Single-Molecule Methods II - in cells

Bo Huang
Macromolecules
2015.03.04
How do deal with moving molecules?
Walking models for kinesin

Hand-over-hand

16 nm

Inchworm

8 nm
Single-molecule localization / tracking

\[ \delta = \frac{\lambda}{2NA} \]

\[ \Delta = \frac{\delta}{\sqrt{N}} \]
Challenge in living cells

- Labeling
- Imaging
- Tracking
- Data Analysis
Labeling live cell targets

• Fluorescent protein

J. Yu, Science 2006
Labeling live cell targets

- Fluorescent protein
  - Low signal level

Mashanov & Molloy, Biophys J. 2007
Labeling live cell targets

• Quantum dot

Chung et al., Nature 2010
Labeling live cell targets

- Organic dyes

β2 adrenergic receptor  Alexa 647 - benzylguanine
Labeling live cell targets

• Organic dyes
  – Enzymatic tags
  – Ligands
  – Small molecule probes
  – Unnatural amino acids
Imaging

• TIRF
  – Plasma membrane proteins
**Imaging**

**Total Internal Reflection Fluorescence Microscopy.** A commercial TIRF microscope (Leica AM TIRF) equipped with an EM-CCD camera (Cascade 512B; Roper Scientific), a 100x oil-immersion objective (HCX PL APO 100x/1.46), and a 635-nm diode laser was used. To avoid photobleaching before image acquisition, cells were searched and focused in bright field and a fine focus adjustment in TIRF mode was performed using only 2% laser power, an intensity insufficient for detecting single molecules. This procedure resulted in negligible photobleaching. Afterward, laser power was set to 83% and image sequences (400–600 frames) were acquired with an exposure time of 50 ms, resulting in the acquisition of an image every 96 ms. The penetration depth of the evanescent field was ~110 nm. Illumination intensity was homogeneous over the imaged area (maximum difference = 10%). Under these conditions, the photobleaching half-life was 6.82 ± 0.12 s. The microscope was equipped with an incubator and a temperature control unit. Experiments were performed at 20.5 ± 0.3 °C. Cells were imaged in a buffer containing 137 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM Hepes, pH 7.3. Only cells with less than 0.45 receptor particle/µm² were analyzed.

**Coverslip Cleaning.** Twenty-four-millimeter glass coverslips were extensively cleaned to remove any background fluorescence. First, they were sonicated in a solution containing 5 M NaOH for 1 h. After three washes with distilled water, they were dried and further sonicated in chloroform for 1 h. Coverslips were then dried and stored in 100% ethanol until use.
Imaging

• High incident angle
  – No official names so far (quasi-TIRF, dirty-TIRF, oblique illumination, HILO, and many more...)
Imaging buffer

• The classical oxygen scavenging system?
  – 1-10 % glucose
  – 1 mg/mL glucose oxidase
  – 0.04 mg/mL catalase
  – 1 % β-mercaptopethanol (~ 140 mM)
Excitation intensity

beams out of the microscope as they were reflected back. The average laser intensities at the specimen plane were $\sim 10 \, \mu W \cdot \mu m^{-2}$ for 488-nm illumination and $\sim 40 \, \mu W \cdot \mu m^{-2}$ for 556-nm illumination.

<table>
<thead>
<tr>
<th>Light intensity (HBO 103 W/2 short-arc lamp), %</th>
<th>Observation conditions</th>
<th>Light energy per observation period ($J , cm^{-2}$)</th>
<th>% cells arrested in mitosis ($n = sample , size$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GFP-MBD cells (460–500 nm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0.5 sec$^a$; 30 sec$^b$</td>
<td>1.434$^c$</td>
<td>100 (10)</td>
</tr>
<tr>
<td>75</td>
<td>0.5 sec; 30 sec</td>
<td>1.194</td>
<td>67 (12)$^d$</td>
</tr>
<tr>
<td>50</td>
<td>0.5 sec; 30 sec</td>
<td>0.790</td>
<td>0 (10)</td>
</tr>
<tr>
<td>10–25</td>
<td>0.5 sec; 1 min</td>
<td>0.081–0.198</td>
<td>0 (&gt;30)</td>
</tr>
<tr>
<td><strong>MBD-DsRed (515–560 nm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>0.5 sec; 30 sec</td>
<td>4.0</td>
<td>31 (13)</td>
</tr>
<tr>
<td>35</td>
<td>0.5 sec; 30 sec</td>
<td>1.866</td>
<td>0 (10)</td>
</tr>
</tbody>
</table>

$^a$Camera exposure time.

$^b$Time-lapse interval.

$^c$The light energy exposure over 1 h of time-lapse microscopy was calculated to allow comparison between the different observation conditions. See Experimental procedures for details about calculation.
Tracking
Track moving particles

- Nearest neighbor matching
- Collision
- Blinking

**Computational Analyses.** Particles were detected and tracked using recently described algorithms (1) implemented in Matlab (The MathWorks). Briefly, the subpixel location and intensity above background of diffraction-limited particles are calculated by fitting a 2D Gaussian with the SD of the spread point function of the microscope around local intensity maxima. Tracking is performed on the basis of an approximation of the multiple-hypothesis tracking approach. Specifically, for each step and each particle a cost is assigned to every potential event (e.g., particle blinking, merging, splitting, appearing, or disappearing) and the solution that minimizes the sum of the costs is selected. This allows us also to track a particle beyond a blinking event, which is not possible with other tracking algorithms (details in ref. 1). The blinking frequency measured in our tracks by dividing the number of blinking events by the total duration of the tracks was low—approximately one event every 1,000 frames per fluorophore—and the duration of a blinking event was relatively short, typically 5–10 frames. The outcome of the tracking anal-
Fast moving molecules

Cytoplastic proteins

- DIC image
- 1000ms exposure
- 10ms exposure

Small molecules

10 ms exposure
What analyses have been done?
Two color tracking
Beyond single-molecule concentration
Fluorescent Speckle Imaging:

Microtubules in the Mitotic Spindle of a Living Epithelial Cell

C.M. Waterman-Storer
A. Desai
J. C. Bulinski
E.D. Salmon
Super-resolution by...

Fluorescence image

Underlying structure
Super-resolution by spatial modulation

Fluorescence image

Underlying structure

Differential modulation of the fluorescence response
Super-resolution by differential depletion

Fluorescence image  ÷  Underlying structure  =  Depletion pattern

STED (Hell 1994, Hell 1999)
GSD (Hell 1995, Hell 2007)
RESOLFT (Hell 2003, Hell 2011)

Diffraction limited PSF  Saturated depletion  Smaller effective PSF
Super-resolution by differential excitation

Fluorescence image

Underlying structure

Excitation pattern

Diffraction limited excitation and emission

Doubled resolution

SIM (Gustafsson / Heintzmann)

SSIM (Gustafsson 2005)
Super-resolution by single-molecule switching

Fluorescence image  \rightleftharpoons \text{Underlying structure}

Photoswitchable molecules

Single molecule image \rightleftharpoons N \text{ photons} \rightarrow \text{Single-molecule localization}

\[ D \approx d / \sqrt{N} \]
Super-resolution by single-molecule switching

STORM = Stochastic Optical Reconstruction Microscopy (Zhuang 2006)
PALM = Photoactivated Localization Microscopy (Betzig & Hess 2006)
FPALM = Fluorescence Photoactivation Localization Microscopy (Hess 2006)
PALMIRA (Hell 2007), GSDIM (Hell 2008), dSTORM (Sauer 2008), SMACM (Moerner 2008)
PAINT (Hochstrasser 2006), SPRAYPAINT (Moerner 2011), SOFI (Weiss 2009)
Reconstructed from 40,000 frames, 3,350,370 localization points

B-SC-1 cell,
Microtubules stained with anti-β tubulin
Cy3 / Alexa 647 secondary antibody

Bates et al., Science, 2007
Imaging subcellular structures

Microtubule

Clathrin + FBP17

Mitochondria + Microtubule

Synapse (brain section)

Huang et al., 2007; Huang et al., 2008; Wu et al., 2010; Dani et al., 2010
Paper discussion for Friday

• Kinetochore
• Focal adhesion
• Nuclear pore complex
Paper discussion for Friday

• How were the structures analyzed?
• How do they compare to other structural analysis methods?
• What are the potentials and other applications?