Single-Molecule Methods
I - *in vitro*

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Macromolecules
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F$_1$-ATPase: a case study

[Diagram showing the structure of F$_1$-ATPase with annotations for F$_0$, Axle, Membrane, ADP, ATP, H$^+$, β$_{DP}$, γ, α$_{TP}$]
Rotation of the axle when hydrolyzing ATP

Kinosita group, 1997-2005
Single Molecule Methods

Noji group, 2005
Why look at molecules, one at a time?
An ensemble picture
Single Object Measurement: Resolving Heterogeneities

Most people are eating... But some are cooking
Single Object Measurement: Resolving Heterogeneities

Conformation states of protein folding

Single Object Measurement: Avoiding Synchronization

Single object movies

- Myosin walk on actin filament
- $F_1$ – ATPase rotation
Single Object Measurement: Avoiding Synchronization
Single Object Measurement: Detecting Rare Species
Single Object Measurement: Detecting Rare Species

RNA polymerase at work

Backtracking (0.1% probability) to correct for base pair mismatch

Abbondanzieri, Greenleaf, Shaevitz, Landick and Block., Nature 2005 (438), p 460
Brief history of single molecule methods

• 1989 - W.E. Moerner - Absorption
• 1990 - M. Oritt - Fluorescence
• 1993 - Room temperature (NSOM)
• 1994 - Confocal
• 1995 - TIRF
• 1998 - Optical trap
• 1996-2001 Single pair FRET
• 2003 - Single molecule localization
• 2000-2006 - Super-resolution microscopy
Single-Molecule Experiments

Fluorescence

Force
Pulling a Molecule

• Atomic force microscope
• Optical trap
• Magnetic tweezers
• Buffer flow
Protein Unfolding by AFM
Optical trap
Magnetic Tweezers
Flow-stretching

Labeled DNA (free)

Labeled DNA (flow)

DNA+nucleosome (flow)
Single-molecule Fluorescence
How to detect single molecule fluorescence?

• Great fluorophores
• Low background
• Sensitive detection
• Single molecule concentration
“Good” Organic Fluorophores
Reducing background: confocal
Reducing background: TIRF
Sensitive detection

High NA Objective
$5,000 ~ $15,000

EMCCD
$20,000 ~ $40,000

APD
$3,000 ~ $10,000
Single molecule concentration

The number of molecules in the detection volume follows Poisson distribution.

Ensuring single molecule detection

No molecule most of the time!

\[ P(k; N_{\text{avg}}) = N_{\text{avg}}^k e^{-N_{\text{avg}}} / k! \]

\[ N_{\text{avg}} = 1 \]
\[ N_{\text{avg}} = 0.5 \]
\[ N_{\text{avg}} = 0.1 \]
Single molecule concentration

Confocal detection volume ≈ 1 µm³ = 1 fL

\[ N_{avg} = 1 \implies 1/(6 \times 10^{23}) \text{ mol} / 1 \times 10^{-15} \text{ L} \approx 2 \text{ nM} \]

\[ N_{avg} = 0.1 \implies 200 \text{ pM} \]

Single molecule concentration ≤ 100 pM

TIRF detection volume ≈ 0.1 fL

\[ N_{avg} = 1 \implies 20 \text{ nM} \]
“Few molecule” spectroscopy

Fluorescence correlation spectroscopy (FCS)

Autocorrelation

Diffusion time

Counts

Time / us
The simplest single molecule experiment: Fluorophore counting

Ulbrich & Isacoff, Nat Methods, 2007
Single-pair FRET
Fluorescence Resonance Energy Transfer (FRET)

FRET efficiency:
Quantum efficiency of energy transfer

\[ E = \frac{k_{\text{FRET}}}{k_{\text{FRET}} + k_{\text{fl}} + k_{\text{nr}}} \]

Often approximated by “proximity ratio”

\[ E \approx \frac{I_{\text{acceptor}}}{I_{\text{donor}} + I_{\text{acceptor}}} \]
FRET efficiency

\[ E = \frac{1}{1 + \left( \frac{R}{R_0} \right)^6} \]
The Förster radius can be calculated from measurable parameters:

\[ R_0^6 = 8.8 \times 10^{23} \kappa^2 n^{-4} Q_0 J \]

Orientation factor \((0 \leq \kappa \leq 4)\) usually 2/3 (caution!)

Refractive index

Donor quantum efficiency

Overlap integral \(J = \int f_D(\lambda) \epsilon_A(\lambda) \lambda^4 \, d\lambda\)

Donor emission \(f_D(\lambda)\) \(\epsilon_A(\lambda)\) Accepter absorption \(\lambda^4\)

For common FRET pairs, \(R_0 \approx 4\text{-}6 \text{ nm}\)
### R₀ values for some Alexa Fluor dyes—Table 1.6

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor Alexa Fluor 488</th>
<th>Acceptor Alexa Fluor 546</th>
<th>Acceptor Alexa Fluor 555</th>
<th>Acceptor Alexa Fluor 568</th>
<th>Acceptor Alexa Fluor 594</th>
<th>Acceptor Alexa Fluor 647</th>
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<tr>
<td>Alexa Fluor 350</td>
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<td>Alexa Fluor 488</td>
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<tr>
<td>Alexa Fluor 647</td>
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<td></td>
<td></td>
<td>NA</td>
</tr>
</tbody>
</table>
Diffusion spFRET

FRET efficiency
Immobilized spFRET

Typical fluorescence FRET images

(a) DNA/DNA 10bp
Cy3 channel
Cy5 channel

(b) DNA/DNA 12bp
Cy3 channel
Cy5 channel

(c) DNA/DNA 15bp
Cy3 channel
Cy5 channel

(d) DNA/DNA 17bp
Cy3 channel
Cy5 channel

(e) DNA/DNA 10bp
Intensity (A. U.)

Time (min)

(f) DNA/DNA 10bp

(g) DNA/DNA 10bp

(h) DNA/DNA 17bp

(h) DNA/DNA 17bp
Spot intensity

Time (s)
What can we measure?

- FRET histogram
- Dwell time distribution
- FRET transition matrix
Typical spFRET setup
Total internal reflection fluorescence microscopy

Total internal reflection:

\[ \theta_1 = 90^\circ \Rightarrow \sin \theta_2 = \sin \theta_c = \frac{n_1}{n_2} \]

\[ n_1 = 1.33 \text{ (water)}, \quad n_2 = 1.52 \text{ (glass)}, \quad \theta_c = 61^\circ \]
The energy of the evanescent wave is localized near the interface.

The strength of the evanescent wave field decreases exponentially.

The penetration depth is a function of the wavelength and the incident angle.

Typical penetration depth: 50-100 nm.
TIRM improves S/N for surface imaging
Prism-type TIRF configuration

Plan Apo
60x Water
NA 1.2

Quartz slide
Spacer
Buffer
Coverglass

CCD
Through-the-objective TIRF
Requirement for TIRF objective

\[ \text{NA} = n_{\text{glass}} \sin \theta_{\text{max}} \]

\[ n_{\text{water}} = n_{\text{glass}} \sin \theta_c \]

\[ \theta_{\text{max}} \geq \theta_c \]

\[ \downarrow \]

\[ \text{NA} \geq n_{\text{water}} \approx 1.33 \]
TIRF compatible objectives

• 1.40 NA
  – “Barely enough” for laser TIRF

• 1.45 / 1.49 NA “TIRF” objective
  – More homogenous illumination field
  – Higher efficiency for lamp TIRF
  – Compromised image quality

• 1.65 NA
  – Sapphire coverglass
  – Toxic oil...
### Prism vs. Objective type TIRF

<table>
<thead>
<tr>
<th>Prism</th>
<th>Objective</th>
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<tbody>
<tr>
<td>More complicated illumination</td>
<td>Simple setup</td>
</tr>
<tr>
<td>Water immersion objective</td>
<td>Oil immersion objective</td>
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<tr>
<td>Quartz slides</td>
<td>Ordinary coverglass</td>
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<tr>
<td>High S/N</td>
<td>Higher signal but even higher background</td>
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</table>
Surface Immobilization

- Streptavidin
- Neutravidin

Biotinylated lipid

Quartz slide
The classic flow channel
The Full Jabłonski Diagram

Absorption

S$_0$ $\rightarrow$ S$_1$

Emission

hv

Emission

Intersystem crossing

Triplet State

T$_1$

Non-radioactive decay

Triplet Quenching

Phosphorescence

Lifetime $\approx \mu$s - ms
Photobleaching

Absorption

$h\nu$

Vibration relaxation

Intersystem crossing

Triplet State

Most common cause: $O_2$
Fight against photobleaching
The oxygen scavenging system

Glucose + O₂ \rightarrow \text{Gluconic acid} + \text{H}_2\text{O}

Glucose oxidase

\text{Gluconic acid} + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O}

Catalase

\beta\text{-mercaptoethanol} \quad \text{HO-CH}_2\text{-CH}_2\text{-SH}

\beta\text{-mercaptoethylamine} \quad \text{H}_3\text{N-CH}_2\text{-CH}_2\text{-SH}

Trolox
Alternated Excitation (ALEX)
Analysis

Raw data
- Noisy
- Crowded-field

Two Tone
- Detection, localization, particle matching
- Profile fitting
- Filtering
  - Automatic
  - Robust
  - Near-optimal signal to noise
  - Works for CW & ALEX

Results
Coming up:

Single-molecule methods in cells