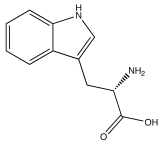
MRC Laboratory of Molecular Biology

Fluorescence Spectroscopy and Microscale Thermophoresis



Stephen McLaughlin
Biophysics
MRC Laboratory of Molecular Biology

Overview

- Phenomena of Fluorescence
- Applications of Fluorescence
  - Stability of proteins
  - Binding studies
  - Kinetics: pre- and steady-state
  - Ligand Screening
- Microscale Thermophoresis

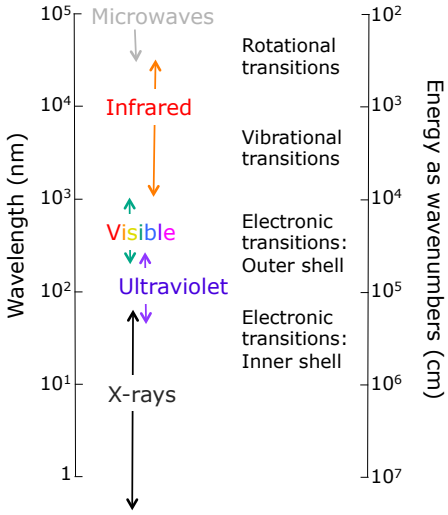
The Energy of Transitions

$$E=h\nu=hc/\lambda$$

- Transitions between electronic states require the energy of light in the UV/visible/X-ray region

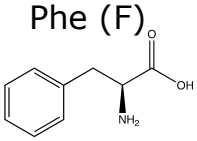
Fluorescence occurs in:

- aromatic compounds ($\pi \rightarrow \pi^*$)
- Lanthinide chelates such as Tb^{3+} and Eu^{3+}

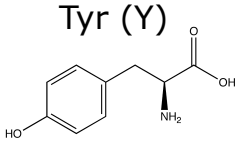


Fluorescence Groups

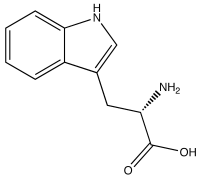
Phe (F)



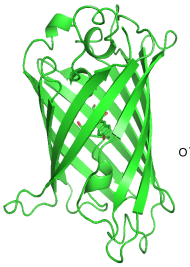
Tyr (Y)

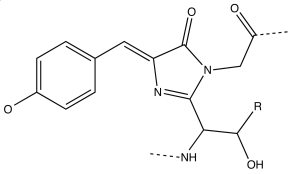


Trp (W)

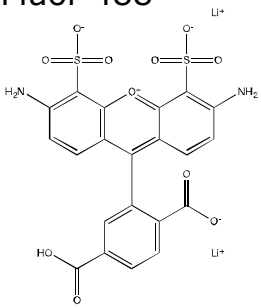


GFP



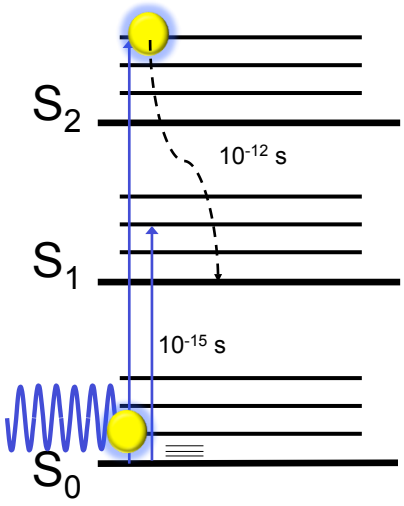


AlexaFluor 488



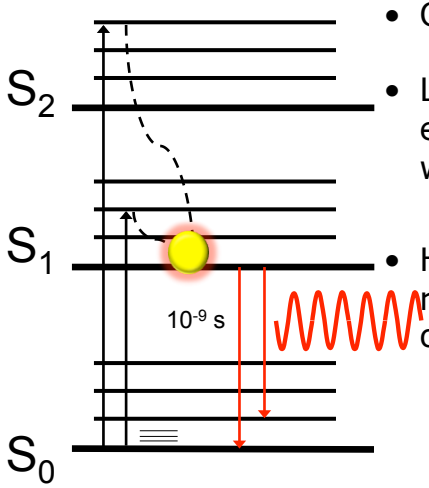
See Ben's talk on labelling for microscopy

Excitation



- Absorption takes place on a time scale 10^{-15} s
- Return to lower vibrational levels of the excited state occurs in 10^{-12} s dissipated as heat
- Excited states last a few nanoseconds

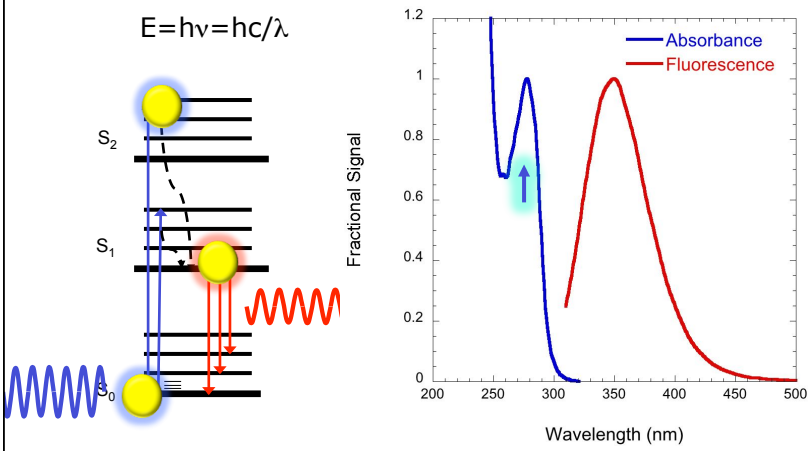
Emission



- Occurs in 10^{-9} s
- Lower energy transition than excitation, hence longer wavelength
- Has wavelength distribution reflecting the vibrational levels of the ground state

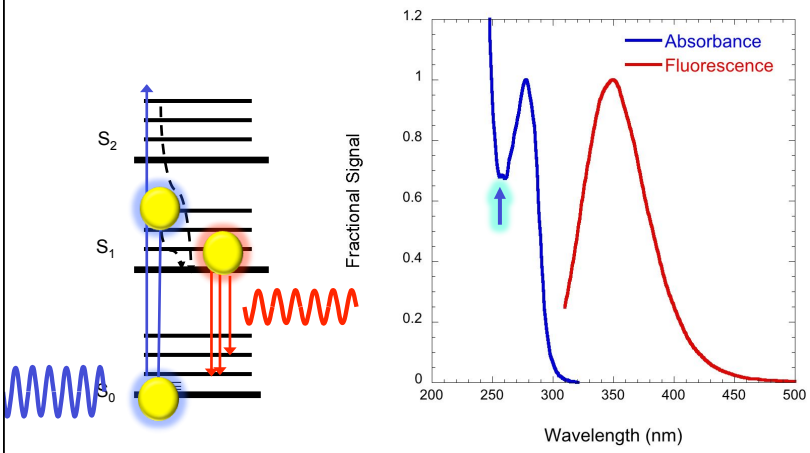
Electronic ground state

Excitation and Emission spectra



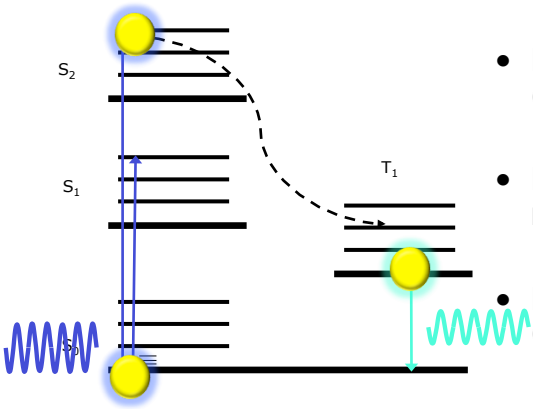
- Emission shows wavelength distribution (structure)
- Wavelength longer than excitation (Stoke's Shift)

Emission is independent of excitation wavelength



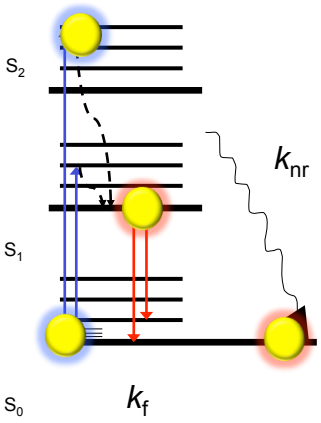
- Independent of the wavelength of exciting light since decay occurs from the same state

What goes up……?



- Quenched by collision
- Energy transferred to (e.g. FRET)
- Bleaching conversion to non-fluorescent species
- Phosphorescence (rearrangement)

Quantum Yield



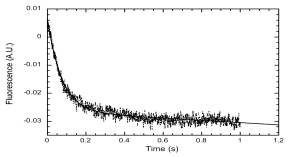
$$Q = \frac{N_{em}}{N_{abs}} = \frac{k_f}{k_f + k_{nr}} \qquad 0 < Q < 1$$

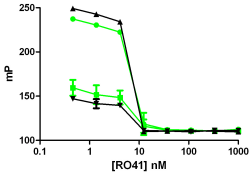
- Reflects the ability to dissipate energy by non-radiative process
- Dependent on the solvent and the exposure of the absorbing group
- Not all fluorophores are equally bright

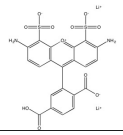
Why Use Fluorescence?

Occurs through electronic transitions in outer shell:

- Sensitive to changes in environment
- Fast response in ns range
- Sensitive
  - pM-µM sensitivity
- Selective
  - Intrinsic fluorescence groups
  - Specific labeling
- Reproducible
  - if bleaching minimised



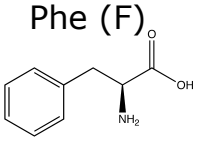




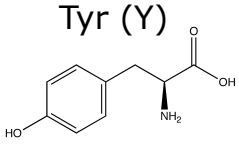
INTRINSIC PROTEIN FLUORESCENCE

Intrinsic Protein Fluorescence

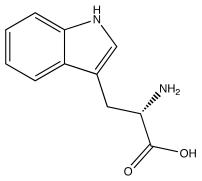
Phe (F)

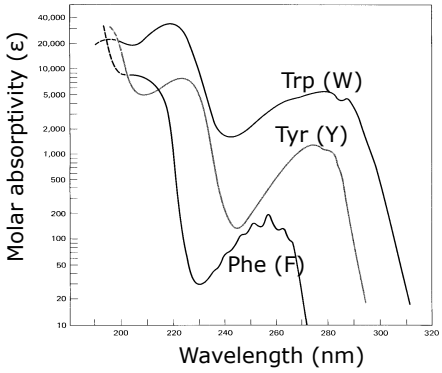


Tyr (Y)



Trp (W)

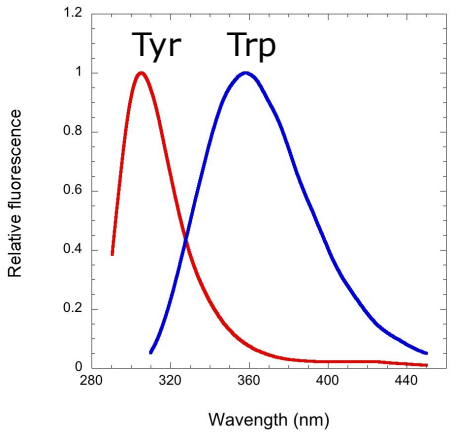




Trp 280nm 5690 M^{-1} cm^{-1}
Tyr 280nm 1290 M^{-1} cm^{-1}

Protein Fluorescence: Emission

Excitation at 280nm



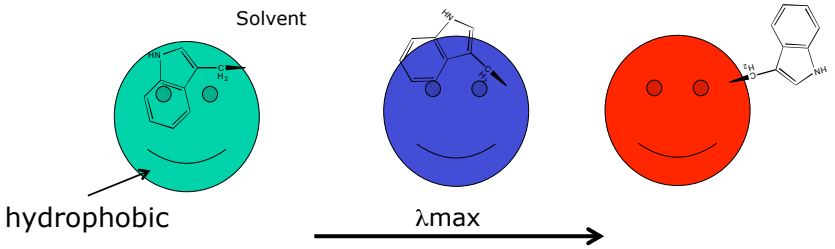
Phe low QE

Tyr 305nm QE 0.14

Trp 350nm QE 0.2

If excitation at >295 nm Tyr fluorescence is barely observed

Environmental Sensitivity of Trp

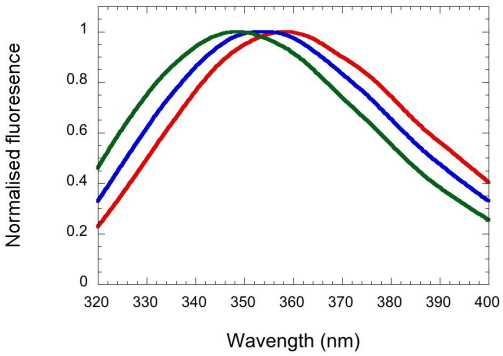


λmax

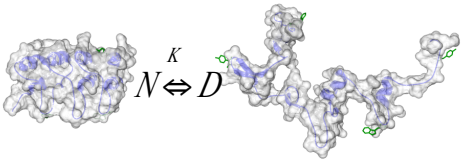
Other factors:

Temperature

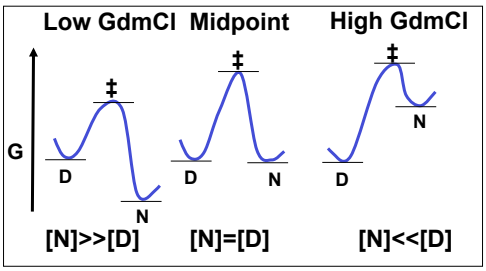
pH

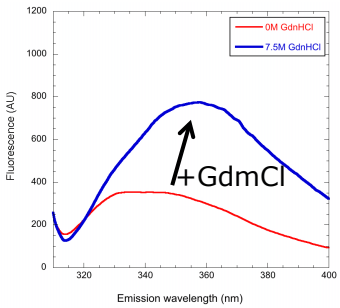


Chemical denaturation of proteins



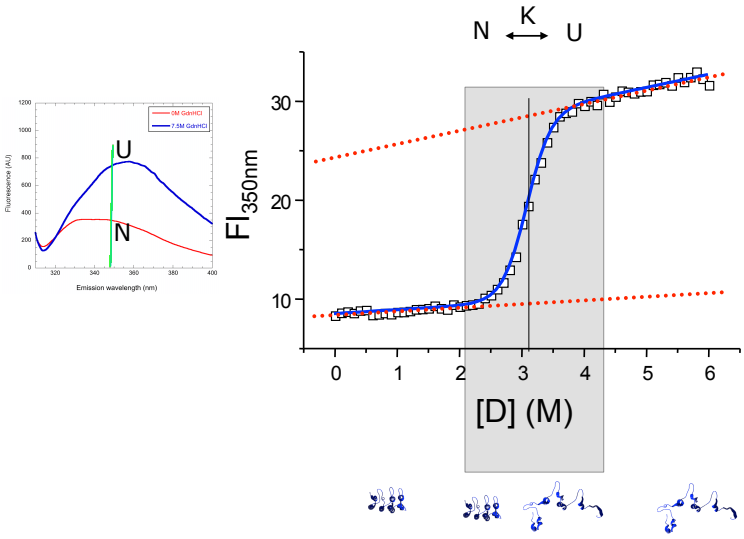
Urea GdmCl



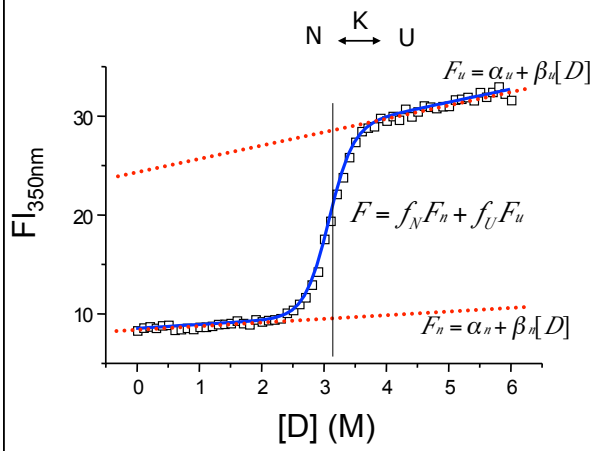


In this case unfolding causes an increase in fluorescence emission and a red-shift in λmax

Chemical denaturation of proteins



Chemical denaturation of proteins



$$K = \frac{[U]}{[N]}$$

$$f_N + f_U = 1$$

$$f_N = \frac{1}{1+K} \qquad f_U = \frac{K}{1+K}$$

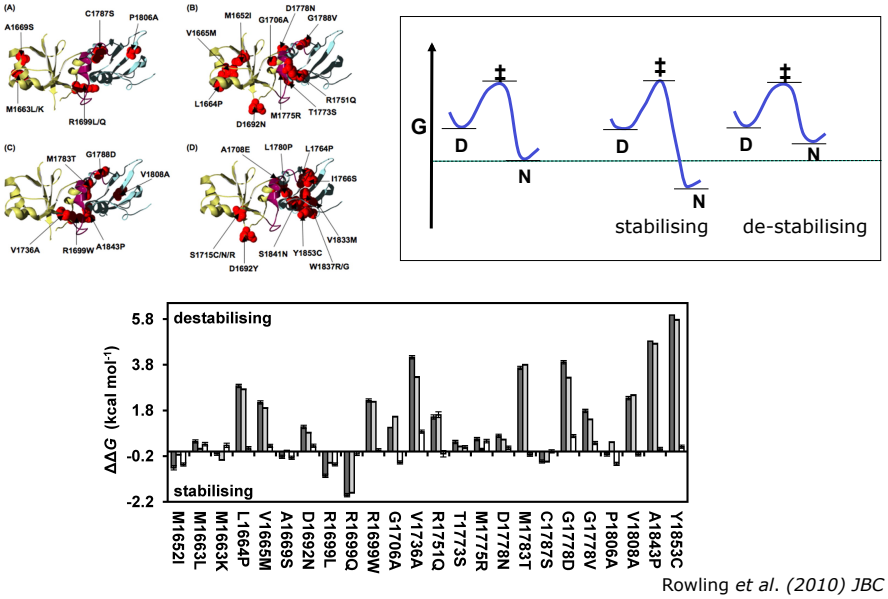
$$\Delta G = -RT\ln K \qquad K = e^{-\frac{\Delta G}{RT}}$$

$$\Delta G = \Delta G^0 - m[D] = m(D_{50\%} - [D])$$

$$\Delta G^0 = mD_{50\%}$$

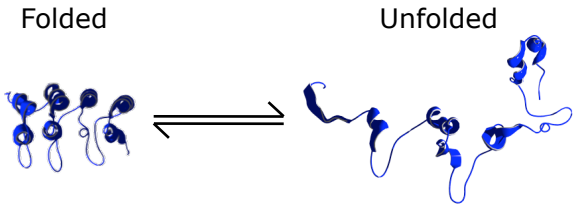
$$F = f_N F_n + f_U F_u = \frac{\alpha_n + \beta_n[D]}{1+K} + \frac{(\alpha_u + \beta_u[D])K}{1+K} = \frac{\alpha_n + \beta_n[D] + (\alpha_u + \beta_u[D])e^{\frac{m(D-D_{50\%})}{RT}}}{1 + e^{\frac{m(D-D_{50\%})}{RT}}}$$

Classification Of BRCA1 Missense Variants

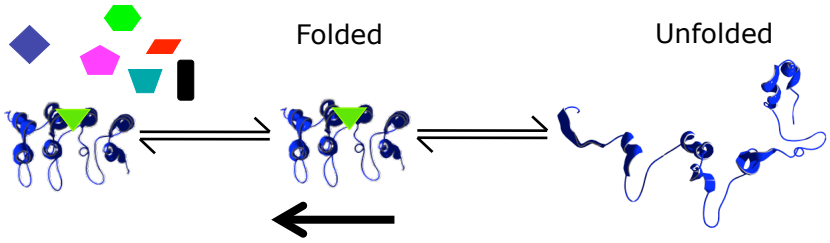


Rowling *et al*. *(2010) JBC*

Differential Scanning Fluorimetry (DSF)

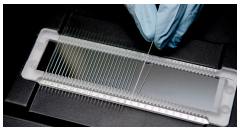


Mutation or buffers may effect protein stability and hence T_m



Ligand binding increases T_m for unfolding

Screening Using Intrinsic Fluorescence: Prometheus



Thermal/Chemical denaturation

Capillary based (48 in parallel)

Small sample volumes (10 µL)

Accurate temperature control

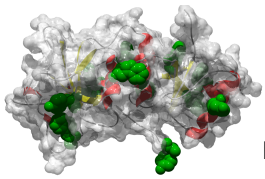
Large dynamic range (5 µg/ml- 250 mg/ml)

Detergent compatible

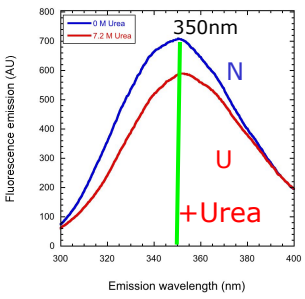
Membrane proteins

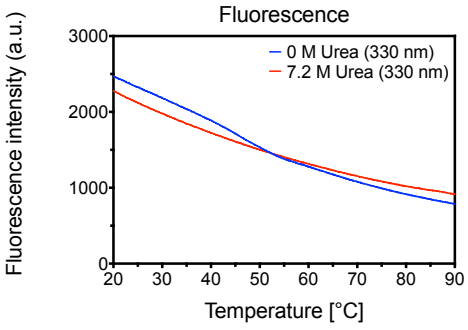
Back-scatter for aggregation

Total fluorescence is sensitive to temperature

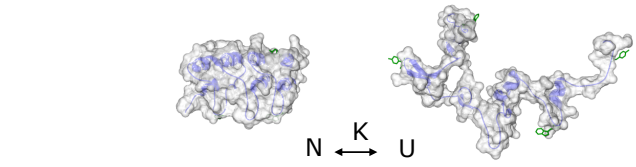


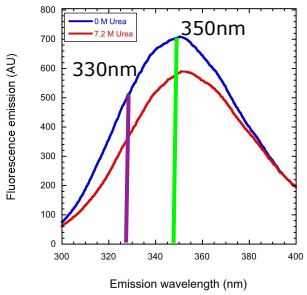
N $\underset{}{\overset{K}{\longleftrightarrow}}$ U

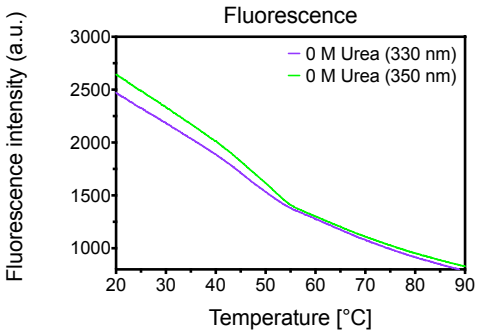




Exploit change in fluorescence spectrum upon unfolding

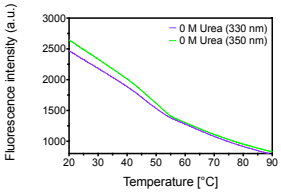




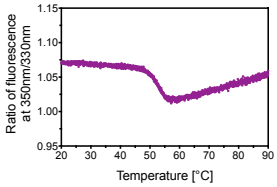


Monitor unfolding *via* 350/330 nm fluorescence

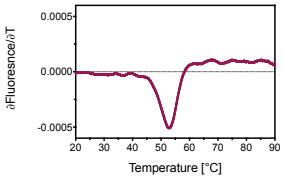
Fluorescence



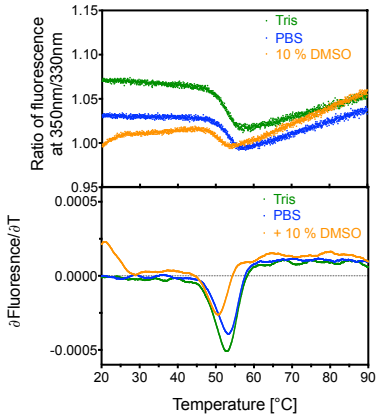
Ratio 350/330



First Derivative



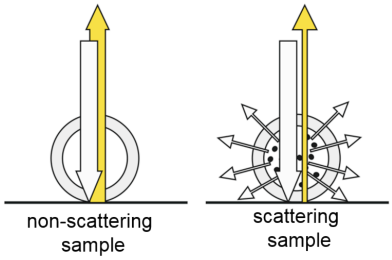
Screening

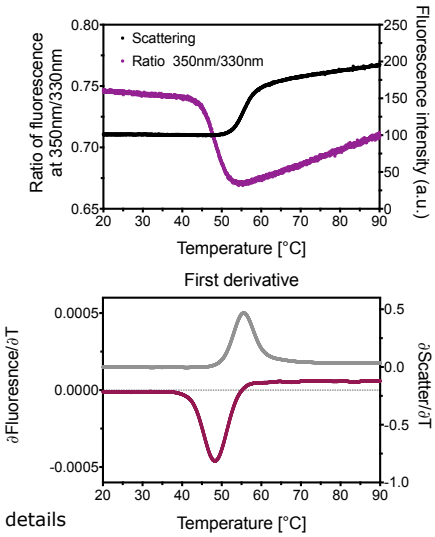


Fit to get T_m, onset of change

Simultaneous measurement of aggregation with scattering

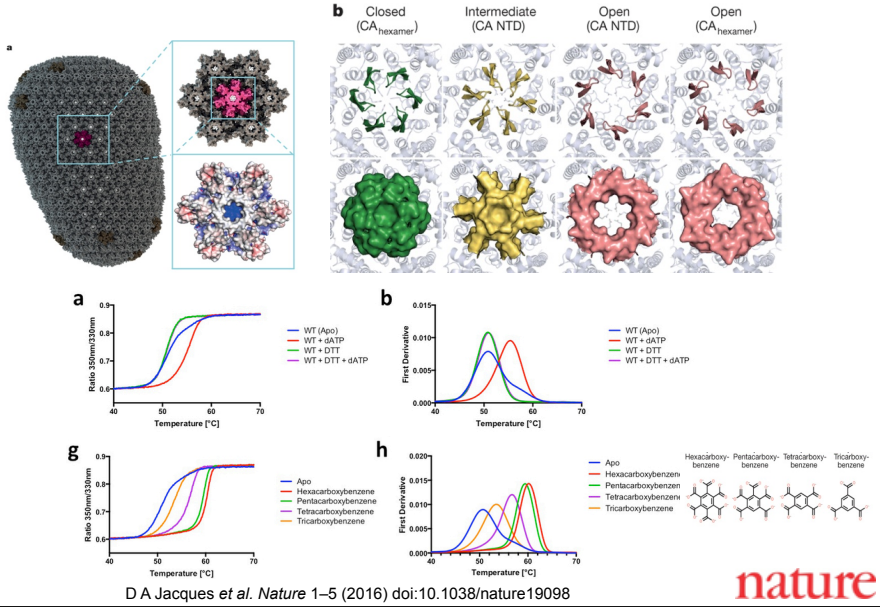
Aggregates will scatter light





See Chris's talk on light scattering techniques for details

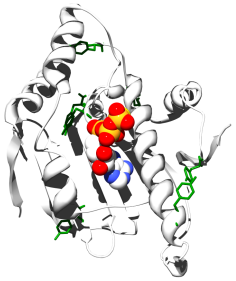
HIV-1 capsid hexamers have a pore at the six-fold symmetry axis



D A Jacques *et al. Nature* 1–5 (2016) doi:10.1038/nature19098

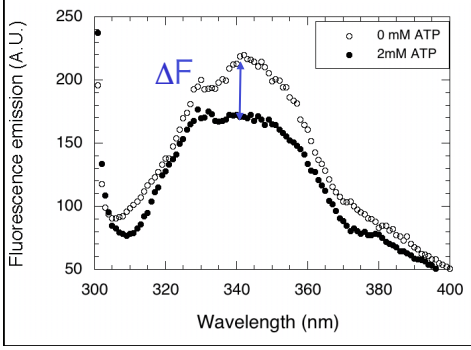
nature

Binding by fluorescence quenching



Protein + ATP $\rightleftharpoons$ Protein.ATP

K_d



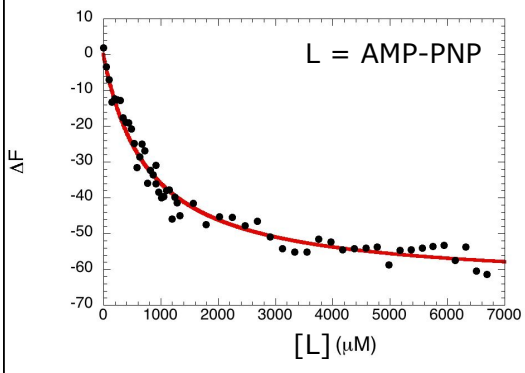
Measure change in intrinsic protein fluorescence (Ex=295nm)

+ nucleotide fluorescence quenched

Analysis of fluorescence changes

P+L $\rightleftharpoons$ PL

K_d



Assume $[PL] \propto \Delta F$

$$K_d = \frac{[P_0].[L_0]}{[PL]}$$

$$K_d = \frac{([P_T]-[PL]).[L]}{[PL]}$$

$[L] >> P_T$, so $[L] \cong [L]_0$

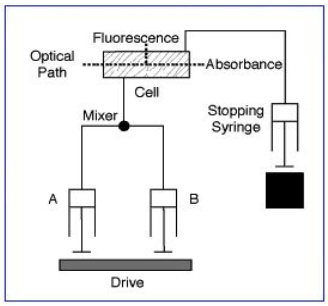
$$[PL] \equiv \Delta F; [P_T] \equiv \Delta F_{max}$$

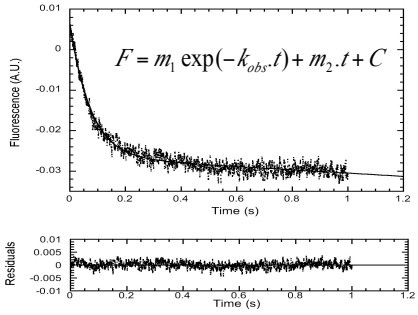
$$K_d = \frac{(\Delta F_{max} - \Delta F).[L]}{\Delta F}$$

$$\Delta F = \frac{\Delta F_{max}.[L]}{K_d + [L]}$$

Reaction kinetics by stopped-flow fluorescence

$$P + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P.ATP \xrightarrow{k_3} P.ADP + Pi \overset{k_4}{\underset{k_{-4}}{\rightleftharpoons}} P + ADP$$



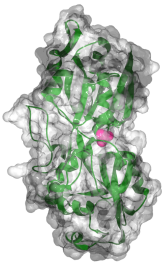


Mix protein and ATP in stopped flow

Fit exponentials: look at residuals

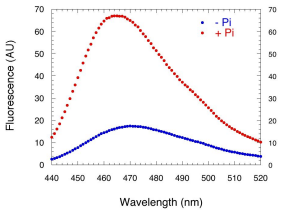
Fluorescence Report Assays for Enzyme Kinetics

$$P + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P.ATP \xrightarrow{k_3} P.ADP + Pi$$

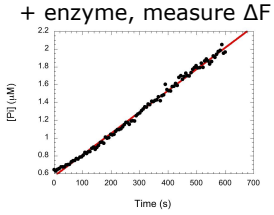


PBP:
Domain movement upon binding Pi reported by site-specific labeling with MDCC

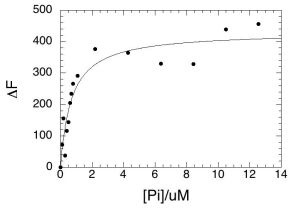
Fluorescence change



+ enzyme, measure ΔF



Standard curve PBP ΔF *vs* [Pi]

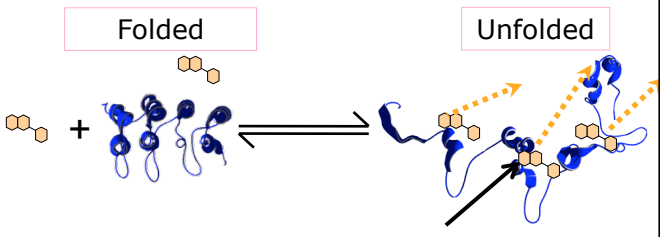


Use Standard curve to convert ΔF to [Pi]
Plot [Pi] *vs* time
Fit initial rates

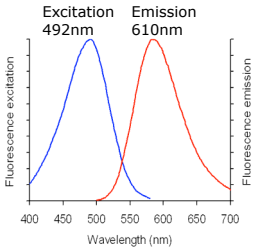
EXTRINSIC FLUOROPHORES

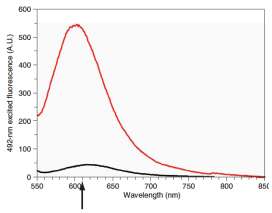
Ligand and Buffer Screening Using an Environmentally Sensitive Fluorophore

Thermally denature protein in presence of dye Sypro Orange



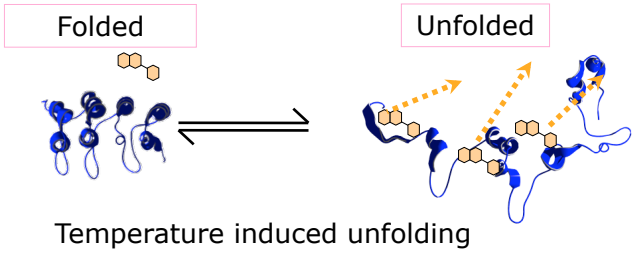
Sypro Orange binds exposed hydrophobic regions



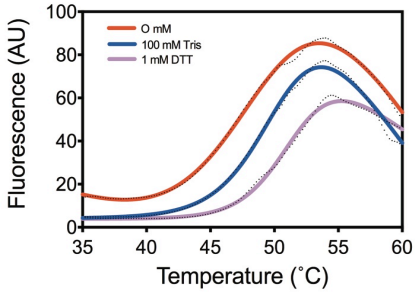


Change in environment of dye causes increase fluorescence

Differential Scanning Fluorimetry (DSF)

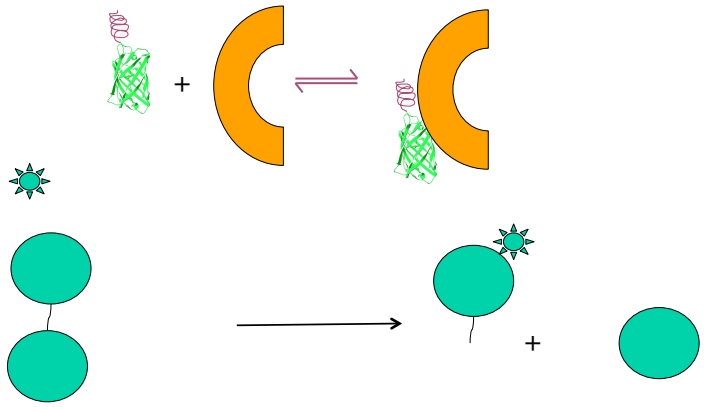


Temperature induced unfolding



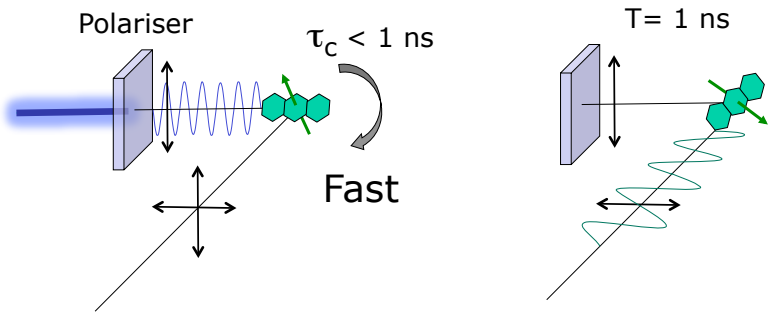
Monitor in RT-PCR instrument FAM (492 nm) and ROX (610 nm) filters

Interaction studies using fluorescence anisotropy and polarisation



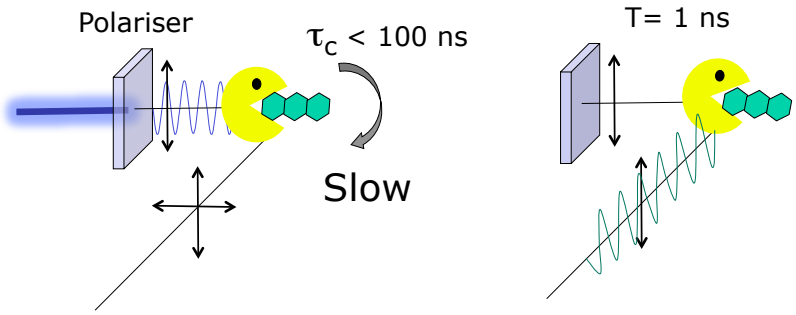
When there is a large difference in the masses of two binding partners or a substrate is cleaved into much smaller products the reaction may be followed by fluorescence polarisation.

Interaction studies using fluorescence anisotropy and polarisation



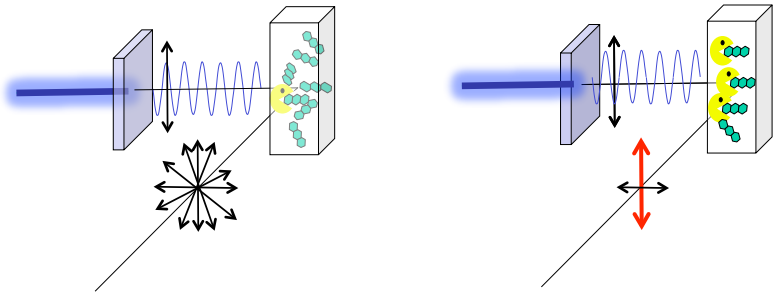
Incident light is vertically polarised
Fluorescence group tumbles fast compared to the life-time of the excited state
Emitted light has lost polarisation of incident light

Fluorescence anisotropy and polarisation



Incident light is vertically polarised
Fluorescence group tumbles slow compared to the life-time of the excited state
Emitted light has the same polarisation of incident light

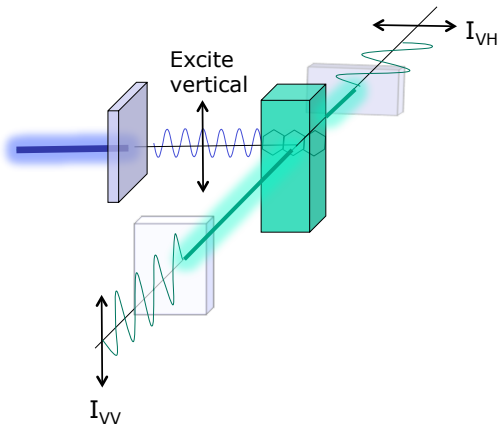
Fluorescence anisotropy and polarisation



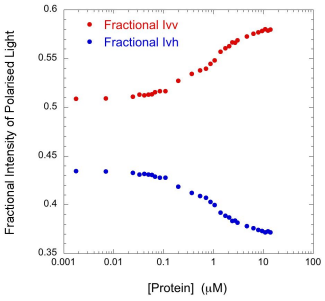
Vertically polarised light excites molecules whose dipoles are aligned perpendicular to the incident light. Molecules tumble fast so the emitted light has a random polarisation (depolarised) .

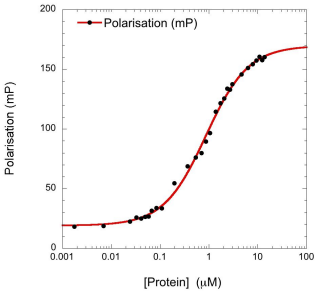
As concentration of interacting protein increases rate of tumbling decreases and emitted light has more light polarised.

Fluorescence anisotropy and polarisation

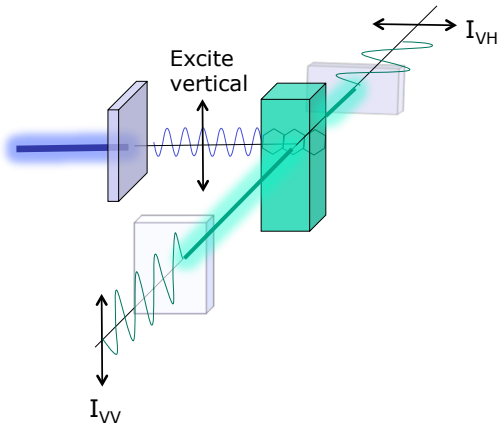


$$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}} \qquad p = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}}$$





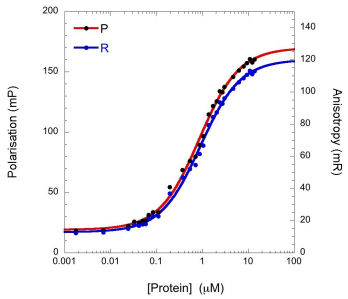
Fluorescence anisotropy and polarisation



Anisotropy changes directly proportional to total incident light.

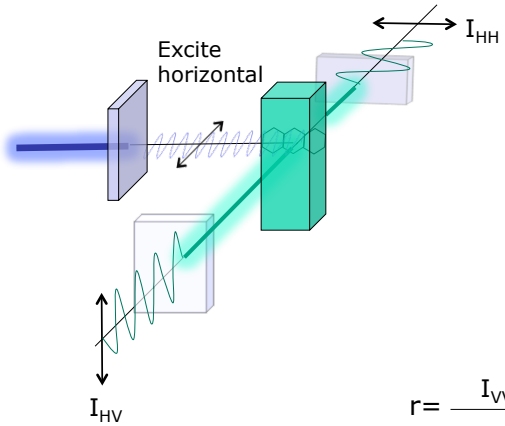
Measuring binding using polarisation may give inaccurate value.

$$r = \frac{2p}{3 - p}$$



$$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}} \qquad p = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}}$$

Fluorescence anisotropy and polarisation



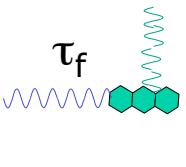
Grating factor corrects for instrument polarisation

$$G = \frac{I_{HV}}{I_{HH}}$$

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \qquad p = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

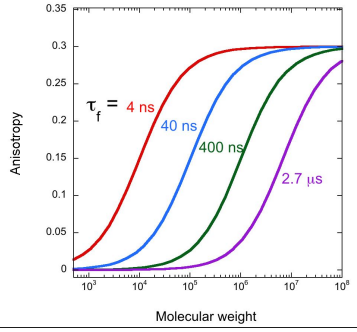
Ensure signal intensities are within appropriate range and instrument is correctly calibrated (beware default on plate reader)

Fluorescence anisotropy depends on size and fluorescence lifetime



$$r = \frac{r_0}{1+\tau_f/\tau_c}$$ Perrin equation

$$\tau_c = \frac{\eta V}{RT} = \frac{\eta M(\bar{v}+h)}{RT}$$



As mass increases r increases to limit

As fluorescence lifetime get longer curves shift to right:

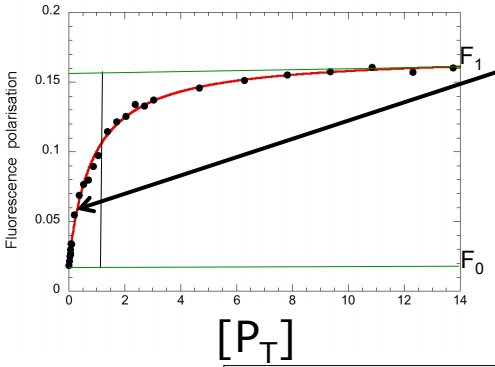
Larger masses needed to increase r

Binding Constants from Anisotropy and Polarisation

$$P + L^* \rightleftharpoons PL^* \quad (K_d)$$

Label small component

[L] << K_d



$$F = F_0 + (F_1 - F_0)\frac{[PL]}{[L_T]}$$

$$K_d = \frac{[P_0].[L_0]}{[PL]}$$

$$[PL] = \frac{([P_T]-[PL]).([L_T]-[PL])}{K_d}$$

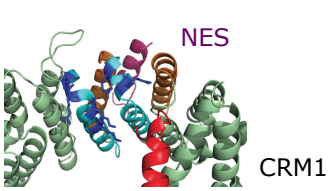
$$[L_T] = [L_0] + [PL]$$

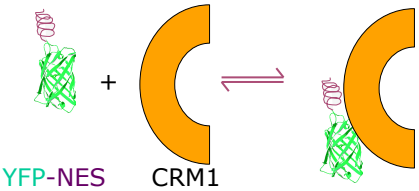
$$[P_T] = [P_0] + [PL]$$

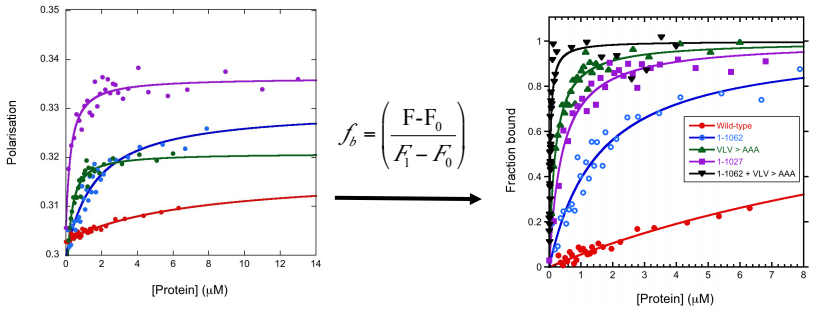
[P] > L_T, so [P] ≠ $[P]_0$

$$F = F_0 + \frac{(F_1 - F_0)\left\{([P_T]+[L_T]+K_d) - \sqrt{([P_T]+[L_T]+K_D)^2 - 4[P_T][L_T]}\right\}}{2[L_T]}$$

Binding Study using Polarisation

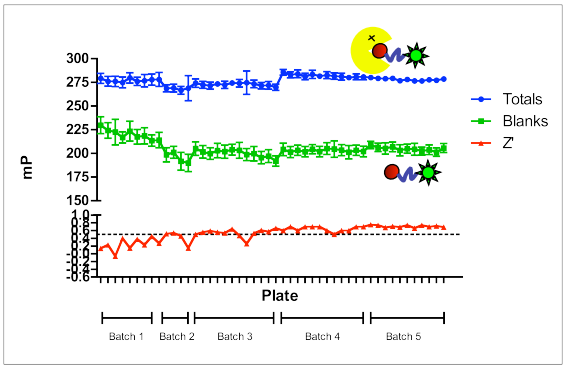






Fox *et al.* (2011) JBC

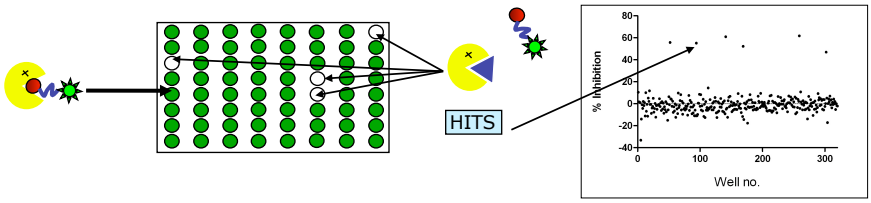
Screening Using Fluorescence Polarisation Assay



Incubate fluorescent probe with binding partner in 384 well plate at sub-saturating conditions.

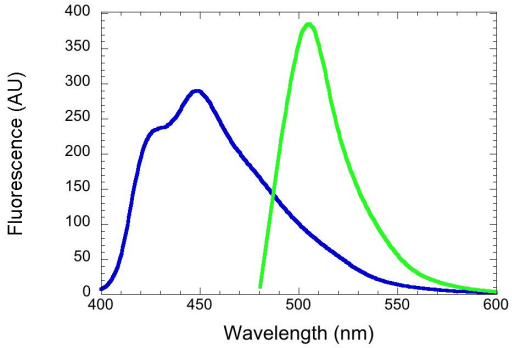
Add compounds and measure FP

Look for loss of FP (displacement)



SPECTROSCOPIC RULERS

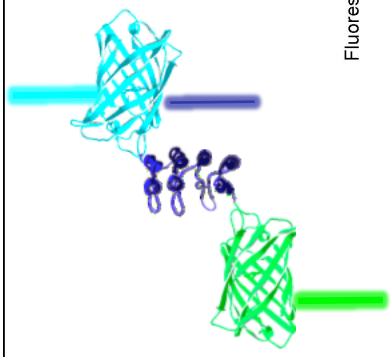
Förster Resonance Energy transfer (FRET)

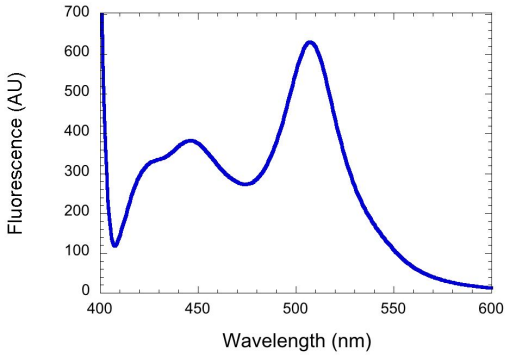


When two fluorophores, whose emission spectra overlap, are in close proximity energy can be transferred through resonance

(See Ben's lecture for more depth)

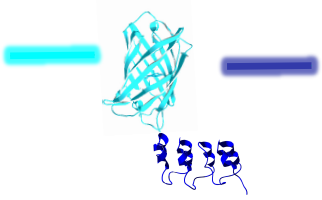
Förster Resonance Energy transfer (FRET)

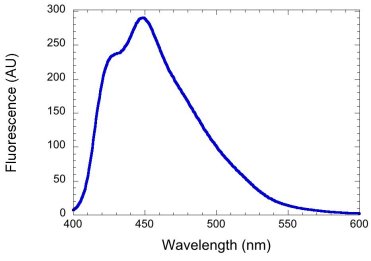




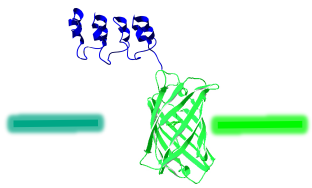
Excitation of donor not only causes donor fluorescence but also excites the acceptor via resonance leading to fluorescence from acceptor

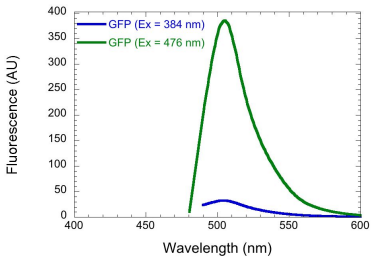
Förster Resonance Energy transfer (FRET)





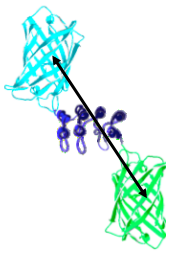
In absence of acceptor some fluorescence at acceptor emission

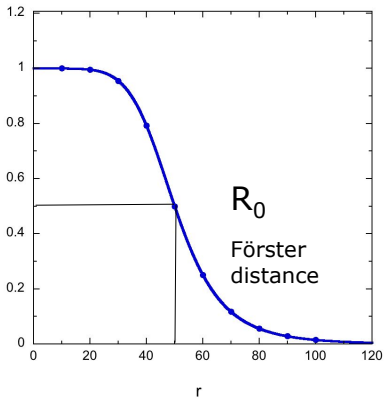




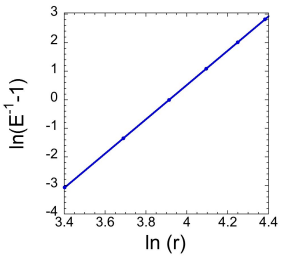
In absence of donor very little fluorescence from acceptor using acceptor excitation wavelength

FRET depends on distance





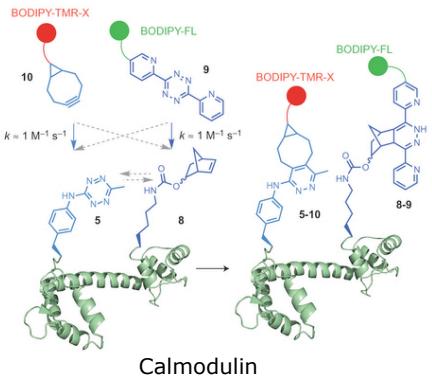
$$E = \frac{R_0^6}{R_0^6 + r^6}$$



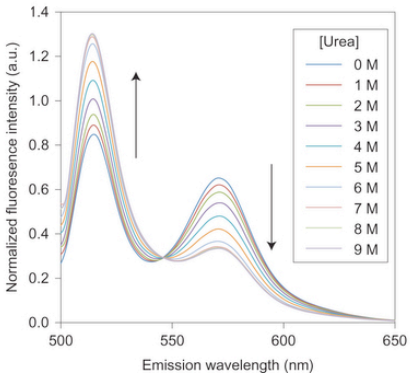
$20\ Å < R_0 < 60\ Å$

Different pairs of acceptors and donors have different R_o

FRET depends on distance



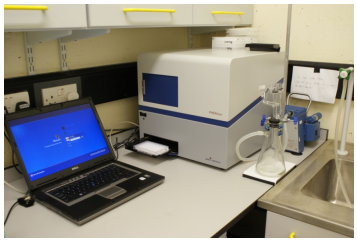
Calmodulin



Decrease in energy transfer indicates increase in distance

Wang et al., Nat Chem (2014) 6, 393–40

Fluorimeters *vs* Plate Readers

	Cuvette-based	Plate-based
		
	Perkin Elmer LS55, Cary Eclipse, Horiba Fluormax	BMG Pherastar
Sample volumes:	4 x 70 µl-1 mL	384/96 x 40/200 µl
Temperature:	precise control	ambient or above
Sensitivity:	nM	pM
Flexibility:	Mono-chromators	Optical modules*
Time:	hour	minutes

See Biophysics website for more information*

Practical Tips

Before Experiment

Buffers and samples filtered to avoid light scattering
Take care of aggregation (check at end)
Clean cuvettes
Concentrations accurate?
Sticking to cuvettes/plates?

Experiment Controls

Good signal change? (Gain setting)
Right settings (e.g. FP in plate reader)
Photo-bleaching?
Background fluorescence?
Inner filter effect?
Accurate titrations?

Practical Tips

Additional controls

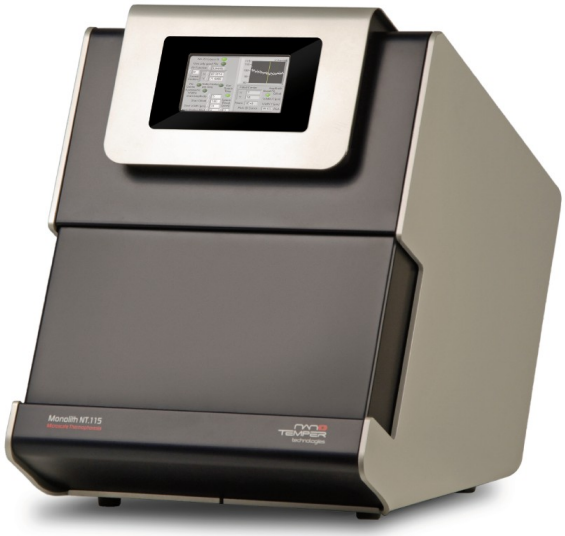
Non-specific binding of fluorphore
Displacement
Temperature control
Time to reach equilibrium

After experiment

Data fitting
Is the range of data good enough?

(see future lecture in series)

Microscale Thermophoresis



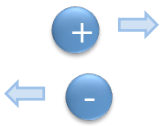
Thermophoresis

Electrophoresis

Electric Field

+ -

charge, (size)

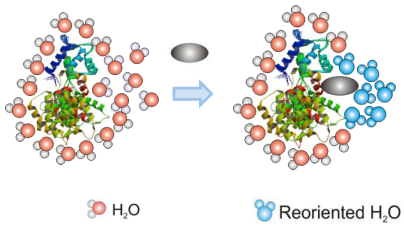


Thermophoresis

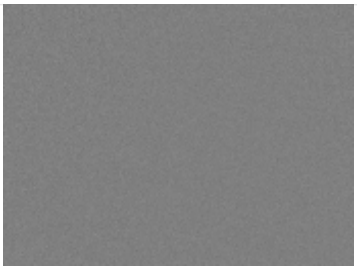
Temperature-Gradient

hot **cold**

charge , **size** and **hydration shell**

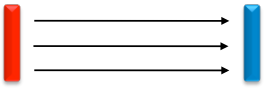


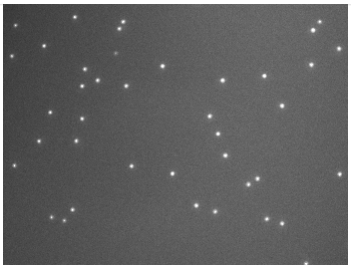
Thermophoresis



DNA

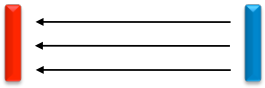
Positive -Thermophoresis





Microbeads

Negative-Thermophoresis

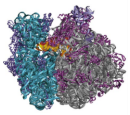


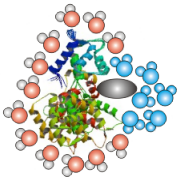
Theory of Thermophoresis

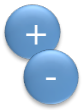
$c_{hot}/c_{cold} = \exp(-\mathbf{S_T}\,\mathbf{\Delta T})$

$$S_T = \frac{A}{kT}\left(-\Delta s_{hyd}(T) + \frac{\beta\sigma_{eff}^2}{4\varepsilon\varepsilon_0 T}\times\lambda_{DH}\right)$$

size — **hydration shell** — **charge ²**



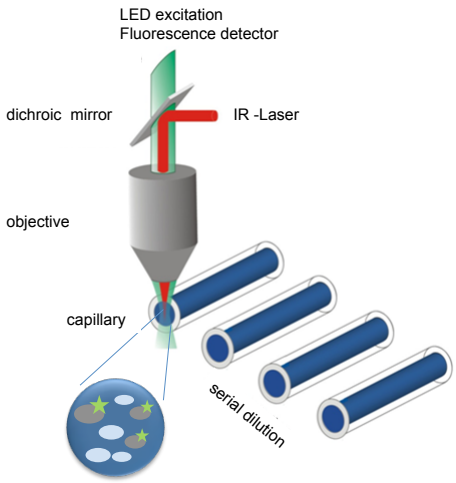




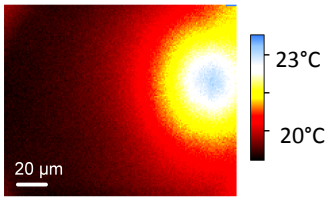
Duhr and Braun PNAS 103, 19678–19682 (2006)
Duhr and Braun PRL 96, 168301 (2006)

Fluorescence Microscale Thermophoresis

Basic setup of the instrument



Example of an IR-Laser induced temperature field (BCECF-dye in TRIS-buffer)



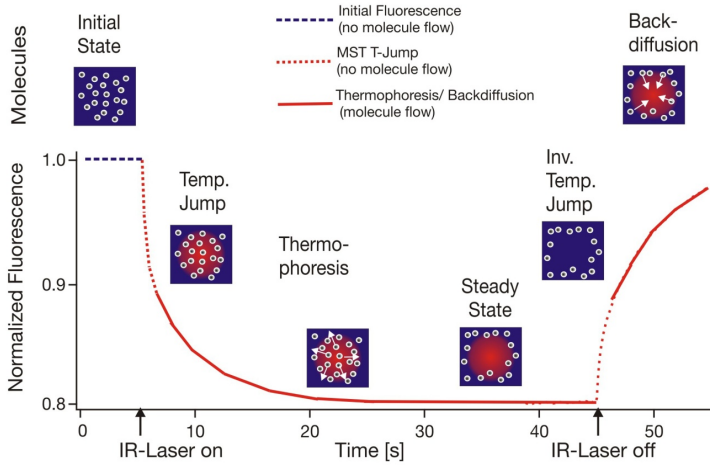
Temperature [°C]

1K /10µm temperature increase corresponds to 1000K/cm

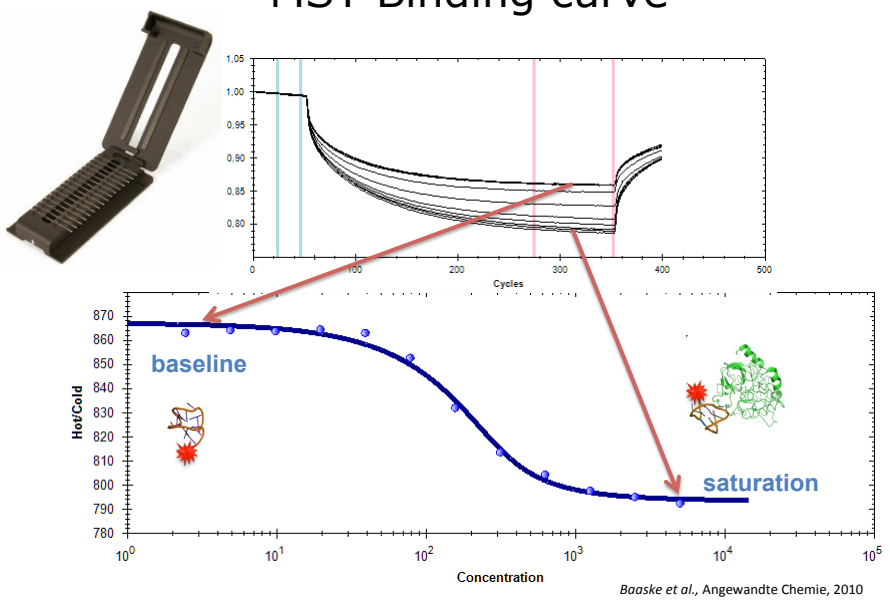
- **4µl of sample** per titration point
- **nM concentrations** of labeled protein

Dyes in Green (Ex 488 nM) or Red (Ex 643 nm) region
Labeling kits for amine, cysteine or hexa-his reactive dyes.

Shape of MST Curve



MST Binding curve



Baaske et al., Angewandte Chemie, 2010

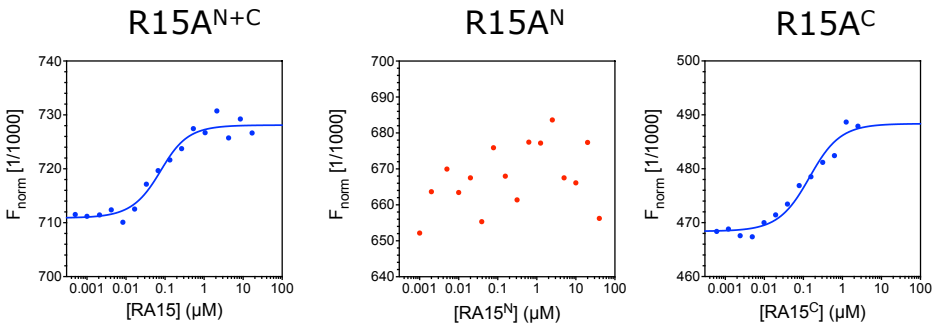
60

Interactions accessible with MST

- protein-protein
- protein-DNA/RNA
- nucleic acid – nucleic acid
- protein-small molecule, peptides, ions
- protein or small molecule – HMW complexes (e.g. ribosome)
- protein or peptide-liposome/vesicle
- membrane receptors embedded in micelles or liposomes
- covalent modifications of nucleic acids, proteins etc.

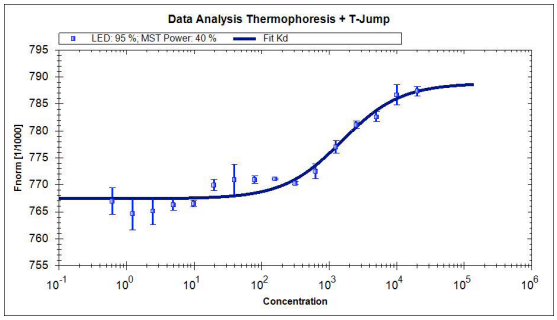
http://www.nanotemper-technologies.com/technologies/mst-technology/publications/

Case Study: Thermophoretic binding curves of PP1 binding to R15A



Carrara, M., Sigurdardottir, A., Bertolotti A. (2017) *Nat Struct Mol Biol.* 24(9):708-716

MST: detergent compatible



0.05 % DDM

Kd Formula (law of mass action)

f(c) = unbound + (bound-unbound) / 2 * (FluoConc + c + Kd - Sqrt((FluoConc + c + Kd)^2 - 4*FluoConc*c)

Fitting for Kd Formula
Fitted Parameter Fitted Value

Dissociation Constant 1530+/-131
Fluo.Conc 30
Bound 788.85
Unbound 767.44
Amplitude 21.41

MST Practical Tips

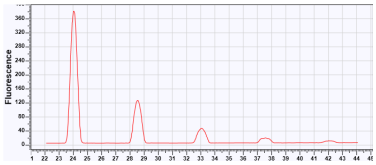
Before Experiment

- Is labeling complete i.e. 1:1 dye to protein?
  - Measure spectrum, correct for dye contribution to absorbance at 280 nm. Calculate concentrations.
- Buffers and samples filtered to avoid light scattering.
  - Is protein/complex stable in buffer?
- Concentrations accurate?
  - Calculated from spectrum, AAA?

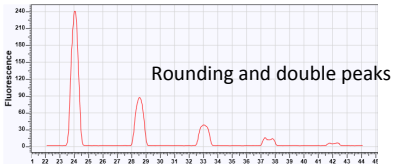
MST Practical Tips

Test Experiments

- Test range of labeled concentrations:



Pre-scan



Post-MST scan

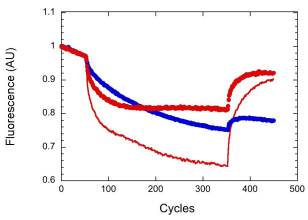
- Is there adequate fluorescence? Use below Kd.
- Is there any association with the capillaries?
  - look for rounding double-peaks

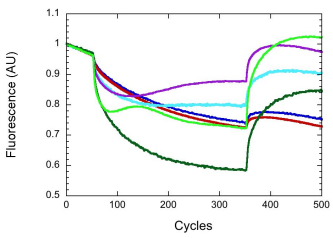
MST Practical Tips

Test Experiments

- Try different capillaries

- Check for aggregation (bumpy curves) in thermophoresis scans
  - Try different buffers
  - Try additions of BSA, Tween

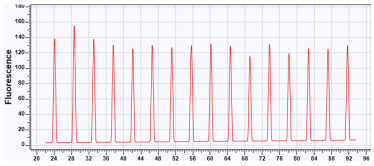


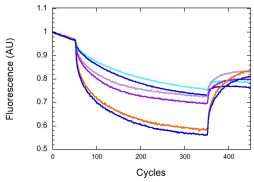


MST Practical Tips

Experimental Setup

- Set-up titration series of unlabeled component
- Add 1:1 to fixed concentration of labeled partner
- Make sure the reaction has enough time to reach equilibrium
- Check fluorescence:
  - Ensure fluorescence doesn't vary randomly (>10%) across capillaries
  - Ensure fluorescence signal is strong enough





- Vary laser power to get optimal thermophoretic changes.

Thanks

Any questions?