Observing ‘Single’ Molecules

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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

A. Crystal structure of wild-type Leishmania major SAS-001 and SAS-002

B. Phased separation difference map (StS strain, strain)

C. Methane selectivity in model

D. Diffusion (and 'crowding')

E. 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 \(~10 \text{ um}^2 \text{ s}^{-1}\) in cytoplasm
- \( T = \frac{x^2}{6D} \) (for 3 dimensional diffusion)
- \( T_{\text{E.coli}} = 10-20\text{ms} \) (~1um)
- \( T_{\text{hela cell}} = 10\text{s} \) (20um)
- \( T_{\text{neuronal cell}} = 10^6\text{s} = 20 \text{ days} \) (1cm)
- \( T_{\text{sciatic nerve}} = \sim 71,000\text{ years} \) (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.
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.

Single molecule experiments should therefore be necessary, be well designed and target specific tractable questions.
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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Scattering based (label free)
- 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 interfere 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

**iSCAT Practicalities**

Commercial instrument (Mass Photometry)

10μL sample 10-100 nM. Label free

- Glass coverslip
- Silicone gasket
- 10μL sample
- Oil objective lens

3 x 10 μm camera field of view

80 nm² 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 80nm².
- 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 stabilization of complexes (cryoEM)
- Measurement of modified proteins or other macromolecules
- Determination of protein-protein interactions in nM range
- Very quick (minutes). Uses uL of nM concentration. **NO LABELS**

References:
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.

Human PI3K heterodimer
Sequence 208 kDa
Observed 205 kDa

Human gcn2 homodimer
Sequence 304 kDa
Observed 292 kDa
LMB iSCAT Applications

Checking homodimer stability on dilution measured 15μL at ~100 & 40nM. Arrows indicate subunit masses.
LMB iSCAT Applications

Checking integrity of CFP complex. 10uL of 100nM

<table>
<thead>
<tr>
<th>Sample</th>
<th>Theoretical MW</th>
<th>Event counts</th>
<th>Average MW</th>
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<tbody>
<tr>
<td>CPF</td>
<td>860 kDa</td>
<td>755 (70%)</td>
<td>856 kDa</td>
</tr>
<tr>
<td>CPFΔFip1</td>
<td>760 kDa</td>
<td>654 (64%)</td>
<td>749 kDa</td>
</tr>
<tr>
<td>CPFΔFip1 + Fip1&lt;sub&gt;1&lt;/sub&gt;</td>
<td>785 kDa</td>
<td>565 (66%)</td>
<td>779 kDa</td>
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<tr>
<td>CPFΔFip1 + Fip1&lt;sub&gt;1&lt;/sub&gt; + Pap1</td>
<td>851 kDa</td>
<td>604 (75%)</td>
<td>844 kDa</td>
</tr>
</tbody>
</table>

Conny Yu, Passmore lab
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Scattering based (label free)

- Interferometric scattering (iSCAT)
Current Confocal Instrument Configuration
Home built: flexible but quirky

- Kineflex fiber coupler
- Aperture control
- Beam collimation
- Dichroic beam splitter

Freely diffusing molecules at pM - nM concentration

\[ \tau_d \approx 1 \text{ ms} \]

\[ \Phi \approx 1 \mu m \]

- Pin hole \( \sim 50-100 \text{ um} \)

FRET APD Detection

FCS APD Detection
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’)?
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

Single molecule FRET in BBL*

- 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 fluorescence 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’ \((\text{photon}_{\text{donor}} + \text{photon}_{\text{acceptor}})\) depending on time binning and background, signifying molecule diffusing through observation volume.
- Calculate FRET efficiency of each burst; \(\text{FE} = \frac{\text{photon}_{\text{acceptor}}}{(\text{photon}_{\text{donor}} + \text{photon}_{\text{acceptor}})}\)
- 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)
Two populations (N and D) are present at equilibrium supporting a conventional barrier limited mechanism.
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Scattering based (label free)
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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

The decreasing size of antibody based therapeutics

Antibody 150 kDa
Domain Antibody 15 kDa
Bicycle peptide 1.5 kDa
Linear peptides library by phage display bi-cyclised before selection
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.

How to measure and analyse fluctuations

- Molecules passing in and out of an 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 ($\tau$).

$$g(\Delta t) = \frac{\langle \partial I(t) \, \partial I(t + \Delta \tau) \rangle}{\langle I \rangle^2}$$
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 ($\tau_D$)
Number of particles at zero time ($N$)

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

\[ y = (1/m_1)(1/(1+(m_0/m_2))) \]

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
<th>Error</th>
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<tbody>
<tr>
<td>$m_1$</td>
<td>67.01</td>
<td>0.23029</td>
</tr>
<tr>
<td>$m_2$</td>
<td>0.83678</td>
<td>0.015865</td>
</tr>
<tr>
<td>Chisq</td>
<td>1.6638e-5</td>
<td>NA</td>
</tr>
<tr>
<td>$R$</td>
<td>0.99857</td>
<td>NA</td>
</tr>
</tbody>
</table>
\( \tau_D \) and Hydrodynamic radius \( Rh \)

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

\[
Rh = \frac{kT}{6\pi\eta D_t}
\]

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

E linear labeled F wt
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

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.
Further examples.....


Questions

• Information, discussions, literature, labels, collaborations ……etc.
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• 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.

Toward dynamic structural biology: Two decades of single-molecule Förster resonance energy transfer

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?