Single Molecule Spectroscopy

Measuring ‘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
  ✓ Cheap(er)
  ✓ Biological buffers, controlled levels of reactants

✓ Solution based. Molecules often freely diffusing and undergo stochastic processes of dynamics, conformational equilibria, binding etc.
✓ Single molecule measurement reveals ensemble heterogeneity
The static view and the real world

Time scales of diffusion

• Time scale (T) to traverse distance (x) in a cell is related to translational diffusion coefficient (D) which for a 30 kDa protein is ~ 10 µm² s⁻¹ in cytoplasm
• T = x² / 6D (for 3 dimensional diffusion)
• T_{E.coli} = 10-20ms (~1 µm)
• T_{hela cell} = 10s (20 µm)
• T_{neuronal cell} = 10⁶s = 20 days (1cm)
• T_{sciatic nerve} = ~ 71,000 years (150cm)
Consequences of diffusion

• Molecular motors... run at around ~ 1 µm 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 µm\(^2\) s\(^{-1}\) than the crowded (high viscosity) environment of the cell

• T for a typical confocal psf of 1 µm is 1 ms (not many photons!)

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. The ‘features’ (shape, width, sub populations, rare events, etc) of the ensemble distribution can be determined.

• Ensemble and single molecule averages should agree 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 ‘one single molecule’: more usually the average of many molecules, but each observed one at a time.
Other Advantages of Single Molecule Spectroscopy 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 μL of nM-pM solution so are economic with material and can measure in the nano - picomole range e.g., for Kd determination.

- No size limit or serious restrictions in solvent.

- Suitable for multi-colour approaches

Practicalities of Single Molecule Spectroscopy Methods

- Spectroscopic (and imaging) methods rely on ‘bright’ fluorescent labels which may perturb the system or undergo complex photophysics .... lots of controls are required.

- Single fluorophore, single site labeling is ‘cleanest’ and may require mutagenesis

- Instrumentation is complex and expensive and requires expertise to build, maintain and in order to obtain optimal data.

- ‘Single molecule’ concentrations may not allow stable complex formation (but can add an excess of unlabeled material) and non-specific absorption onto surfaces may be a problem.

- 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.
Non-structural 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

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); diffusion of dye and/or dye side chain or solvent quenching for diffusion times (size), or time resolved intrachain dynamics.

§ Next Generation Biophysics Meeting, LMB, 26/09/2018

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

Current Confocal Instrument Configuration

Freely diffusing molecules at pM – nM concentration

Beam collimation

Pin hole ~ 50-100 µm

FRET APD Detection

FCS APD Detection

APD

Dichroic

Kinflex fiber coupler

Aperture control

Beam splitter

Ø ~ 1 µm

t ~ 1 ms

633
Population distributions and 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. The ‘features’ (shape, width, sub populations) of the ensemble distribution can be determined.

• Types of heterogeneity
  – Static (mixtures)
  – Dynamic (equilibrium)
  – Temporal (reaction)
Static Ensemble Heterogeneity

- Measuring properties of single molecules reveals ensemble heterogeneity commonly shown in the form of a probability histogram.

Dynamic Ensemble Heterogeneity

- Depending on the time scale of interconversion ($\tau_{\text{int}}$) and the time scale of measurement ($\tau_{\text{meas}}$) ... which depends on the time step within the diffusion/observation time ... we will observe two populations, a single broad distribution or a single sharp distribution.

- $\tau_{\text{meas}} \sim 50 \mu s$ is possible so many biological equilibria of interest can be resolved.
Accessing fast and slow dynamic heterogeneity

- **Fast**: fluctuation correlation techniques have faster $\tau_{mac}$ (10ns) and can resolve inter-conversion of states from ms to ns as well as allowing measurement of $\tau_{int}$

- **Slow**: immobilising molecules allows measurements on single molecules for much longer than ms diffusion (up to 10's seconds) allowing slower interconversion to be observed. Equilibrium distributions can also be inferred from the dwell time in each conformation.

- Immobilisation can also avoid temporal ensemble desynchronisation of multi step reactions / pathways allowing complex kinetic pathways and their component rates to be determined.

Fluorescence based Single Molecule FRET and FCS

Data collection and analysis
Samples

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

Fluorescence resonance energy transfer; FRET

- FRET is a non-radiative transfer through dipole-dipole interactions of donor and acceptor groups between 1 and 10 nm apart with consequential fluorescence from the acceptor
- Fret efficiency proportional separation and so can yield ‘distance’ information
- Forster distance (Ro) is specific for each FRET pair and at that separation, FE = 50%
  \[ E = \frac{1}{1 + (r/R_o)^6} \]
- Fret requires spectral overlap between donor emission and acceptor absorption

![Diagram of FRET](image)

Excitation wavelength | donor | “FRET” acceptor
--- | --- | ---
420 | 430 | 500
440 | 450 | 550
525 | 570 | 600

Absorption and Emission Spectra (%)
**FRET data and analysis**

- Measure photon counts of donor and acceptor fluorescence averaging over a predefined bin interval.
- 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 (LMB example I).
- Zero peak from donor only label or non fluorescing acceptor can be eliminated in more complex two colour excitation methods (TCCD, PIE, ALEX).

**FCS data and analysis**

- Molecules passing in and out of the observation volume cause fluctuations in the signal.
- Fluctuations can be analysed \(I(t)\) by calculating the autocorrelation of the signal for different offsets in time \((\tau)\).

\[
G(\tau) = \frac{\langle \delta I(t) \delta I(t + \tau) \rangle}{\langle I(t) \rangle^2}
\]
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”

$G(t) = \frac{1}{N} \left[ \frac{1}{1 + \frac{\tau}{\tau_D}} \right]^{1/2}$

FCS and ACF analysis

- Intensity (bleaching, quenching, labeling)
- Analysis of the autocorrelation function

Translational diffusion time ($t_D$)

Number of particles* $(N)$

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

*Since this is a Poissonian process at zero delay we have the average of the square in fluctuations = average divided by average squared = 1/average

Although we study micro ensembles of molecules at nM concentrations (10-100), the fluctuations from individual molecules only correlate with themselves making FCS a single molecule method.
**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, 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

**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 chain

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

- Independent diffusion times and populations in mixtures. Fitting multiple species requires 3-5 fold difference in size (fiber or polymer growth)
- Amplitude of ACF is inversely proportional to the number of molecules and can be used to determine stoichiometries of interaction
- Average photon intensity reflects the concentration of dye so in comparison with the number of molecules indicates the labeling efficiency.
- Equilibrium (non perturbation) method for measuring fast kinetics on the us to ns time scale and changes in these with conditions or upon binding
- Two dye studies using two colour cross correlation ‘isolates’ particles containing two dyes, e.g. in binding experiments.
- FRET FCS observes diffusion of acceptor labeled molecules that are undergoing FRET from donor
- Inverse FCS observes diffusion of unlabeled particles (vesicles?) in low background of dye in solution

Fluorescence based Single Molecule FRET and FCS
Examples from LMB projects

I FRET static heterogeneity
II PET FCS to monitor dynamics in cyclized peptides
Example I; FRET static heterogeneity. Protein folding, barrier limited or not?

- Do small ultra fast folding proteins such as BBL fold by a barrier (transition state) limited process between distinct states (ensembles) or via a non-cooperative gradual accretion 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 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*

- Labels introduced through N and C-terminal cysteine mutants
- Stability is unaffected
- Excite at 532 nm CW 150uW
- Detect donor and acceptor fluorescence in separate APD channels using dichroic filters

Alexa Fluor 647 acceptor
Alexa Fluor 546 donor

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

FRET distribution two discrete states

Two populations (N and D) are present at equilibrium supporting a conventional barrier limited mechanism (although arguments continue)
Example II. PET FCS. Intrachain dynamics in linear and constrained peptides.

Pre-constrained peptides have less entropy loss on binding and bind tighter because they are less dynamic?

Bicyclic Kallikrein inhibitor

- 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
- Cleaved only ‘slowly’ by Kallikrein at Arg7
- But 2D proton NMR NOESY shows lack of long range NOE’s that is inconsistent with rigid structure

See http://bicycletherapeutics.com/
PET FCS Labeling strategy

Also linear labeled + trp and linear labeled + WT phe

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


Further examples.....


Backbone-driven collapse in unfolded protein chains. Teufel DP, Johnson CM, Lum JK, Neuweiler
Questions

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

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Further Reading

• Talks 1,3 and 4 Nick, Ben and Jon

Current setup

• Freely diffusing fluorescence based
• FRET dye-dye quenching interactions
• FCS diffusion and/or dye-side chain or solvent quenching

Developments?

• .....Two colour techniques?
• ..... Microfluidics?
• ..... Immobilised molecules?

Themed Issue: Single-molecule optical spectroscopy
Chemical Society Reviews, 21 February 2014, Issue 4, Page 963 to 1340