Optical Spectroscopies: UV-Vis, CD, Fluorescence

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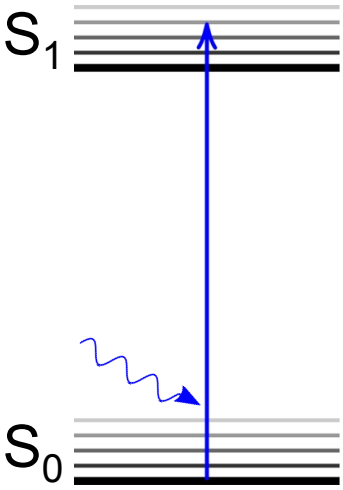
MEDCH 528

Jan 19/24, 2018

Outline

- UV-Vis Absorbance
  - » intrinsic vs. extrinsic chromophores
- Circular Dichroism
  - » far UV, near UV
- Fluorescence
  - » steady-state, lifetime, anisotropy, single-molecule

Electronic Spectroscopy



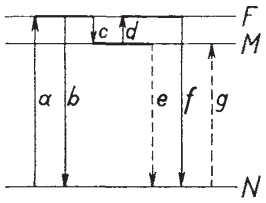
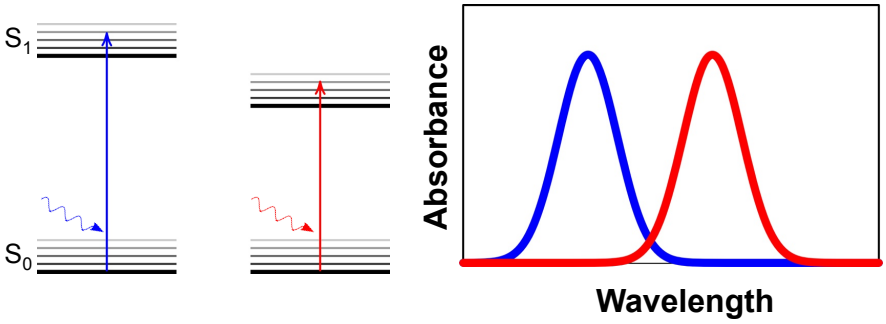


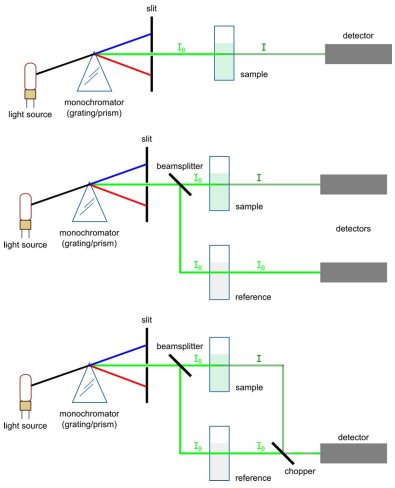
FIG. 1.—Energy levels in a phosphorescent molecule. *a*–absorption, *b*–fluorescence, *c*–transition to metastable level, *d*–thermal excitation, *e* and *f*–phosphorescence, *g*–absorption of very small transition probability.

Jablonski, *Nature* 131:839 (1933)

Ideal Absorbance Spectra



Spectrophotometer Schematics



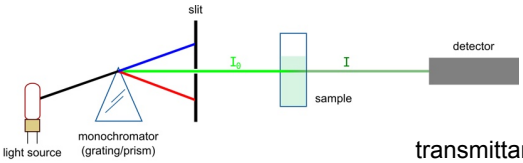
single-beam

dual-beam,
dual detector

dual-beam,
single detector

adapted from Wikipedia

What is Absorbance, Anyway?



transmittance

$$T = \frac{I}{I_0}$$

$$A \approx OD = \log_{10}\left(\frac{I_0}{I}\right)$$

absorbance

optical density

Beer's Law:
(aka Beer-Lambert Law)

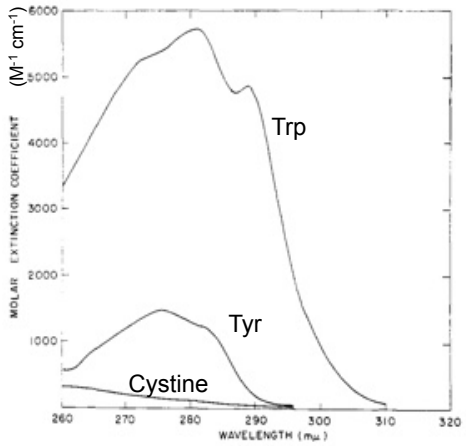
$$A = \varepsilon bc$$

pathlength

concentration

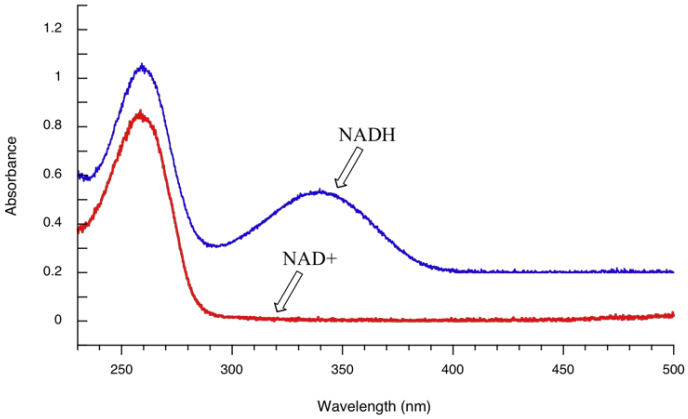
extinction coefficient
[conc^{-1}][length^{-1}]

Intrinsic Chromophores in Proteins



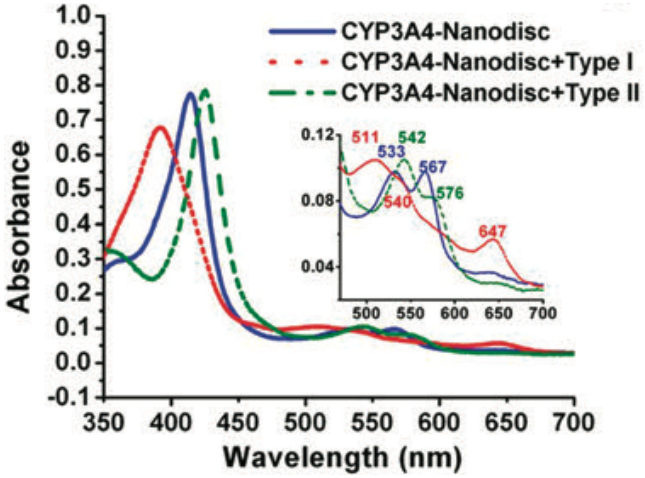
20 mM phosphate, pH 6.5, 6.0 M Gdn HCl Edelhoch, *Biochemistry* 6:1948 (1967)

Extrinsic Chromophores: Substrates/Products



ChemWiki, chemwiki.ucdavis.edu
Soderberg, *Organic Chemistry With a Biological Emphasis*

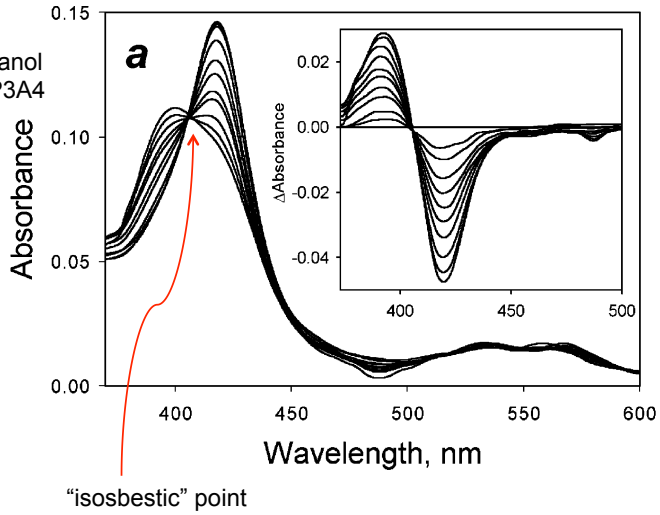
Extrinsic Chromophores: Prosthetic Groups



Das *et al.*, *Anal. Chem.* 81:3754 (2009)

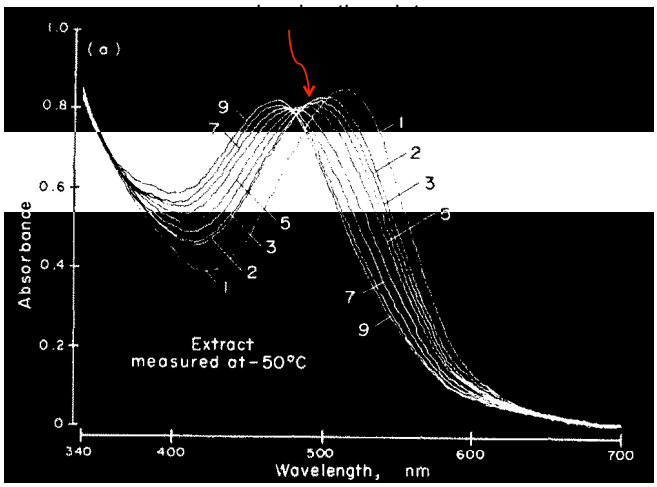
Spectral Titrations

1-pyrenebutanol binding CYP3A4



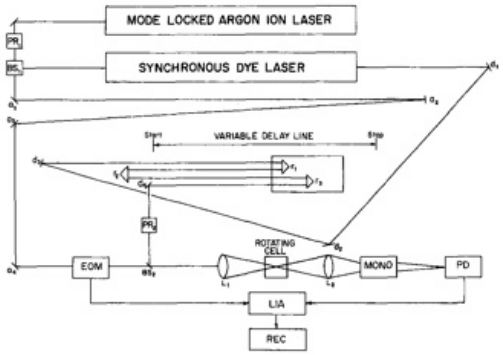
Fernando *et al. Biochemistry* 45:4199 (2006)

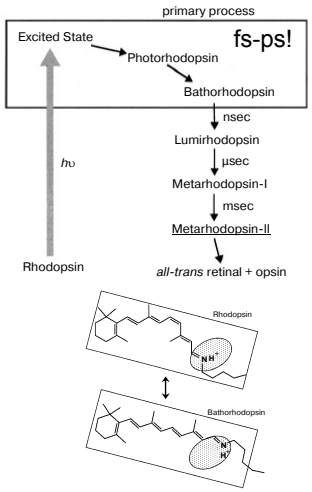
Non-2-State Systems: Rhodopsin Photobleaching



Kawamura *et al. Vision Res.* 17:991 (1977)

Transient Absorbance: Pump-Probe Spectroscopy





Lytle *et al. Appl. Spectr.* 39:444 (1985)

Kandori *et al. Biochemistry (Mosc.)* 66:1197 (2001)

Summary of Absorbance

- convenient probe of concentration
- label-free steady-state & kinetic insight
- can interrogate very short timescales

Caveats

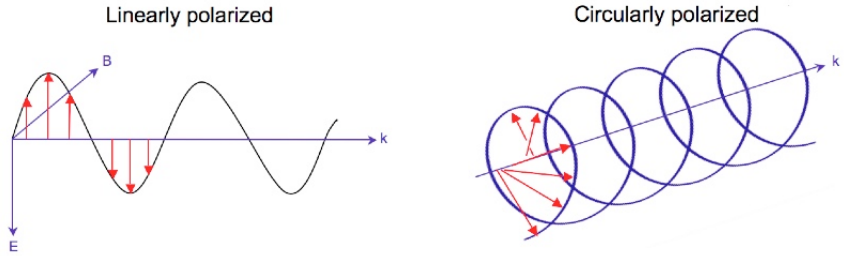
- low resolution
- intrinsic chromophores not very environment-sensitive

Outline

- UV-Vis Absorbance
  - » intrinsic vs. extrinsic chromophores
- Circular Dichroism
  - » far UV, near UV
- Fluorescence
  - » steady-state, lifetime, anisotropy, single-molecule

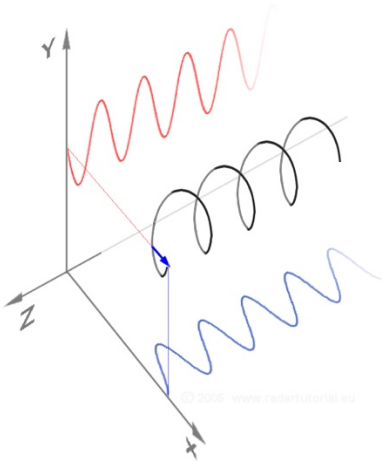
Circular Dichroism

- differential absorption of <u>circularly polarized</u> light by an <u>optically active</u> system



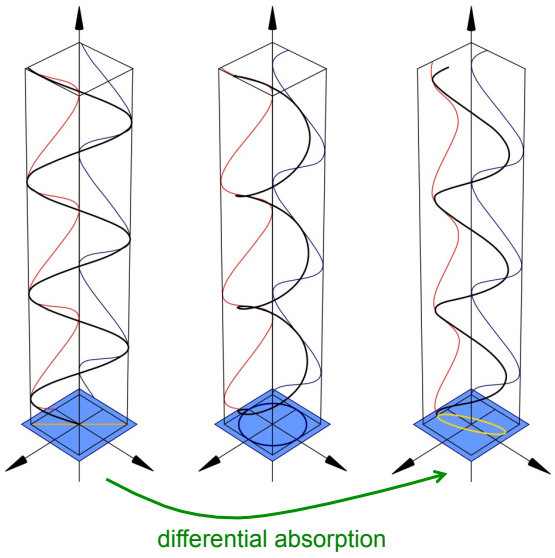
Wikipedia

Circularly Polarized Light (CPL)



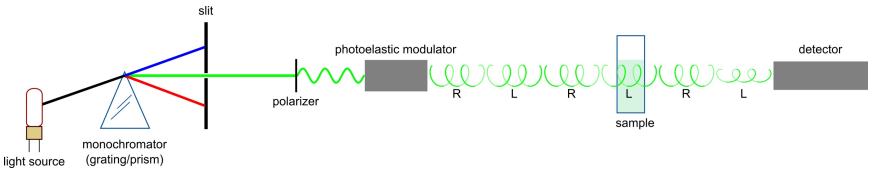
Wikipedia

Linear vs. Circular vs. Elliptical Polarizations



Wikipedia

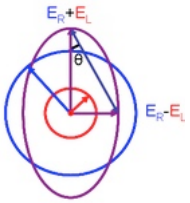
Measuring Circular Dichroism



Δabsorbance: $\Delta A = A_L - A_R$

molar circular dichroism: $\Delta\varepsilon = \dfrac{\Delta A}{bc}$

molar ellipticity: (in °cm²/dmol) $[\theta] \approx \dfrac{\tan\theta}{bc} = \dfrac{1}{bc}\left(\dfrac{I_R - I_L}{I_R + I_L}\right) = 3298\Delta\varepsilon$



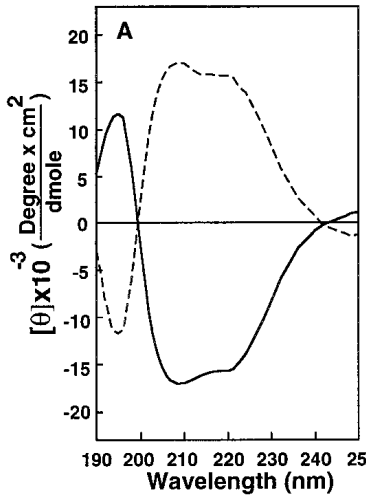
commonly reported as mean residue ellipticity:
molar ellipticity divided by # of amino acids in protein

chemwiki.ucdavis.edu

Optically Active Molecules

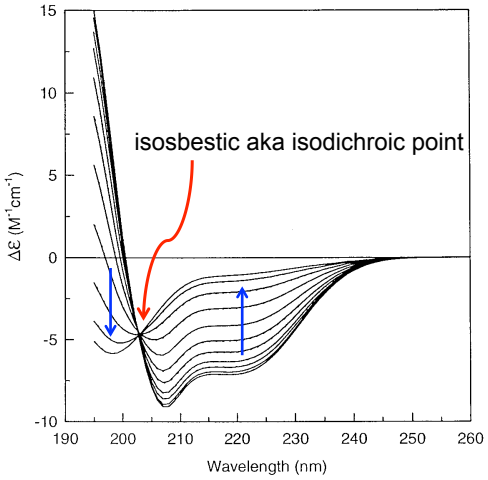
- chiral molecules interact differentially with circularly polarized light
  - » differential refraction: optical rotation
  - » differential absorbance: circular dichroism
- (any asymmetric single molecule is optically active, even without a chiral center)

Far-UV CD of Protein 2' Structure



Pritzker *et al. PNAS* 95:7287 (1998) Gratzer, *P. Roy. Soc. Lond. A.* 297:163 (1967)

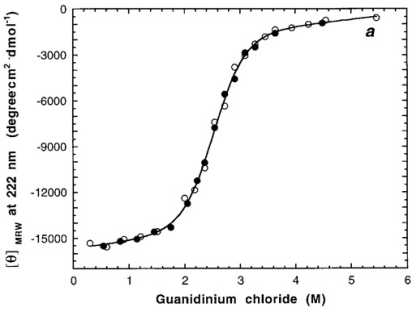
Measuring Protein Folding by CD



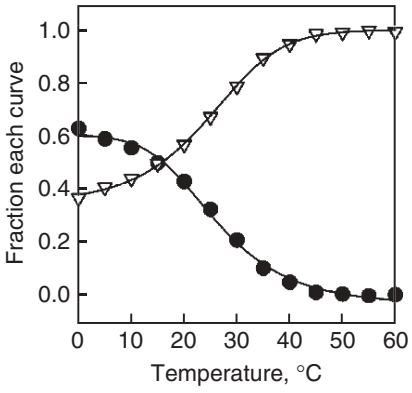
Krittanai & Johnson. *Anal. Biochem.* 253:57 (1997)

Measuring Protein Folding by CD

single-wavelength MRE



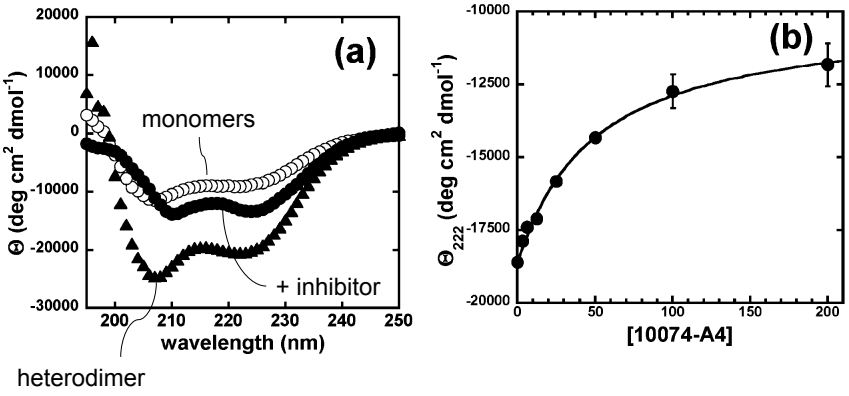
spectral deconvolution



Hornemann & Glockshuber. *J. Mol. Biol.* 262:614 (1996)

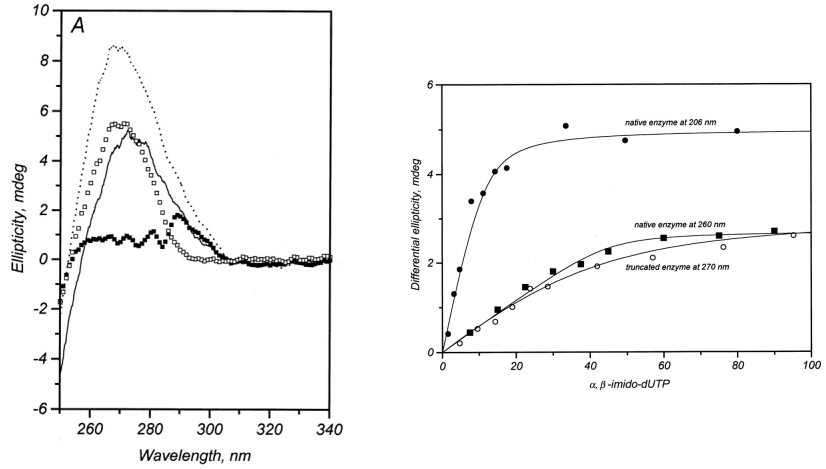
Greenfield. *Nat. Prot.* 1:2527 (2006)

Binding Measured by CD: Disruption of the c-Myc/Max Dimer



Hammoudeh *et al. J. Am. Chem. Soc.* 131:7390 (2009)

Near-UV CD: Local Structure Around Aromatic Side-Chains



Vertessy *et al. FEBS Lett.* 421:83 (1998)

Summary of CD

- label-free probe of secondary structure
- rich insight into folding mechanisms

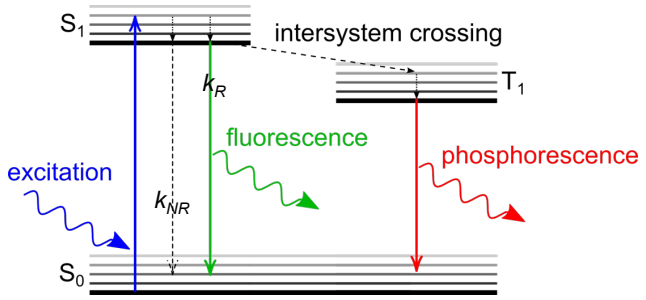
Caveats

- low resolution
- can be tricky to assign changes, esp. near-UV

Outline

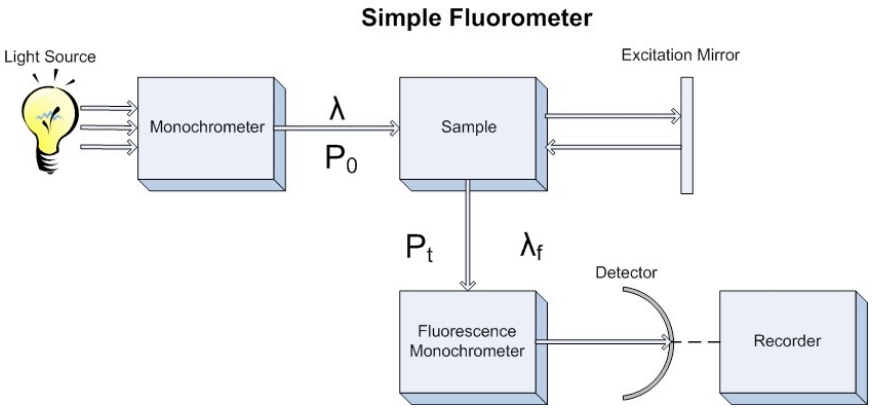
- UV-Vis Absorbance
  - » intrinsic vs. extrinsic chromophores
- Circular Dichroism
  - » far UV, near UV
- Fluorescence
  - » steady-state, lifetime, anisotropy, single-molecule

Fluorescence



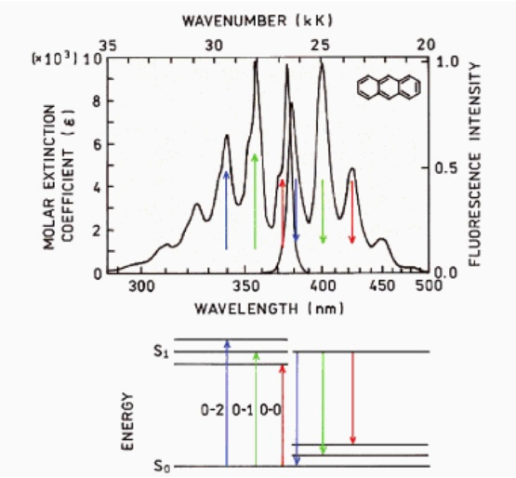
- intensity/quantum yield $\Phi = \frac{N_{emitted}}{N_{absorbed}} = \frac{k_R}{k_R + k_{NR}}$
  - » quenching, FRET
- spectral shifts
- excited-state lifetime
- anisotropy/polarization

Fluorimeter Schematic



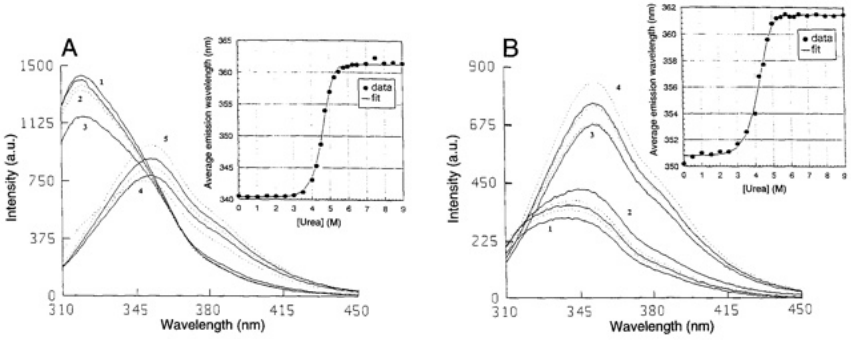
chemwiki.ucdavis.edu

Excitation & Emission Spectra



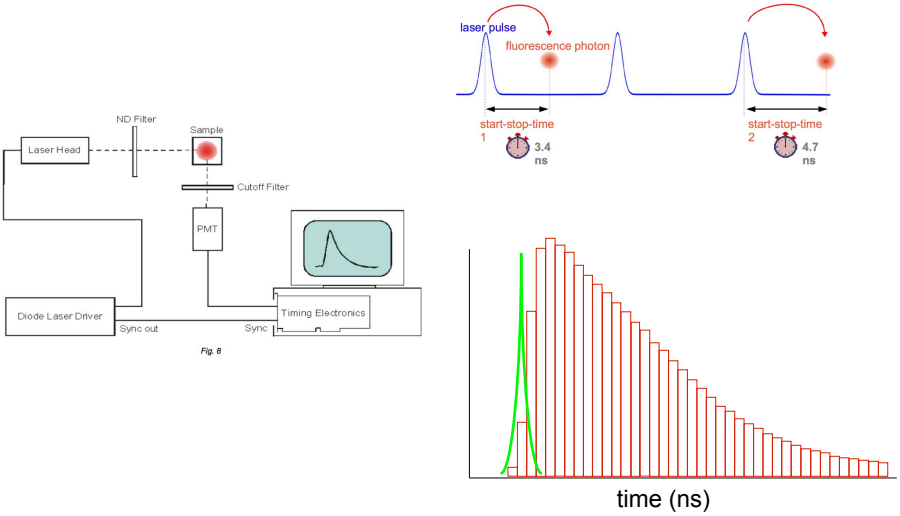
Lakowicz, *Principles of Fluorescence Spectroscopy*, 3rd Ed.

Intrinsic Trp Fluorescence: Folding



Royer *et al. Protein Sci.* 2:1844 (1993)

Excited State Lifetimes by TCSPC
(Time-Correlated Single Photon Counting)



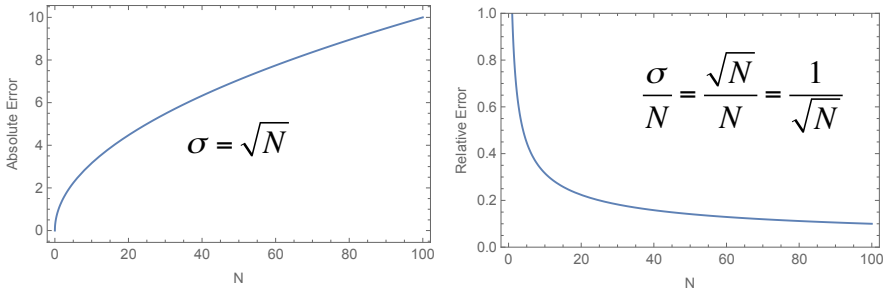
Wahl, Technical Note: TCSPC v2.1, Picoquant

Sidebar: Shot Noise

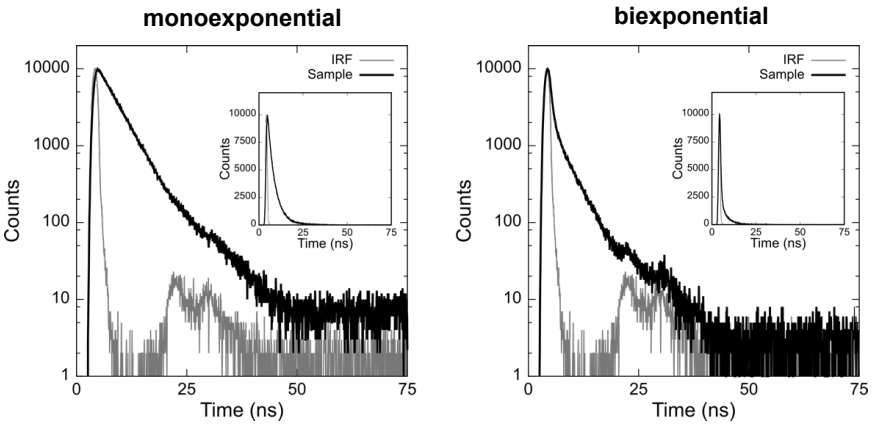
- For any counting process where the trials or intervals are independent of each other, the *variance* is equal to the *number of observations*.

$$\sigma^2 = N$$

$$\Rightarrow \sigma = \sqrt{N}$$



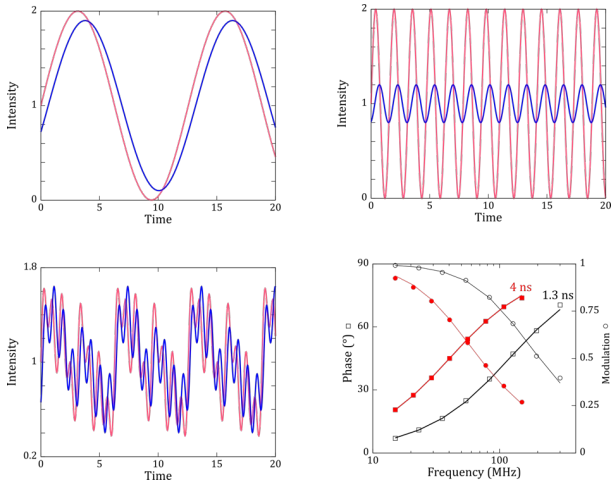
TCSPC: Mixed Systems



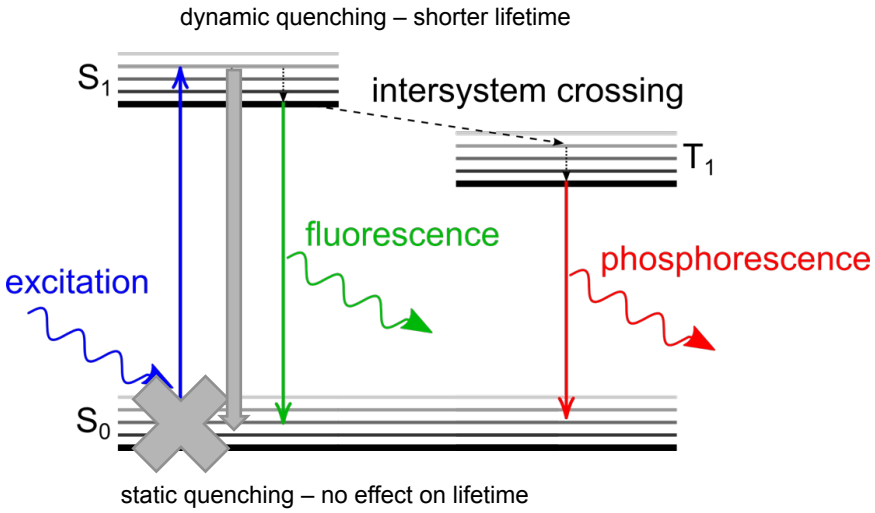
$$n(t) = n_0 \exp\left[-\frac{t}{\tau_F}\right]$$

$\langle t \rangle = \tau_F$ for a single decay event

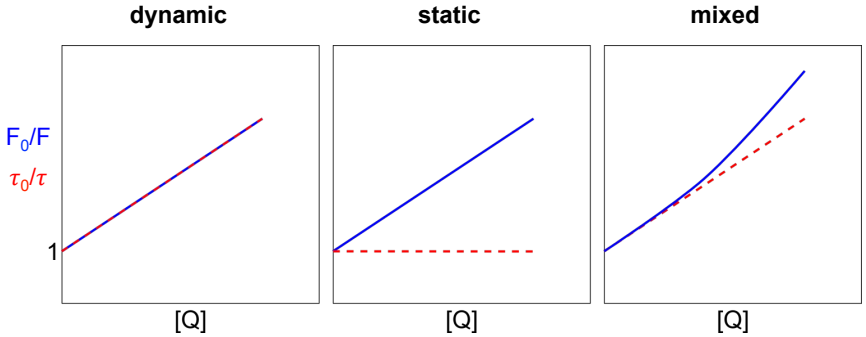
Frequency-Domain Lifetime Measurements



Fluorescence Quenching



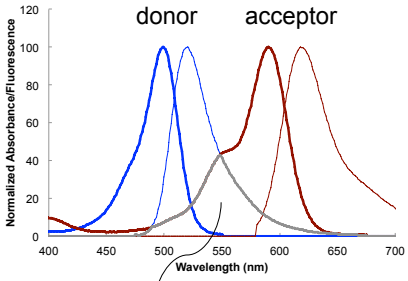
Fluorescence Quenching: Stern-Volmer Plots



dynamic $\frac{F_0}{F} = \frac{\tau_0}{\tau} = 1 + K_{SV}[Q] = 1 + k_a \tau_0 [Q]$ -- kinetics!

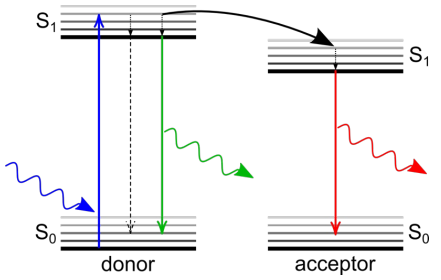
static $\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + \frac{[Q]}{K_D}$ -- affinity!

Förster Resonance Energy Transfer:
A Special Case of Dynamic Quenching



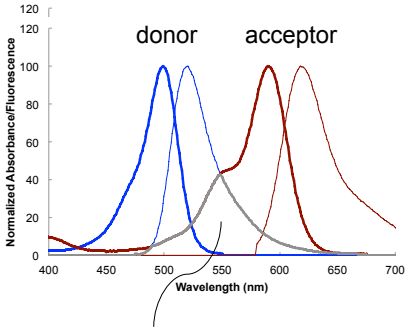
$J(\lambda)$
overlap in donor emission and acceptor excitation

non-radiative
(dipole-dipole coupling)



decrease in donor lifetime

Förster Resonance Energy Transfer:
A Special Case of Dynamic Quenching



$J(\lambda)$
overlap in donor emission and acceptor excitation

Förster equation describes distance dependence of energy transfer efficiency, ET_{eff}:

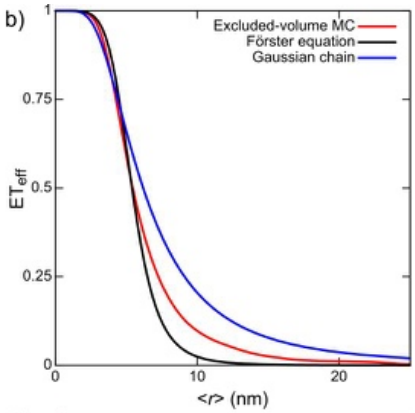
$$ET_{eff} = \frac{A}{A+D} = \frac{1}{1+\left(r/R_0\right)^6}$$

$$R_0 \propto \kappa^2 \Phi_D J\left(\lambda\right) n^{-4}$$

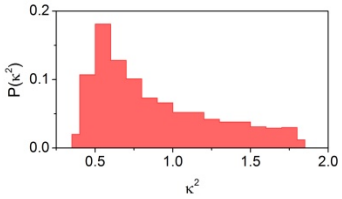
Förster radius R_0 depends on:
- orientational parameter $\kappa^2 \approx 2/3$
- donor quantum yield Φ_D
- overlap integral $J(\lambda)$
- refractive index n

Distance Measurement by FRET

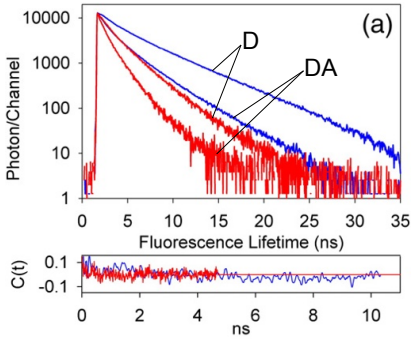
$$ET_{eff} = \frac{1}{1+\left(r/R_0\right)^6}$$

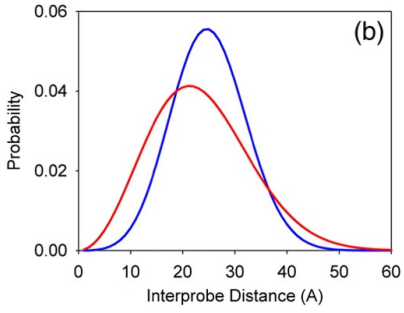


- very sensitive to distances ~ R_0 (typically 20-60Å)
- rapid dynamics can flatten the distance dependence
- κ^2 = 2/3? not always!



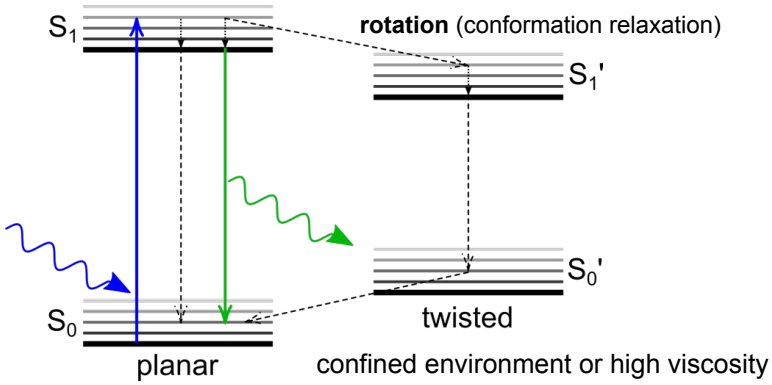
Distance Distributions by TR-FRET





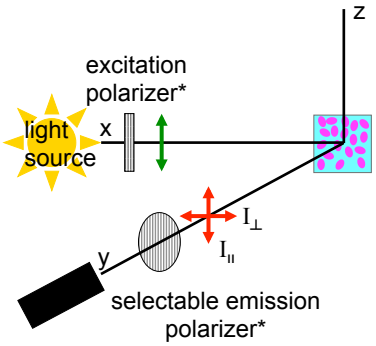
Grupi & Haas. *J. Mol. Biol.* 411:234 (2011)

Environment-Sensitive Extrinsic Fluors:
the Twisted Intramolecular Charge-Transfer Mechanism



examples: ANS, TNS, Nile Red, Thioflavin T…

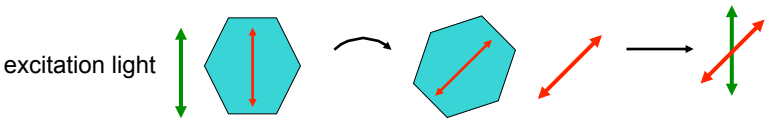
Fluorescence Anisotropy



sample excited with linearly polarized light – emission will also be polarized.
how polarized? depends on how quickly the fluorophore is tumbling.

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$ **anisotropy**

$$\left(p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \right)$$ **polarization**



The Perrin Equation

$$\frac{r_0}{r} = 1 + \frac{\tau}{\theta}$$

τ = fluorescence lifetime
r_0 = fundamental anisotropy
θ = rotational correlation time

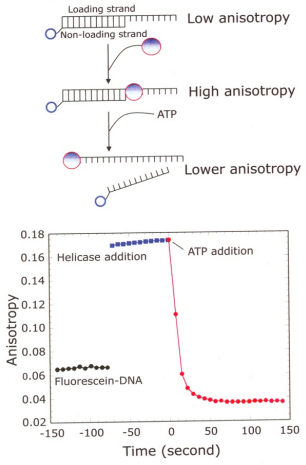
$$\theta = \frac{\eta V_H}{RT} = \left(6D_R\right)^{-1}$$

R=gas constant
T=temperature
η=viscosity
V_H=volume of molecule
D_R=rotational diffusion coefficient

$$r_0 = \frac{3\cos^2\xi - 1}{5} \le 0.4$$

where ξ is the angle between excitation and emission dipole moments

Helicase Activity Monitored by Anisotropy



anisotropy jumps upon helicase binding to labeled DNA

ATP addition triggers unwinding – anisotropy progressively decreases as strands unwind

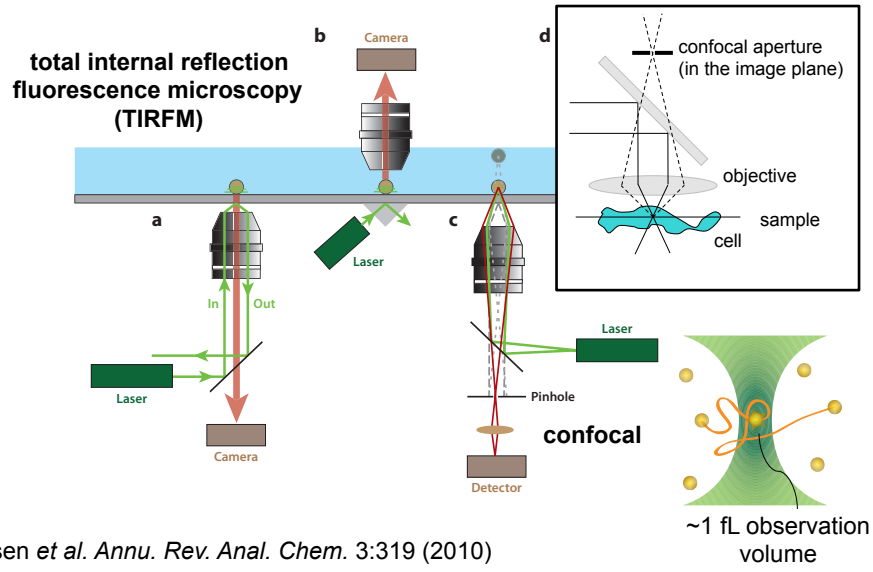
low final anisotropy reflects lower molecular weight and higher flexibility of ssDNA product vs. dsDNA substrate

Xu *et al. Nuc. Acids Res.* e70 (2003)

Outline

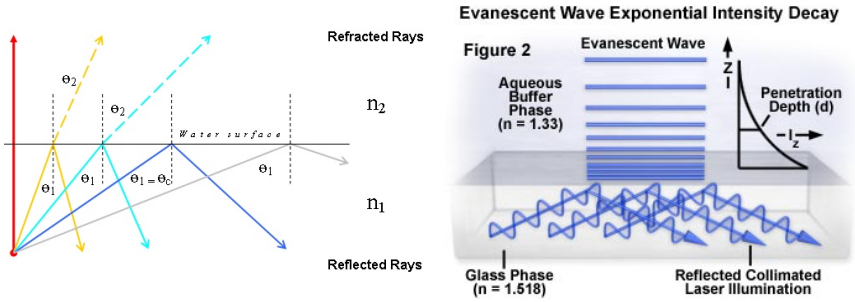
- UV-Vis Absorbance
  - » intrinsic vs. extrinsic chromophores
- Circular Dichroism
  - » far UV, near UV
- Fluorescence
  - » steady-state, lifetime, anisotropy, single-molecule

Single-Molecule Geometries



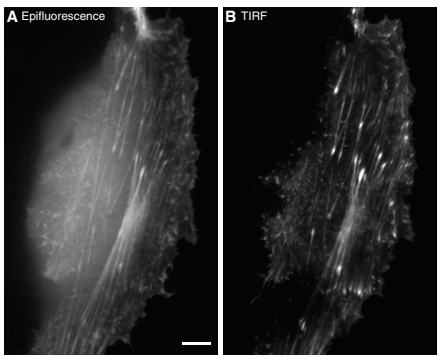
Claessen *et al. Annu. Rev. Anal. Chem.* 3:319 (2010)

TIRFM Selective Probes Surface-bound Molecules

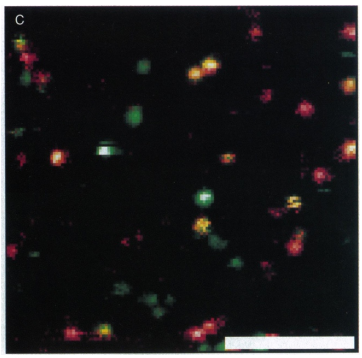


Olympus, http://www.olympusmicro.com/primer/

Single Molecules Are Resolved as Diffraction-Limited Spots

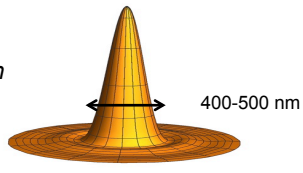


Mattheyses *et al. J. Cell. Sci.* (2010) 123:3621

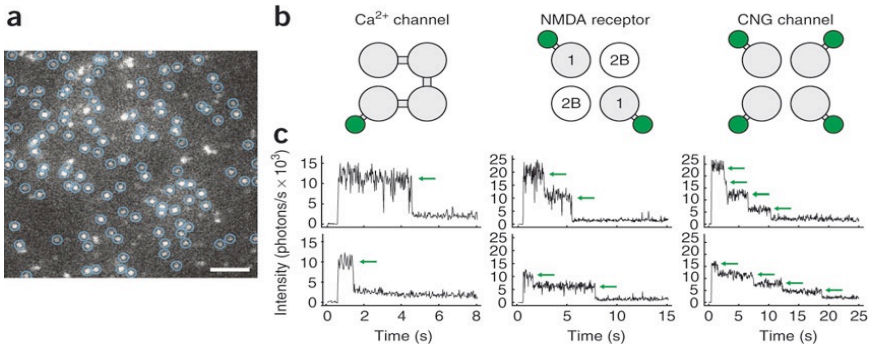


Ha *et al. PNAS* (1996) 93:6264

Airy disk: the *point spread function* that determines the diffraction limit



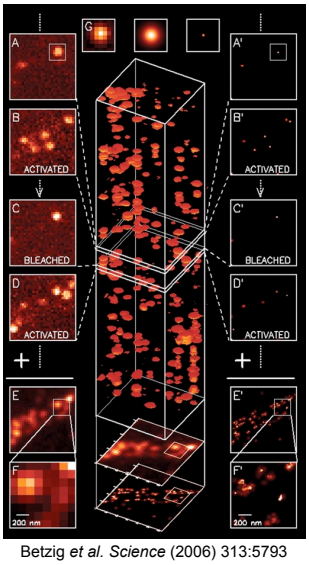
Measuring Stoichiometry by Single-Molecule Photobleaching



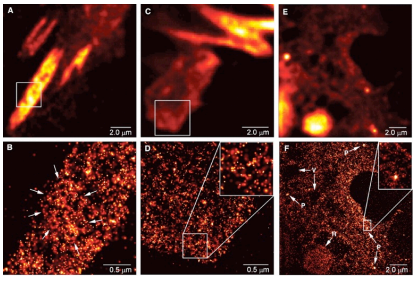
Ulbrich & Isacoff *Nature Methods* (2007) 3:319

Super-Resolution Microscopy :

(STORM, PALM, FPALM, PAINT, SHRIMP, FIONA…)

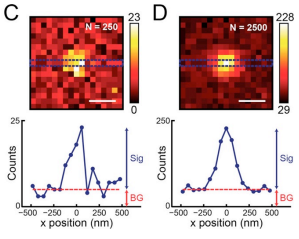


Betzig *et al. Science* (2006) 313:5793



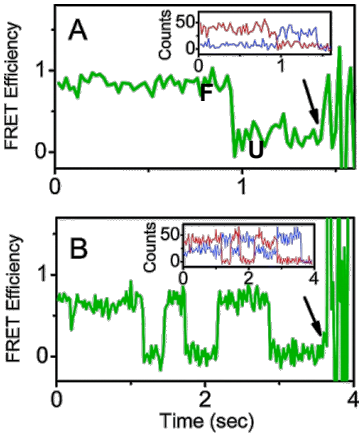
resolution depends on number of photons detected per molecule:

$$\sigma \propto \frac{1}{\sqrt{N}}$$



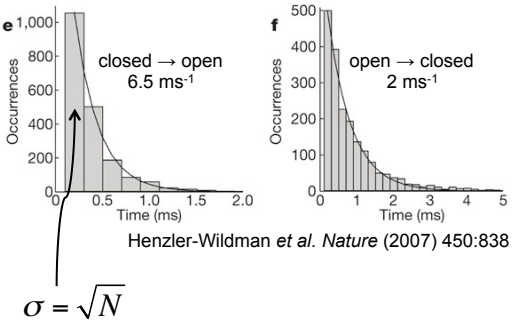
von Diezmann *et al. Chem. Rev.* (2017) 117:7244

Conformational Dynamics of Immobilized Protein Molecules



Rhoades *et al. JACS* (2004) 126:14686

residence time histograms:
kinetic information

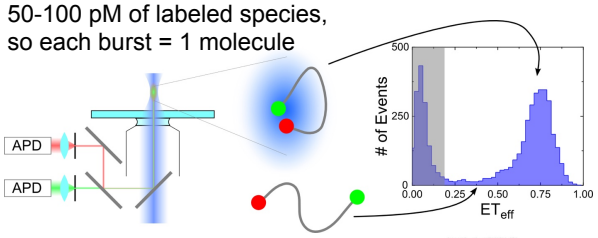


Henzler-Wildman *et al. Nature* (2007) 450:838

$$\sigma = \sqrt{N}$$

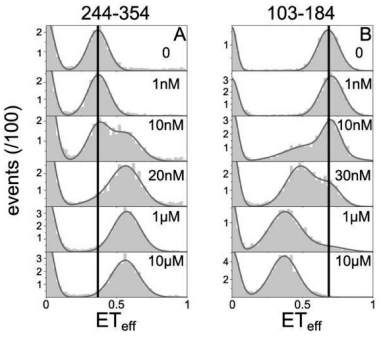
Confocal Single Molecule-FRET

50-100 pM of labeled species,
so each burst = 1 molecule



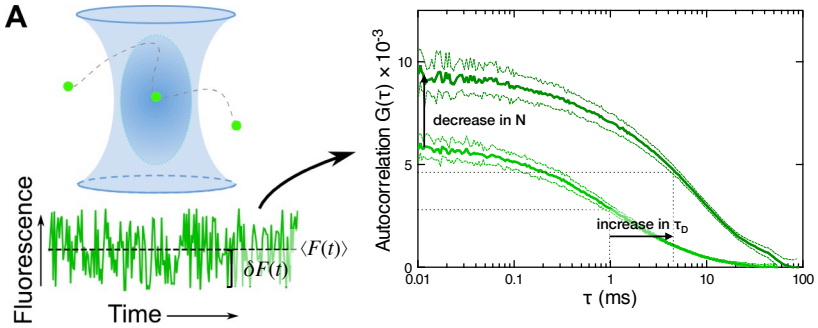
compile statistics
for 1000s of bursts

intrinsically disordered protein tau
binding heparin:



Elbaum-Garfinkle & Rhoades,
J. Am. Chem. Soc. 134:16607 (2012)

Fluorescence Correlation Spectroscopy



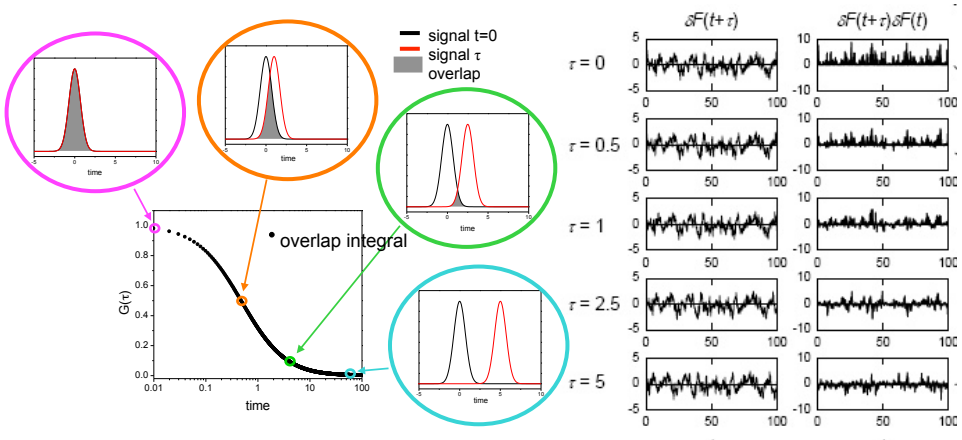
$$\delta F(t) = F(t) - \langle F(t) \rangle$$

$$\langle F(t) \rangle = \frac{1}{T}\int_0^T F(t)dt$$

normalized autocorrelation

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

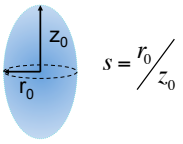
Why Does Autocorrelation Decay?



Adapted from *Fluorescence Correlation Spectroscopy: An Introduction to its Concepts and Applications*, by Petra Schwille and Elke Haustein

Diffusion Measurement by FCS

in 3 dimensions, $G(\tau) = \dfrac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} = \dfrac{1}{N} \dfrac{1}{\left(1+\frac{\tau}{\tau_D}\right)} \dfrac{1}{\left(1+s^2 \frac{\tau}{\tau_D}\right)^{1/2}}$



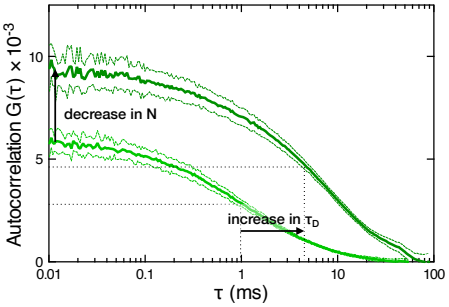
$s = r_0 / z_0$

focal volume

N : average number of molecules in focal volume (an absolute measure of concentration!)

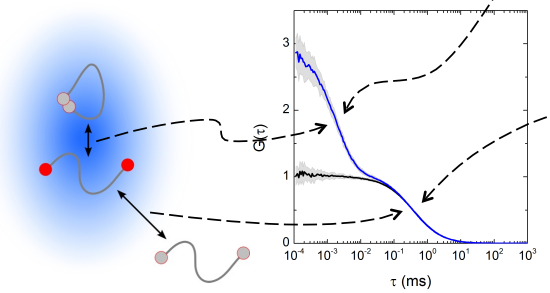
τ_D : diffusion time of molecules

$$\tau_D \propto R_h \propto 1/D$$



Other Uses of FCS

$$G(\tau) = \left(\frac{1 - A + A\exp\left(-\tau/\tau_A\right)}{1-A}\right)\left(\frac{1}{N}\frac{1}{\left(1+\frac{\tau}{\tau_D}\right)}\frac{1}{\left(1+s^2\frac{\tau}{\tau_D}\right)^{1/2}}\right)$$



Fast (sub-ms) components of the autocorrelation signal can be used to probe conformational dynamics, chemical reactions, and rapid photophysical processes.

Summary of Fluorescence

- sensitive, selective and versatile probe of:
  - » conformation, local environment, intramolecular distances
- broad dynamic range for kinetics:
  - » excited-state lifetime, anisotropy, FCS
- insight into molecular heterogeneity, population distributions
  - » SM-FRET, TR-FRET, TIRF

Caveats

- many factors can affect intrinsic fluorescence
- extrinsic fluorophores may perturb the system
- distance determination – low resolution, many potential artifacts