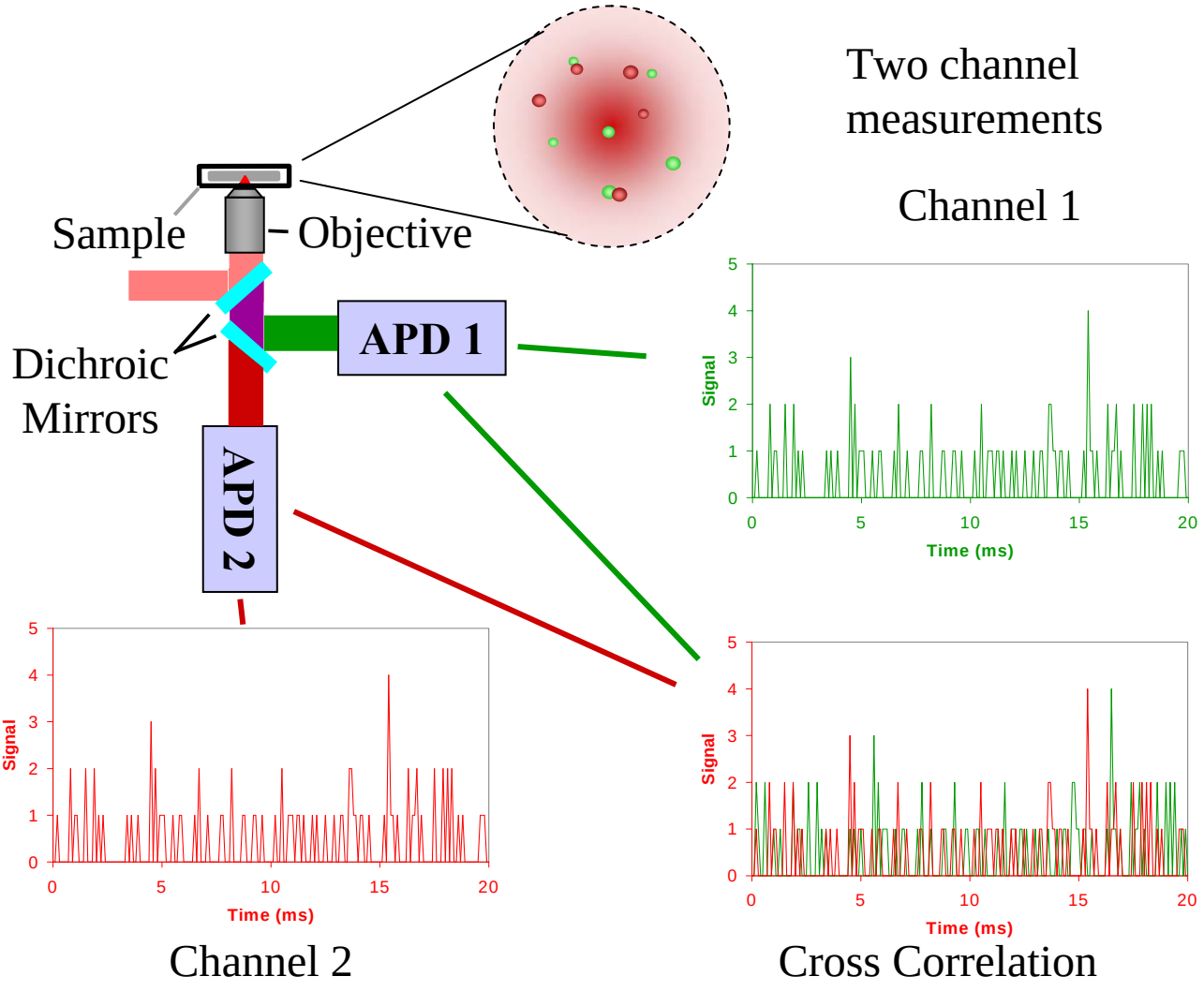
Low Intensity Limit:

Uncertainty dominated by number of photons:
Signal-to-noise is proportional to $I_T\, G(0)$
(both I_T and $G(0)$ depend on the gate width)

Calculation of the S/N vs Gate Width for a mixture of TMR and ANS with Equal Fluorescence Intensities



Cross-Correlation Spectroscopy



Type of Experiment	Beamsplitting Optics	Channel 1	Channel 2
Fast Correlation Measurements	50/50 Beamsplitter	50% of the total Signal	The remaining Signal
Two-Color Experiments	Dichroic Mirror	Green Photons	Red Photons
Polarization Measurements	Polarizing Beamsplitter	⊥ Polarized light	|| Polarized light

Cross-Correlation Spectroscopy

Two-Channel Detection

$$F_1(t) = \kappa_1 Q_1 \int d\mathbf{r}\, W(\mathbf{r}) C_1(\mathbf{r},t)$$

$$F_2(t) = \kappa_2 Q_2 \int d\mathbf{r}\, W(\mathbf{r}) C_2(\mathbf{r},t)$$

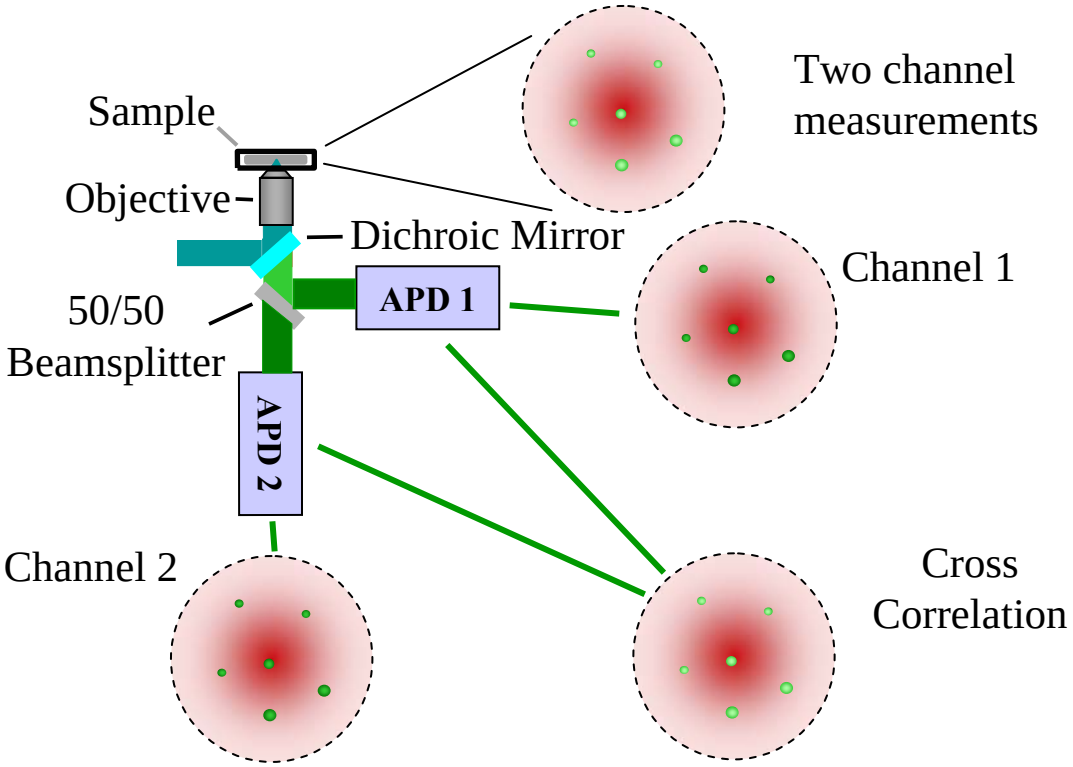
$$G_{ij}(\tau) = \frac{\left\langle \delta F_i(t)\delta F_j(t+\tau)\right\rangle}{\left\langle F_i(t)\right\rangle\left\langle F_j(t)\right\rangle}$$

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

The product $\left\langle \delta C_i(\mathbf{r},\tau)\delta C_j(\mathbf{r}',0)\right\rangle$ is only non-zero if *i* and *j* are the same particle or if they diffuse together.

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

Fast Cross-Correlation Spectroscopy



$$N_1 = N_2 \quad \rightarrow \quad G_{1,2}(\tau) = \frac{\langle N \rangle G_D(1, D, \tau)}{\langle N \rangle \langle N \rangle} = G_D(N, D, \tau)$$

Same information as ACF, but without detector artifacts

Signal-to-noise considerations:
The S/N ratio is proportional to molecular brightness, ε

$$\varepsilon_{CCF} \rightarrow \varepsilon_{ACF}/2$$

$$(S/N)_{CCF} = (S/N)_{ACF}/2$$

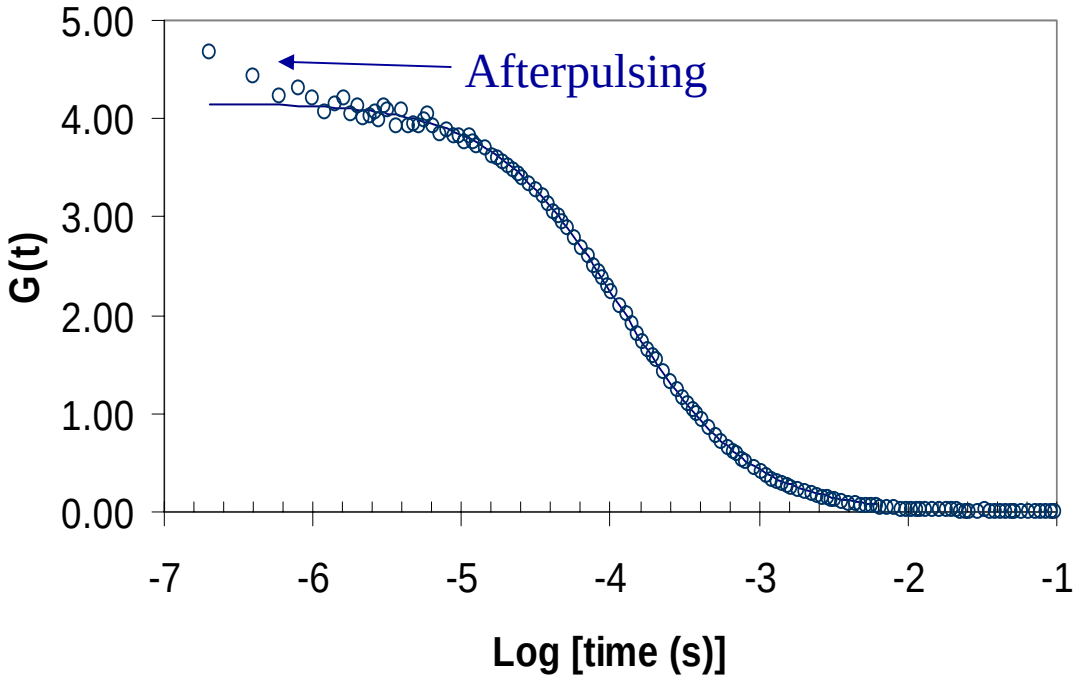
For symmetric CCFs,

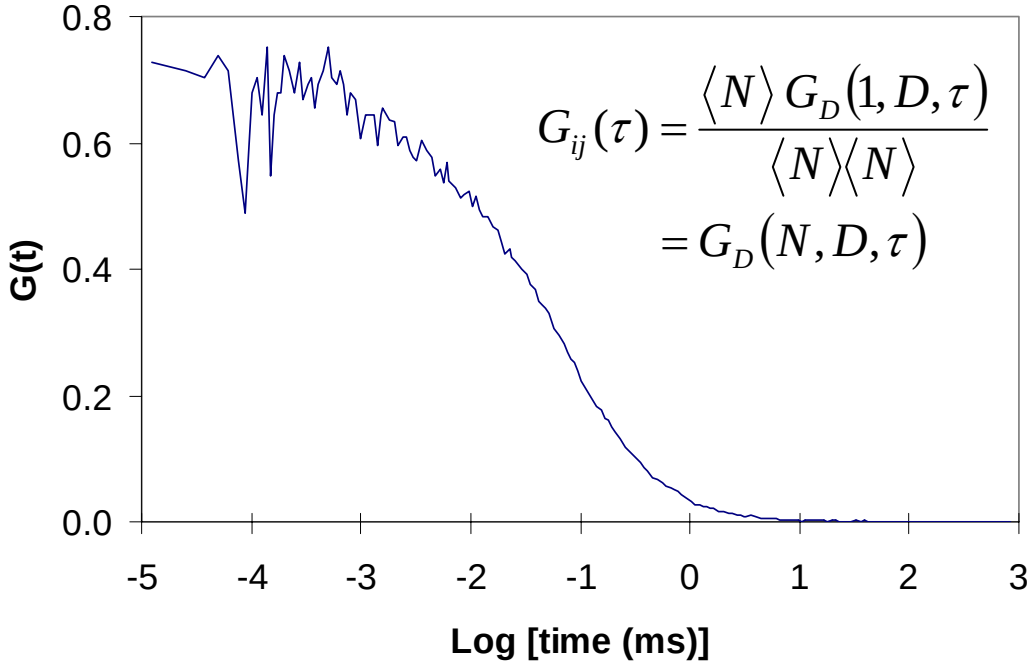
$$G_{1,2}(\tau) = G_{2,1}(\tau),$$

and the S/N ratio of the total CCF, $\{G_{1,2}(\tau) + G_{2,1}(\tau)\}/2$, is $\sqrt{½}$ that of the ACF

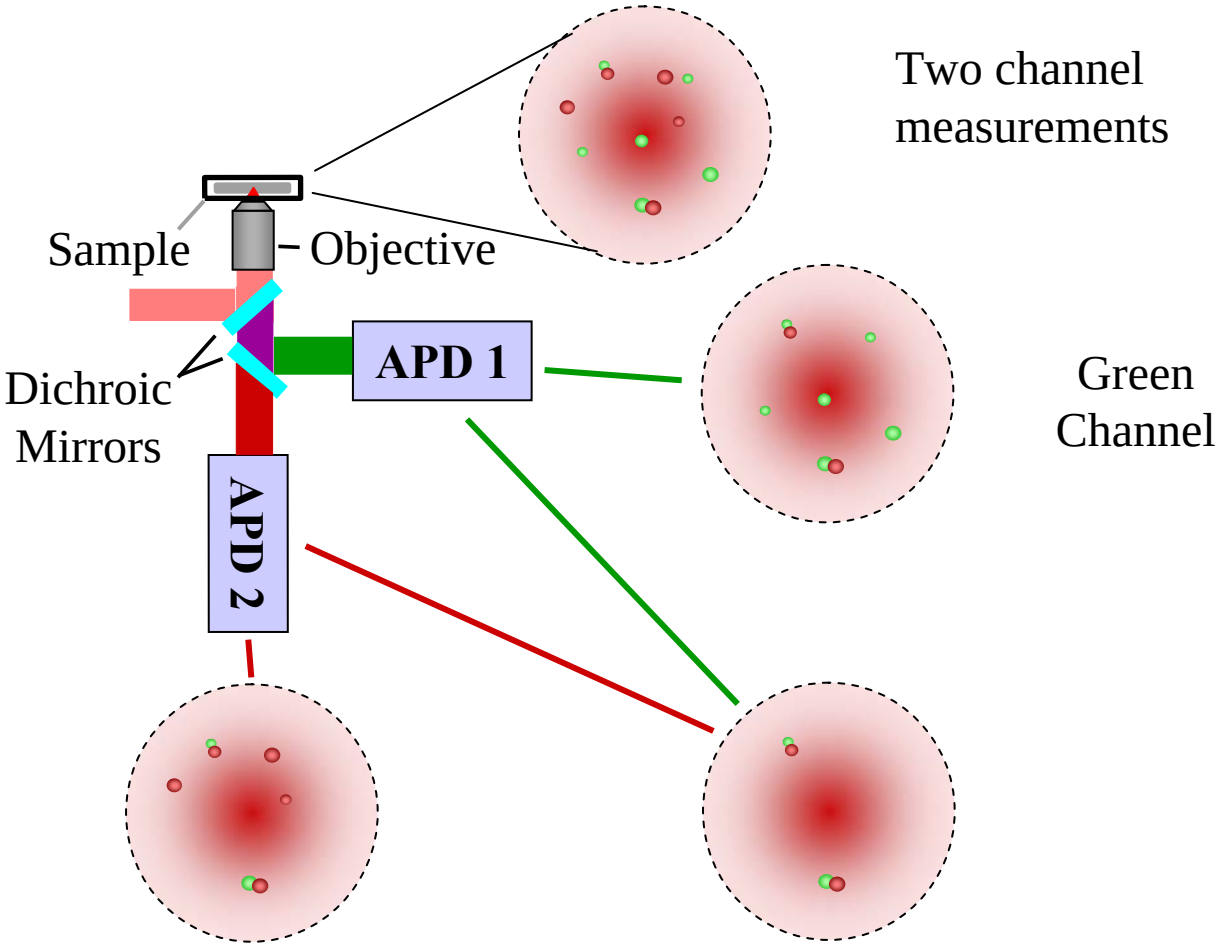
Fast Cross-Correlation Spectroscopy

Compensation for detector dead time and removal of afterpulsing artifacts





Two-Color Cross-Correlation Spectroscopy



$$G_{GR}(\tau) = \frac{\langle F_G(t)F_R(t+\tau)\rangle - \langle F_G(t)\rangle\langle F_R(t)\rangle}{\langle F_G(t)\rangle\langle F_R(t)\rangle}$$

The sample consists of three species, N_G, N_R, and N_{GR}
Ideally, only the N_{GR} cross correlate

$$G_{GR}(\tau) = \frac{N_{GR}\, G_D(1, D_{GR}, \tau)}{\langle N_G + N_{GR}\rangle\langle N_R + N_{GR}\rangle}$$

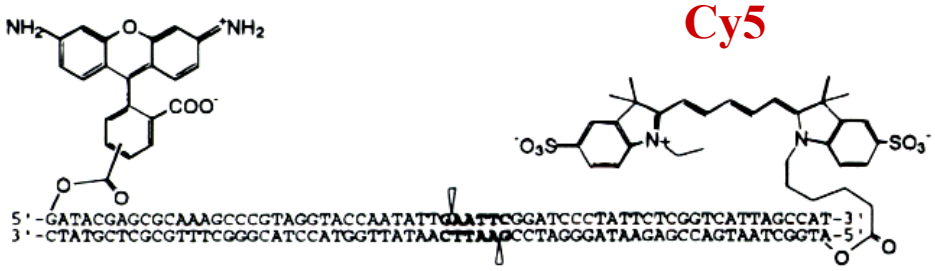
Amplitude is *proportional* to concentration of N_{GR}!!!

Two-Color Cross-Correlation Spectroscopy

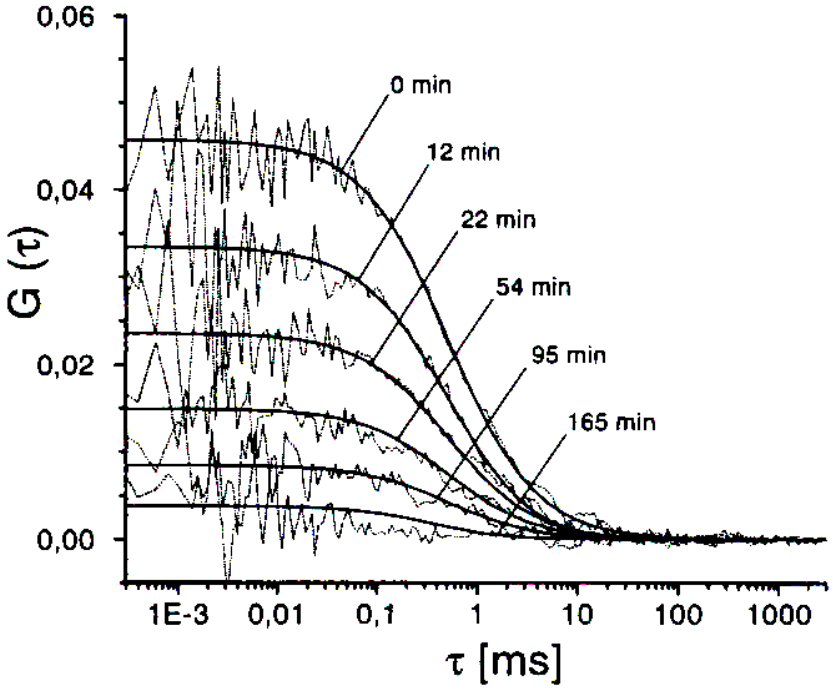
Labeled double stranded DNA

Rhodamine Green

Cy5

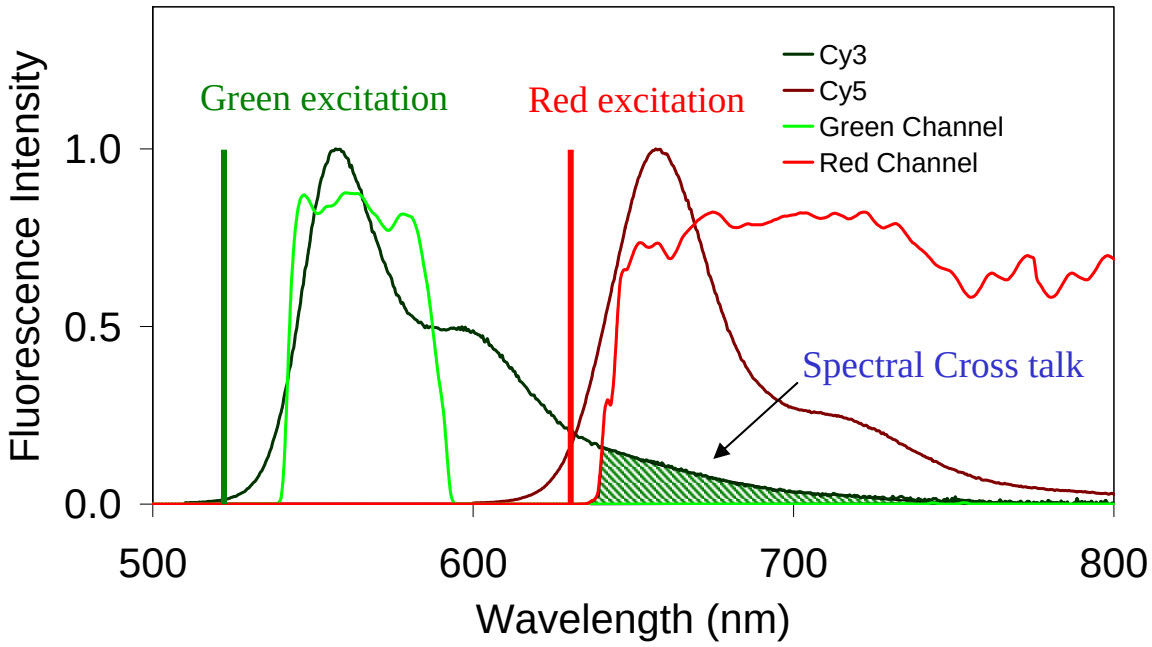


Reaction of restriction endonuclease *Eco*RI with dsDNA



Kettling, Koltermann, Schwille, Eigen *PNAS* (1998) 95:1416

Spectral Cross Talk



Multiple Molecular Brightnesses

$\varepsilon_{G,GG}$, $\varepsilon_{G,GR}$: Brightness of the Green dye with Green Excitation in the Green and Red channels

$\varepsilon_{R,GG}$, $\varepsilon_{R,GR}$: Brightness of the Red dye with Green Excitation in the Green and Red channels

$\varepsilon_{G,RG}$, $\varepsilon_{G,RR}$: Brightness the Green dye with Red Excitation in the Green and Red channels

$\varepsilon_{R,RG}$, $\varepsilon_{R,RR}$: Brightness of the Red dye with Red Excitation in the Green and Red channels

Typically, $\varepsilon_{R,GG} = \varepsilon_{R,RG} = 0$; The Red dye does not fluoresce in the Green channel

$\varepsilon_{G,RG} = \varepsilon_{G,RR} = 0$; The Green dye does not absorb Red excitation

Spectral Cross Talk

Assuming three species, C_G, C_R, and C_{GR}, where the double labeled molecules have the properties of both of the Red and Green molecules, we have:

$$F_G(t) = \int d\mathbf{r}\, W_G(\mathbf{r})\left[\varepsilon_{G,GG}\left(C_G(\mathbf{r},t) + C_{GR}(\mathbf{r},t)\right)\right]$$

$$F_R(t) = \int d\mathbf{r}\, W_G(\mathbf{r})\left(\varepsilon_{G,GR}C_G(\mathbf{r},t) + \varepsilon_{R,GR}C_R(\mathbf{r},t) + \left(\varepsilon_{G,GR} + \varepsilon_{R,GR}\right)C_{GR}(\mathbf{r},t)\right)$$
$$+ \int d\mathbf{r}\, W_R(\mathbf{r})\varepsilon_{R,RR}\left(C_R(\mathbf{r},t) + C_{GR}(\mathbf{r},t)\right)$$

For identical probe volumes

$$F_G(t) = \int d\mathbf{r}\, W(\mathbf{r})\left[\varepsilon_{G,GG}\left(C_G(\mathbf{r},t) + C_{GR}(\mathbf{r},t)\right)\right]$$

$$F_R(t) = \int d\mathbf{r}\, W(\mathbf{r})\left[\varepsilon_{G,GR}C_G(\mathbf{r},t) + \left(\varepsilon_{R,GR} + \varepsilon_{R,RR}\right)C_R(\mathbf{r},t)\right)$$
$$+ \left(\varepsilon_{G,GR} + \varepsilon_{R,GR} + \varepsilon_{R,RR}\right)C_{GR}(\mathbf{r},t)\Big]$$

$$G_{GR}(\tau) = \Im_G^R \frac{\gamma}{\langle N_G + N_{GR}\rangle}\left(\frac{1}{1+\tau/\tau_{D_G}}\right)\left(\frac{1}{1+\left(w_r/w_z\right)^2\tau/\tau_{D_G}}\right)$$
$$+ \Im_{GR}^R \frac{\gamma}{\langle N_G + N_{GR}\rangle}\left(\frac{1}{1+\tau/\tau_{D_{GR}}}\right)\left(\frac{1}{1+\left(w_r/w_z\right)^2\tau/\tau_{D_{GR}}}\right)$$

The terms add linearly with the fractional intensity!

Non-Aligned Volumes

For two concentric probe volumes of different dimensions:

$$W_G(\mathbf{r}) = I_0 \exp\left[-\frac{2\left(x^2+y^2\right)}{w_{r_G}^2} - \frac{2z^2}{w_{z_G}^2}\right]$$

$$W_R(\mathbf{r}) = I_0 \exp\left[-\frac{2\left(x^2+y^2\right)}{w_{r_R}^2} - \frac{2z^2}{w_{z_R}^2}\right]$$

$$F_G(t) = \int d\mathbf{r}\, W_G(\mathbf{r})\left[\varepsilon_{G,GG}\left(C_G(\mathbf{r},t) + C_{GR}(\mathbf{r},t)\right)\right]$$

$$F_R(t) = \int d\mathbf{r}\, W_G(\mathbf{r})\left(\varepsilon_{G,GR}C_G(\mathbf{r},t) + \varepsilon_{R,GR}C_R(\mathbf{r},t) + \left(\varepsilon_{G,GR}+\varepsilon_{R,GR}\right)C_{GR}(\mathbf{r},t)\right)$$
$$+ \int d\mathbf{r}\, W_R(\mathbf{r})\varepsilon_{R,RR}\left(C_R(\mathbf{r},t) + C_{GR}(\mathbf{r},t)\right)$$

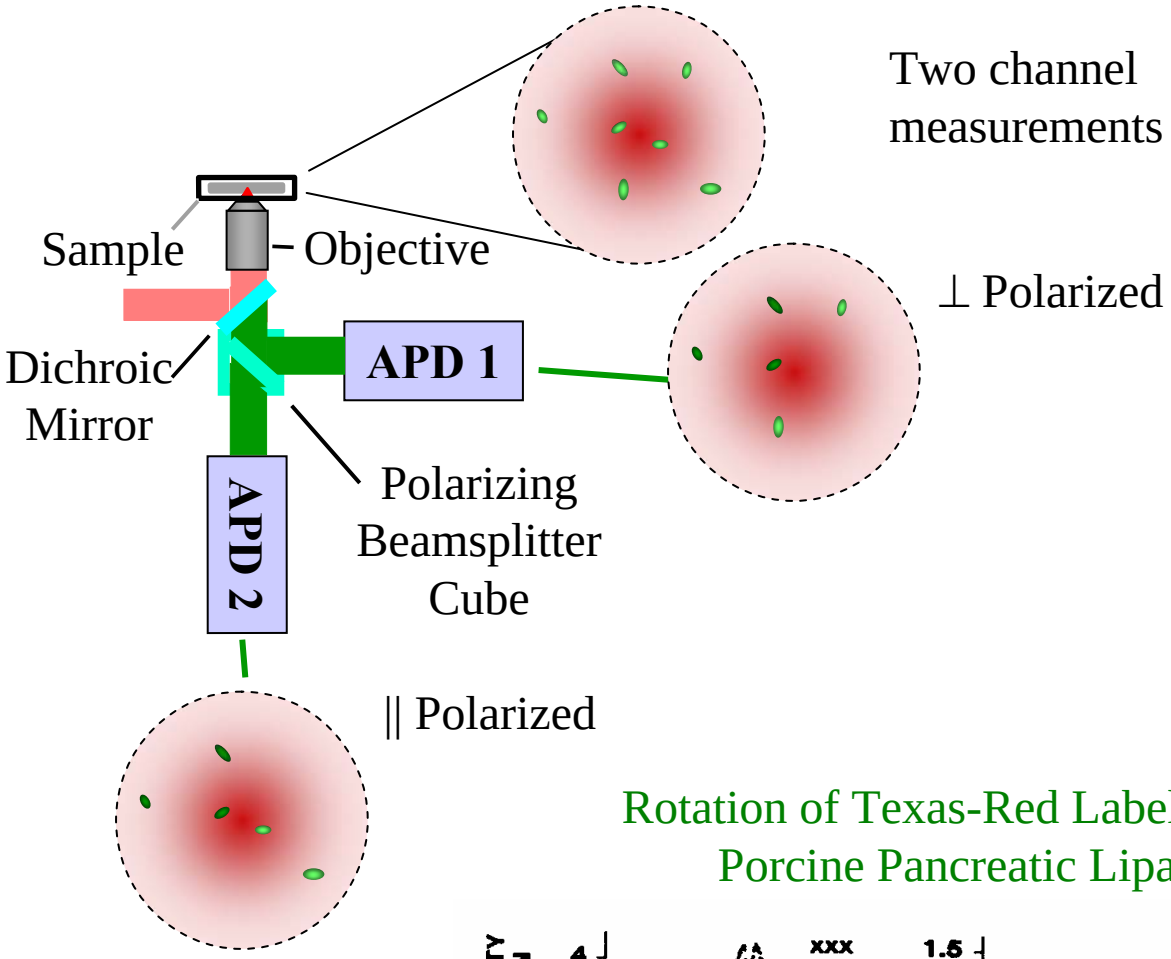
The CCF is given by:

$$G_{GR}(\tau) = \Im_G^R \frac{\gamma}{V_{eff}\left\langle C_G + C_{GR}\right\rangle}\left(\frac{1}{1+\tau/\tau'_{D_G}}\right)\left(\frac{1}{1+\left(w_r/w_z\right)^2\tau/\tau'_{D_G}}\right)$$
$$+ \Im_{GR}^R \frac{\gamma}{V_{eff}\left\langle C_G + C_{GR}\right\rangle}\left(\frac{1}{1+\tau/\tau'_{D_{GR}}}\right)\left(\frac{1}{1+\left(w_r/w_z\right)^2\tau/\tau'_{D_{GR}}}\right)$$

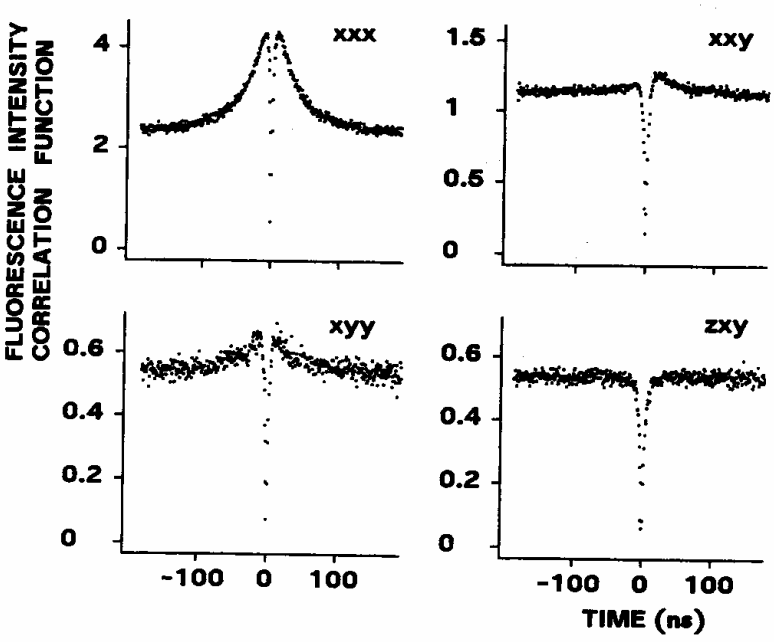
$$V_{eff} = \left(\frac{\pi}{4}\right)^{\frac{3}{2}}\left(w_{r_G}^2 + w_{r_R}^2\right)\left(w_{z_G}^2 + w_{z_R}^2\right)^{\frac{1}{2}}$$

$$\tau'_{D_i} = \frac{\left(w_{r_G}^2 + w_{r_R}^2\right)}{8D_i}$$

Rotational Diffusion



Rotation of Texas-Red Labeled Porcine Pancreatic Lipase



Kask et al, Biophys J (1989) 55:213

The CCF for G_{xxy} and G_{xyx} are Asymmetric!!

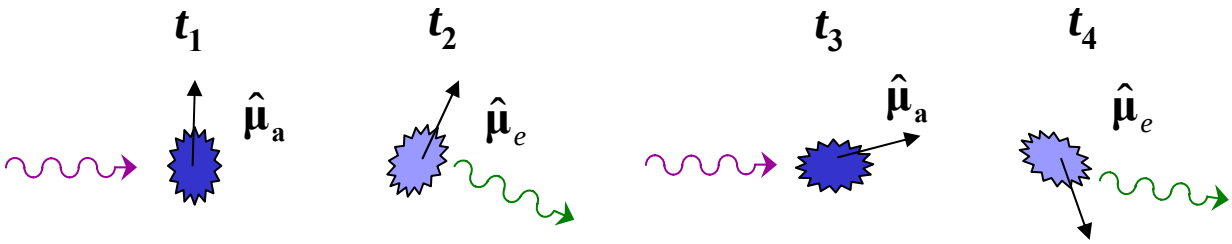
Rotational Diffusion

Orientation of the fluorophore must be incorporated.

Absorption and detection efficiency depend upon the polarization:

$$\sigma(t) = A^2(\boldsymbol{\Omega}(t)) = 3(\hat{\boldsymbol{\mu}}_a \cdot \hat{\mathbf{a}})^2$$
$$\kappa(t) = E^2(\boldsymbol{\Omega}(t)) = 3(\hat{\boldsymbol{\mu}}_e \cdot \hat{\mathbf{e}})^2$$

The probability of a molecule absorbing a photon at t_1, emitting the first photon at t_2, absorbing a second photon at t_3, and emitting a second photon at t_4 is:



$$\tilde{G}(t_1,t_2,t_3,t_4) = \left\langle A^2(\boldsymbol{\Omega}(t_1))F_a'(\mathbf{Q}(t_1))E^2(\boldsymbol{\Omega}(t_2))F_e'(\mathbf{Q}(t_2))\right.$$
$$\left. A^2(\boldsymbol{\Omega}(t_3))F_a'(\mathbf{Q}(t_3))E^2(\boldsymbol{\Omega}(t_4))F_e'(\mathbf{Q}(t_4))\right\rangle$$

where $F'(\mathbf{Q}(t))$ includes all non-orientation properties of the fluorophore (e.g. position, electronic state, chemical state, …)

Assuming $\boldsymbol{\Omega}$ and $\mathbf{Q}$ are independent:

$$\tilde{G}(t_1,t_2,t_3,t_4) = \left\langle A^2(\boldsymbol{\Omega}(t_1))E^2(\boldsymbol{\Omega}(t_2))A^2(\boldsymbol{\Omega}(t_3))E^2(\boldsymbol{\Omega}(t_4))\right\rangle$$
$$\left\langle F_a'(\mathbf{Q}(t_1))F_e'(\mathbf{Q}(t_2))F_a'(\mathbf{Q}(t_3))F_e'(\mathbf{Q}(t_4))\right\rangle$$

Rotational Diffusion

The normal autocorrelation function measures the probability of measuring a second photon with a delay τ

Define $t_2 = 0$ and $t_4 = \tau$

$$\tilde{G}(\tau) = \int_{-\infty}^{0} dt_1 \int_{t_1}^{\tau} dt_3 \left\langle A^2(\mathbf{\Omega}(t_1)) E^2(\mathbf{\Omega}(0)) A^2(\mathbf{\Omega}(t_3)) E^2(\mathbf{\Omega}(\tau)) \right\rangle \left\langle F_a'(\mathbf{Q}(t_1)) F_e'(\mathbf{Q}(0)) F_a'(\mathbf{Q}(t_3)) F_e'(\mathbf{Q}(\tau)) \right\rangle$$

Assuming an mono-exponential decay of lifetime τ_{fl} and taking the limit as $\tau_{fl} \rightarrow 0$ (*i.e.* we are interested in t >> τ_{fl}), we obtain:

$$\tilde{G}(\tau) = \left\langle F_a'(\mathbf{Q}(0)) F_a'(\mathbf{Q}(\tau)) \right\rangle \left[1 - e^{-\tau/\tau_{fl}}\right] \left\langle A^2(\mathbf{\Omega}(0)) E^2(\mathbf{\Omega}(0)) A^2(\mathbf{\Omega}(\tau)) E^2(\mathbf{\Omega}(\tau)) \right\rangle$$

Assuming the excitation and absorption dipoles are equivalent (*i.e.* $\mu_a = \mu_e = \mu$) and molecule is a rigid sphere undergoing rotational diffusion:

$$\tilde{G}(\tau) \approx \left\langle F_a'(0) F_a'(\tau) \right\rangle \left[1 - e^{-\tau/\tau_{fl}}\right] \left(1 + c_1 e^{-6\theta\tau} + c_2 e^{-20\theta\tau}\right)$$

or more precisely:

$$\tilde{G}(\tau) = \left\langle F_a'(0) F_a'(\tau) \right\rangle \left[1 - e^{-\tau/\tau_{fl}}\right] \left(\sum_{\ell} B_\ell(\hat{\mathbf{a}}, \hat{\mathbf{e}}_1, \hat{\mathbf{e}}_2)\, e^{-\ell(\ell+1)\theta\tau} \right)$$

where

$$B_\ell(\hat{\mathbf{a}}, \hat{\mathbf{e}}_1, \hat{\mathbf{e}}_2) = \sum_{m=-\ell}^{\ell} \frac{81}{4\pi} \int d\boldsymbol{\mu}_1 \int d\boldsymbol{\mu}_2 (\hat{\boldsymbol{\mu}}_1 \cdot \hat{\mathbf{a}})^2 (\hat{\boldsymbol{\mu}}_1 \cdot \hat{\mathbf{e}}_1)^2 (\hat{\boldsymbol{\mu}}_2 \cdot \hat{\mathbf{a}})^2 (\hat{\boldsymbol{\mu}}_2 \cdot \hat{\mathbf{e}}_2)^2 Y_{\ell m}(\boldsymbol{\mu}_1) Y_{\ell m}^*(\boldsymbol{\mu}_2)$$

and the $Y_{\ell m}(\mu)$ are the spherical harmonics