MRC | Laboratory of Molecular Biology

Observing 'Single' Molecules

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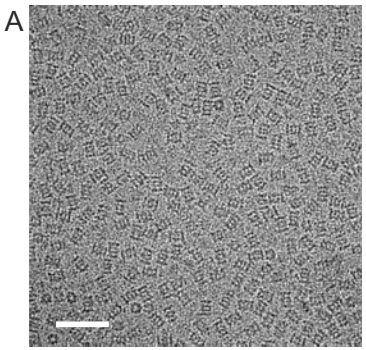
Feb 2020

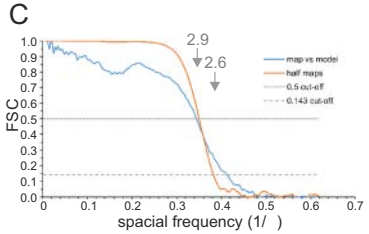
Observing 'Single' Molecules

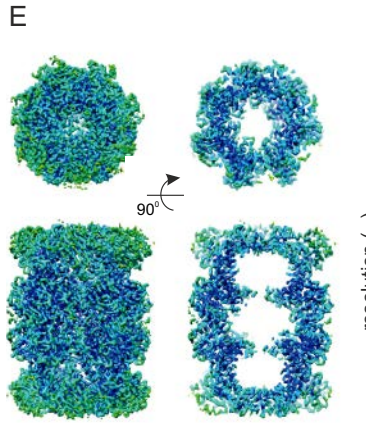
- Direct visualisation … Cryo EM
- Ordering … X-ray crystallography
- Localisation…. Imaging & Super resolution techniques …
- Purification and measurement of their physical properties one molecule at a time… **single molecule 'techniques'**

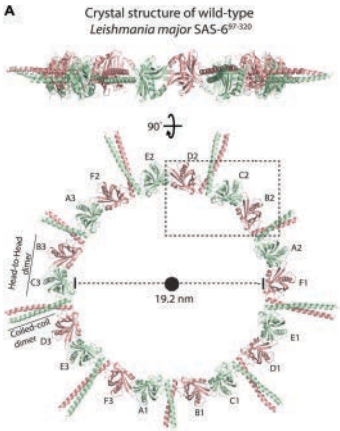
- Although we loose atomic 'resolution' we gain the following
  - ✓ Cheap(er)
  - ✓ Physiological conditions of buffer and temperature, controlled levels of reactants
  - ✓ **Solution based. Molecules diffusing and undergoing stochastic processes of dynamics & conformational equilibria as well as directed processes of binding, catalysis etc.**
  - ✓ **Single molecule measurements reveal ensemble heterogeneity**

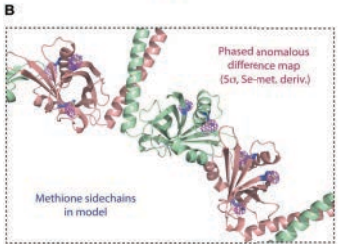
The static view and the dynamic world

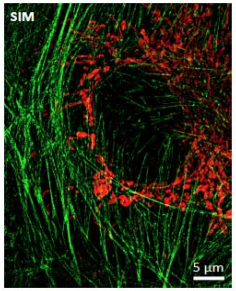








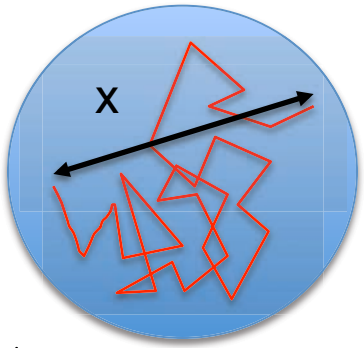




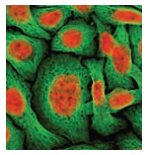
Diffusion (and 'crowding')

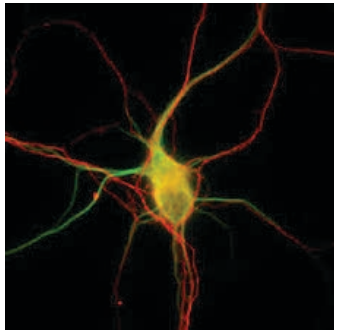
Dynamics

Time scales for stochastic diffusion



- Time scale (T) to traverse distance (x) is related to the translational diffusion coefficient (D) which for a 30KDa protein is ~ 10 um^2 s^{-1} in cytoplasm
- $T = x^2 / 6D$ (for 3 dimensional diffusion)
- $T_{E.coli}$ = 10-20ms (~1um)
- $T_{hela\ cell}$ = 10s (20um)
- $T_{neuronal\ cell}$ = 10^6s = 20 days (1cm)
- $T_{sciatic\ nerve}$ = ~ 71,000 years (150cm)



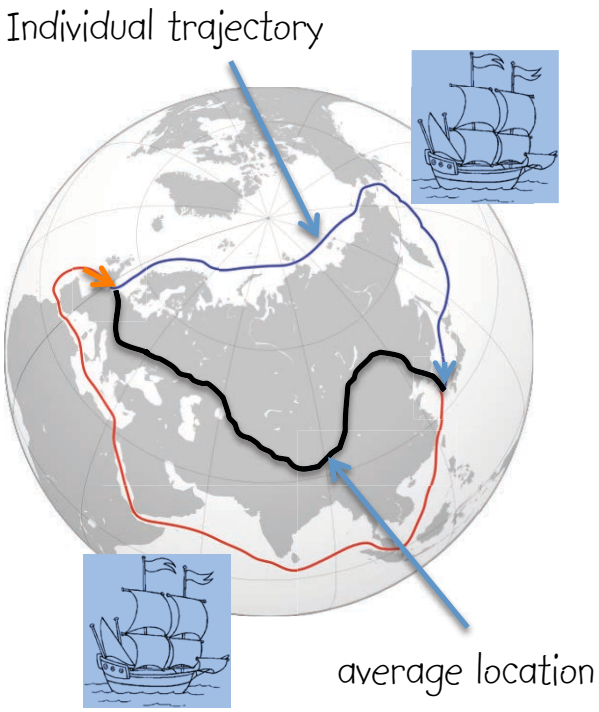


Consequences of diffusion

- Molecular motors... run at around ~ 1 um s^{-1} usually in one direction and can transport large cargo which would diffuse slowly
- In buffer, D is an order of magnitude faster ~ 100 um^2 s^{-1} than the crowded (high viscosity) environment of the cell
- T for a typical confocal psf of 1 um in buffer is ~ 2 ms so for a labeled sample with count rate 10 kHz yields 20 photons..... Single molecule experiments are technically challenging !

Ensemble Heterogeneity

- **Since individual molecules are observed we can determine the statistical distribution (heterogeneity) of a given property which is averaged during bulk ensemble measurements.**
- **'Features' of the ensemble distribution may be determined (populations, shape, width, rare events, etc).**
- However, there should be an equivalence of ensemble and the single molecule averages following the ergodic principal. "Averaging many molecules (ensemble spatial average, 'bulk measurement') is equal to averaging the behaviour of individual single molecules for a long time (temporal average) or many individual single molecules each for a short time (numerical average)".
- Measurements in solution are typically not of a single molecule observed over time : more usually the average of many molecules each observed individually for a short time because of rapid diffusion and photophysics of the labels.



Other Advantages of Single Molecule Methods

- Non perturbative, in solution measurement of diffusion and dynamics occurring because of statistical thermal fluctuations.
- Single molecule spectroscopy measurements typically require 10's of uL of nM-pM solution so are economic with material and usually measure in the nano - picomole range.
- No size limit or serious restrictions in solvent.
- Suitable for multi-colour approaches.

However……Practicalities of Single Molecule Methods

- Fluorescence based methods rely on 'bright' fluorescent labels which can perturb the system or undergo complex photophysics at high illumination intensity …. lots of controls are required.
- For purified materials, single fluorophore and single site labeling is 'cleanest' and may require mutagenesis or fusion proteins. (Talk 3)
- Lab built instrumentation is complex, 'expensive' and requires expertise to build, maintain and in order to obtain optimal data.
- 'Single molecule' concentrations may not allow stable complex formation and non-specific absorption onto surfaces is a problem if diffusion is of interest.
- Some systems may require immobilisation depending on the time scale of events and / or the process.

<u>Single molecule experiments should therefore be necessary, be well designed and target specific tractable questions.</u>

Single Molecule 'Techniques'

Force or Manipulation based

- Atomic Force Microscopy; 'Tapping' for scanning and imaging. 'Pulling' force extension methods
- Optical and Magnetic tweezers; Polymer or magnetic bead held under constant force or in constant position. Lumicks C-trap

Scattering based (label free)

- Interferometric scattering (iSCAT)

Fluorescence based

- Imaging and localisation (Talks 1-4)
- 3rd Generation single molecule sequencing*
- Fluorescence resonance energy transfer (FRET; Talks 3 and 5); dye-dye 'quenching' interactions for FRET populations and distances
- Fluctuation correlation spectroscopy (FCS); diffusional times (properties) of labeled species. Also (PET-FCS) dye side chain quenching for time resolved intrachain dynamics.

*Single molecule real-time (SMRT) sequencing comes of age: applications and utilities for medical diagnostics. *Nucleic Acids Research*, 1/02/2018

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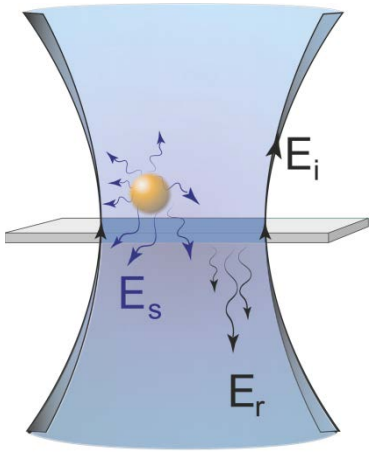
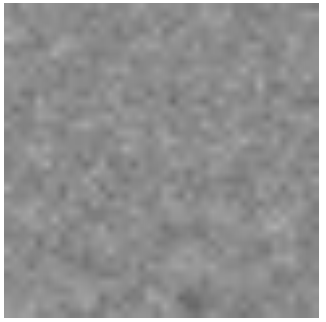
- Interferometric scattering (iSCAT)

iSCAT ; interferometric scattering microscopy for single molecules

- Macromolecules in solution approaching and 'landing' at a glass surface scatters incident light (Ei). The scattering (Es) can be optimised to interfer with the light being reflected from the glass liquid interface (Er).
- Careful 'NA-filtering'* of the total reflected signal allows the 'contrast' generated by individual single molecule events to be measured and contrast scales linearly with mass.
- Thus macromolecular mass and its heterogeneity can be determined from a single measurement of landings over time.
- Light scattering is a universal property of all proteins (and other macromolecules) being a function of their polarizability (Lecture 13 Light Scattering)
- iSCAT is therefore universal and requires no label



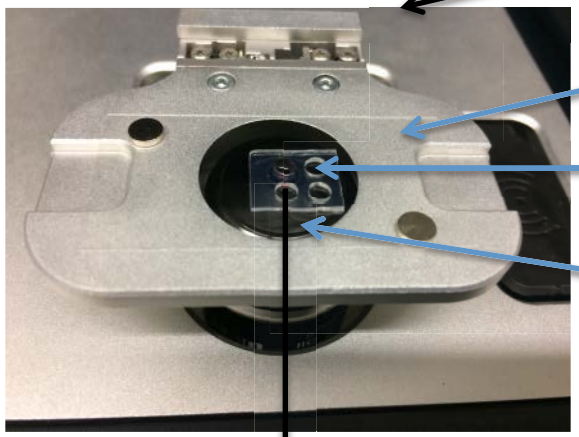


*Cole et al., Label-Free Single-Molecule Imaging with Numerical-Aperture-Shaped Interferometric Scattering Microscopy ACS Photonics. 2017 Feb 15; 4(2): 211–216.

iSCAT Practicalities

Commercial instrument (Mass Photometry)

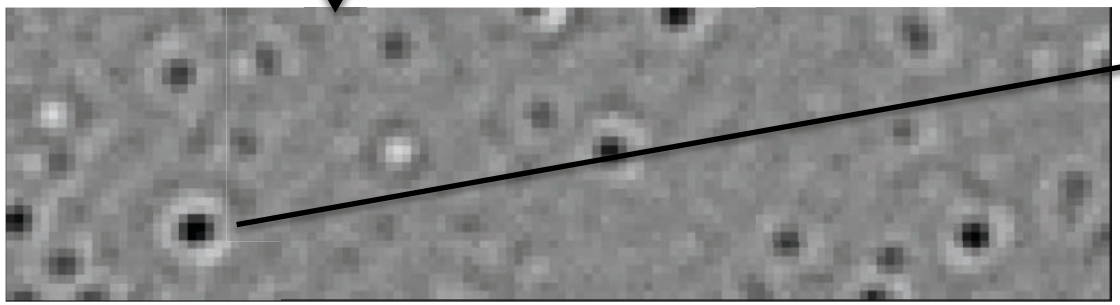
10uL sample 10-100 nM. Label free



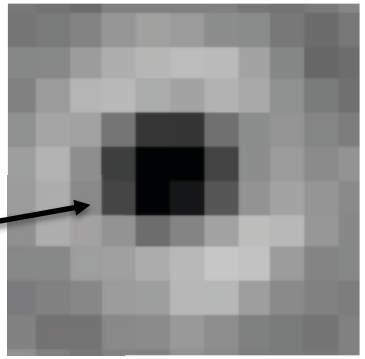
Glass coverslip

Silicone gasket
10uL sample

Oil objective lens



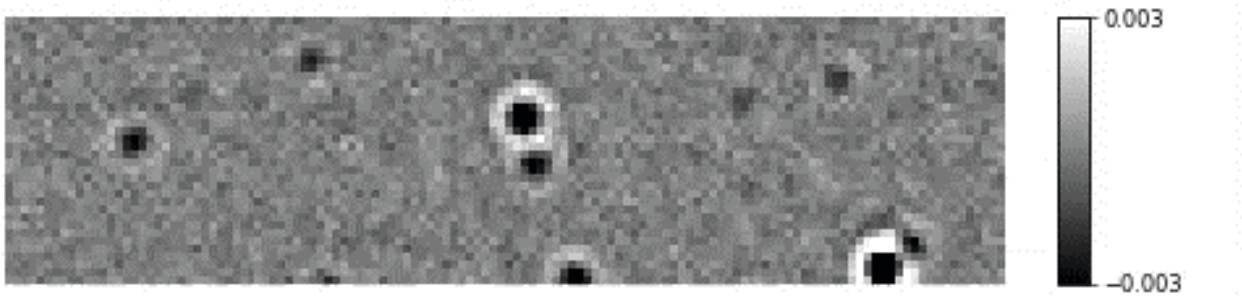
3 x 10 um camera field of view



80 nm^2 pixel
Diffraction limited ~320nm event
White halo is Airy ring

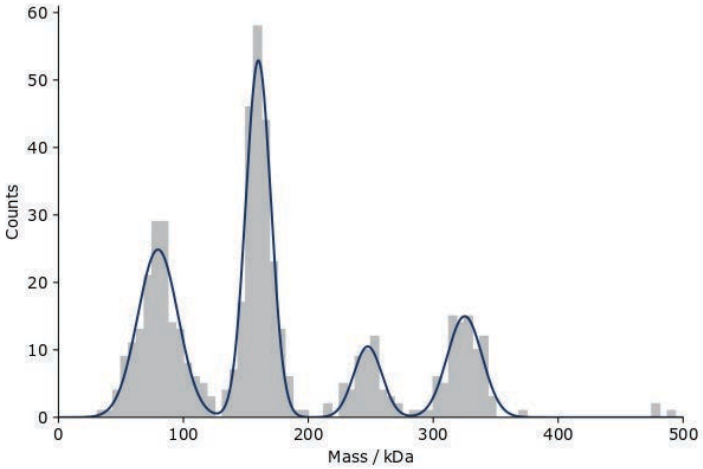
Characterising "Landings"

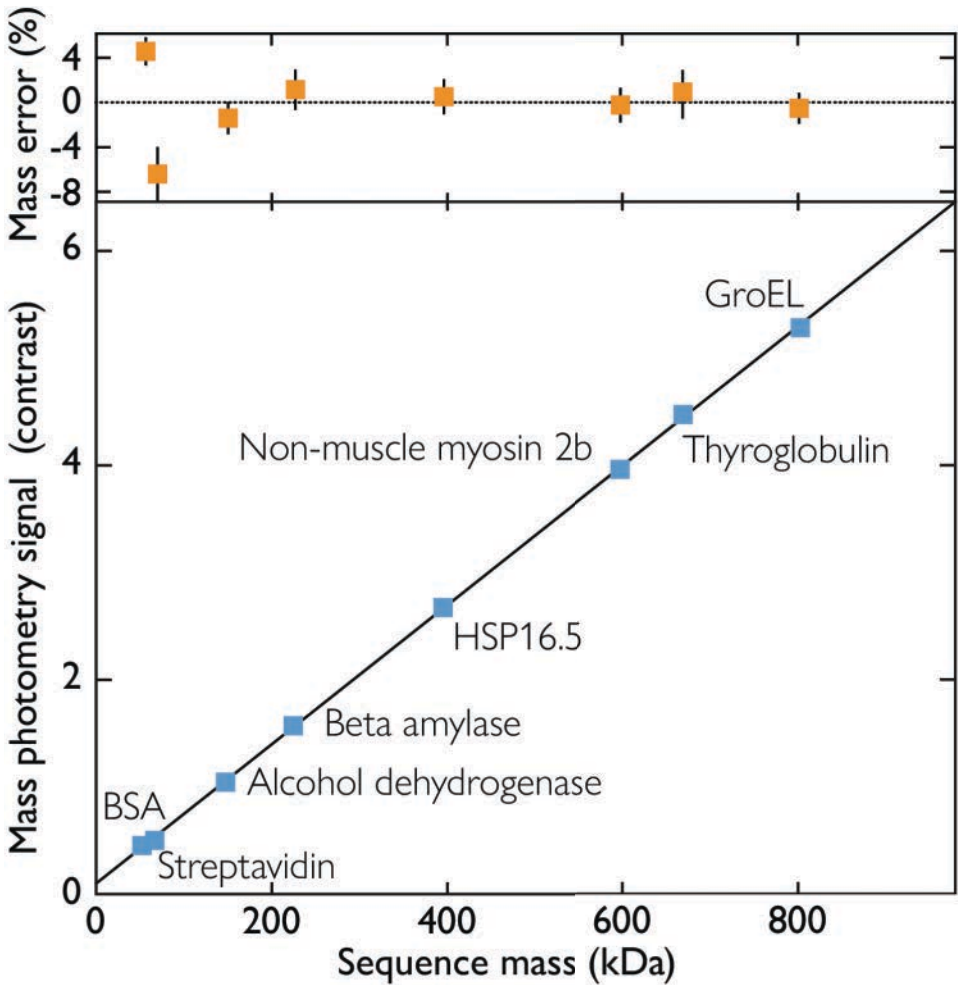
- Many landing events are observed in a movie at 100fps for 1 min
- Frames are averaged (5:1) and a running ratio calculated comparing frames before and after a time point. Ratiometric view
- Macromolecules appear, land and then disappear (since when sticking they are in frames both before and after)
- Contrast generated is negative since destructive interference with the bulk reflected light is optimised
- Multiple events can occur later in the movie at the same location since pixel is 80nm^2.
- Software seeks discrete non-overlapping events and evaluates maximum contrast for analysis



Converting Ratiometric Contrast to Mass

- Mass is determined from a calibration curve of standards
- All proteins should be well determined between 50kDa and 2MDa
- Different calibrations are probably required for modified proteins, nucleic acids, lipids etc when absolute mass is required





iSCAT Applications

- Mass determination in solution and check on heterogeneity
- Assembly and stabalisation of complexes (cryoEM)
- Measurement of modified proteins or other macromolecules
- Determination of protein-protein ineteractions in nM range
- Very quick (minutes). Uses uL of nM concentration. NO LABELS

1: Young G, Kukura P. Interferometric Scattering Microscopy. Annu Rev Phys Chem. 2019 Jun 14;70:301-322.

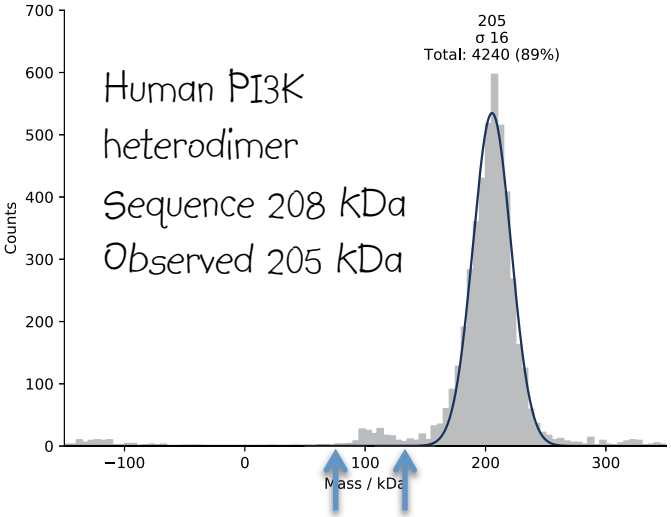
2: Wu D et al. Measuring the affinity of protein-protein interactions on a single-molecule level by mass photometry. Anal Biochem. 2020 ;592:113575.

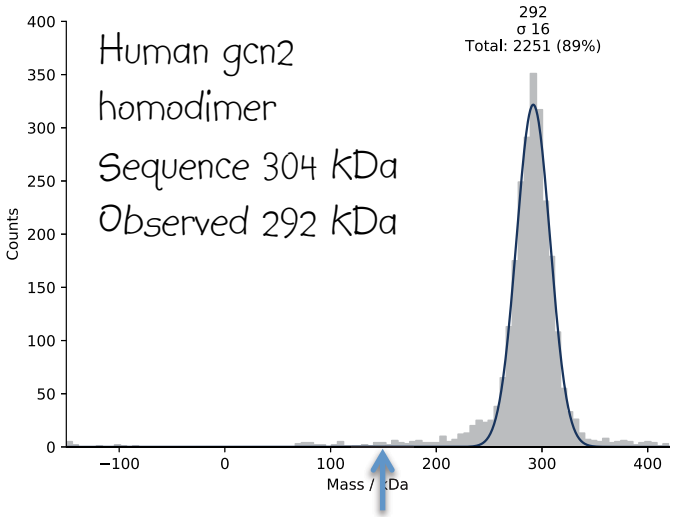
3. Single molecule mass photometry of nucleic acids. Yiwen Li, Weston B. Struwe, Philipp Kukura. bioRxiv 2020.01.14.904755

4. Quantitative mass imaging of single biological macromolecules Young et al., Science 360, 423–427 (2018)

LMB iSCAT Applications

Verifying purity, dispersity and integrity of two dimers measured 15uL at 50 nM
Arrows indicate subunit masses. Kd of dimerisation thus X10 (or more) lower than 50nM.

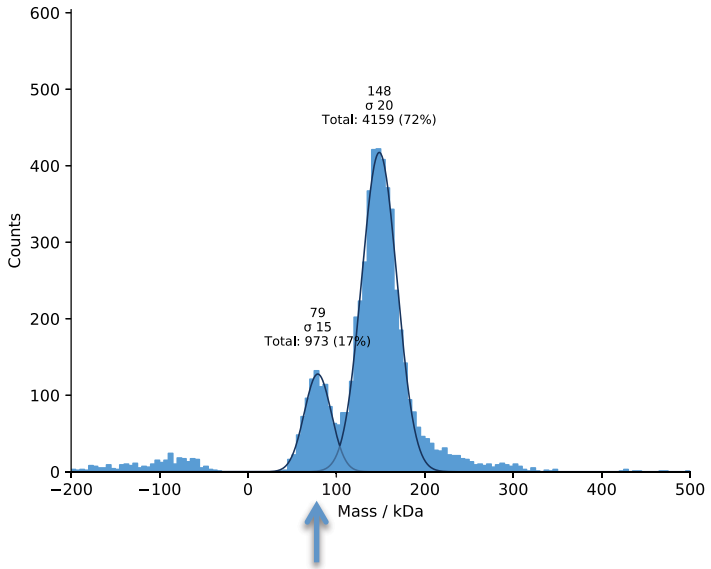


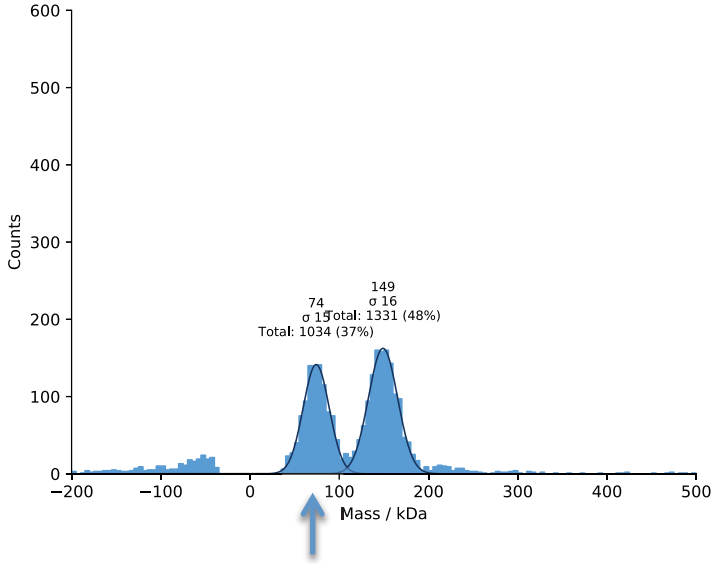


Olga Perisic, Williams lab

LMB iSCAT Applications

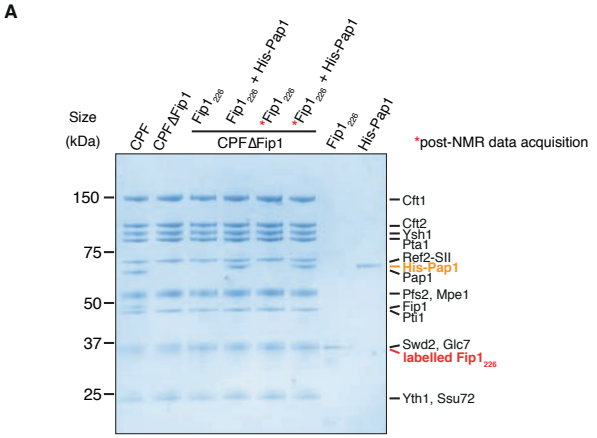
Checking homodimer stability on dilution measured 15uL at ~100 & 40nM
Arrows indicate subunit masses.



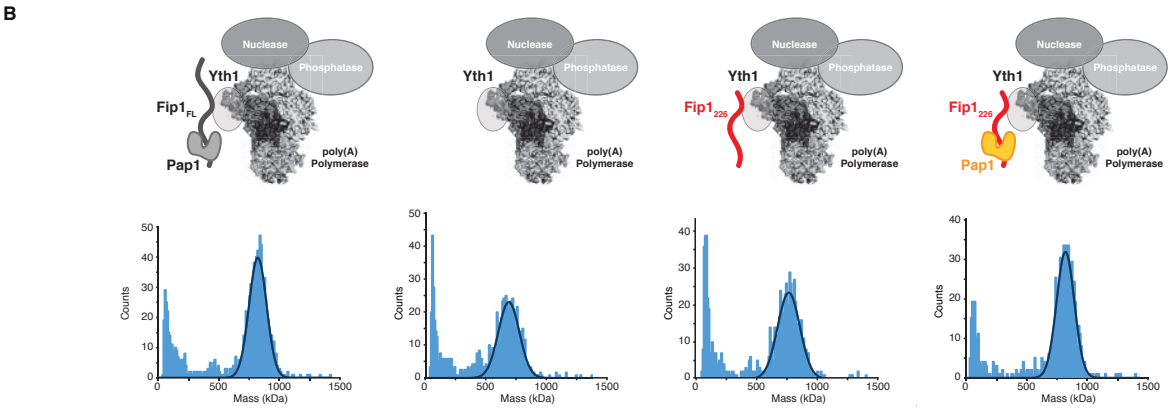


Sami Chaaban, Carter lab

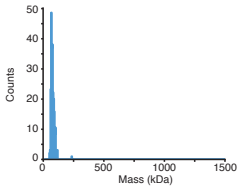
LMB iSCAT Applications



Checking integrity of CFP complex. 10uL of 100nM



Sample	CPF	CPFΔFip1	CPFΔFip1 + Fip1226	CPFΔFip1 + Fip1226 + Pap1
Theoretical MW	**860 kDa**	**760 kDa**	**785 kDa**	**851 kDa**
Event counts	**755 (70%)**	**654 (64%)**	**565 (66%)**	**604 (75%)**
Average MW	**856 kDa**	**749 kDa**	**779 kDa**	**844 kDa**



Sample **Buffer**

Conny Yu, Passmore lab

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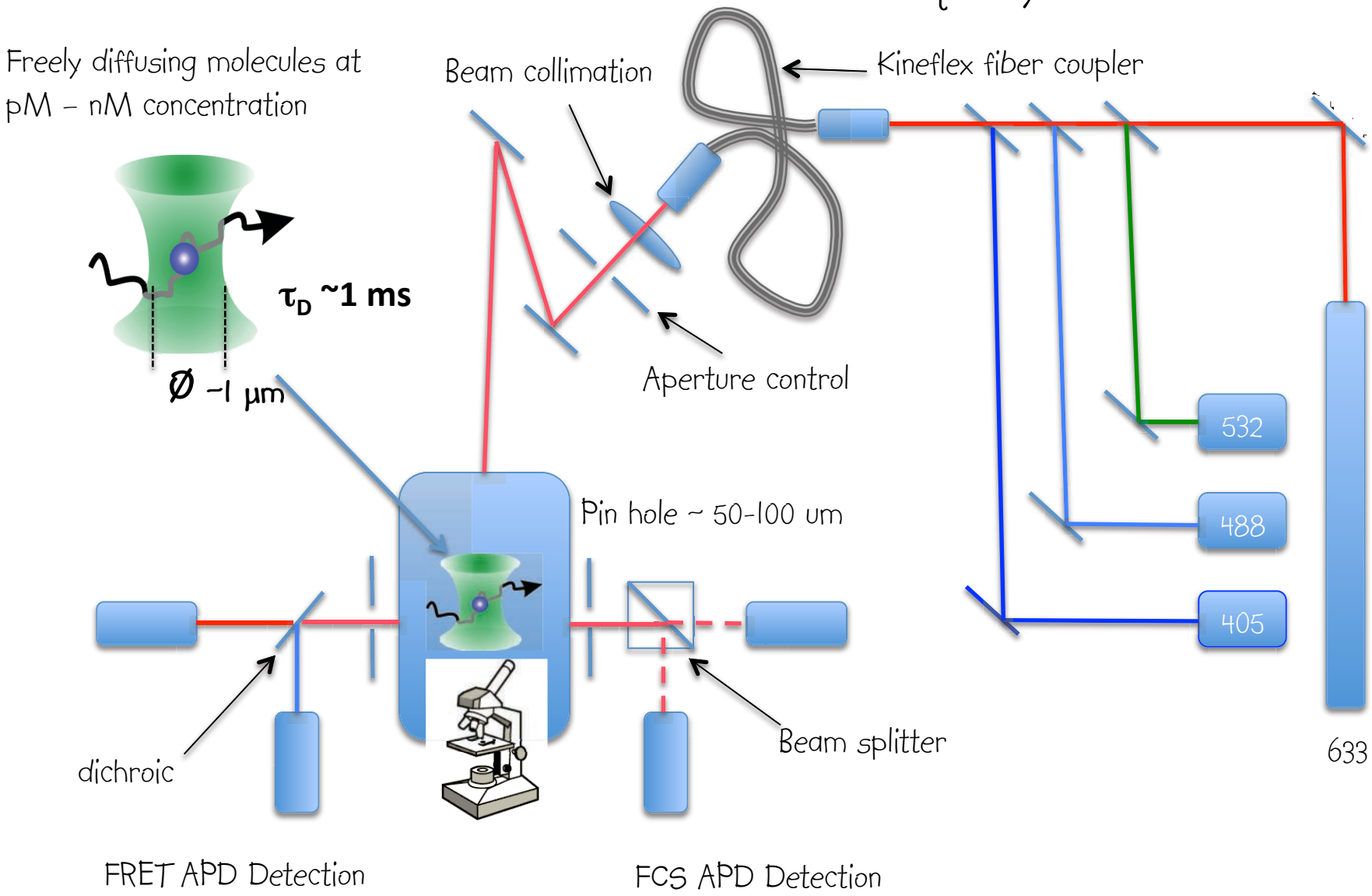
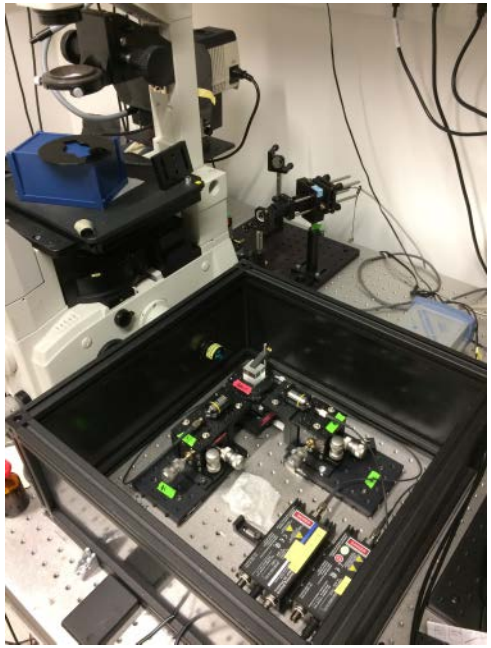
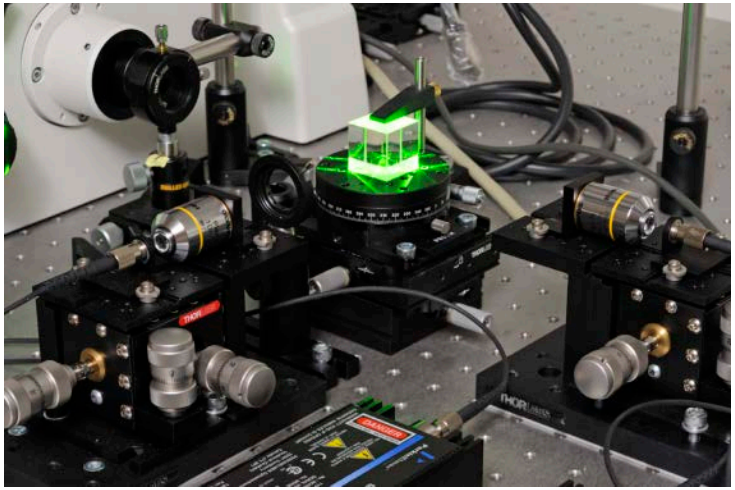
Current Confocal Instrument Configuration

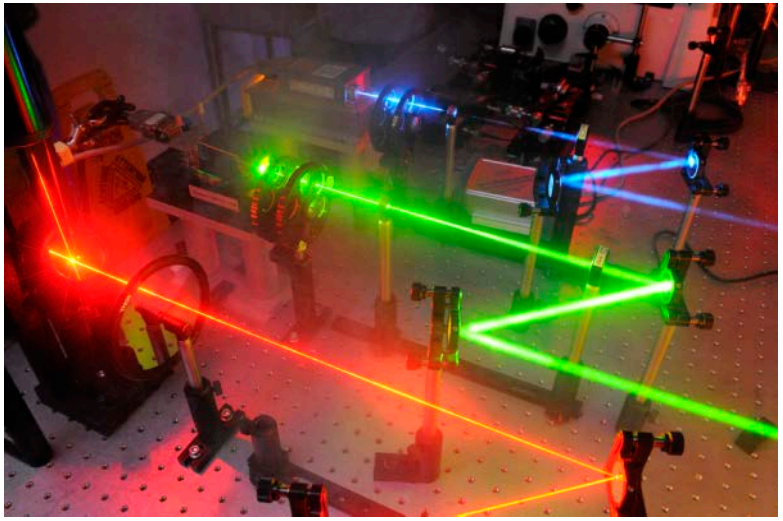
Home built : flexible but quirky





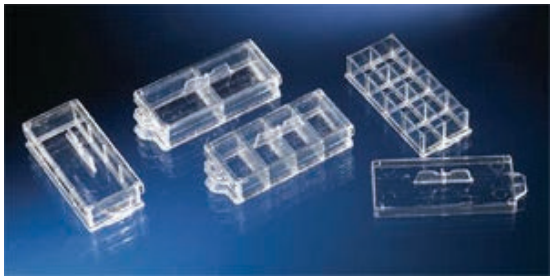


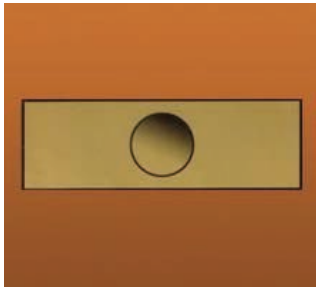

LASER ON
DO NOT ENTER

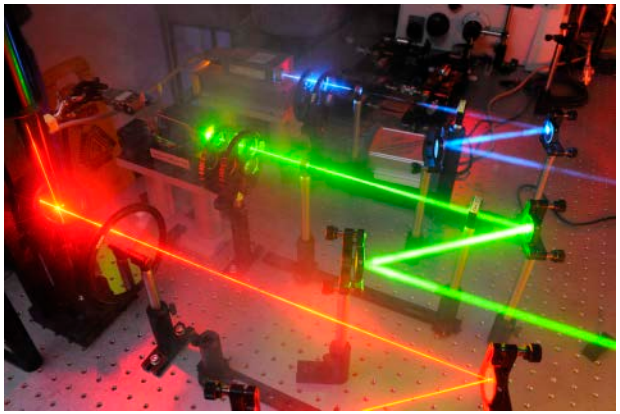


Samples

- Compatible with any buffer; typically include 0.05% Tween 20 and 0.1mg/ml BSA
- Slides (40ul) or 8 well Tek-trays (200ul)
- Trays good for surface pre-treatment (passivation polylysine) and for use in titrations, additions, mixing etc







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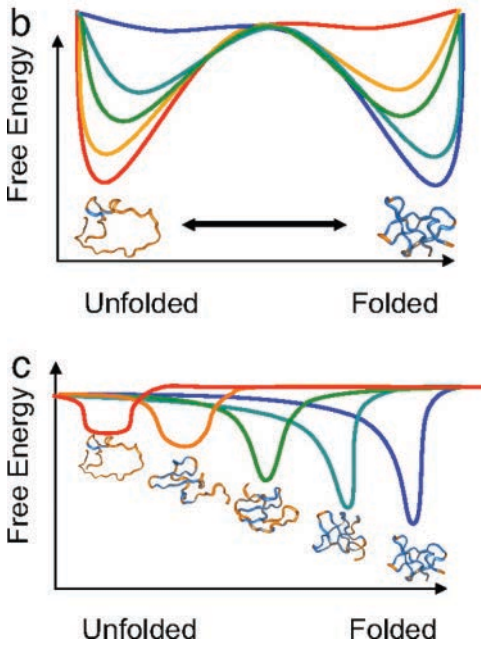
Protein folding, barrier limited or not?

- Do small ultra fast folding proteins fold via a barrier (transition state) limited process between distinct states (ensembles) or via a non-cooperative gradual acretion of structure ('downhill one state folding')?



PLoS One. 2013 Oct 28;8(10):e78044.
Proc Natl Acad Sci U S A. 2012 Jan 3;109(1):179-84.
J Mol Biol. 2009 387:993-1001.
J Mol Biol. 2009 387:975-85.
J Mol Biol. 2009 387:986-92.
Proc Natl Acad Sci U S A. 2009 106:103-8.
Nature. 2006 442 :317-21.
J Mol Biol. 2004 344:295-301.
Science. 2002 298:2191-5.

Barrier(s) in PSBD BBL?

Barrier limited

- Many proteins fold slowly over large energetic barriers
- Equilibria and kinetics consistent with an activated (barrier limited) cooperative process
- Biology requires active/inactive binary states for evolution

No barrier; 'Downhill'

- A prediction of energy landscape theory under extreme native conditions
- Small proteins fold ultrafast near diffusion controlled
- 'Attractive' because folding mechanism could be mapped at equilibrium

Debate has continued because ensemble equilibrium and kinetic measurements are ambiguous

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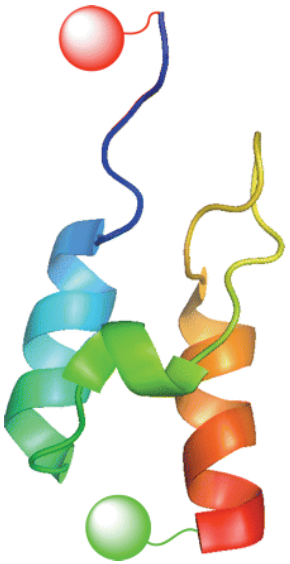
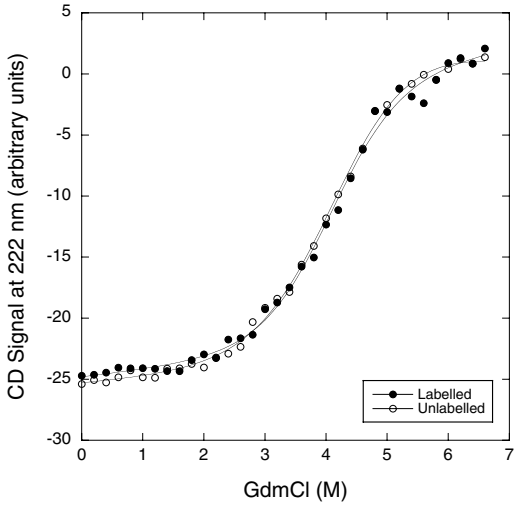
Single molecule FRET in BBL*

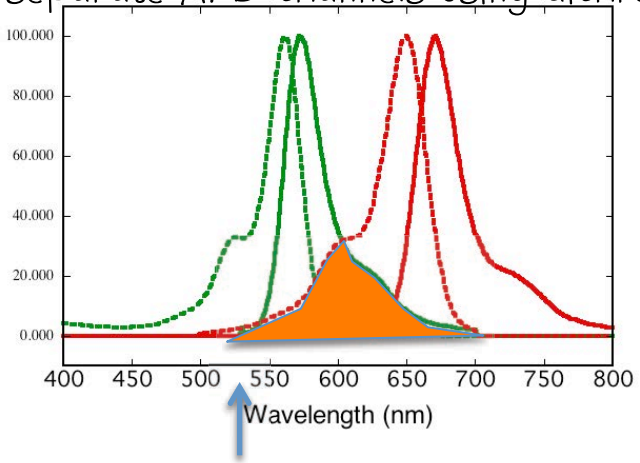
Alexa Fluor 647 acceptor



Alexa Fluor 546 donor

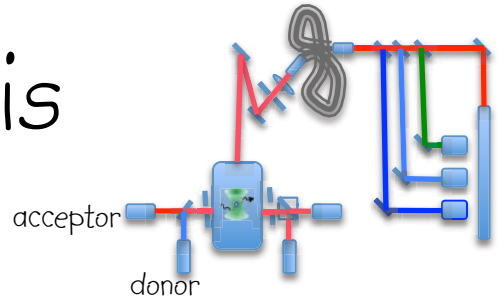


- Denturants (GdmCl or urea) used to perturb system between folded and unfolded (talk 5)
- Labels introduced through N and C-terminal cys mutants (crude but works)
- Stability is unaffected
- Excite 532 nm CW 150uW
- Detect donor and acceptor fluoresecence in separate APD channels using dichroic

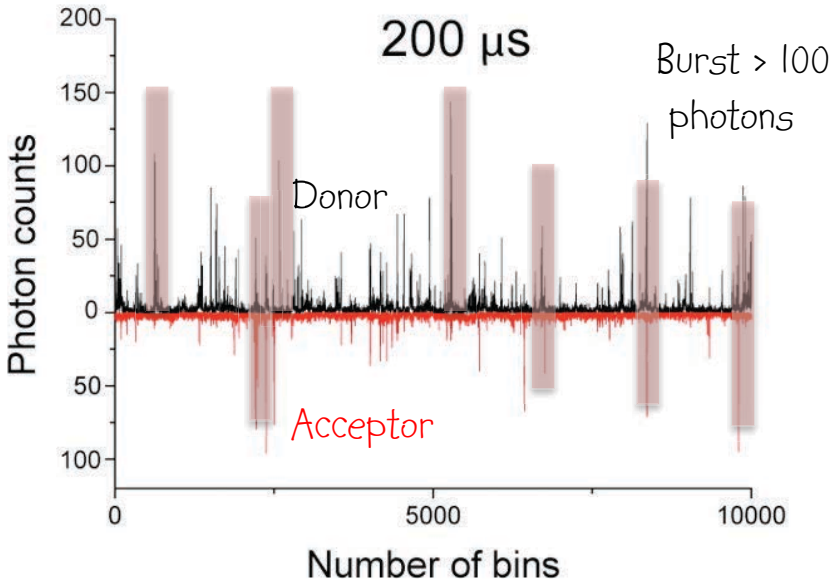


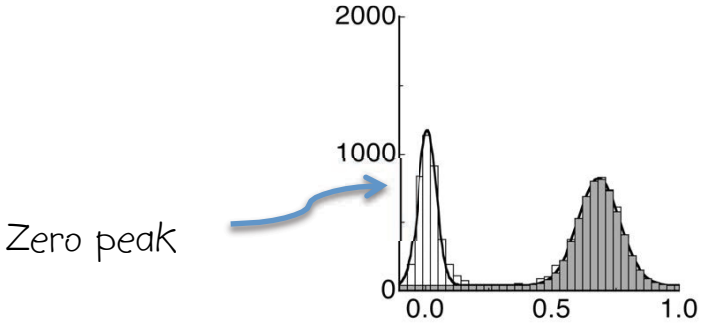
*Huang F, Ying L, Fersht AR, PNAS (2009) 106, 16239-16244

FRET data and analysis

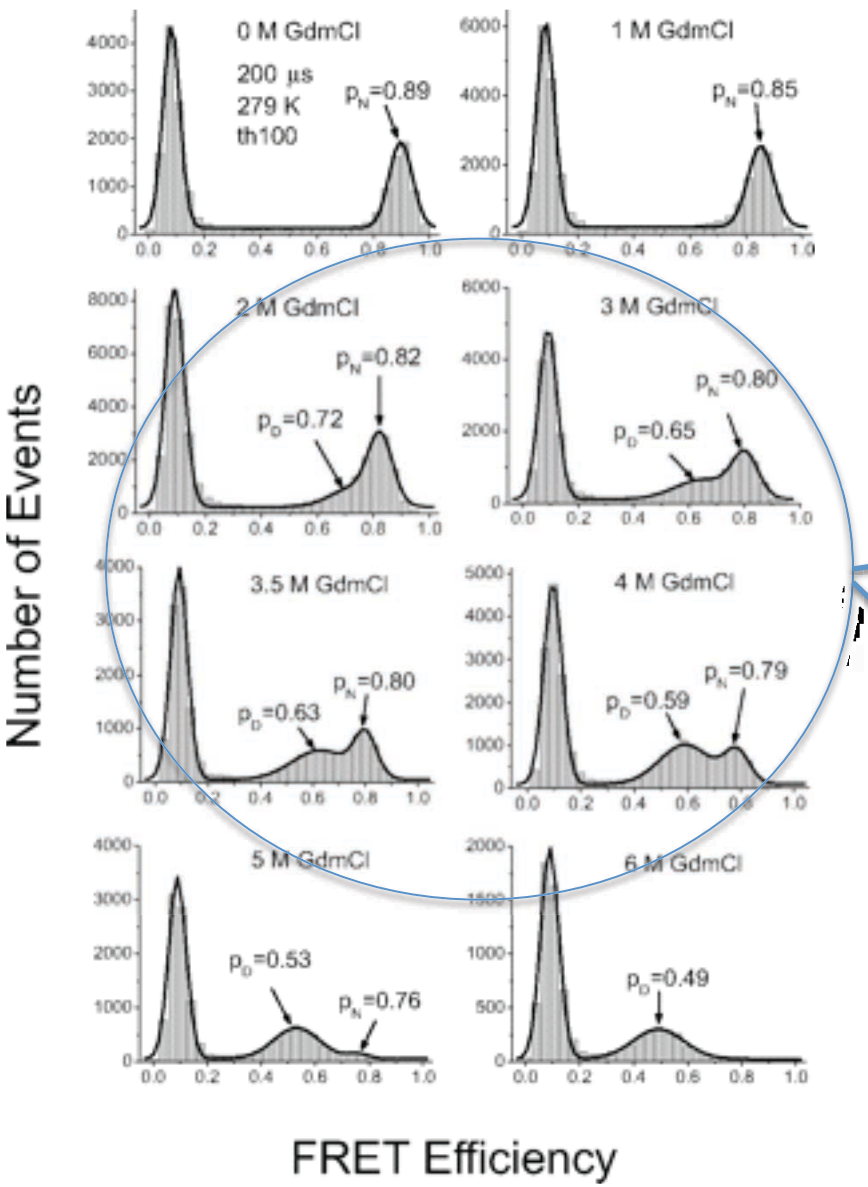


- Measure photon counts of donor and acceptor fluorescence averaging over a predefined bin interval...200us.
- Set count threshold for a diffusion event 'burst' ($photon_{donor}$ + $photon_{acceptor}$) depending on time binning and background, signifying molecule diffusing through observation volume
- Calculate FRET efficiency of each burst; FE = $photon_{acceptor}$ / ($photon_{donor}$ + $photon_{acceptor}$)
- Construct **FRET histogram** from many single molecules
- Zero peak from donor only label or non fluorescing acceptor can be eliminated in more complex two colour excitation methods (TCCD, PIE, ALEX)

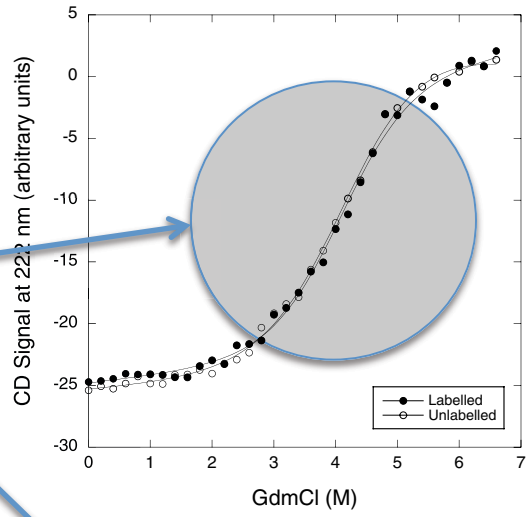


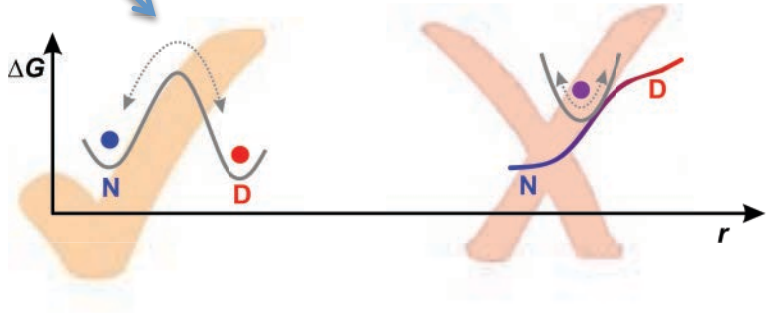


FRET distribution two discrete states



Two populations (N and D) are present at equilibrium supporting a conventional barrier limited mechanism





Single Molecule Techniques

Force or Manipulation based

- Atomic Force Microscopy; 'Tapping' for scanning and imaging. 'Pulling' force extension methods
- Optical and Magnetic tweezers; Polymer or magnetic bead held under constant force or in constant position. Lumicks C-trap

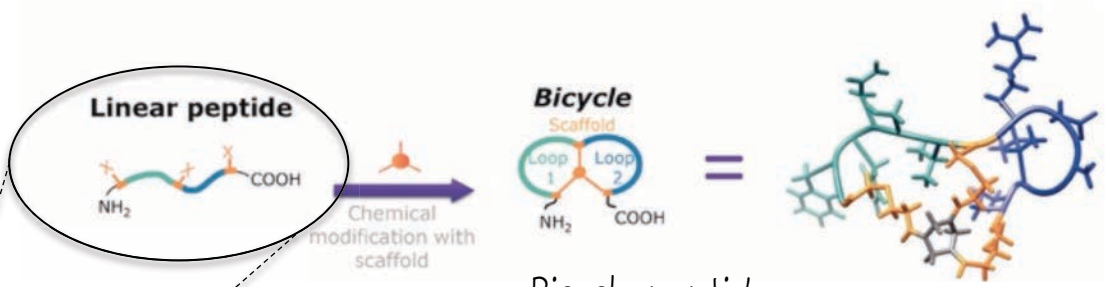
Scattering based (label free)

- Interferometric scattering (iSCAT)

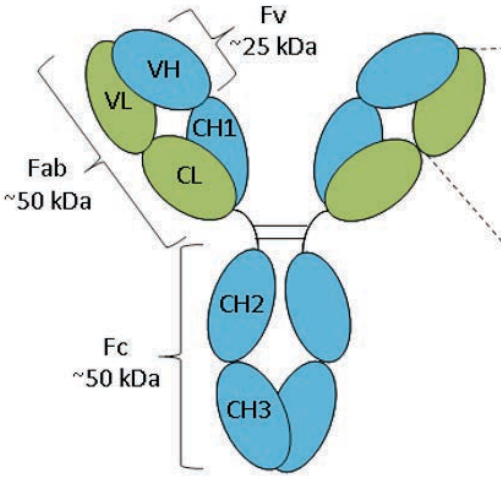
Fluorescence based

- Imaging and localisation (Talks 1-4)
- 3rd Generation single molecule sequencing*
- Fluorescence resonance energy transfer (FRET); dye dye 'quenching' interactions for FRET populations and distances
- **Fluctuation correlation spectroscopy (FCS); diffusional times (properties) of labeled species. Also (PET-FCS) dye side chain quenching for time resolved intrachain dynamics.**

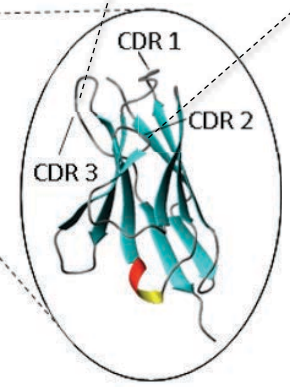
The decreasing size of antibody based therapeutics



Bicycle peptide
1.5 kDa



Antibody
150 kDa



Domain Antibody
15 kDa

Linear peptides library by phage display bi-cyclised before selection

Bicycle Peptides

More constrained / less dynamic? ..... FCS

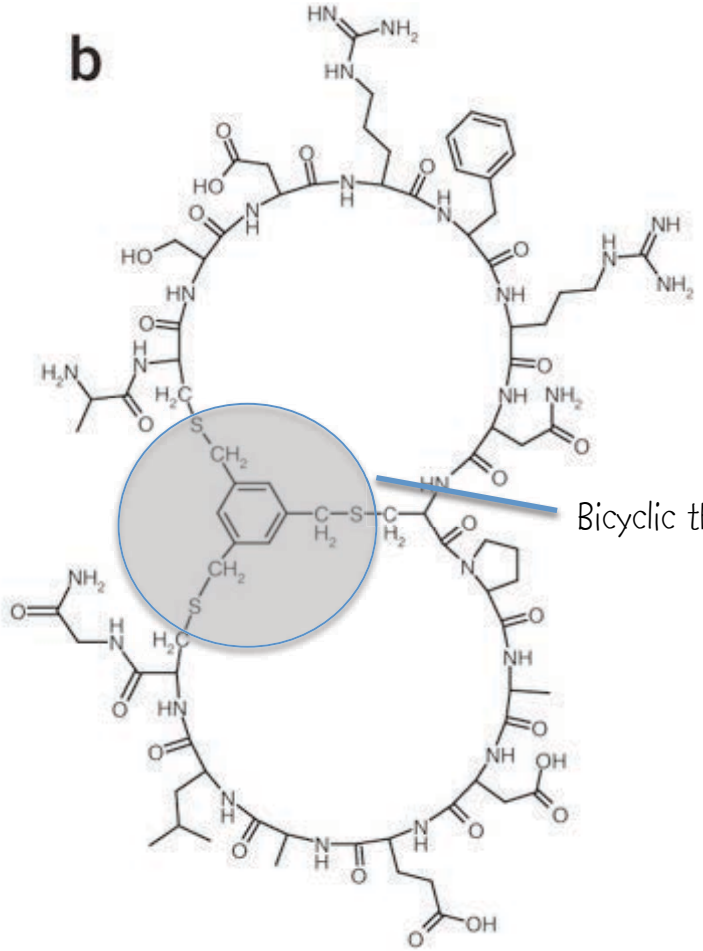
Smaller entropic penalty so tighter binding?..... ITC

Structured so more protease resistant?

Better tissue penetration

Conformation and dynamics in constrained peptides

- Bicyclic peptide inhibitor of plasma Kallikrein (PK15) evolved using phage display*
- IC 50 2 nM compared to > 10 uM for the linear peptide. Inhibits coagulation ex vivo
- Pre-constrained peptides have less entropy loss on binding and bind tighter because they are less dynamic?
- But 2D proton NMR NOESY shows lack of long range NOE's that is inconsistent with rigid structure
- FCS and PET-FCS could reveal 'conformation' and dynamics



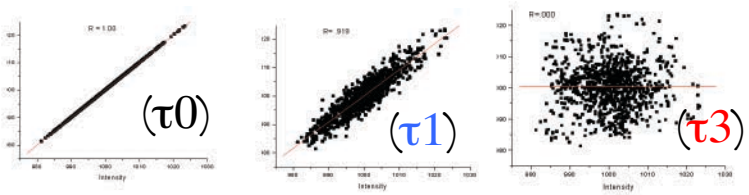
H-ACSDRFRNCPADEALCG-NH_2

*Nat Chem Biol. 2009 (7):502-7. doi: 10.1038/nchembio.184

See http://bicycletherapeutics.com/

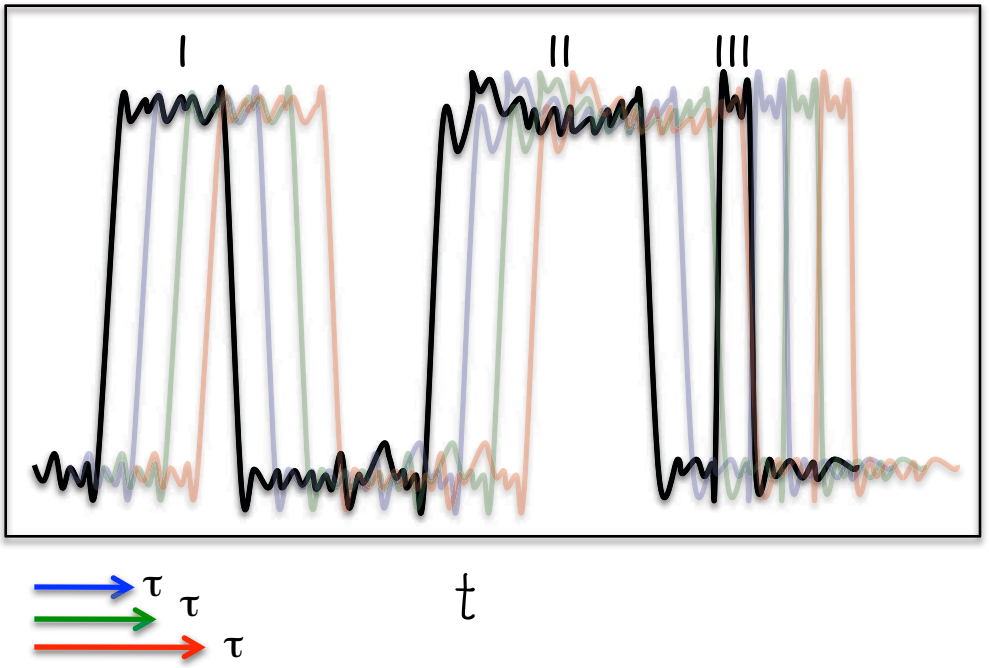
How to measure and analyse fluctuations

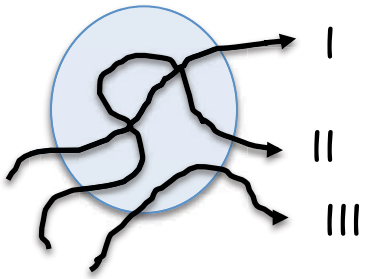
- Molecules passing in and out of a observation volume cause **fluctuations** in the signal at the detector(s)
- Fluctuations can be analysed by calculating the autocorrelation of the signal for different offsets in time (**τ**)



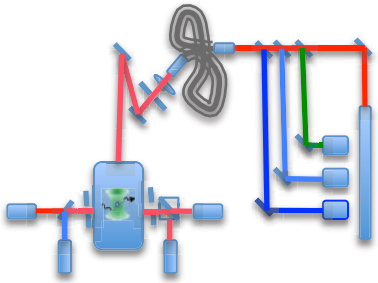
$$g(\Delta t) = \frac{\left\langle \partial I(t)\ \partial I(t+\Delta\tau) \right\rangle}{\left\langle I \right\rangle^2}$$

Measured signal intensity



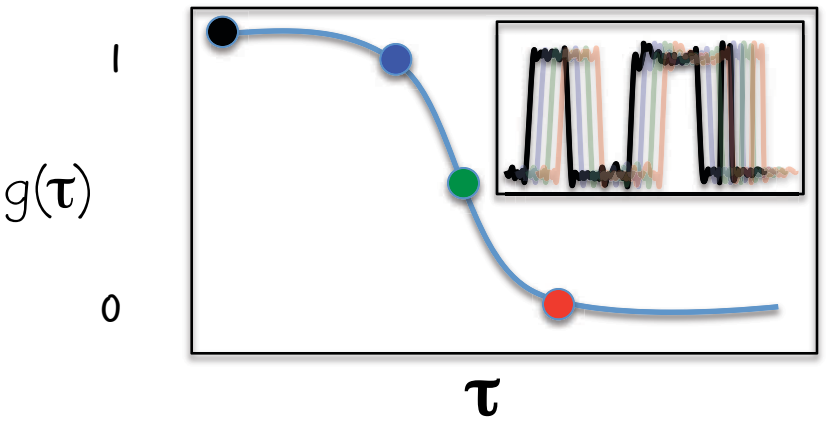


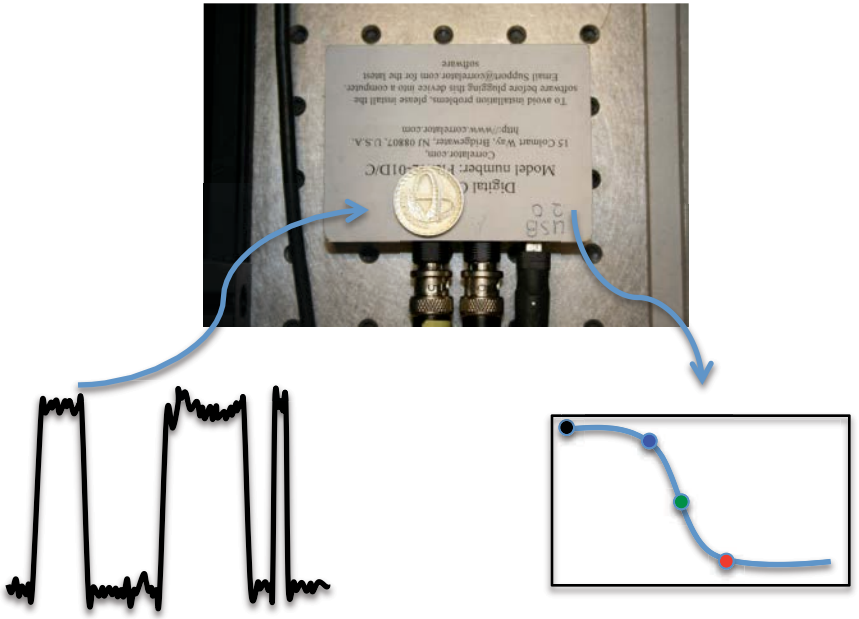
Calculating the Autocorrelation function (ACF)



- Resultant autocorrelation function varies between 1 (for t = 0) to 0 for long times of t (depending on correlation time)

- Hardware correlators do all the numerical work in real time
- Cross correlation between two APD detectors increases the time resolution of single detector system limited by microsecond "after pulsing"



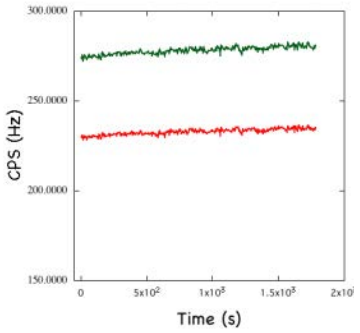


FCS and ACF analysis

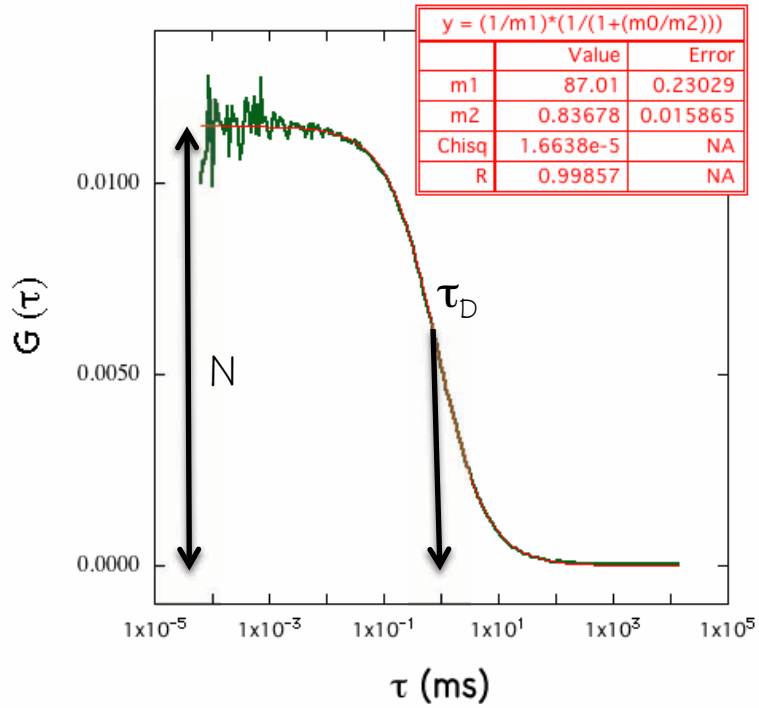
- Average intensity plot (bleaching, quenching, labeling efficiency)
- Analysis of the autocorrelation function

  Translational diffusion time (τ_D)

  Number of particles at zero time (N)



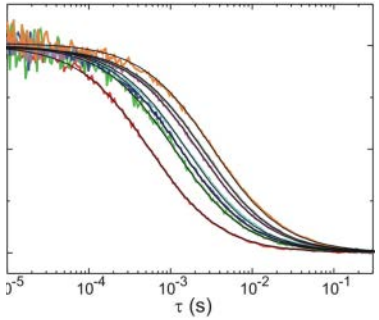
$$G(\tau) = \frac{1}{N} \cdot \left[\frac{1}{1+\frac{\tau}{\tau_D}} \cdot \frac{1}{\sqrt{1+\frac{r_o^2}{z_o^2} \cdot \frac{\tau}{\tau_D}}} \right]$$



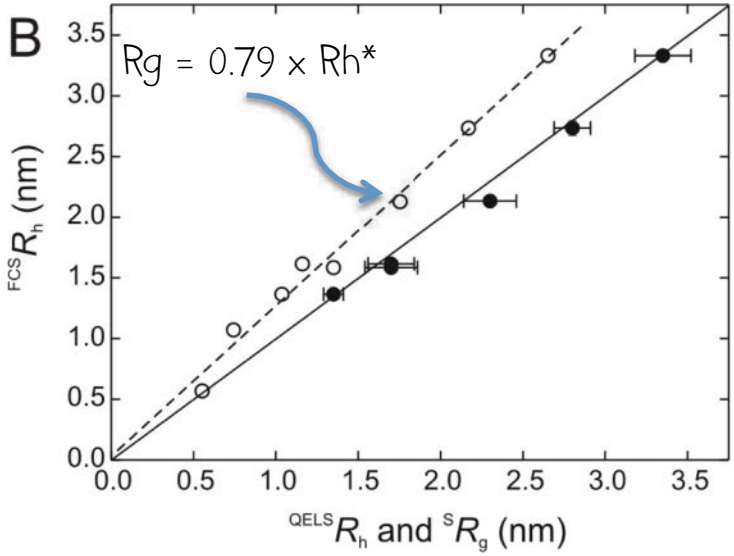
$$g(\Delta t) = \frac{\langle \partial I(t)\ \partial I(t+\Delta\tau) \rangle}{\langle I \rangle^2}$$

τ_D and Hydrodynamic radius Rh

- τ_D can be used to follow changes in diffusion, for example on binding.
- τ_D can give the translational diffusion coefficient (D_t) if the observation volume is known, or we measure τ_D of a known standard
- From this we get hydrodynamic volume (Rh) using Stokes-Einstein equation.
- Shape, asymetry, chain flexibility, excluded volume effects, and solvation all contribute to Dt (and thus Rh) so changes may not reflect corresponding changes in physical dimension.



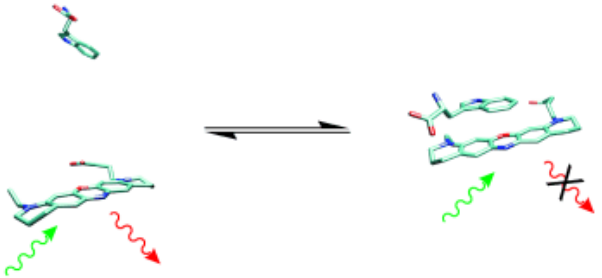
$$Rh = \frac{kT}{6\pi\eta Dt}$$

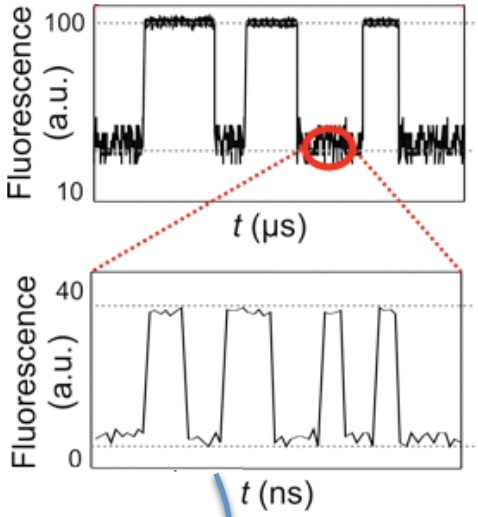


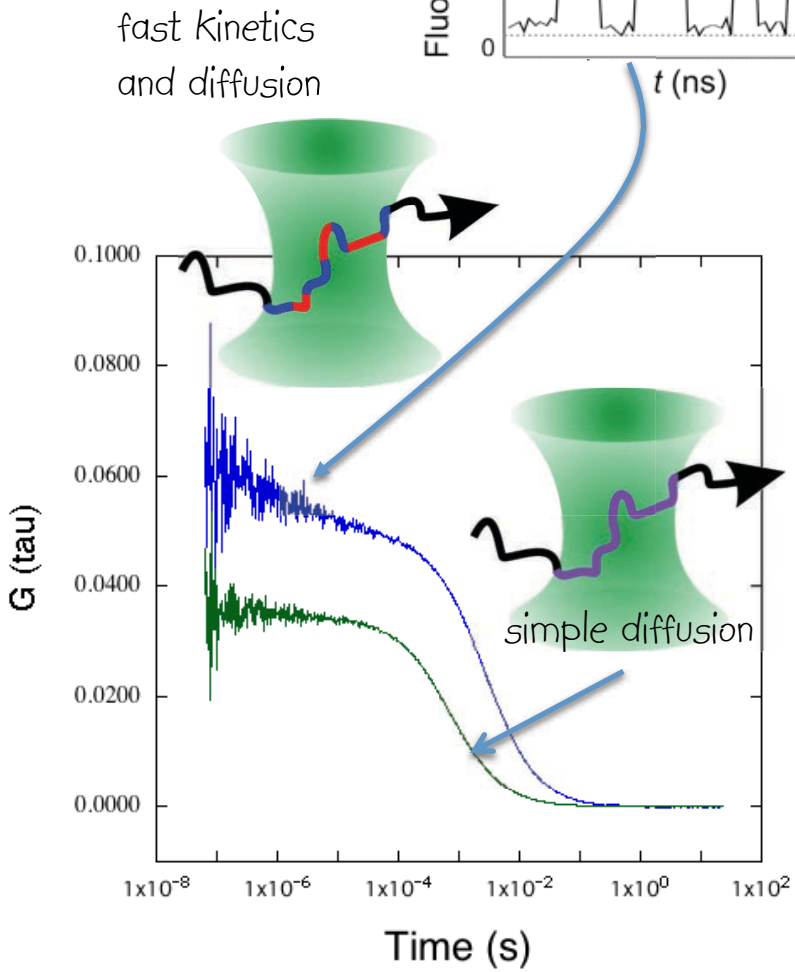
*Expected m for globular proteins 0.77 Burchard et al. (1980) Macromolecules, 13, 1265-1272

FCS analysis contd.

- Additional dynamics that occur **during** the diffusion of molecules can be resolved in the ACF if they give rise to fluorescence fluctuations
- PET FCS* (photo induced electron transfer) is quenching requiring VDW contact between oxazine based dyes and trytophan or guanine and can be used to probe dynamics and intra/inter chain contact formation.



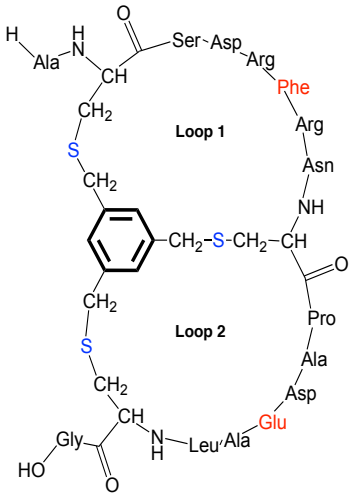




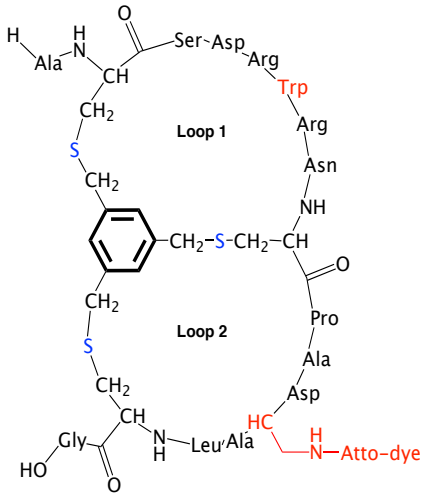
*Chemphyschem. 2009;10:1389-98
http://www.atto-tec.com

PET FCS Labeling strategy

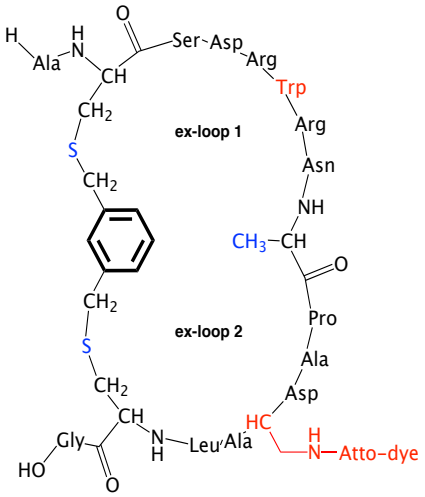
A WT Bicyclic



B Bicyclic +Atto +Trp



C Monocyclic +Atto +Trp



D linear labeled W mutant

Atto 655

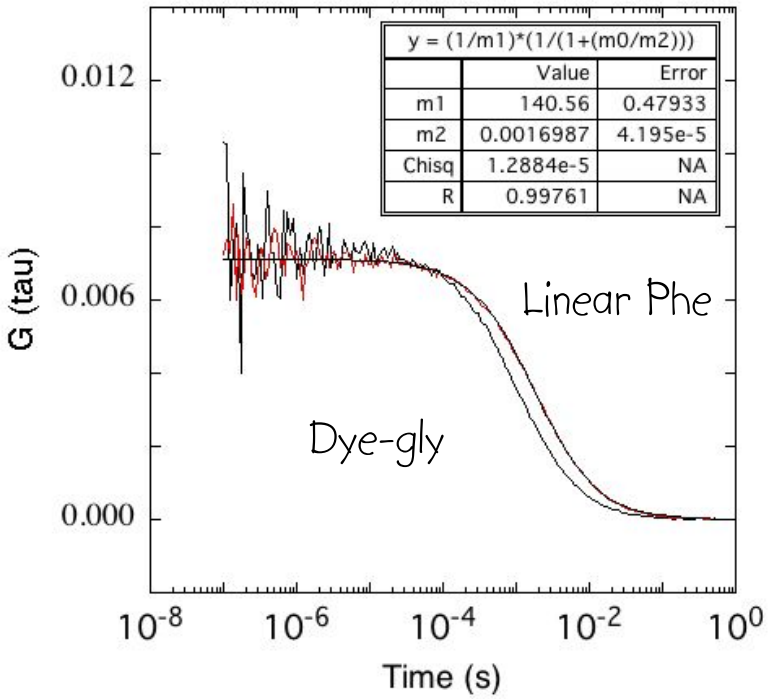
H-ACSDRWRNCPADEALCG-NH_2

E linear labeled F wt

Atto 655

H-ACSDRFRNCPADEALCG-NH_2

Dye and linear phe control



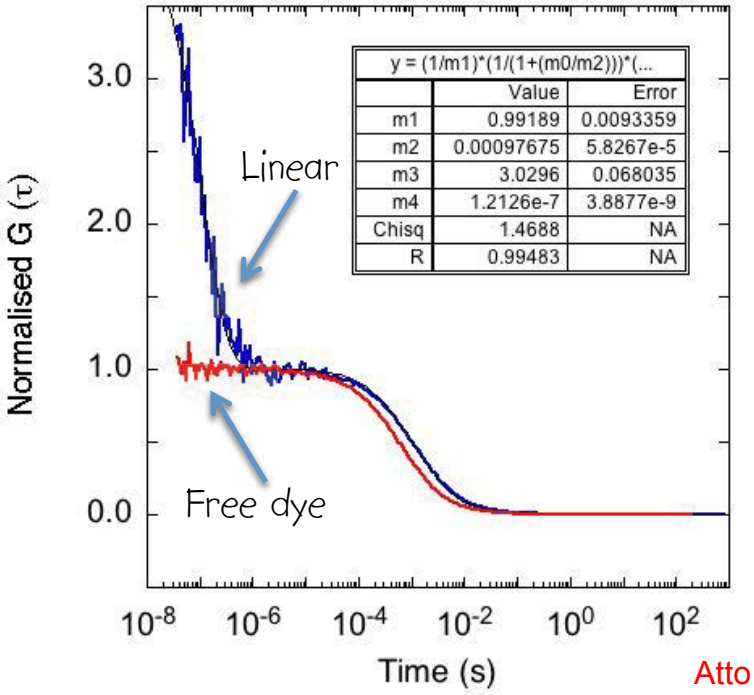
No intensity fluctuations other than diffusion

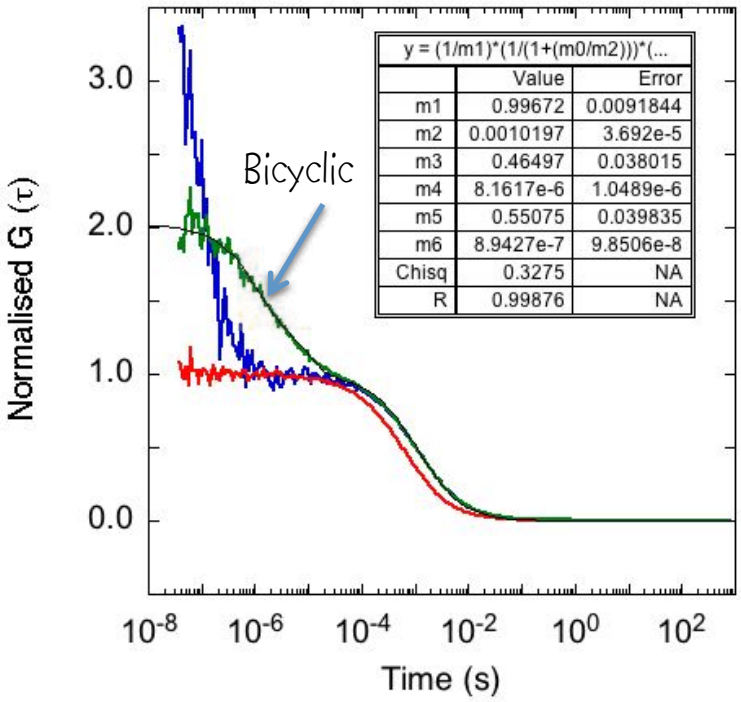
Note longer diffusion of peptide 17 mer compared to dye-amino acid

Linear and bicyclic with trp PET quencher

Linear trp large amplitude PET fluctuations on time scale of 100 ns consistent* with i – i+7 sequence separation but with proline

Bicyclic moderate amplitude PET fluctuations on slower time scale of 1-10 us
Still fast enough to explain the lack of NMR NOE's



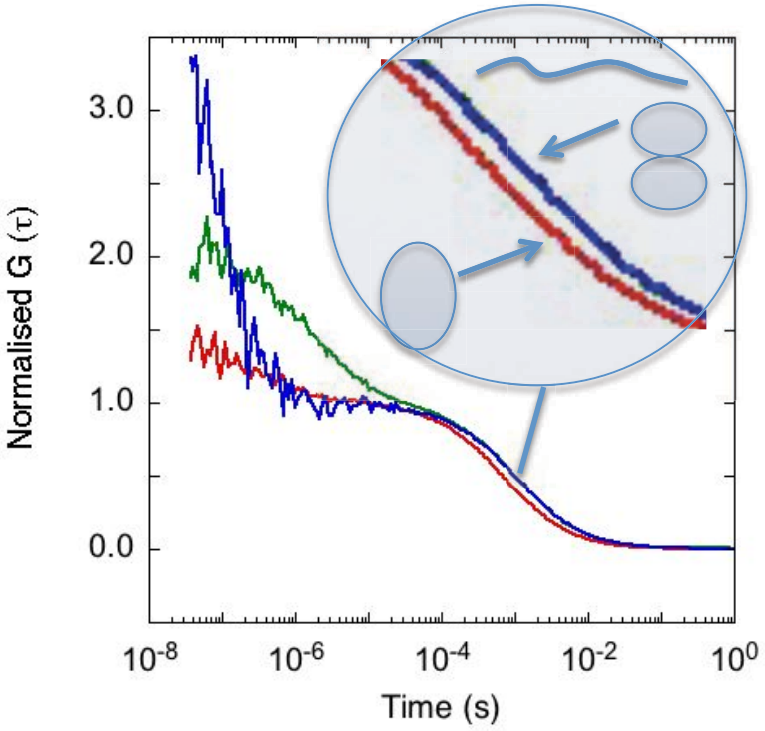


Atto

H-ACSDRWRNCPADEALCG-NH_2

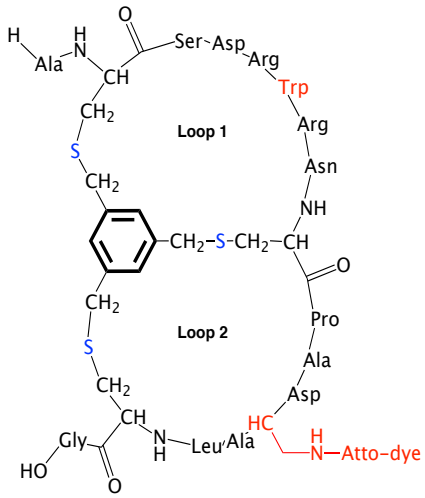
*J Mol Biol. 2003;332:265-74.

Monocyclic with trp PET quencher

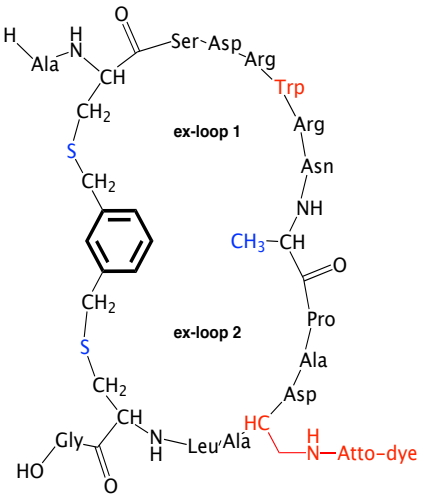


Monocyclic peptide has reduced amplitude fluctuations on an intermediate timescale
Diffusion is faster than bicyclic and linear peptide possibly indicating a 'collapsed' conformation

B

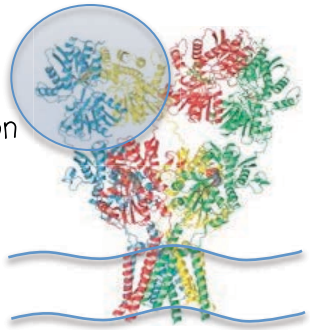


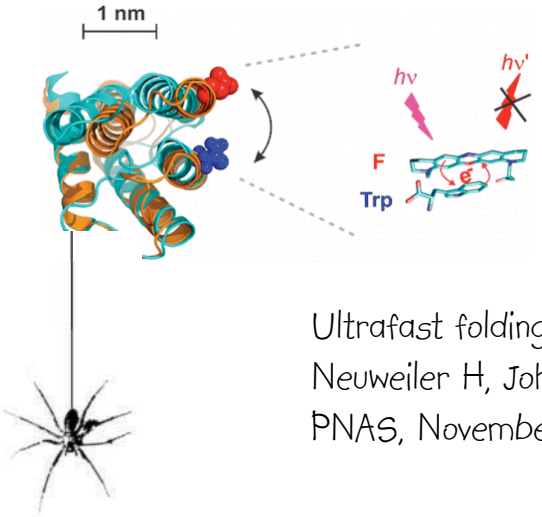
C



Further examples.....

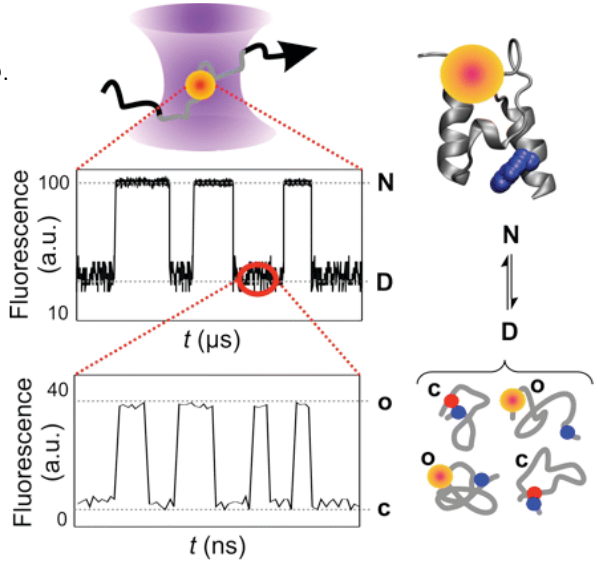
Dynamics in the N-terminal domain of an ionotropic glutamate receptor. Jensen MH, Sukumaran M, Johnson CM, Greger IH, Neuweiler H.
J Mol Biol. 2011 414:96-105



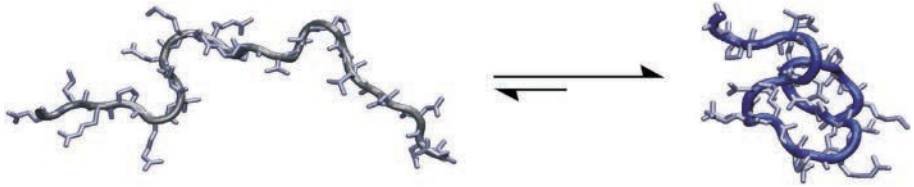


Microsecond folding and domain motions of a spider silk protein structural switch. Ries J, Schwarze S, Johnson CM, Neuweiler H. J Am Chem Soc. (2014) 136(49):17136-44

Ultrafast folding and denatured state dynamics using PET-FCS.
Neuweiler H, Johnson CM, Fersht AR.
PNAS, November 3, 2009, 106, 18569-18574.



Backbone-driven collapse in unfolded protein chains.
Teufel DP, Johnson CM, Lum JK, Neuweiler H.
J Mol Biol. 2011 Jun 3;409(2):250-62.



Questions

- Information, discussions, literature, labels, collaborations .....etc.
- Room 2S005
- cmj@mrc-lmb.cam.ac.uk
- stephenm@mrc-lmb.cam.ac.uk

Further Reading

- Talks 1,3 and 4 on imaging and labeling

Recent advancement of light-based single-molecule approaches for studying biomolecules.
Croop et al. Rev Syst Biol Med. 2019 Feb 6:e1445.

Toward dynamic structural biology: Two decades of single-molecule Förster resonance energy transfer.
Lerner E, et al. Science. 2018 Jan 19;359(6373).

Current setups

- iSCAT label free mass photometry
- Freely diffusing fluorescence based FRET (dye-dye interactions) and FCS for diffusion and/or dye-side chain or solvent quenching

Developments?

- .....Two colour FCS techniques?
- .... Inverse FCS for vesicles
- ..... Microfluidics?
- ..... Immobilised or trapped molecules?