Time-resolved fluorescence spectroscopy: An old technique to monitor protein higher order structure changes

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Outline

- Introduction
- Experimental Setup and Data Analysis
- Results
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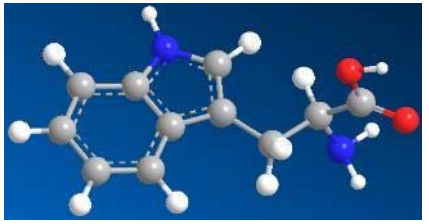
  - Case Study 2
  - Case Study 3
- Conclusions



Statement

Fluorescence spectroscopy cannot provide quantitative information about HOS

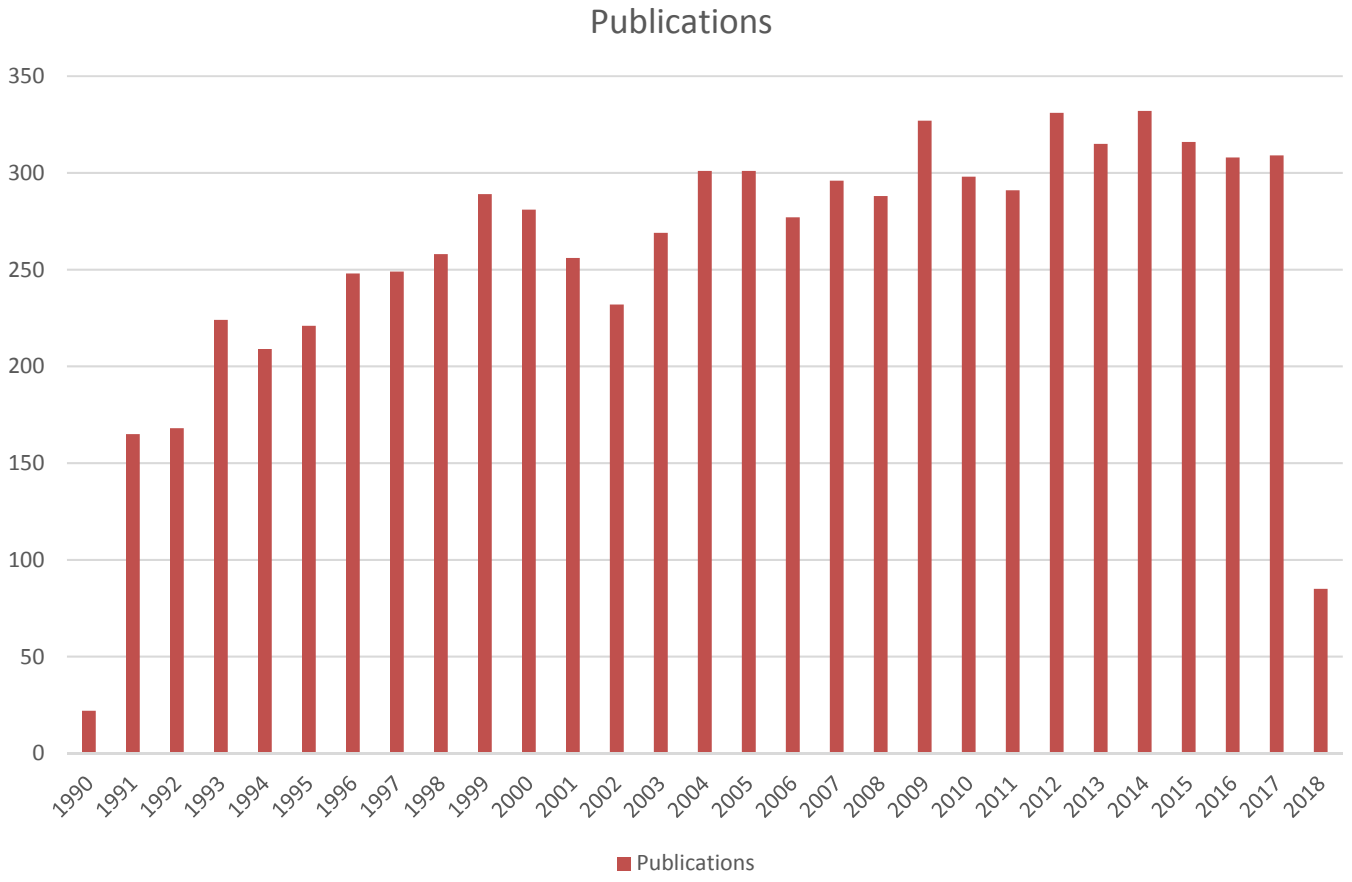
- No numeric values can be determine for α-helix and β-sheet

Fluorescence spectroscopy is sensitive to change in HOS

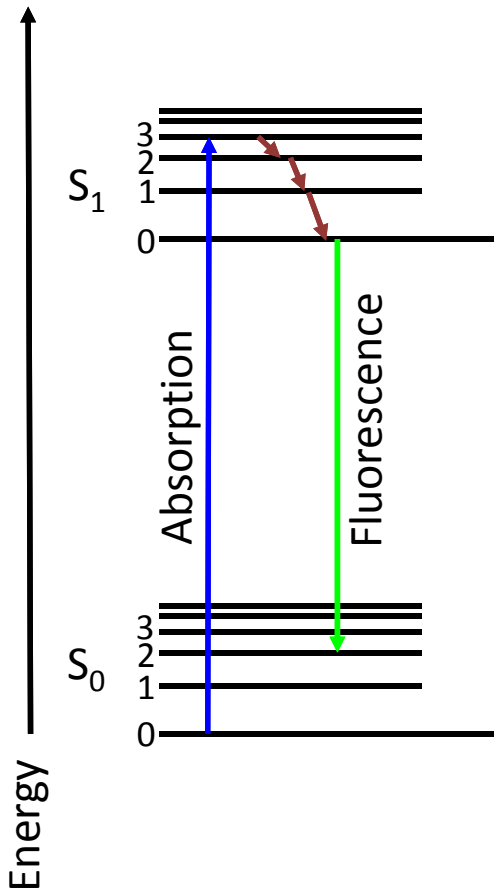
- Any change in fluorescence is indicative of a microenvironment change around tryptophan residues

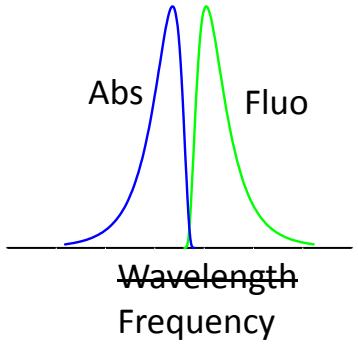
Web of Science

Search: tryptophan fluorescence protein



Introduction to Fluorescence

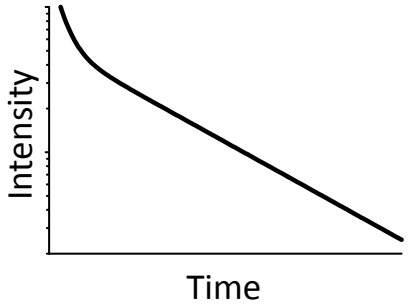




Log-normal function

$$I(x) = \frac{1}{x\sigma\sqrt{2\pi}} e^{-\frac{(lnx-\mu)^2}{2\sigma^2}}$$

ν_{peak} ; σ



Q - quantum yield

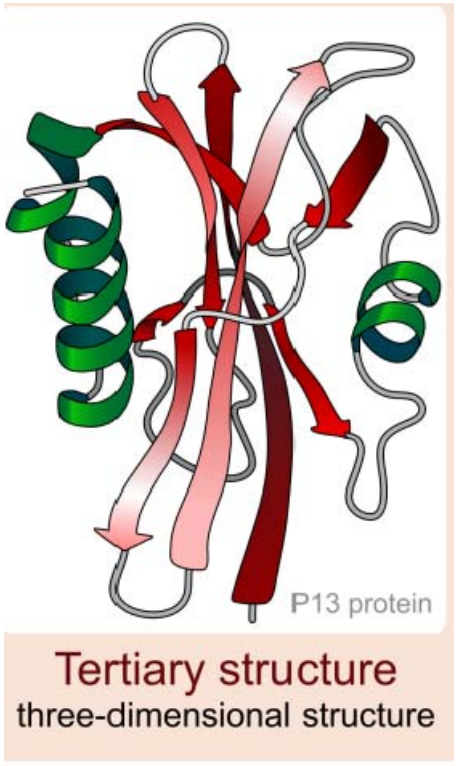
τ – fluorescence lifetime

τ_n – natural lifetime

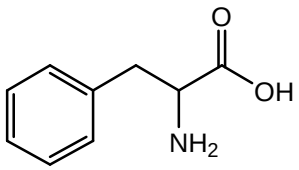
$\tau_n = \tau / Q$

✓ Time-resolved fluorescence provides information not available from steady-state data

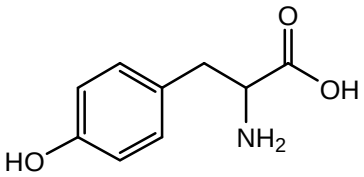
Intrinsic Protein Fluorescence



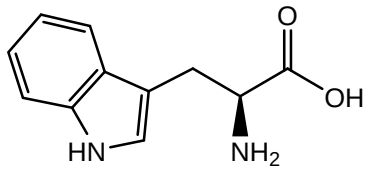
Tertiary structure
three-dimensional structure



phenylalanine



tyrosine

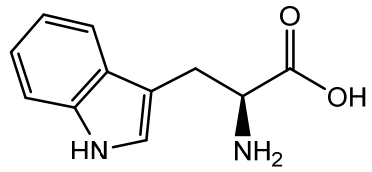


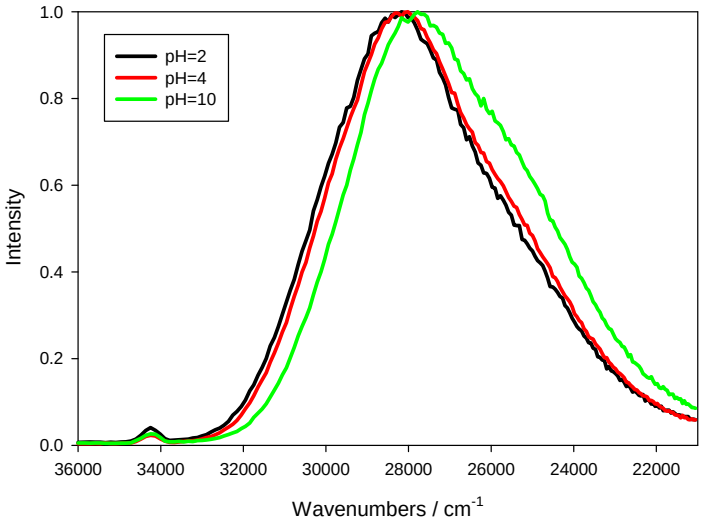
tryptophan

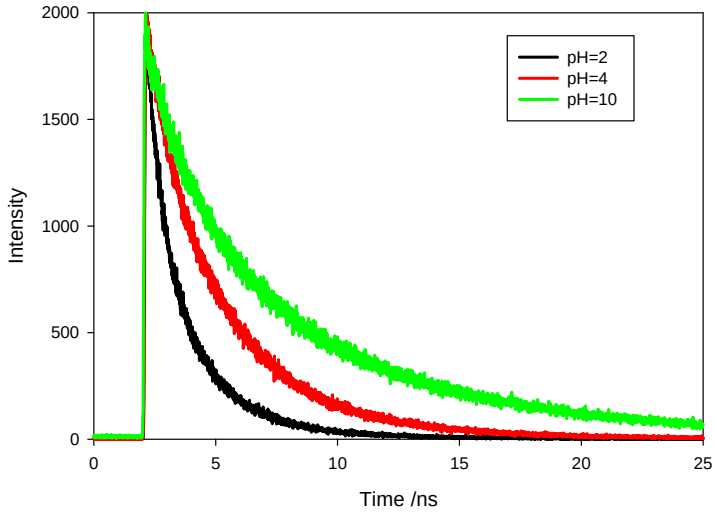
		Absorption		Fluorescence	
	τ (ns)	λ (nm)	ε	λ (nm)	Yield, Q
Tryptophan	3.1	280	5600	348	0.2
Tyrosine	3.6	274	1400	303	0.14
Phenylalanine	6.4	257	200	282	0.04

✓ Intrinsic fluorescence can be used to monitor changes in HOS

Tryptophan







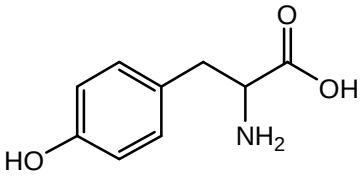
	pH=2	pH=4	pH=10
<τ>	1.98	3.2	7.06

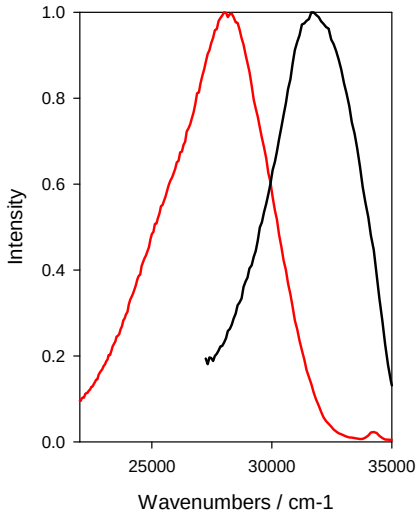
Tryptophan Fluorescence is sensitive to

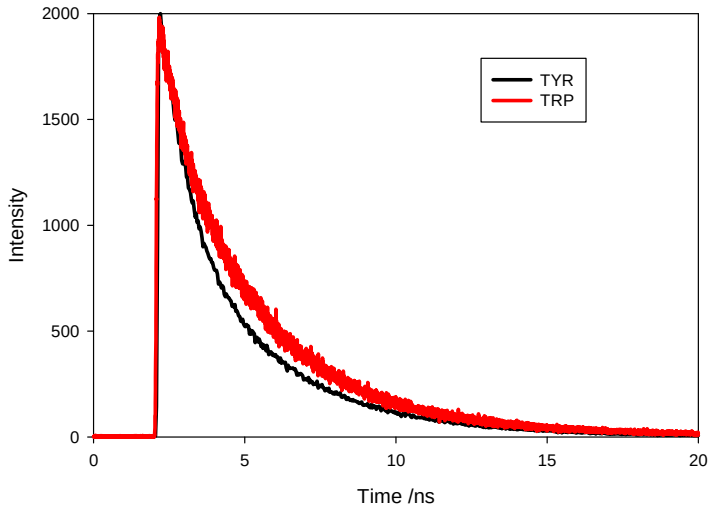
✓ pH

✓ Solvent Polarity

Tyrosine







- ✓ Distinguishable from tryptophan fluorescence
- ✓ Less sensitive to microenvironment

Resonance Energy Transfer

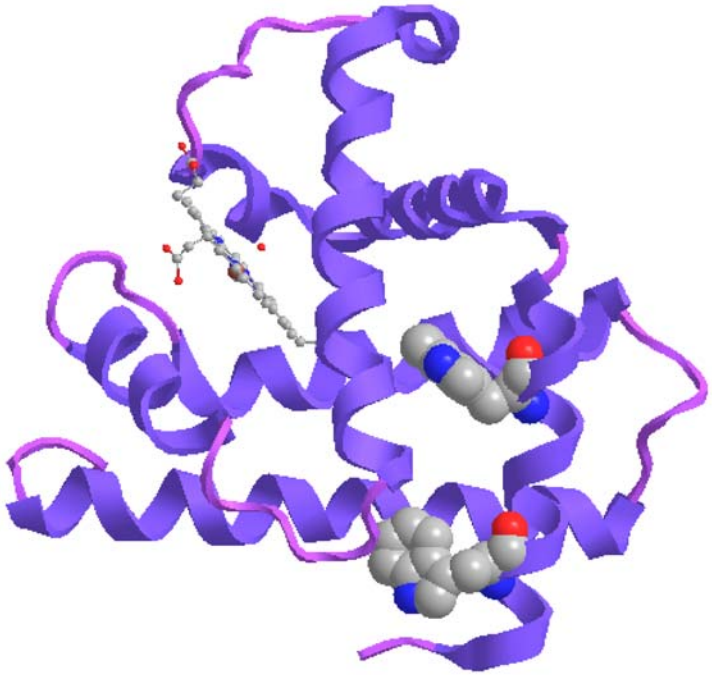
$$D^* + A \rightarrow D + A^*$$

$$k_T(r) = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6$$

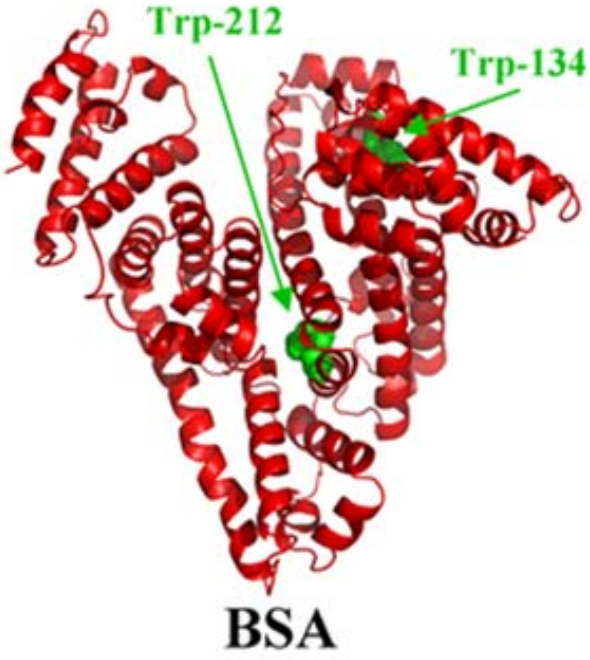
Donor	Acceptor	R_0(A)
Phe	Tyr	11.5-13.5
Tyr	Tyr	9-16
Tyr	Trp	9-18
Trp	Trp	4-16

✓ Tryptophan is acceptor for all three amino acids: phe, tyr, trp

Sensitivity

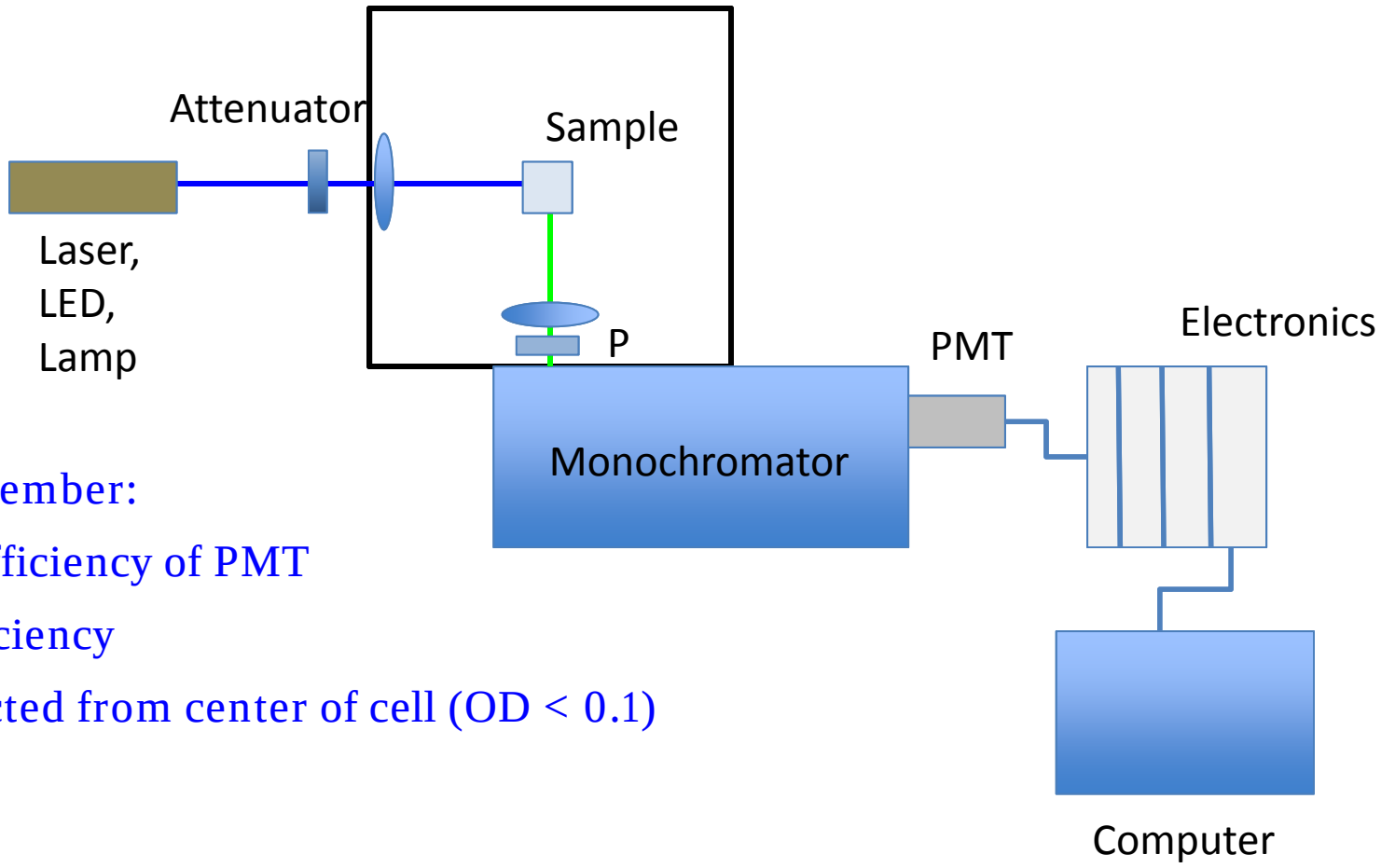


Myoglobin
Average Fluorescent Lifetime:
1.06 ns → 2.44 ns



Albumin (Bovine)
Average Fluorescent Lifetime:
4.38 ns → 2.3 ns

Spectrofluorometer



Things to remember:

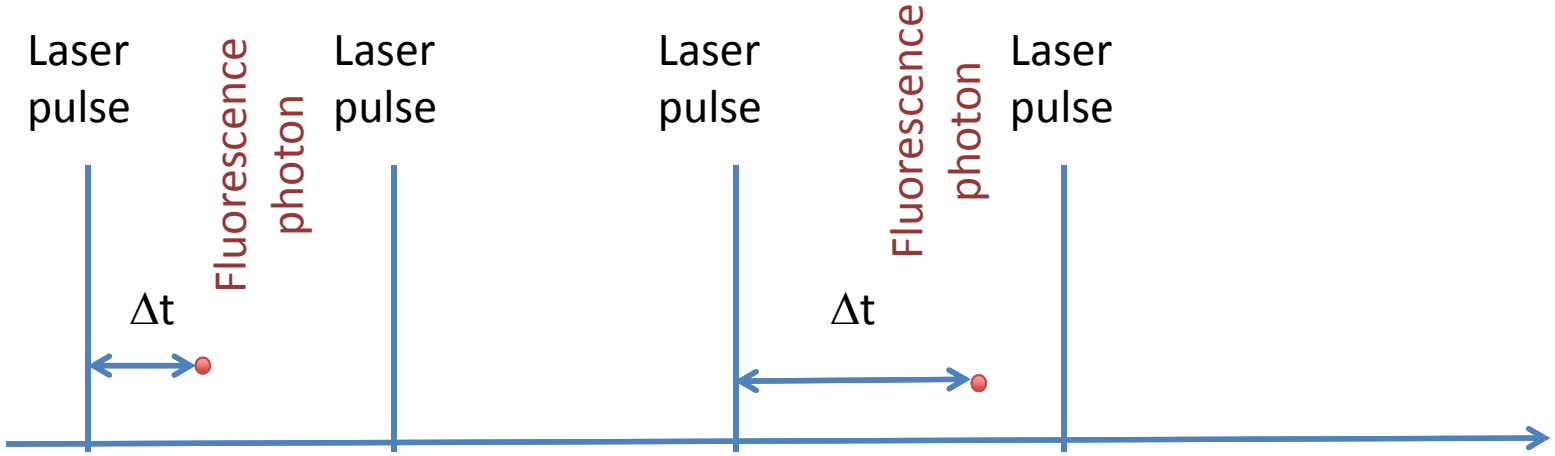
✓Quantum Efficiency of PMT

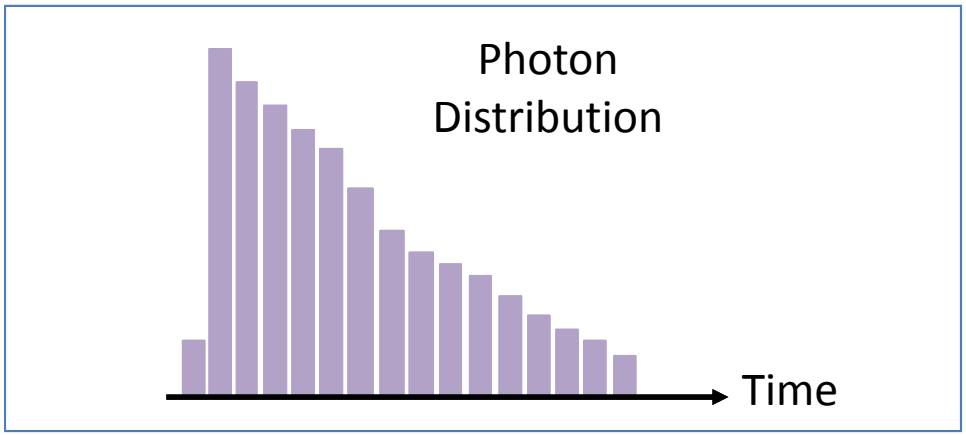
✓Grating Efficiency

✓Signal collected from center of cell (OD < 0.1)

✓Simple design, robust method

Time correlated single photon counting





✓Statistics

Deconvolution

Convolution

$$N(t) = \int_0^t L(t')I(t-t')dt'$$

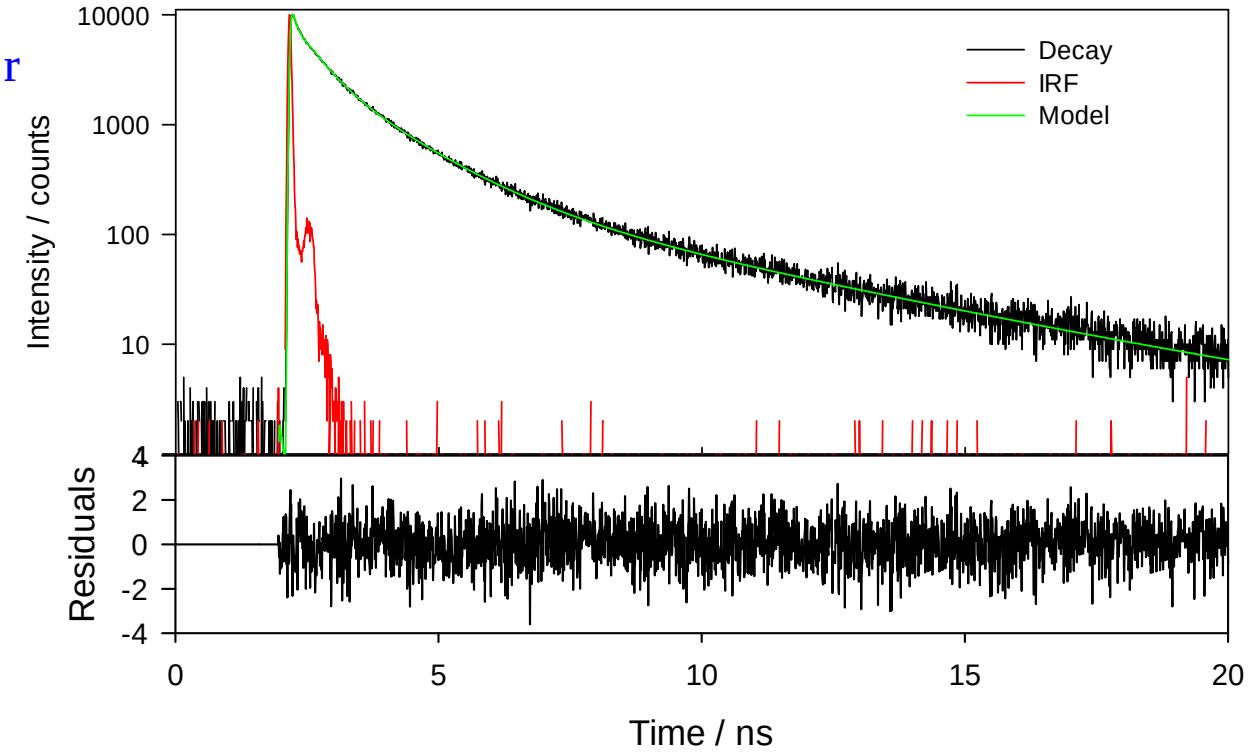
Model

$$I(t) = \sum_i A_i e^{-t/\tau_i}$$

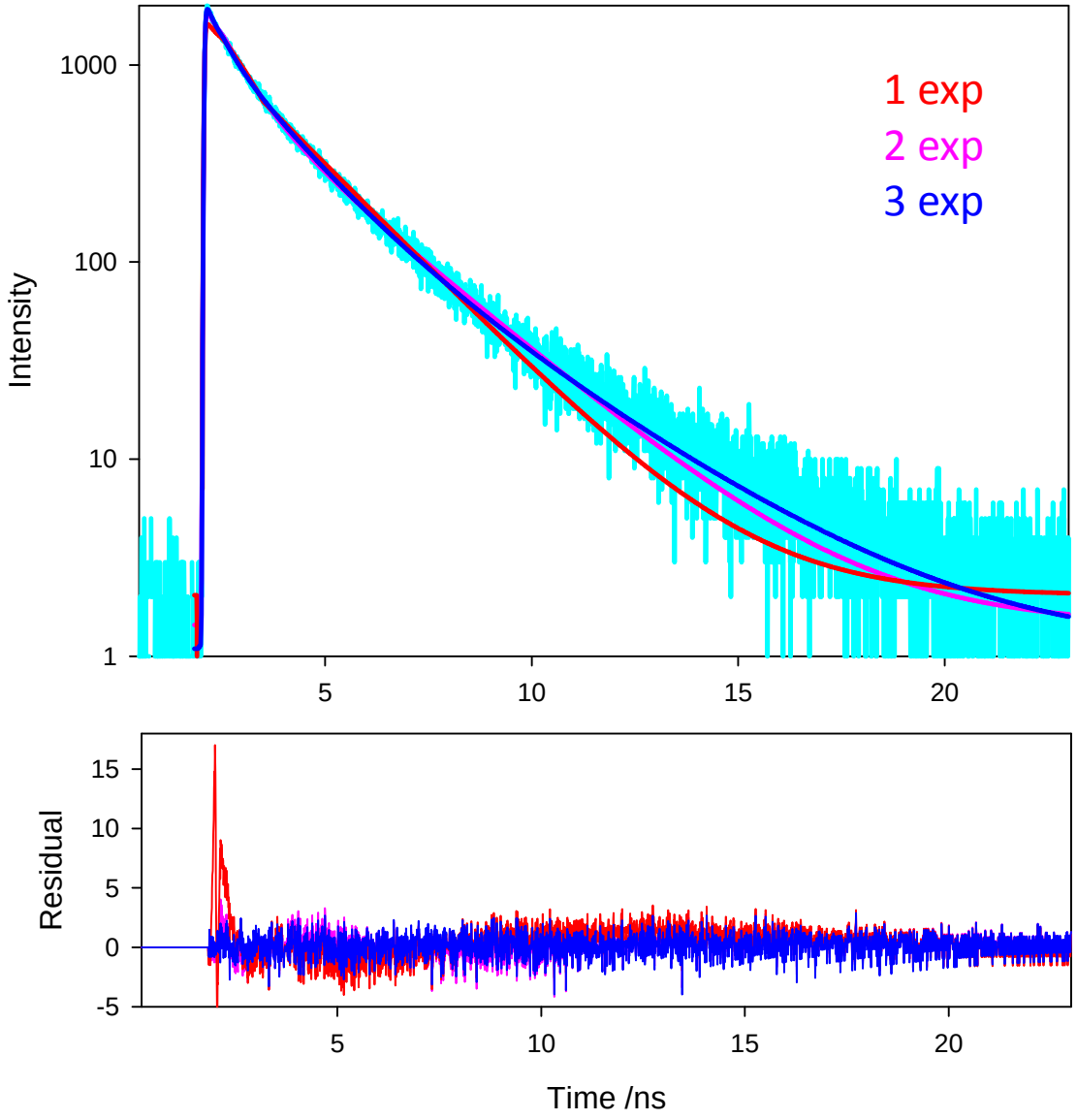
Goodness-of-fit parameter

$$\chi^2 = \sum_{k=1}^{n} \frac{|N(t_k) - N_c(t_k)|^2}{N(t_k)}$$

$$\chi_R^2 = \frac{\chi^2}{n-p}$$



Data Analysis



Models

Multiexponential model: $I(t) = \sum_i A_i e^{-t/\tau_i}$ Independent species

Stretched exponential model: $I(t) = \sum_i A_i e^{-(t/\tau_i)^{\beta_i}}$

Heterogeneous environment

Distribution model: $I(t) = \int_{-\infty}^{\infty} \rho(\tau) e^{-\frac{t}{\tau}} d\tau$

Dynamic environment

✓ Model should describe the system

Example of Models

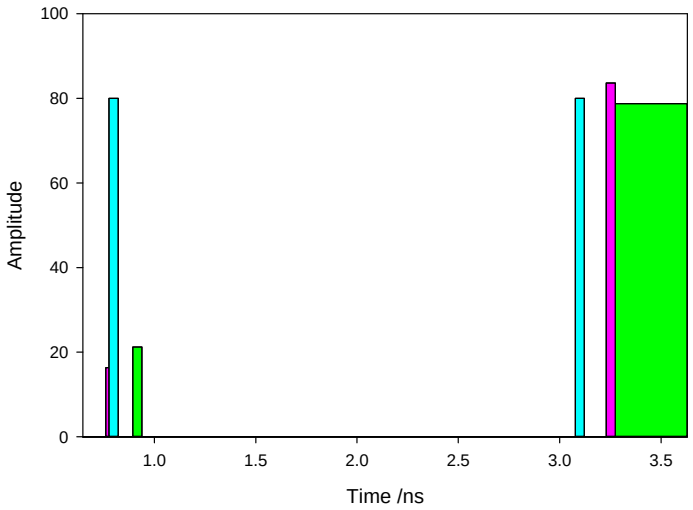
Multiexponential model: $\chi^2 = 0.9879$

τ=2.95 ns

Stretched exponential model: $\chi^2 = 0.9500$

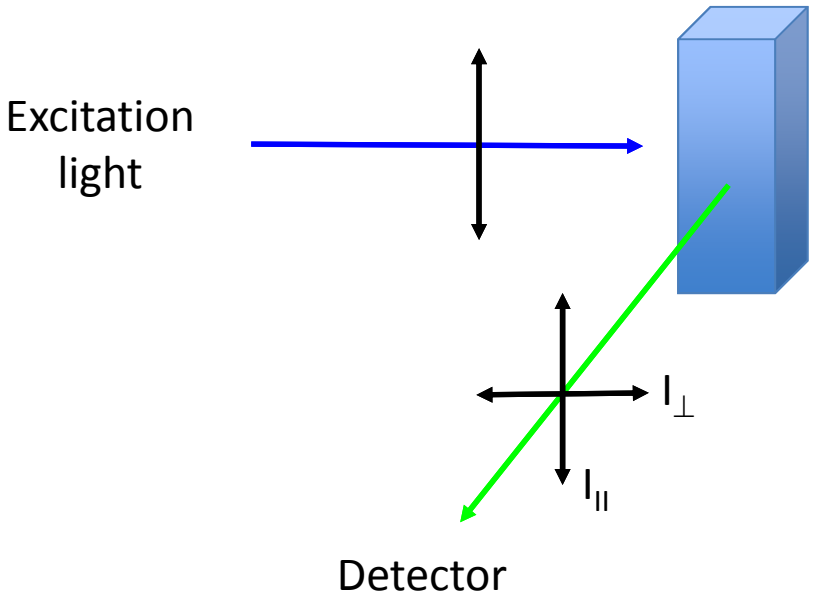
Distribution model: $\chi^2 = 0.9832$

τ=2.96 ns



✓ Model should describe the system

Fluorescence anisotropy



Anisotropy

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} = r_0 e^{-\frac{t}{\theta}}$$

Initial Anisotropy

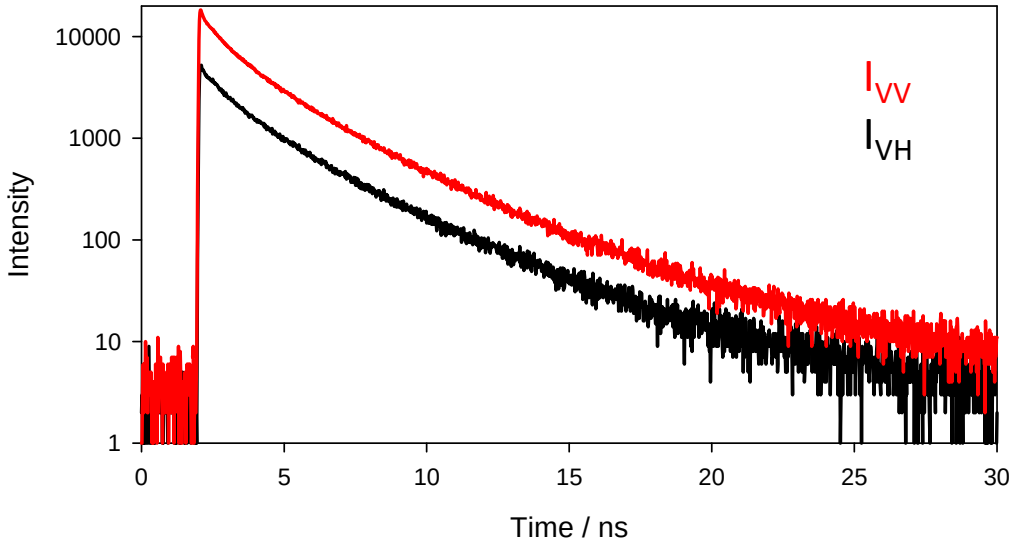
$$r_0 = \frac{2}{5}\left(\frac{3cos^2\beta - 1}{2}\right)$$

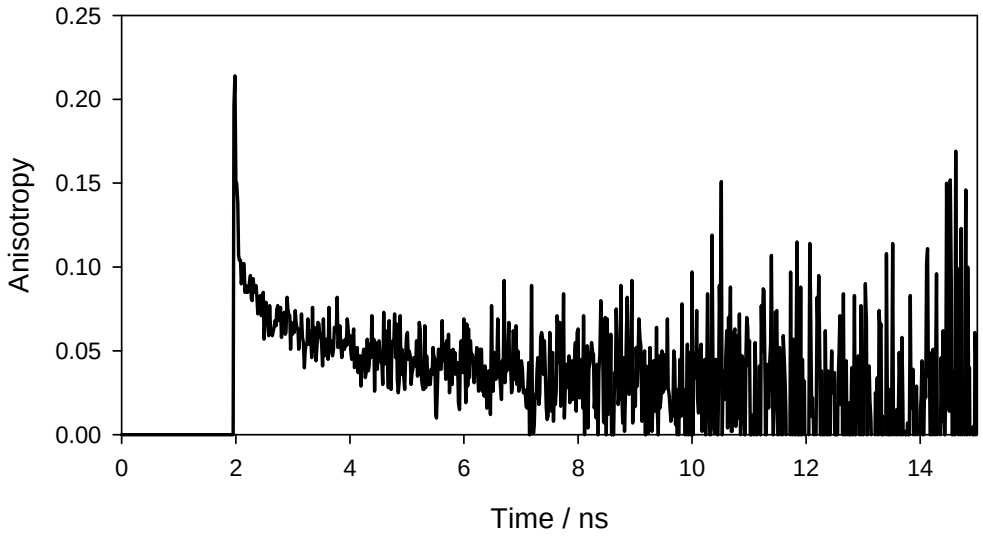
Rotational Correlation Time

$$\theta = \frac{\eta V}{RT}$$

✓Anisotropy measurement can provide information about size of the protein

Time-resolved Anisotropy





Two methods:

- Direct calculation
- Deconvolution

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)}$$

$$r(t) = \frac{I_{VV}(t) - \mathrm{G}I_{\mathrm{VH}}(t)}{I_{\mathrm{VV}}(t) + 2\mathrm{G}I_{VH}(t)}$$

✓ Correction by G factor

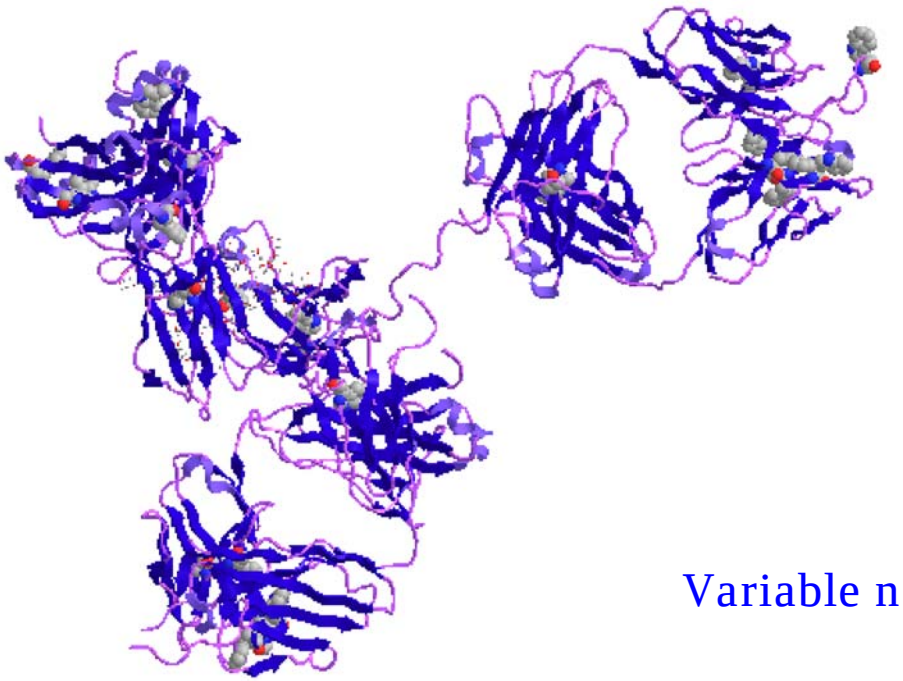
Case Study 1

Model protein in solution under stressed condition

Protein	Total #AA	Total # Trp	Location of Tryptophan		Average Fluorescent Lifetime, ns	with SDS	2M GuHCl
Albumin (bovine)	583	2	W-134 α-helix	W-212 α-helix	4.38	2.3	2.45
Myoglobin	152	2	W-7 α-helix	W-14 α-helix	1.06	2.44	2.32

Case Study 2

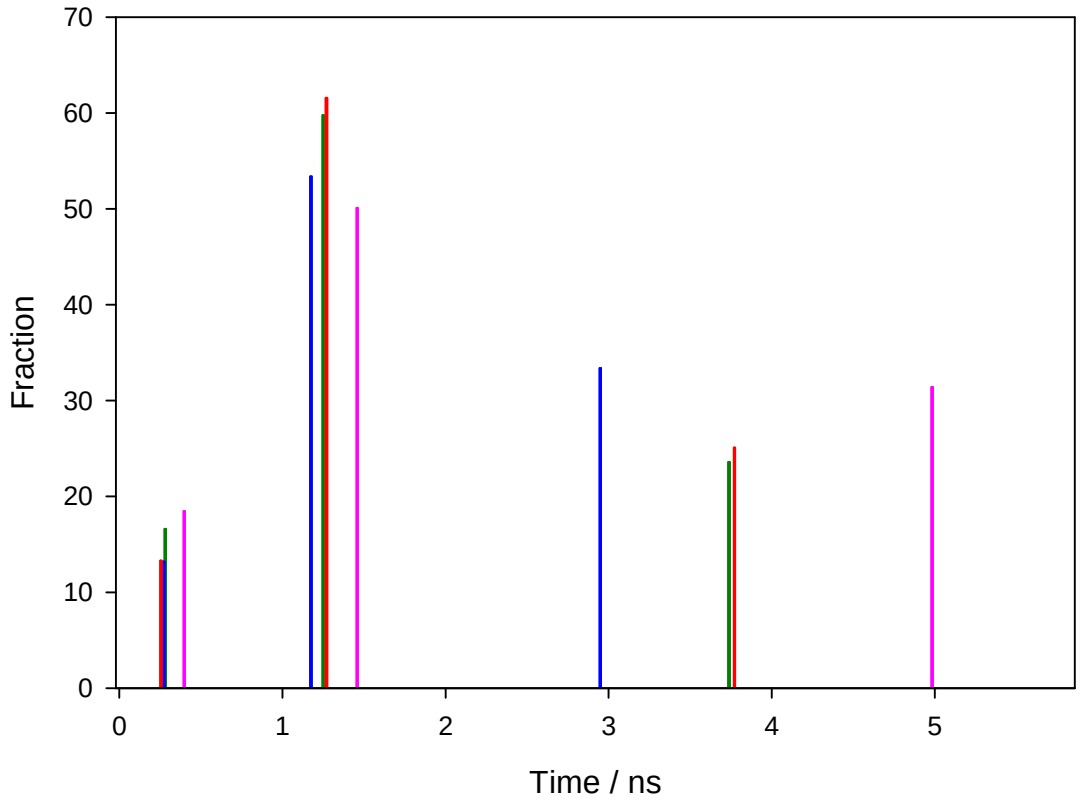
Monoclonal Antibody



Variable number of TRP

Case Study 2

4 Monoclonal Antibodies

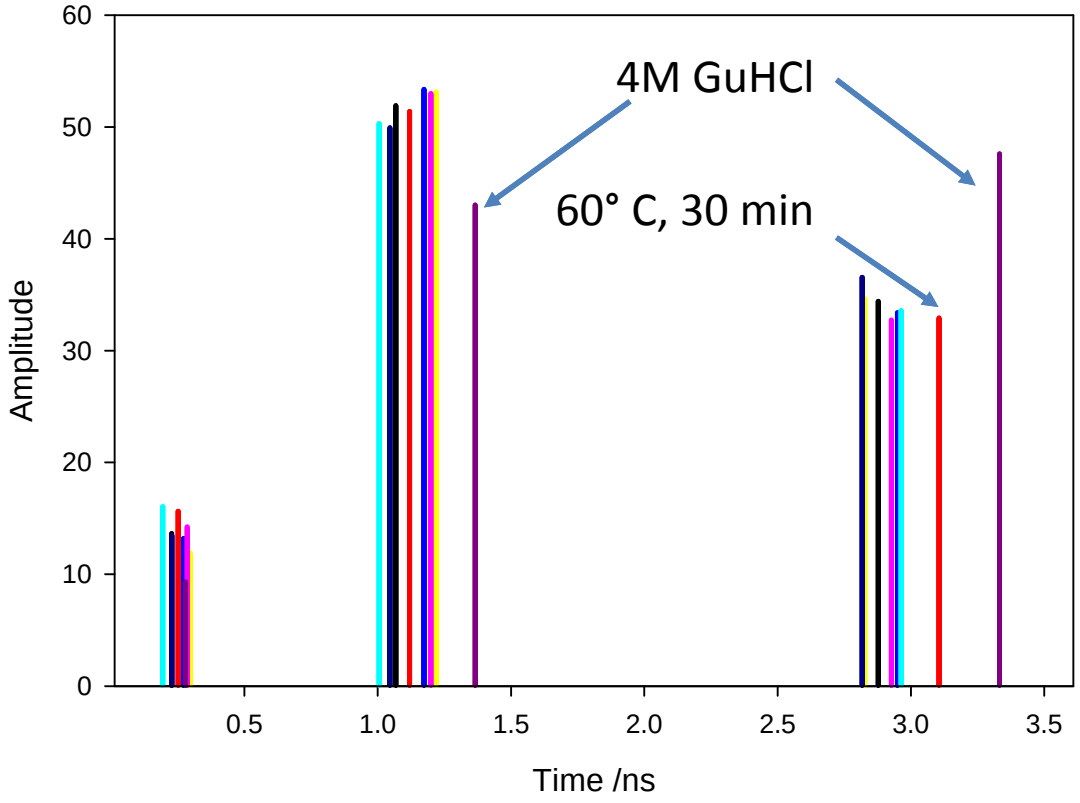


<τ>, ns
1.68
1.65
1.76
2.37

✓ Time-resolved fluorescence can distinguish mAbs

Case Study 3

Monoclonal Antibody under stressed condition



$\langle\tau\rangle$=1.62 ± 0.05 ns

τ_{heat}=1.64 ns

τ_{GuHCl}=2.20 ns

✓ Time-resolved fluorescence is sensitive enough to determine degradation of mAb

Conclusions

- Time-resolved fluorescence data provides information about changes in HOS, which is not available from steady-state data
- “Know before you measure”

Acknowledgements

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- Stephanie Capsuto


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