Single molecule experiment
Why single molecule experiment?

- To study the property or function of a single individual molecule
- Answer to fundamental biological questions, which would be impossible by conventional methods on an ensemble or bulk collection of molecules
- A sufficient number of observations from a single molecule is equivalent to a standard population-averaged snapshots: Statistical mechanics
  - A single molecule experiment contains all information of the molecular ensemble

- Exact distribution of molecular property (ex. color of the balls) by looking at one molecule at a time
- Keep track of the change in the property of individual molecules with time, measuring the entire time trajectories including multiple pathways and intermediates
Use and potential of single molecule experiment

- Heterogeneity and disorder in a sample
- Quantitative measurement of the kinetics (microseconds to seconds) or statistics of complex biological processes: variances in kinetic rates, conformational dynamics, and lifetimes of transient intermediates
- Work at the low numbers of molecules (typically < 1,000) with no enrichment
- Precise localization (with nanometer accuracy) and counting of molecules (~ $10^5$ molecules / $\mu$m$^2$) in spatially distributed samples such as a living cell

Expand the understanding and knowledge of biological processes:
- Structural and chemical biology, enzymology and systems biology
Toolkits for single molecule experiments

Forster resonance energy transfer (FRET)

Super-resolution microscopy

Optical tweezers

Magnetic tweezers

AFM
Optical tweezers

- Optical trap of a single nanoparticle by focusing a laser to a diffraction-limited spot with a high numerical aperture (NA) microscope objective.
- Most versatile single-molecule manipulation technique:
  - Spatial resolution: 0.1 ~ 2 nm
  - Temporal resolution: 10^{-4} sec
- Force range: 0.1 ~ 100 pN on particles ranging in size from nanometers to micrometers.
- Low noise and low-drift.
- Dielectric particle in the vicinity of the focus experiences three-dimensional restoring force directing toward the focus.
- For small displacements of the trapped object from its equilibrium position, the force is linearly proportional to the displacement, and the optical trap can be well approximated as a linear spring.
  - The spring constant, or stiffness, depends on the steepness of the original gradient (how tightly the laser is focused), laser power and the polarizability of the trapped object.
- Particles ranging in size from ~ 20 nm to several micrometers can be stably trapped.
  - Single nanoparticle, single cell, lipid vesicles, polystyrene or silica microspheres.
**Principle**

- Nanometer and micron-sized dielectric particles are trapped by extremely small forces via a highly focused laser beam.
- **Beam is typically focused by sending it through a microscope objective.**
  - The narrowest point of the focused beam, known as the beam waist, contains a very strong electric field gradient.
  - Dielectric particles are attracted along the gradient to the region of strongest electric field, which is the center of the beam.

- Refraction of rays at the particle surface.
- For a particle centered on the beam axis, momentum transfer is in the direction of propagation: Axial trapping
- A particle off the axis is pulled into the beam: lateral trapping
- Dielectric objects are attracted to the center of the beam, slightly above the beam waist.
- The force applied on the object depends linearly on its displacement from the trap center just as with a simple spring system

\[ F_{\text{trap}} = k_{\text{trap}} x \]
(a) Measurement of the force and displacement of optically trapped kinesin-coated beads moving along fixed microtubules

(b) Measurement of translocation and force generation of individual RNA polymerase molecules as they transcribe DNA
   - Stall force (~30pN), transcriptional pausing, mechanism of polymerase translocation

Mechanical unfolding of proteins and nucleic acid structures
Magnetic tweezer

- The most straightforward method to implement
- A magnetic particle in an external magnetic field experiences a force proportional to the gradient of the square of the magnetic field.
- The field generated by sharp electromagnetic tips or small permanent magnets: \( \sim 200 \text{ pN} \) on micrometer-sized magnetic particles
- Used to study mechanical properties of biological macromolecules like DNA or proteins in single-molecule experiments
If the bead leaves its equilibrium position on the x-axis by $dx$, it will be subjected to a restoring force $F_x$ that increases linearly with $dx$ in the first order approximation. Considering only absolute values of the involved vectors, it is geometrically clear that the proportionality constant is the force exerted by the magnets $F$ over the length $l$ of the molecule that keeps the bead anchored to the tethering surface: $F_x = dx/l \cdot F$
3-D position of magnetic bead by imaging

Principle of vertical position determination by comparing the bead diffraction image at one position with a library of images of the bead at different focal positions of the objective.
Applications: Study of DNA topology and topoisomerase

(c) To measure DNA topology with magnetic tweezers, extension is measured as a function of rotation for a 1 μm super-paramagnetic bead tethered to a surface by a 3-kb molecule of DNA under 0.4 pN of pulling force. As the DNA is over- or under-wound (supercoiled) there is a slight decrease in extension near zero turns, which is due to the accumulation of twist in the DNA molecule. At ± 4 turns the DNA buckles, forming a plectoneme loop. Each subsequent turn increases the plectoneme by another loop, leading to a linear decrease in extension from 4 to 12 turns. Removal of the plectonemes by the activity of a topoisomerase can be directly observed in real time by monitoring the extension of a supercoiled DNA molecule. Schematics are not drawn to scale.
Helicase moves directionally along a nucleic acid phosphodiester backbone, separating two annealed nucleic acid strands (i.e., DNA, RNA, or RNA-DNA hybrid) using energy derived from ATP hydrolysis.
Forster resonance energy transfer (FRET)

- A non-radiative energy transfer from an excited donor fluorophore to a nearby acceptor through long-range dipole-dipole interactions
- Energy transfer efficiency:
  \[ E = \frac{I_A}{I_D + I_A} \]
  
  \[ E = \frac{I_A}{I_D + I_A} \]

- Spectroscopic ruler: Inter-fluorophore distance at low nanometers (2 ~ 8 nm)

\[ E = \frac{1}{1 + (R/R_o)^6} \]

- Localization of biomolecules
- Assay of enzyme activity
- Study of biomolecular interactions

Oh et al. *JACS* (2005)
Protein conformational dynamics

- Proteins are in motion, sampling the ensemble of different conformations
- Energy landscape: Conformational space that a protein can explore
  - Relative population of different conformational states
  - Rates of inter-conversion

Key role in biological processes:
- Recognition of cognate ligands
- Signal transduction
- Allosteric regulation of proteins
- Enzyme catalysis

Relatively long lifetimes: μs to ms scale
- Individual states and kinetics of inter-conversion can be detected experimentally.

How do protein dynamics affect their functions?

**Molecular recognition**

Forty-six crystal structures of ubiquitin; conformational dynamics control ubiquitin-protein interactions and influence *in vivo* signaling.

**Signal transduction**

Signals such as ligand binding, mutation, PTSs alter energy landscape, affecting relative population of conformations.

**Enzyme catalysis and allosteric regulation**

- Active (green) and inactive (blue, red, yellow, and gray) conformations.
- Allosteric ligand binding (cyan star) stabilizes the inactive conformation (red), inhibiting the enzyme activity.

*Lee and Craik, Science (2009)*
Maltose binding protein (MBP)

- ATP-binding cassette (ABC) transporter in *E. coli*: active transport and chemotaxis
- Large conformational change upon ligand binding: Rotation > 30° at hinge region
- The highest binding affinity for maltotriose ($K_d = \sim 1.6 \times 10^{-7} \text{M}$)
- $M_r = 40 \text{kDa}$

Maltose uptake in *E. coli*

N-terminal domain

C-terminal domain
Conformational change in MBP

Crystal structure difference between ligand-free and ligand-bound forms: open and closed conformations

Existence of the minor partially closed species (~5%) in the absence of a ligand by NMR relaxation experiment

Ligand-free

Ligand-bound

Sharff et al. *Biochem.* (1992)

Scheme for smFRET measurements of MBP

Labeling of MBP with a FRET pair and a biotin

Prism-type Total Internal Reflection Fluorescence (TIRF) Microscope
TIRF microscope for single molecule FRET measurements

- TIRFM uses an evanescent wave to selectively illuminate and excite fluorophores in a restricted region of the specimen immediately adjacent to the glass-water interface.
- The evanescent wave is generated only when the incident light is totally internally reflected at the glass-water interface.
- The evanescent field occurs if incident light is totally reflected at the interface of two transparent media with different refractive indices.
- The evanescent electromagnetic field decays exponentially from the interface, and thus penetrates to a depth of only approximately 100 nm into the sample medium.
- TIRFM enables a selective visualization of surface regions such as the basal plasma membrane.
sm FRET measurements for analyzing Conformational dynamics

Real-time kinetic analysis of the conformational transition of a protein at the single-molecule level

Heterogeneous population

Active, real molecules

Ensemble FRET
(Bulk measurement)

Counts
FRET efficiency

Single-molecule FRET
(Histogram of FRET)

Counts
FRET efficiency

Dynamic behavior

FRET efficiency
Time (s)

smFRET enables real-time traces of fluorescence intensity and FRET efficiency from individual molecules
Intrinsic conformational dynamics of wt-MBP

- The intrinsic transition was too fast to detect owing to a low time resolution (~2 ms)
- Maltose-induced structural change in wt-MBP
MBP variants with hinge region mutations

Changes in domain closure angles and binding affinity of the MBP by hinge region mutations

Introduction of bulky amino acids into the hinge region

→ Slow down the intrinsic conformational dynamics
→ Allow the analysis of conformational dynamics using smFRET

Conformational dynamics of single and double mutants of MBP

Mutant construction

- MBP (Ligand-free) (0°)
- MBP-I329W (Ligand-free) (9.5°)
- A96W I329W
- MBP-A96W/I329W (28.4°)

Real-time traces of MBP mutants

- Wild type
- Single mutant (SM) (I329W)
- Double mutant (DM) (A96W/I329W)

- Detection of intrinsic dynamics of the mutants in the absence of a ligand
- Transitions between two distinct conformational substates

High FRET: Partially closed state, Low FRET: Open form
Molecular recognition

- Role of conformational dynamics in protein function:
  - Recognition of cognate ligands
  - Signal transduction
  - Allosteric regulation of proteins
  - Enzyme catalysis

- Function of proteins depends on the recognition and binding of specific ligands
  \(\rightarrow\) Central to all biological processes

- **Two textbook models**: The protein exists in a single, stable conformation.
  - Lock-and-key model by Fischer (1894)
  - Induced-fit model by Koshland (1963)

Enzyme and the substrate possess specific complementary shapes that fit exactly into one another.

Initial interaction between enzyme and substrate is relatively weak, but these weak interactions rapidly induce conformational changes in the enzyme that strengthen binding.
Conformational dynamics and ligand binding

- **Existence of conformational substates**: Interplay between protein dynamics and molecular recognition
- **Conformational selection model**: Dynamics-coupled recognition mechanism

**Induced-fit model**
Conformational change to a ligand-bound form occurs only after interaction with a ligand

**Conformational selection model**
Ligand-binding conformation is pre-existing before the addition of a ligand

The kinetic and thermodynamic rate constants can determine most probable recognition mechanism

**Induced-fit model**
The closing rate will be faster in the presence of a ligand than in its absence and will be accelerated by increasing ligand concentration: Shorter $T_{\text{closing}}$

**Conformational selection model**
The closing rates in the presence of a ligand will be the same as those with no ligand because the population of a ligand-binding conformer is limited by the intrinsic conformational transition rate.
Kinetic rates by dwell time analysis

Real-time changes in FRET efficiency

\[ E_{\text{FRET}} = \frac{I_A}{I_A + I_D} \]

\( I_D \): Donor intensity
\( I_A \): Acceptor intensity

The opening and closing rates

Closing rate = \( \frac{1}{\text{Open dwell time} (\tau)} \)
Opening rate = \( \frac{1}{\text{Closed dwell time} (\tau)} \)
Kinetic rates of the mutants

Representative time traces of the intensity and FRET efficiency for the single mutant

- The closing rate increased linearly with an increase in the ligand (maltose, maltotriose) concentration.
- The opening rate remained almost constant over the range of tested ligand concentrations.

- Ligands bind preferentially the open state, inducing a structural change to a closed form.
- Direct evidence for the induced-fit mechanism.

Kinetic rates in the absence and presence of a ligand

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Closing rate</th>
<th>Opening rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Single mutant

Double mutant

Wild-type
Three-color smFRET for analysis of ligand binding preference

- Direct quantification of the ligand binding preference to a conformer using a Cy7-ligand
- Alternative excitation of Cy3 and Cy5 using a mechanical shutter

Monitoring of the conformational transition between the open and partially closed states through Cy3-Cy5 FRET or Cy5 intensity

Tracking the ligand binding through Cy7 intensity
Synthesis of Cy7-maltose

Synthesis of PEG-amine-linked maltose followed by conjugation with Cy7-mono NHS ester

Mass spectrometry analyses

PEG-amine maltose

Cy7-maltose

Cy7-mono NHS ester
Three-color smFERT for the double mutant with Cy7-maltose

Simultaneous tracking of the conformational dynamics and binding of Cy7-maltose

- Binding of Cy7-maltose:
  Jumps of Cy7 signal and concurrent drops of Cy5 signal
- Dissociation of Cy7 maltose: Decrease of Cy7 signal

Relative distribution of the FRET efficiencies (Cy3-Cy5) before the binding and after the dissociation of Cy7-maltose:
479 binding and dissociation events of 52 molecules

- Cy7-maltose binds to both the open and partially closed states of the mutant
- More than 80% of binding and dissociation events occurred in the open state
smFRET analysis: Extended induced-fit model

- Protein undergoes conformational transition between a highly populated open state and a minor populated partially closed state.
- Ligand binds to both a highly populated and weakly populated substates, inducing a structural transition to a ligand-bound, closed form.
Interplay between conformational dynamics and binding affinity

Binding affinity (Dissociation constant ($K_d$)) for a ligand: crucial role in biological processes

A correlation between the intrinsic opening rate of MBP variants and their binding affinity for a ligand

Hinge mutants of MBP: perfect model to investigate the role of conformational dynamics in the ligand binding affinity by smFRET measurements
MBP variants with mutations at hinge region

<table>
<thead>
<tr>
<th>MBP variants</th>
<th>Mutation</th>
<th>$K_d$ (nM) by ITC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wile-type</td>
<td>A96 I329</td>
<td>1166</td>
</tr>
<tr>
<td>96A/329W</td>
<td>A96 I329W</td>
<td>17±1</td>
</tr>
<tr>
<td>A/Y</td>
<td>A96 I329Y</td>
<td>22±6</td>
</tr>
<tr>
<td>A/F</td>
<td>A96 I329F</td>
<td>35±8</td>
</tr>
<tr>
<td>A/R</td>
<td>A96 I329R</td>
<td>905±50</td>
</tr>
<tr>
<td>A/K</td>
<td>A96 I329K</td>
<td>987±25</td>
</tr>
<tr>
<td>W/I</td>
<td>A96W I329</td>
<td>81±18</td>
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<tr>
<td>Y/I</td>
<td>A96Y I329</td>
<td>218±23</td>
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<tr>
<td>F/I</td>
<td>A96F I329</td>
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<tr>
<td>W/A</td>
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<td>515±67</td>
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<tr>
<td>Y/A</td>
<td>A96Y I329A</td>
<td>741±22</td>
</tr>
<tr>
<td>F/A</td>
<td>A96F I329A</td>
<td>989±37</td>
</tr>
</tbody>
</table>

Kinetic analysis for MBP variants with different binding affinities for maltose
Kinetic analysis for MBP mutants using smFRET measurements

Representative time-trace of intensity and FRET in the absence of a ligand

Kinetic rates by dwell time analysis

Linear relation between the opening rate and binding affinity (dissociation constant)

Intrinsic dynamics (opening rate) dominantly dictates the binding affinity for a ligand
Single molecule FRET

• Simple and easy to implement in biological systems

• Enables the understanding and demonstrating the interplay between protein conformational dynamics and its function

• Can be used for designing drugs with greater potency and proteins with desired function