SINGLE MOLECULE TECHNIQUES

Dr. B. Christoffer Lagerholm
Facility Manager
Wolfson Imaging Centre - Oxford
Weatherall Institute of Molecular Medicine,
University of Oxford,
John Radcliffe Hospital

christoffer.lagerholm@imm.ox.ac.uk
Outline

• Single Molecule Techniques
  • FCS (Dynamics)
  • Single Particle Tracking (Dynamics)
  • PALM/STORM (Localization)

• Equipment requirements

• Single Molecule Probes

• Single Particle Tracking
Fluorescence Correlation Spectroscopy (FCS)

Fluctuations in fluorophore concentrations due to diffusion in and out of the local volume provide information on the mobility of the labeled probe from which a diffusion coefficient can be obtained.

Concentration range:

? ← nM → ~1μM

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

$$= \frac{C}{V_{eff}} \frac{1}{1 + \frac{4D\tau}{\omega_z^2}} \frac{1}{\sqrt{1 + \frac{4D\tau}{\omega_z^2}}}$$

http://www.drbio.cornell.edu/Infrastructure/Apparatus_WWW/fluorecorrspec.html
Single Particle Tracking
Required Equipment

**Light/Fluorescence Microscope**
- Light source (Hg arc lamp/Laser)
  - Epi-fluorescence
  - TIRF
- Condenser (for brightfield imaging)
  - Gold particles
- Microscope objective
  - High NA
- Sensitive camera
  - EMCCD
Technical Considerations for Single Molecule Imaging

- Diffraction
- Microscope resolution
- Signal detection
- Data analysis
Because of diffraction, the image of point source as focused by a microscope is an **Airy pattern**

The center of the Airy pattern is known as the **Airy disk**

\[ r_{\text{Airy}} = \frac{0.61 \lambda}{\text{NA}_{\text{objective}}} \]

- \( \lambda \) = wavelength of light
- \( \text{NA} \) = numerical aperture = \( n \sin \alpha \)
- \( n \) = index of refraction of immersion media
- \( \alpha \) = half-angle of angular aperture

The Airy disk represents 84 percent of the total luminous energy
• NA ranges from <0.95 for air, <1.29 for water, <1.45 for oil

• For 100X magnification, 1.4 NA oil immersion objective and illumination with green light (500 nm)

\[
r_{\text{Airy}} = \frac{(0.61)(500 \text{ nm})}{1.4} = 220 \text{ nm}
\]
Microscope Resolution

• The diffraction of light causing the Airy disk is also the limiting factor of the resolution of a microscope.

• The resolution of a microscope is defined as the minimum distance two objects have to be separated by to be resolved as two separate objects.

Airy patterns of two point sources

• For 100X magnification, 1.2 NA oil immersion objective and illumination with green light (500 nm)

Resolution (Rayleigh limit) = \( \frac{(0.61)(500 \text{ nm})}{1.4} \) = 220 nm
Signal Detection

• Single Molecule Imaging typically relies on EMCCDs which utilizes arrays of square pixels for detection.

• For optimum resolution data should be sampled at \( \frac{1}{2} \times \) the resolution. This is known as **Nyquist Sampling**

\[
\text{Nyquist Sampling} = \frac{(0.61) \times (500 \text{ nm})}{2 \times 1.4} = 110 \text{ nm}
\]

• Hence optimal sampling for 100X magnification, 1.4 NA oil immersion objective and illumination with green light (500 nm) would require a detector with 100 X 110 nm = 11 \( \mu \text{m} \) pixels.
Data Analysis

• Obtain sub-pixel resolution by curve fitting to determine centroids of single molecules

• Approximate Airy pattern with a 2D spatial Gaussian and fit each image

$$A + \frac{B}{2\pi w^2} \exp\left[-\frac{1}{2\pi w^2}((x - x_o)^2 + (y - y_o)^2)\right]$$

where $\omega = \text{width of the PSF of the microscope (\sim 220 \text{ nm})}$
Single Particle Tracking

- Time lapse imaging of spatially resolved single molecules, particles or subcellular structures

- Technique results in time trajectories of sub-pixel positions of single objects

- Such trajectories contain information about:
  - diffusion coefficients
  - velocities
  - step sizes
  - spatial and temporal confinement
SPT Approach

Gold Ø 40nm

Qdot Ø 20nm

Fluorescent dye Ø 1nm

membrane ṫ 5nm
Diffusion in membranes and solution

**Saffman-Delbruck:**

\[
D_M = \frac{k_B T}{4\pi \mu_M h} \left( \ln \left( \frac{\mu_M h}{\mu_S R} \right) - \gamma \right)
\]

\(\mu_S = 1.002\) cP

\(h = 4\) nm

\(T = 293\) K

\(\mu_M/\mu_S = 100\) (solid line)

\(\mu_M/\mu_S = 80\) (short dashed line)

\(\mu_M/\mu_S = 60\) (long dashed line).

**Stokes-Einstein:**

\[
D_S = \frac{k_B T}{6\pi \mu_S R_H}
\]

\(\mu_S = 1.002\) cP

\(T = 293\) K.
Dynamics are analyzed with respect to that predicted by Brownian (random) motion in a 2D (or 3D) fluid.

For 2D, the mean square displacement (MSD) is

\[
<r^2> = \sum_{n=1}^{q} \sqrt{(x_{t_1} - x_{t_2})^2 + (y_{t_1} - y_{t_2})^2} = 4D \Delta t \quad \Delta t = t_2 - t_1
\]

where D is the diffusion coefficient.

Brownian motion \(< r^2 > = 4D t\)

Anomalous diffusion \(< r^2 > = 4D t^\alpha \quad \alpha < 1\)

Diffusion with flow \(< r^2 > = 4D t + (Vt)^2\)

Confined diffusion \(< r^2 > \approx < r_C^2 > [1 - A_1 \exp(-4A_2Dt / < r_C^2 >)]\)

Quantum Dot single molecule imaging

189 Hz (5.3 ms integration) with 160X magnification and Andor EMCCD

anti-CD73 Fab’-biotin + sAv-605 Qdot

Playback 100 Hz, 2000 frames
Scale bar = 1mm
Diffusion Coefficient

Brownian Diffusion
\[ D \sim 0.25 \text{ } \mu m^2/sec \]
A few comments on diffusion and SPT

- Diffusion is stochastic where the displacements, \( r \), during a time lag, \( t_{\text{lag}} \), in the case of free diffusion in an infinite 2D plane is given by a Raleigh distribution

\[
P (r, t_{\text{lag}}) = \frac{2r}{4Dt} e^{-\frac{r^2}{4D t_{\text{lag}}}}
\]

- Localization errors (precision) for SPT is typically 10-30 nm

\[
\frac{\partial}{\partial r} [P (r, t_{\text{lag}})] = 0 \text{ for } r_{\text{peak}} = \sqrt{2Dt_{\text{lag}}}
\]

![Graph showing probability distribution of displacements over time lag for different diffusion coefficients and time lags.](image)

- D=10 \( \mu \text{m/s} \) (blue) \hspace{1cm} D=5 \( \mu \text{m/s} \) (green) \hspace{1cm} D=1 \( \mu \text{m/s} \) (red)

  - \( t_{\text{lag}}=25 \mu \text{s} \) (solid) \hspace{1cm} \( t_{\text{lag}}=100 \mu \text{s} \) (dashed) \hspace{1cm} \( t_{\text{lag}}=1 \text{ ms} \) (dotted)

- Localization errors (precision) for SPT is typically 10-30 nm
Simulating diffusion

![Simulated particle trajectories and MSD plots](image)

Monte Carlo simulation of 2D Brownian motion. Simulated data: 1000 particle trajectories of 10 displacements (black), 200 particle trajectories of 50 displacements (grey).

a) All simulated particle trajectories.
b) MSD plot for each particle trajectory and best fit to the mean MSD of all displacements (dashed lines).
c) Histogram of single trajectory $D_5$. 
Single molecule probes
Probe Considerations

- Very bright and stable
- Small
- Monovalent (i.e. one probe per molecule of interest)
- Low non-specific binding
## Typical Single Molecule Imaging Probes

<table>
<thead>
<tr>
<th></th>
<th>Gold</th>
<th>Cy3</th>
<th>Quantum dots</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Means of detection</strong></td>
<td>Scattering</td>
<td>Fluorescence</td>
<td>Fluorescence</td>
</tr>
<tr>
<td><strong>Size (diameter)</strong></td>
<td>40 nm</td>
<td>~1-2 nm</td>
<td>~10-20 nm</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>Photostable</td>
<td>Small</td>
<td>Photobleaching resistant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monovalent</td>
<td></td>
</tr>
<tr>
<td><strong>Disadvantages</strong></td>
<td>Immobilization</td>
<td>Photobleaching (~5 s)</td>
<td>Non-specific binding Blinking</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Multivalent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Gold

• Detectable by brightfield microscopy

• Rayleigh scatterer, intensity \( \sim d^6 \) where \( d = \text{diameter of particle} \)

• Particles > 30 nm diameter are detectable

• Conjugation of protein to gold particles depends upon
  (a) ionic attraction between negatively charged gold and positively charged protein
  (b) hydrophobic attraction between the antibody and the gold surface;
  (c) dative binding between the gold conducting electrons and sulphur atoms which may occur within amino acids of the protein.
Fluorescent dyes

Cy3

Properties
Reactive towards primary amines (lysines)
Excitation maximum (nm) 548
Emission maximum (nm) 562
Extinction coefficient (M-1 cm-1) 150 000 *
Quantum yield 0.04 *
Fluorescence lifetime (ns) <0.3

(source www4.amershambiosciences.com)
Quantum dot properties

- Unique optical properties
  - strongly fluorescent (high absorptivity and quantum yields)
  - tunable, size dependent emission wavelengths
  - narrow emission spectra (FWHM in the 20-35 nm range)
  - single wavelength excitation of multiple color quantum dots
- photostable
- electron dense
Qdots are fluorescent nanocrystals composed of semi-conductor cores, i.e. CdSe. The size of the Qdot cores ≤ Excitation Bohr Radius, resulting in discrete, size-tunable band gap energies.

The diagrams and graphs illustrate the fluorescent properties of Qdots with varying wavelengths and their corresponding fluorescence intensities.
Fluorescence spectra

Fluorescein Ab conjugate, pH 8

Qdot655 sAv conjugate, pH 7.2
Brightness

$\sim \varepsilon \phi$

where $\varepsilon$ = extinction coefficient (measure of how strongly the dye absorbs light)

$\phi$ = quantum yield (ratio of photons emitted as fluorescence relative to absorbed photons)

**Extinction coefficients.** Streptavidin conjugates of black - Alexa488, blue - Cy3, red - Alexa568 and Qdot 605 (green - Qdot 605).

**Emission intensity.** Streptavidin conjugates of black - Alexa488, blue - Cy3, red - Alexa568 and Qdot 605 (green - Qdot 605). (532 nm excitation)

Images from Quantum Dot Corporation (www.qdots.com)
Stability/Photoresistance

Image from Quantum Dot Corporation (www.qdots.com)
Commercially available types Qdots (Invitrogen) and Sizes
Brightness and Intermittency Comparison
5 ms integration, 100 W Hg lamp (470/40 nm)

Qdot are very bright ... but blink
Study 1:

Parallel multi-color single molecule imaging of 3 lipid raft markers in the same sub-regions of live MEFs
Multicolor Single Molecule Imaging with Qdots

Our set-up

QuadView

Acquisition speed limits: 4 colors ~30 Hz (50x50µm)

2 colors ~ 500 Hz (50x2 µm)
IA32 with 1μg/ml biotin-cap-DPPE (BSA loaded) and combination of sAv-QD565, sAv-QD605, sAv-QD655, and sAv-QD705 and 50μM βMe
150X 1.45NA, 10 ms integration, 24.86 Hz
Parallel Targeting Schematic with Qdots
Hydrodynamic size of Qdot conjugates

![Diagram showing hydrodynamic size of Qdot conjugates]
Mouse NIH3T3 co-transfected with BirA-KDEL, EGFR-BLAP, ACP-GPI, and YFP-K-Ras2

Cells were grown ON in 1 mM biotin and labeled with 1nM sAv-QD605, 1nM CoA-QD655, and 200pM ChTox-QD705

10 ms integration, ~25Hz acquisition rate
Differential effects of cholesterol depletion

Free diffusion model, $1 \leq n_{\text{lag}} \leq 5$, corresponding to $40 \leq t_{\text{lag}} \leq 200$ ms
Controls

No Blocking

(EGFR<sup>AP</sup>, SAV-QD605)

<n>351</n>

<avg><i>D</i>(p) = 0.015 μm<sup>2</sup>/s</avg>

Blocking

(EGFR<sup>AP</sup>, SAV-QD605)

<n>1779</n>

<avg><i>D</i>(p) = 0.035 μm<sup>2</sup>/s</avg>

CD56<sup>AP</sup>

(Do-QD655)

<n>511</n>

<avg><i>D</i>(p) = 0.074 μm<sup>2</sup>/s</avg>

CD56<sup>AP</sup>

(Do-QD605)

<n>1397</n>

<avg><i>D</i>(p) = 0.072 μm<sup>2</sup>/s</avg>

No β-ME

50 μM β-ME

500 μM β-ME

(EGFR<sup>AP</sup>, SAV-QD605) (EGFR<sup>AP</sup>, SAV-QD605) (EGFR<sup>AP</sup>, SAV-QD605)

<n>173</n>

<avg><i>D</i>(p) = 0.036 μm<sup>2</sup>/s</avg>

<n>1779</n>

<avg><i>D</i>(p) = 0.038 μm<sup>2</sup>/s</avg>

<n>1025</n>

<avg><i>D</i>(p) = 0.043 μm<sup>2</sup>/s</avg>
Conclusions

- Demonstration that up to 4-color parallel multi-color SPT is possible with Qdots.

- A large majority of analyzed trajectories at investigated time lags, $40 \leq t_{\text{lag}} \leq 200$ ms are statistically best described by a free diffusion model with a diffusion coefficient, $D$, of $0.01 - 0.05 \, \mu m^2/s$.

- Cholesterol depletion by mβCD results in slower diffusion for all molecules however at different extents. This is an indication that supposed lipid raft markers show differential sensitivity to cholesterol depletion.

Future

- Improved Qdot conjugates.
Study 2:

High-speed sequential single molecule imaging of a biotinylated lipid (DPPE), a biotinylated lipid anchored protein (BLAP-CD59), and a biotinylated transmembrane protein (BLAP-EGFR) with same preparation of sAv-QD655
Hop diffusion?

Super fast, 1700 Hz, 0.5 msec integration, sAv-QD655 attached to Biotin-cap-DPPE in MEF

Scale bar = 1 μm

Δt=1.52 sec  Δt=0.36 sec
Our SPT analysis

1. Identification of single particle positions and trajectory linking
2. Calculation of Mean Squared Displacements by:

\[ MSD_m(n\tau) = \frac{1}{N-n} \sum_{i=1}^{N-n} \left[ (x_m((i+n)\tau) - x_m(i\tau))^2 + (y_m((i+n)\tau) - y_m(i\tau))^2 \right] \]

3. Curve fitting to three nested diffusion models:

- \( MSD = 4Dt + c \) (free diffusion)
- \( MSD = 4D_\mu \tau (1 - \exp[-t/\tau]) + 4D_{macro}t \) (mixed diffusion)
- \( MSD = 4D_\mu \tau (1 - \exp[-t/\tau]) + c \) (confined diffusion)

\( D_{macro} \) is the long term diffusion coefficient, \( D_\mu \) is the short term diffusion coefficient within a confinement area \( L \) given by:

\[ L = \sqrt{12D_\mu \tau} \]  
(confineent size)

\( \tau \) is the time constant at which the confinement boundary restricts free diffusion.

The lifetime of the confinement time zones, \( \tau_{conf} \), is given by:

\[ \tau_{conf} = \frac{L^2}{D_{macro}} \]  
(confineent time)

For each trajectory, the fits of the three diffusion models were statistically compared by an F-test and divided into sub-populations corresponding to the diffusion behavior.
IMMOBILIZED sAV-QD655

A) Superimposed centroid positions (18 trajectories, N=6083 positions) and geometric mean of the centroid positions (red) of sAv-QD655 that were non-specifically adsorbed to a glass surface and imaged (1760 Hz, t_Aq = 0.52 ms). The standard deviation, δ_xy, of the positions were δ_x~30 nm and δ_y~26 nm. This is the minimum precision by which we can determine the position of single QDs in these measurements. B) Single trajectory example where the color scheme is a linear progression from blue to red as a function of the elapsed time from when the trajectory originated to when it ended. C) MSD plot for trajectory in B and best fit to Eqs. 1. D) Best fit parameters of example in B.

<table>
<thead>
<tr>
<th>sAv-QD655</th>
<th>B. Immobilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>D_{macro} (µm²/sec)</td>
<td>0.0 +/- 0.0</td>
</tr>
<tr>
<td>τ_{Conf} (ms)</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>D_{Micro} (µm²/sec)</td>
<td>0.9 +/- 0.2</td>
</tr>
<tr>
<td>L² (nm²)</td>
<td>(80 +/- 7)²</td>
</tr>
<tr>
<td>τ (ms)</td>
<td>0.6 +/- 0.1</td>
</tr>
</tbody>
</table>
**Trajectories:** Representative examples of trajectories categorized according to the three different types of diffusion. The spatial precision of each position is ~30 nm.

# Summary of High-speed SPT data

<table>
<thead>
<tr>
<th>Molecule</th>
<th>( N_{total} )</th>
<th>Diffusion model</th>
<th>( N/N_{total} ) (%)</th>
<th>( D_{macro} ) (( \mu \text{m}^2/\text{s} ))</th>
<th>( D_\mu ) (( \mu \text{m}^2/\text{s} ))</th>
<th>( \tau ) (ms)</th>
<th>( L ) (nm)</th>
<th>( \tau_{conf} ) (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPE</td>
<td>124</td>
<td>Free</td>
<td>10 %</td>
<td>0.23</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Confined</td>
<td>31 %</td>
<td>-</td>
<td>0.20</td>
<td>6.7</td>
<td>129</td>
<td>( \infty )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed</td>
<td>59 %</td>
<td>0.047</td>
<td>0.38</td>
<td>2.5</td>
<td>131</td>
<td>96</td>
</tr>
<tr>
<td>CD59</td>
<td>444</td>
<td>Free</td>
<td>5 %</td>
<td>0.14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Confined</td>
<td>27 %</td>
<td>-</td>
<td>0.21</td>
<td>4.8</td>
<td>109</td>
<td>( \infty )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed</td>
<td>68 %</td>
<td>0.050</td>
<td>0.33</td>
<td>2.3</td>
<td>102</td>
<td>62</td>
</tr>
<tr>
<td>EGFR</td>
<td>272</td>
<td>Free</td>
<td>8 %</td>
<td>0.091</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Confined</td>
<td>28 %</td>
<td>-</td>
<td>0.14</td>
<td>6.9</td>
<td>99</td>
<td>( \infty )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed</td>
<td>64 %</td>
<td>0.034</td>
<td>0.21</td>
<td>2.3</td>
<td>84</td>
<td>53</td>
</tr>
</tbody>
</table>
Distributions of D and L from High-speed SPT data
Conclusions

- Demonstration that high-speed SPT is possible with Qdots at rates up to \( \sim 1700 \) Hz.

- The results show heterogeneous trajectories for all investigated molecules where \( \sim 10\% \) of molecules are freely diffusing, \( \sim 20-30\% \) of molecules are confined, and \( 60-70\% \) of molecules are confined to domains of \( L^2 \approx 100\text{nm}^2 \) for 50-100 ms. The diffusion coefficient within the domains, \( D_{\mu} \), is \( \sim 0.3-0.4 \mu\text{m}^2/\text{s} \). The diffusion coefficient between domains, \( D_{\text{macro}} \), is \( \sim 0.05 \mu\text{m}^2/\text{s} \).
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