SINGLE MOLECULE TECHNIQUES

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UNIVERSITY OF OXFORD
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Outline

- **Single Molecule Techniques**

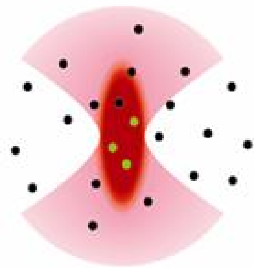
    - **FCS (Dynamics)**
    - **Single Particle Tracking (Dynamics)**
    - **PALM/STORM (Localization)**

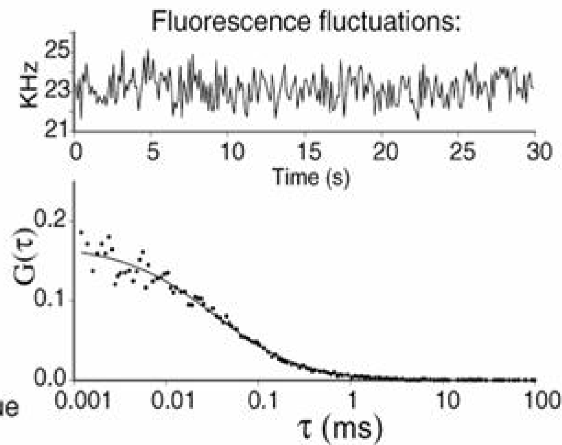
- **Equipment requirements**

- **Single Molecule Probes**

- **Single Particle Tracking**

Fluorescence Correlation Spectroscopy (FCS)





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

Concentration range:

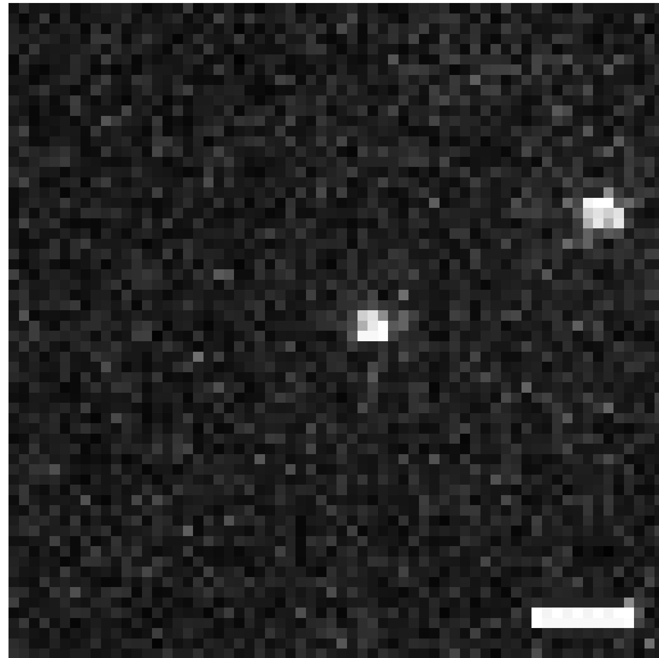
? ⟵ nM ⟶ ~1μM

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

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

http://www.drbio.cornell.edu/Infrastructure/Apparatus_WWW/fluorecorrspec.html

Single Particle Tracking



Required Equipment

Light/Fluorescence Microscope

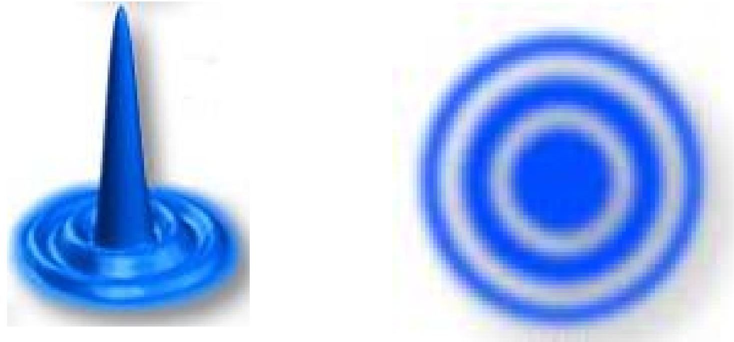
- Light source (Hg arc lamp/Laser)
  - Epi-fluorescence
  - TIRF
- Condenser (for brightfield imaging)
  - Gold particles
- Microscope objective
  - High NA
- Sensitive camera
  - EMCCD

Technical Considerations for Single Molecule Imaging

- Diffraction
- Microscope resolution
- Signal detection
- Data analysis

Diffraction

• 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**

$$r_{Airy} = \frac{0.61\ \lambda}{NA_{objective}}$$

λ = wavelength of light
NA=numerical aperture=n sin α
n = index of refraction of immersion media
α = half-angle of angular aperture

•The Airy disk represents 84 percent of the total luminous energy

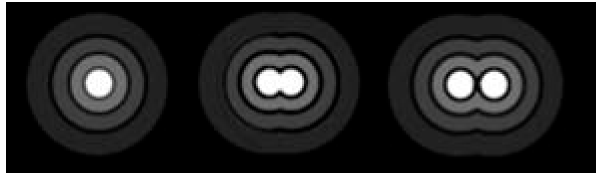
- NA ranges from <0.95 for air, <1.29 for water, <1.45 for oil
- For 100X magnification, 1.4 NA oil immersion objective and illumination with green light (500 nm)

$$r_{Airy} = \frac{(0.61)\,(500\text{ nm})}{1.4} = 220\text{ nm}$$

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)

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

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 ½ X the resolution. This is known as **Nyquist Sampling**

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

• 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 μm pixels

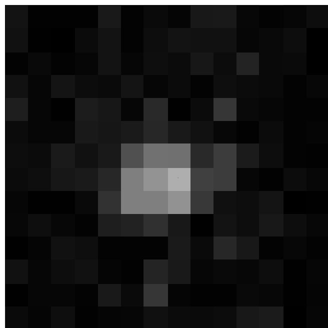
Data Analysis

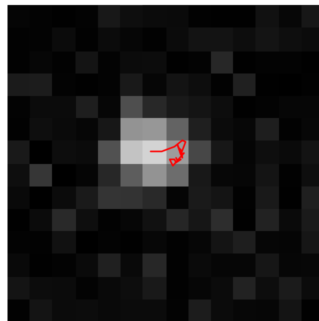
• **Obtain sub-pixel resolution by curve fitting to determine centroids of single molecules**

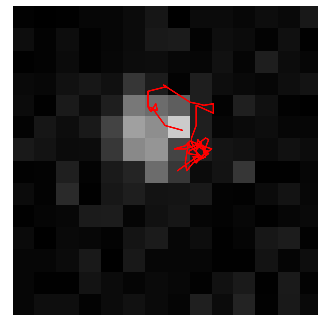
•**Approximate Airy pattern with a 2D spatial Gaussian and fit each image**

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

where ω = width of the PSF of the microscope (~220 nm)







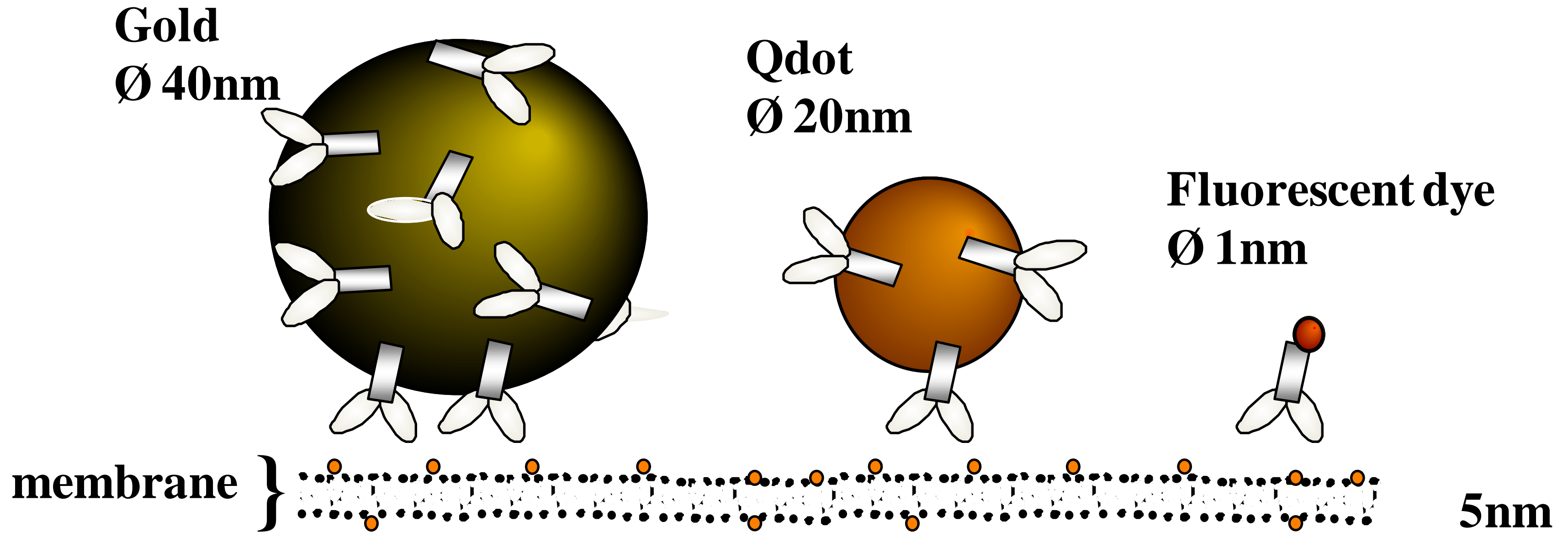
Single Particle Tracking

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

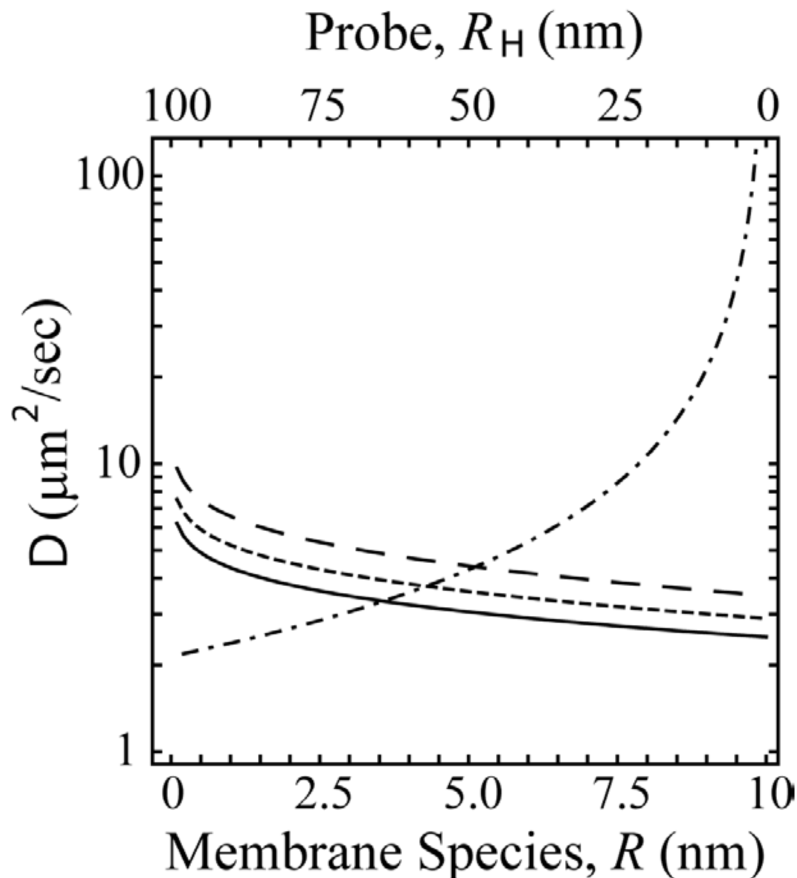
- **Technique results in time trajectories of sub-pixel positions of single objects**

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

SPT Approach



Diffusion in membranes and solution



Saffman-Delbruck:

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

μ_S = 1.002 cP
h = 4 nm
T = 293 K
μ_M/μ_S = 100 (solid line)
μ_M/μ_S = 80 (short dashed line)
μ_M/μ_S = 60 (long dashed line).

Stokes-Einstein:

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

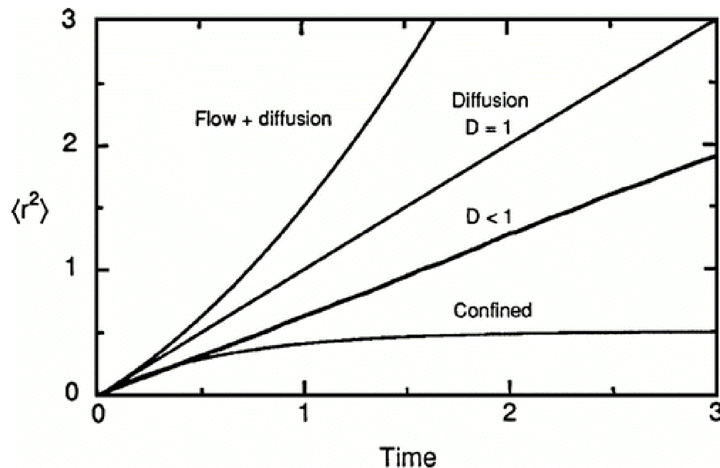
μ_S = 1.002 cP
T = 293 K.

• Dynamics are analyzed with respect to that predicted by Brownian (random) motion in a 2 D (or 3D) fluid

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

$$<r^2>=\frac{\sum_{n=1}^{q}\sqrt{(x_{t1}-x_{t2})^2+(y_{t1}-y_{t2})^2}}{q}=4D\,\Delta t \qquad \Delta t=t_2-t_1$$

where D is the diffusion coefficient



$$\textit{Brownian motion} <r^2>=4Dt$$

$$\textit{Anomalous diffusion} <r^2>=4Dt^{\alpha} \quad \alpha<1$$

$$\textit{Diffusion with flow} <r^2>=4Dt+(Vt)^2$$

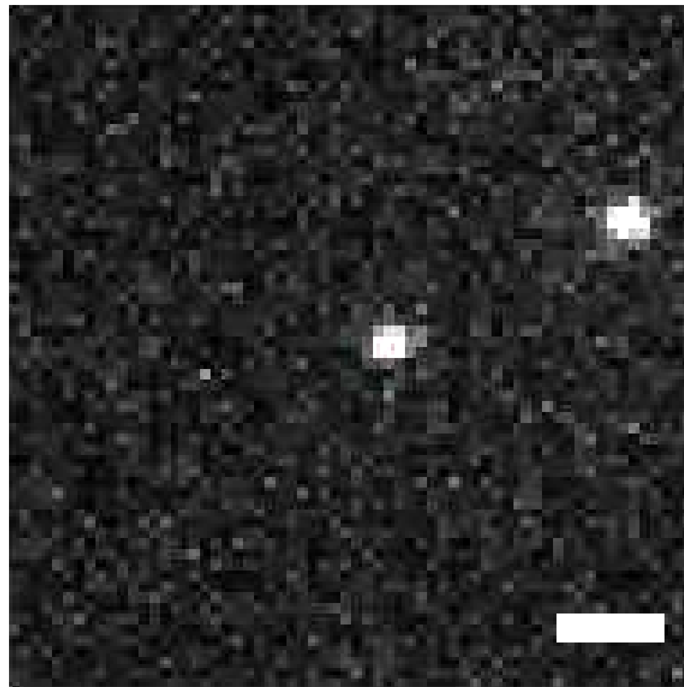
$$\textit{Confined diffusion} <r^2>\approx<r_C^2>[1-A_1\exp(-4A_2Dt/<r_C^2>)]$$

Saxton and Jacobson (1997) Annu. Rev. Biophys. Biomol. Struct. 26: 373-99

Quantum Dot single molecule imaging

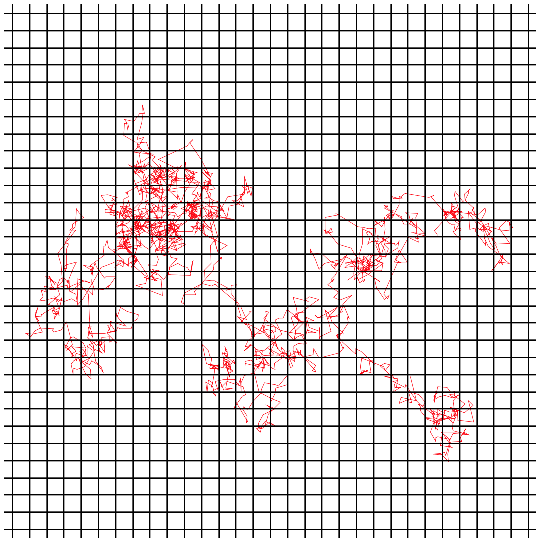
189 Hz (5.3 ms integration) with 160X magnification and Andor EMCCD

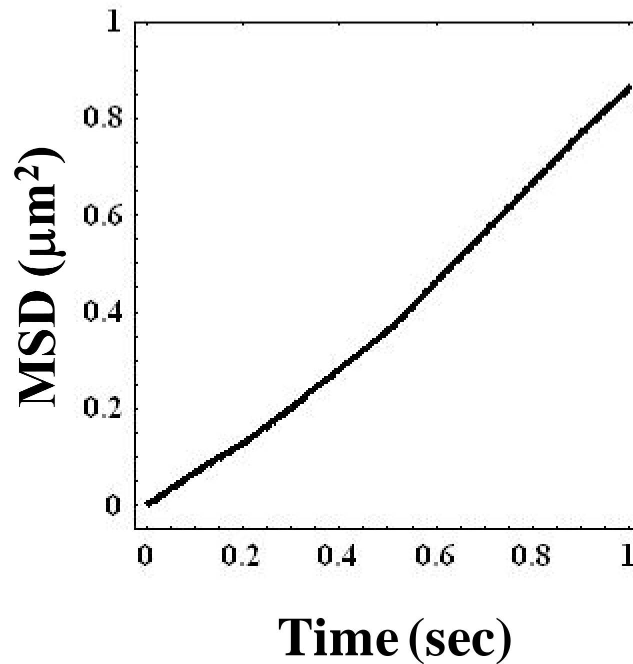
anti-CD73 Fab'-biotin + sAv-605 Qdot



Playback 100 Hz, 2000 frames
Scale bar = 1mm

Diffusion Coefficient





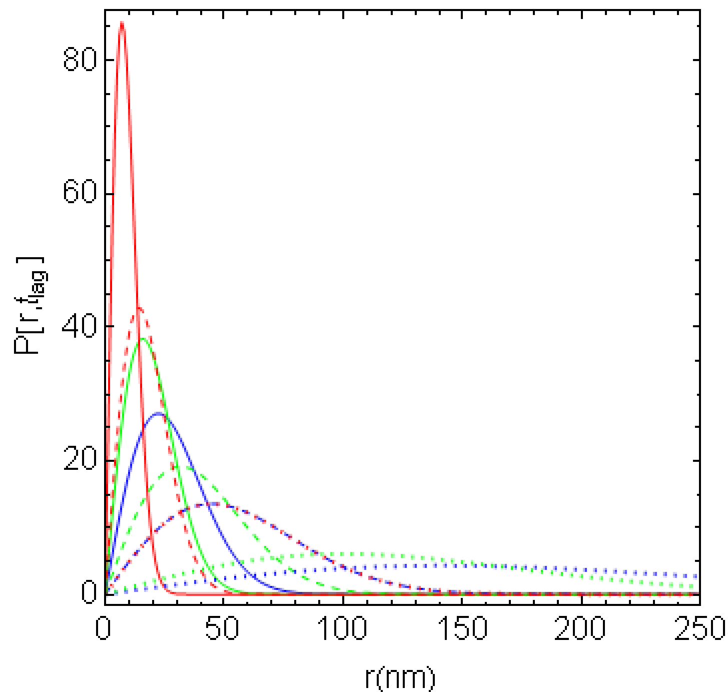
Brownian Diffusion
D ~ 0.25 μm^2/sec

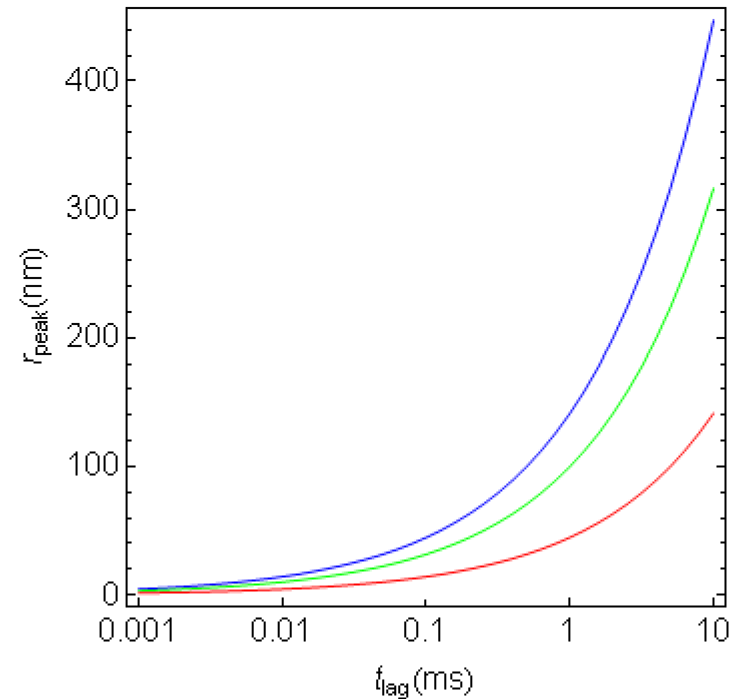
A few comments on diffusion and SPT

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

$$P\left(r, t_{lag}\right) = \frac{2r}{4\,D\,t}\,e^{-\frac{r^2}{4\,D\,t_{lag}}}$$

$$\frac{\partial}{\partial r}\left[P\left(r, t_{lag}\right)\right] = 0 \; for \; r_{peak} = \sqrt{2\,D\,t_{lag}}$$



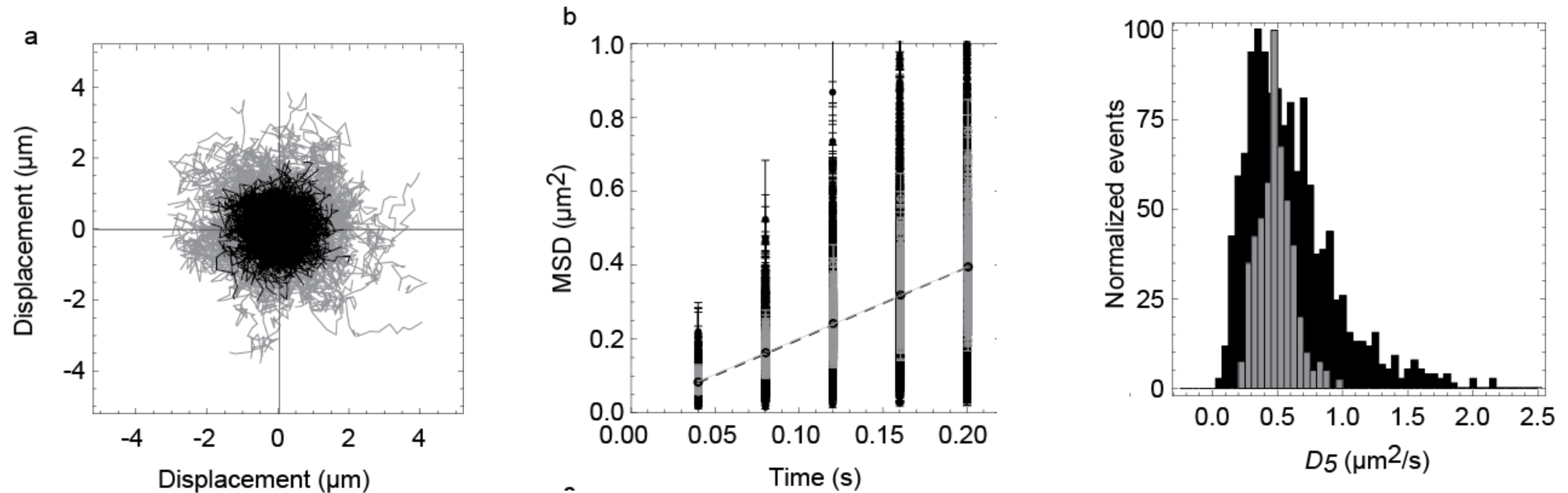


D=10 μm/s (blue) D=5 μm/s (green) D=1 μm/s (red)

t_{lag}=25 μs (solid) t_{lag}=100 μs (dashed) t_{lag}=1 ms (dotted)

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

Simulating diffusion



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

	Gold	Cy3	Quantum dots
Means of detection	Scattering	Fluorescence	Fluorescence
Size (diameter)	40 nm	~1-2 nm	~10-20 nm
Advantages	Photostable	Small Monovalent	Photobleaching resistant
Disadvantages	Immobilization Large Multivalent	Photobleaching (~5 s)	Non-specific binding Blinking

Gold

- Detectable by brightfield microscopy

- Rayleigh scatterer, intensity $\sim d^6$ where d = diameter of particle

- Particles > 30 nm diameter are detectable

- Conjugation of protein to gold particles depends upon

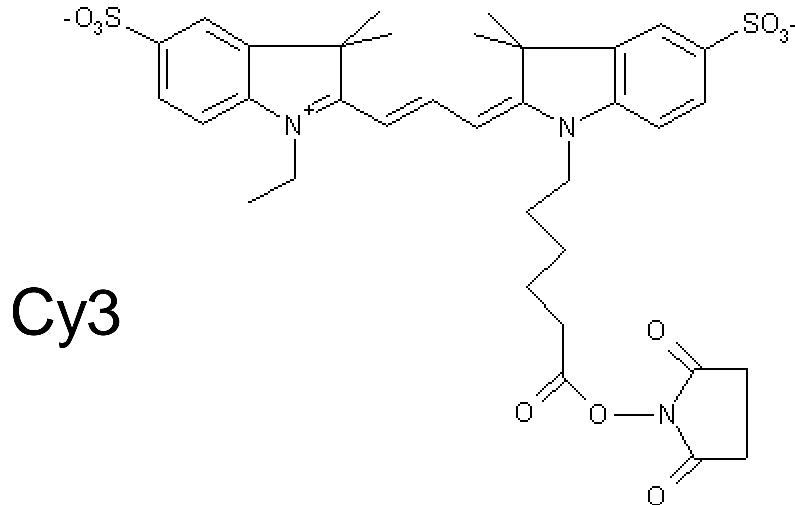
(a) ionic attraction between negatively charged gold and positively charged protein

(b) hydrophobic attraction between the antibody and the gold surface;

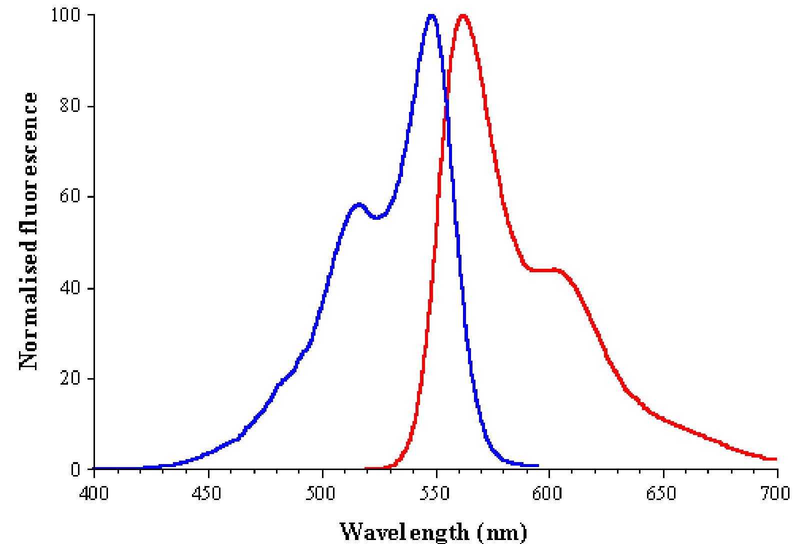
(c) dative binding between the gold conducting electrons and sulphur atoms which may occur within amino acids of the protein.

Fluorescent dyes

Cy3



N-hydroxy succinimide ester



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)

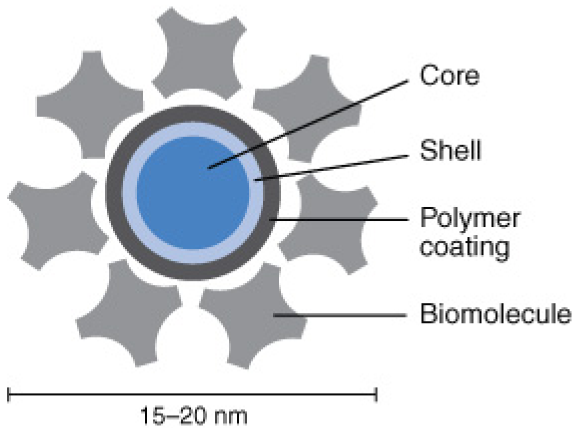
  single wavelength excitation of multiple color quantum dots

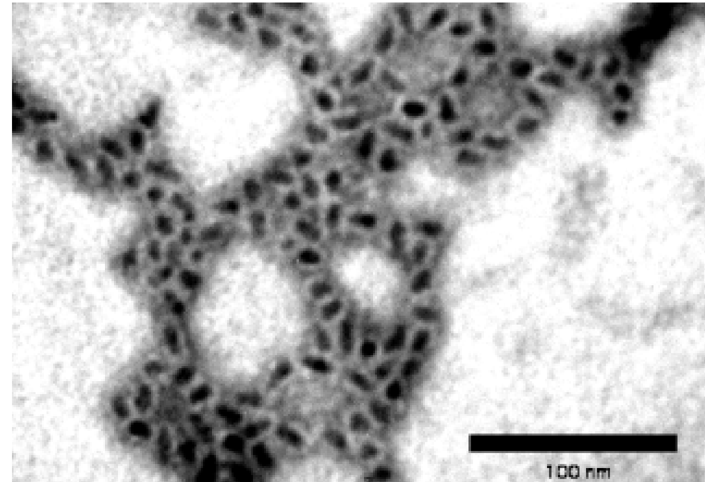
  photostable

  electron dense

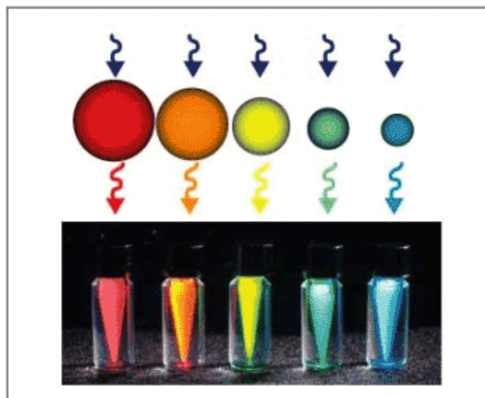
Quantum dot (QDot) basics

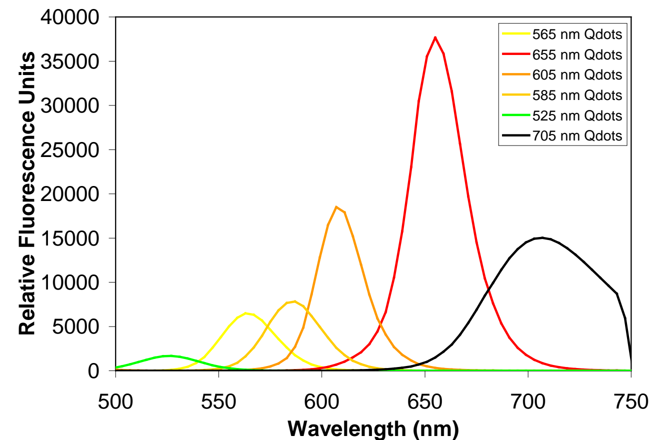
Qdots are fluorescent nanocrystals composed of semi-conductor cores, i.e. CdSe



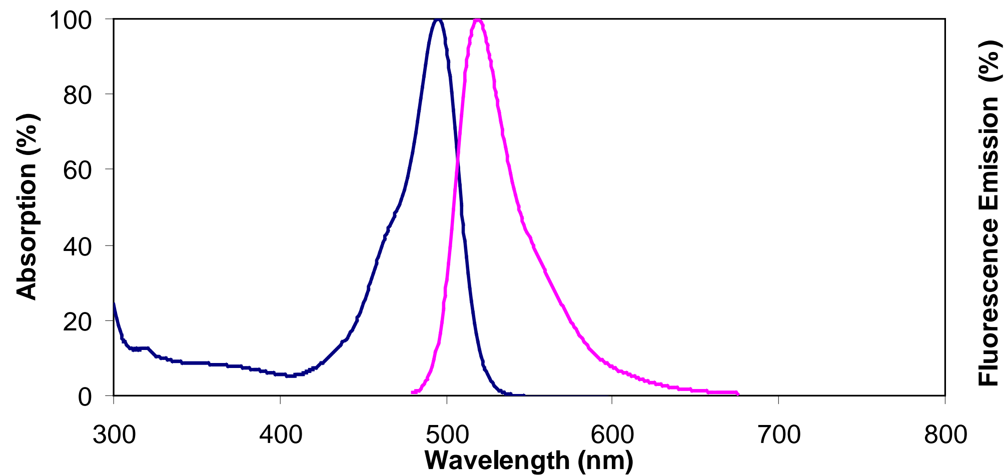


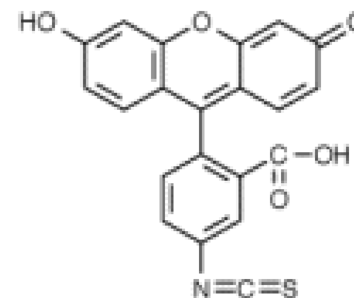
The size of the Qdot cores ≤ Excition Bohr Radius, This results in discrete, size-tunable band gap energies

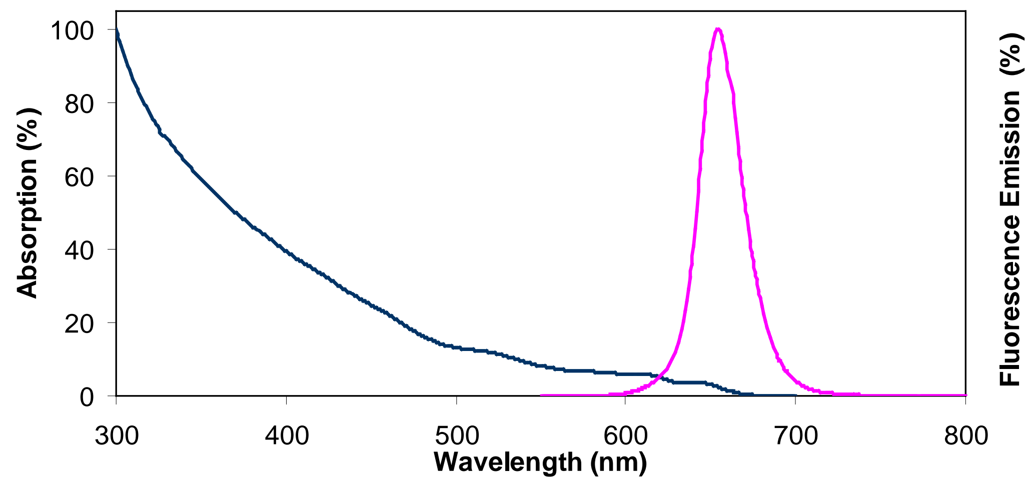




Fluorescence spectra





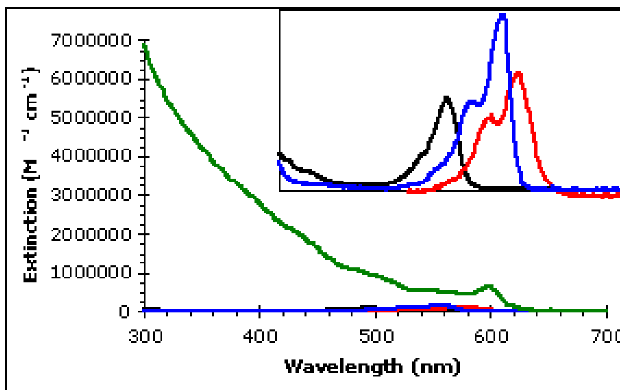


Brightness

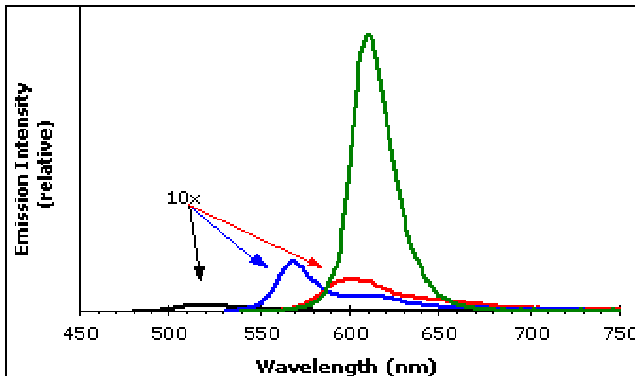
$\sim\varepsilon^*\varphi$

where ε = extinction coefficient (measure of how strongly the dye absorbs light)

φ = 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

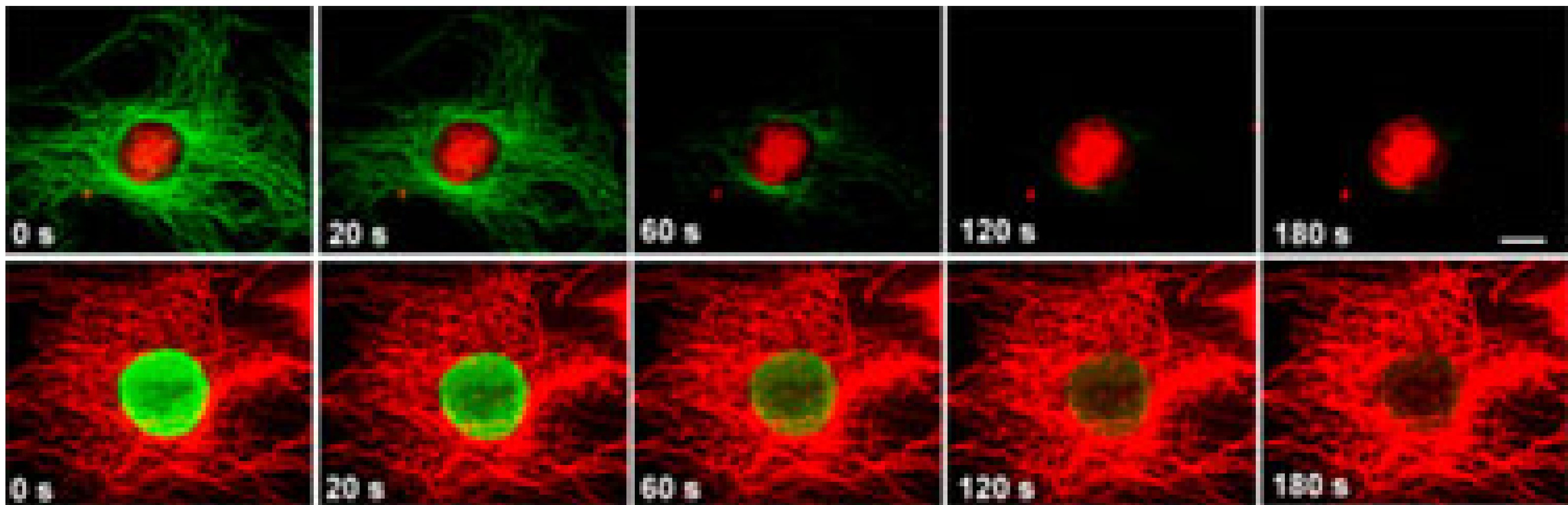
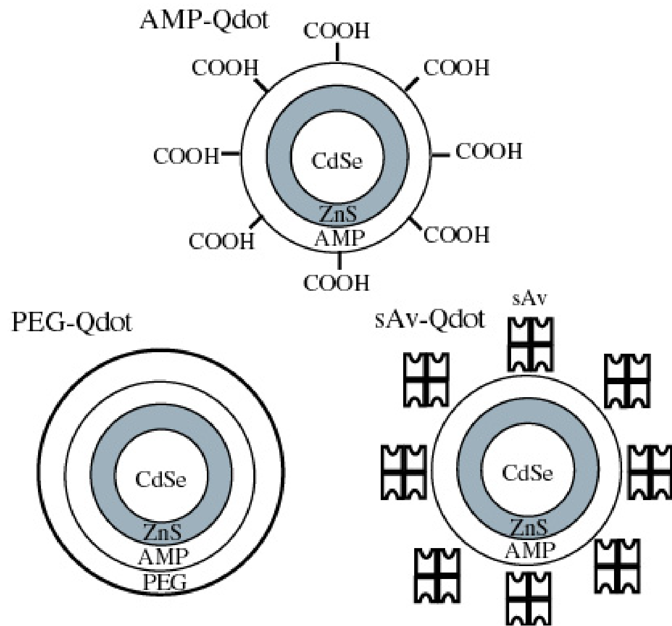
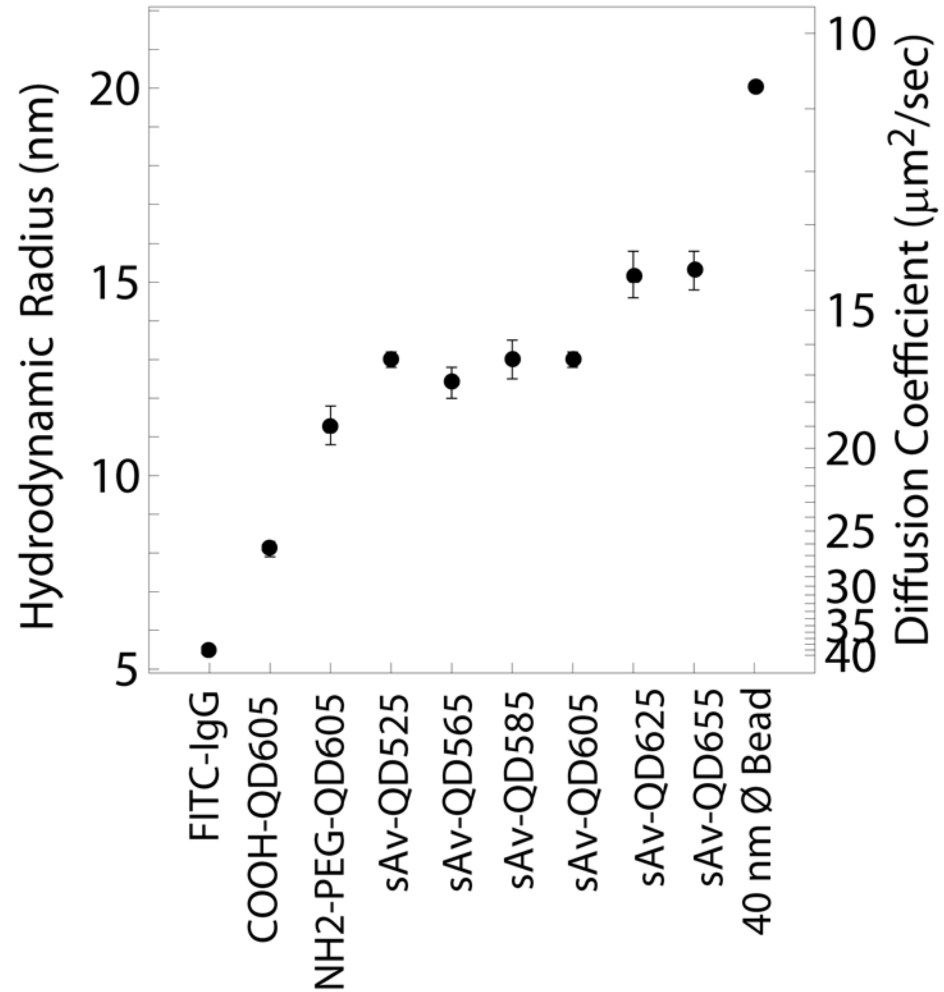


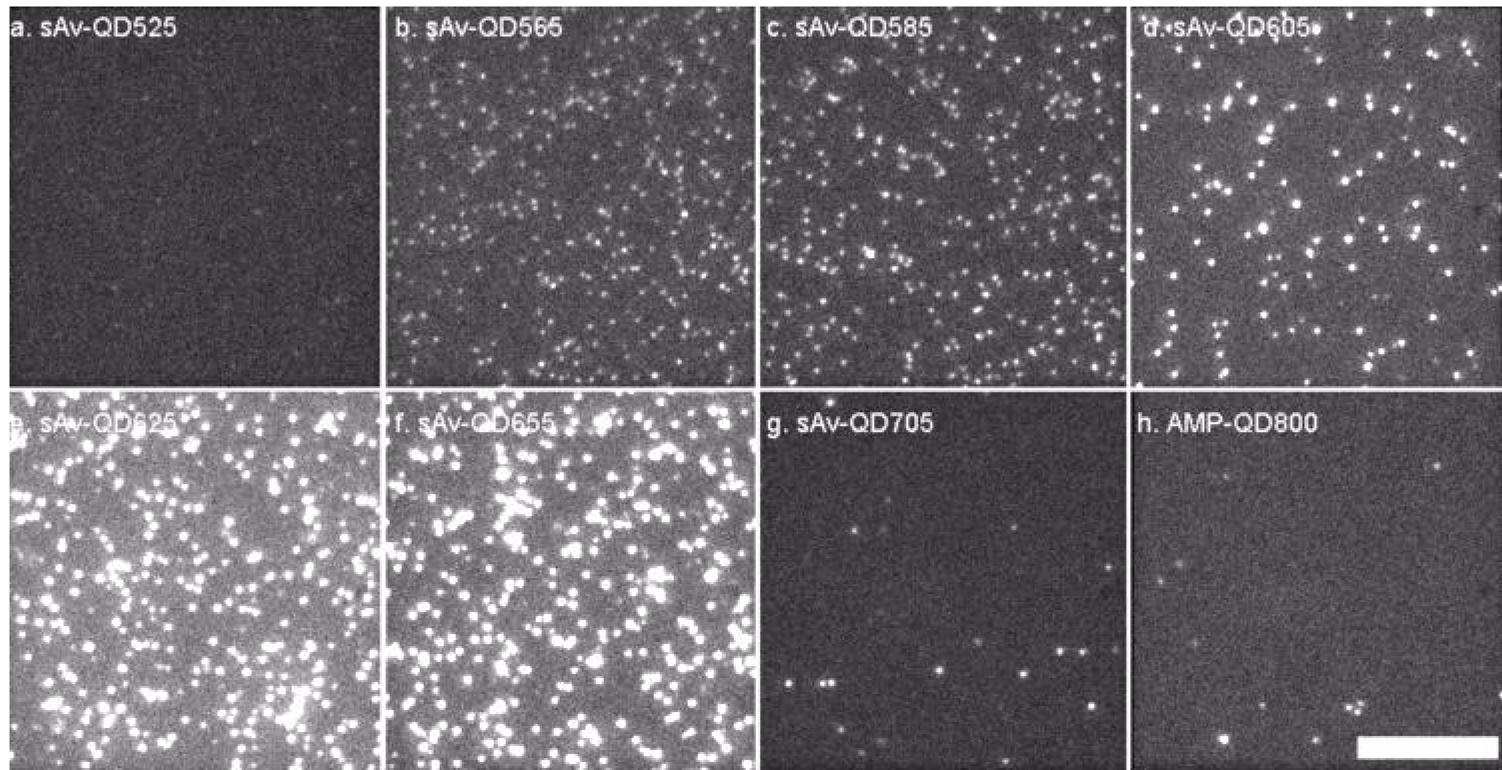
Image from Quantum Dot Corporation (www.qdots.com)

Commercially available types Qdots (Invitrogen) and Sizes





Brightness and Intermittency Comparison
5 ms integration, 100 W Hg lamp (470/40 nm)



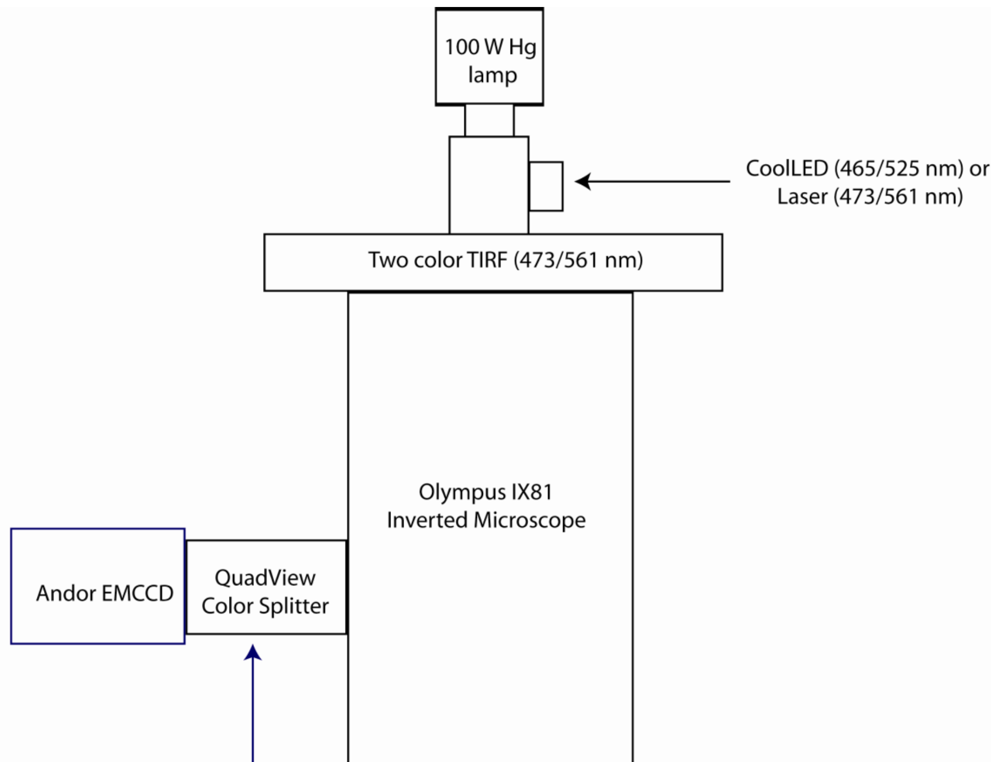
Qdot are very bright … but blink

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

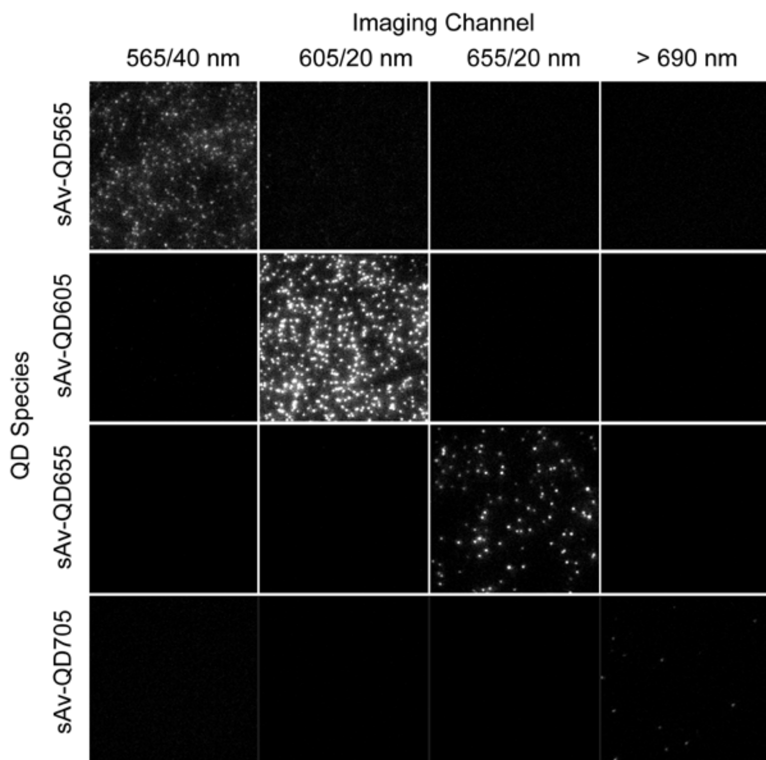


QuadView

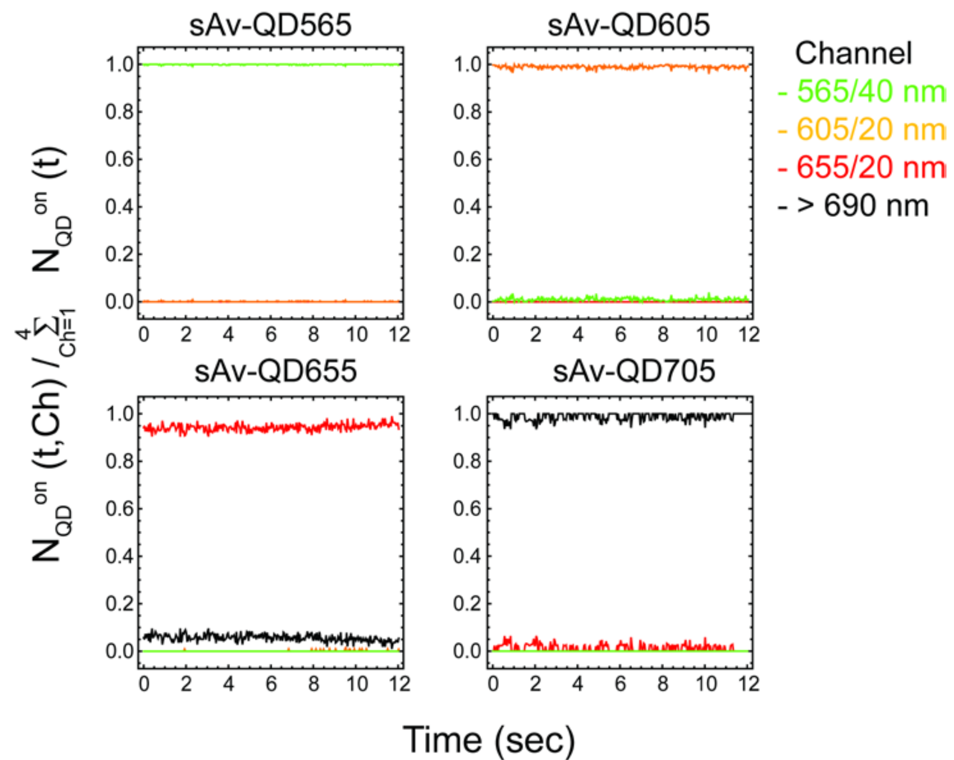
Acquisiton speed limits: 4 colors ~30 Hz (50x50μm)

2 colors ~ 500 H

(50x2 μm)

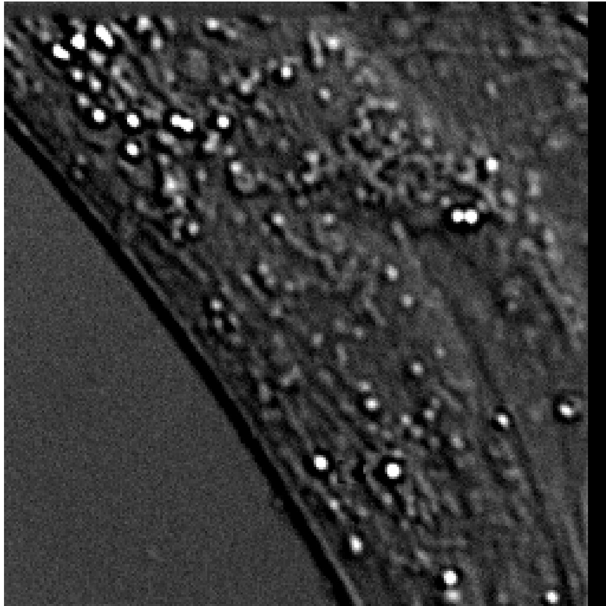


Imaging Channel
565/40 nm
605/20 nm
655/20 nm
> 690 nm
QD Species
sAv-QD565
sAv-QD605
sAv-QD655
sAv-QD705

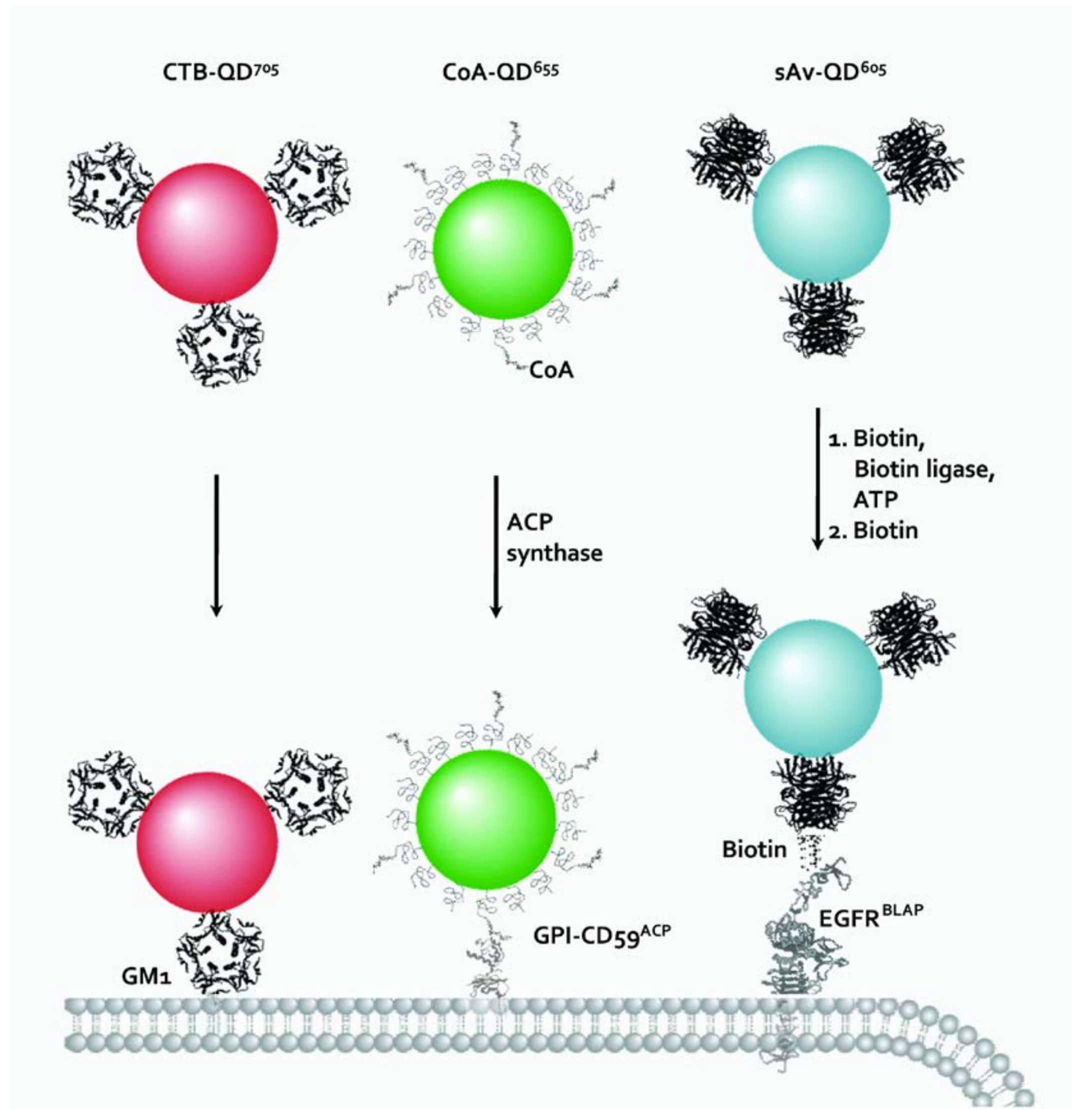


sAv-QD565
sAv-QD605
sAv-QD655
sAv-QD705
Channel
- 565/40 nm
- 605/20 nm
- 655/20 nm
- > 690 nm
$N_{QD}^{on}(t,Ch) / \sum_{Ch=1}^{4} N_{QD}^{on}(t)$
Time (sec)

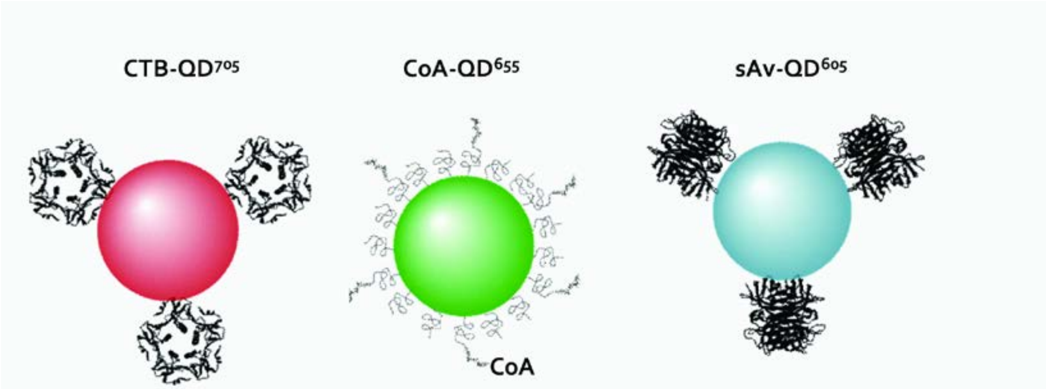
IA32 with 1ug/ml biotin-cap-DPPE (BSA loaded) and combination of sAv-QD565, sAv-QD605, sAv-QD655, and sAv-QD705 and 50uM βMe 150X 1.45NA, 10 ms integration, 24.86 Hz

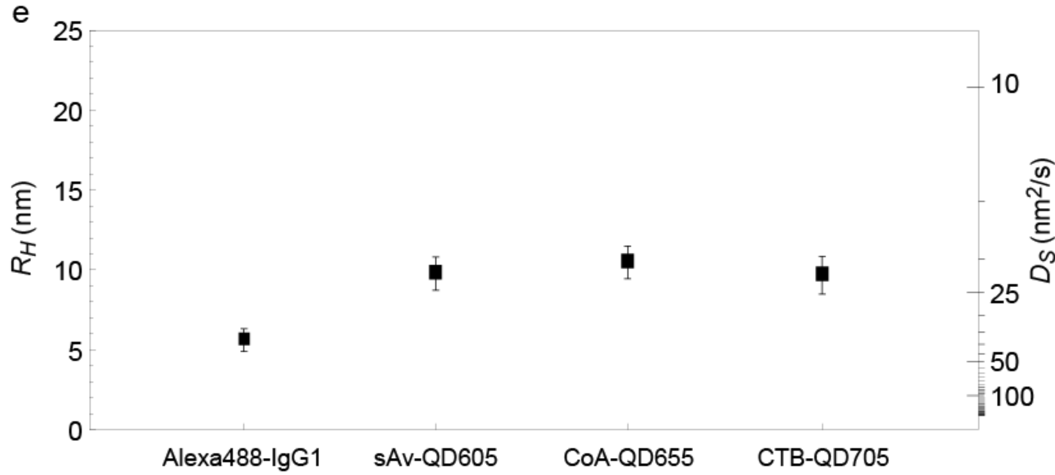


Parallel Targeting Schematic with Qdots



Hydrodynamic size of Qdot conjugates

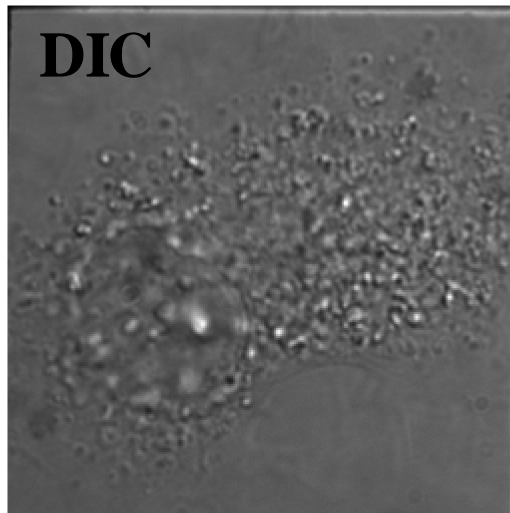


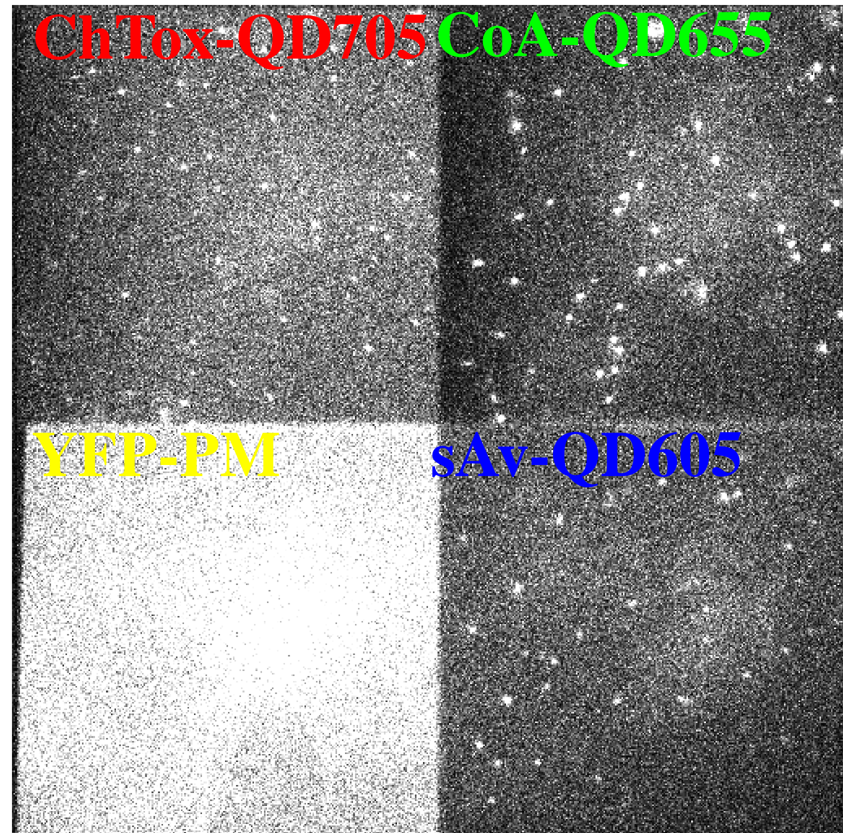


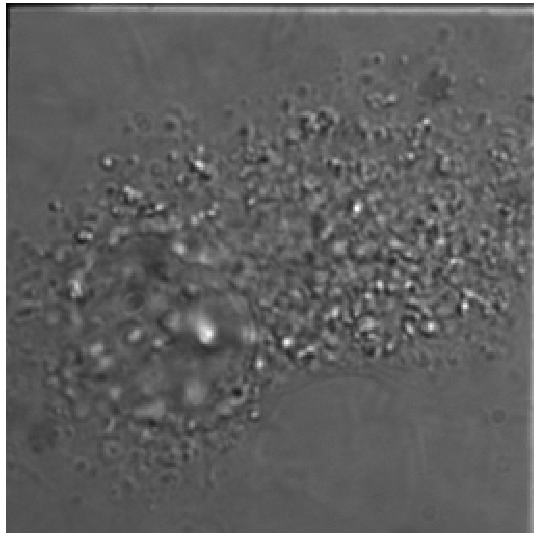
Mouse NIH3T3 co-transfected with BirA-KDEL, EGFR-BLAP, ACP-GPI, and YFP-K-Ras2

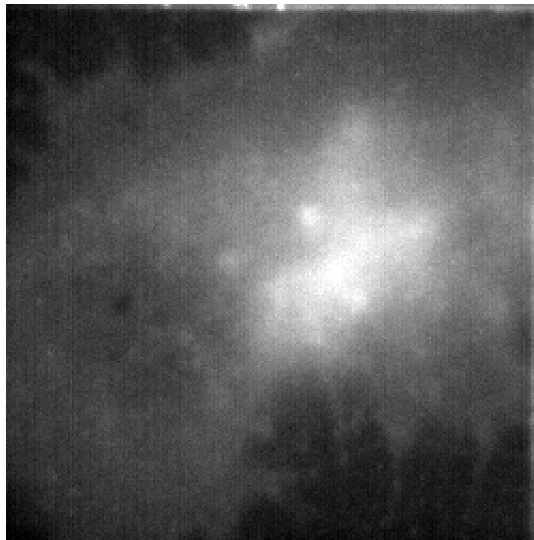
Cells were grown ON in 1 mM biotin and labeled with 1nM sAv-QD605, 1nM CoA-QD655, and 200pM ChTox-QD705

10 ms integration, ~25Hz acquisition rate





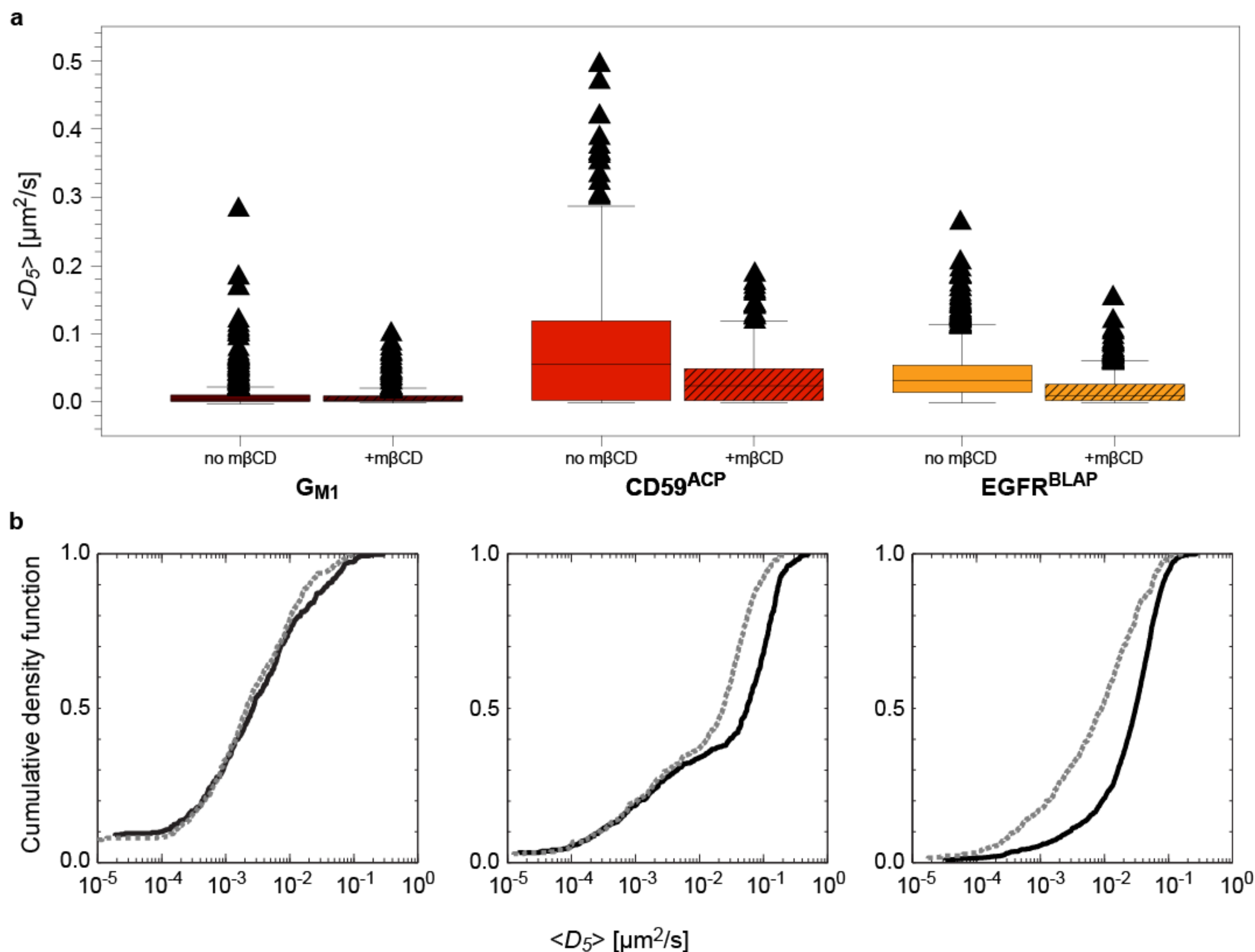




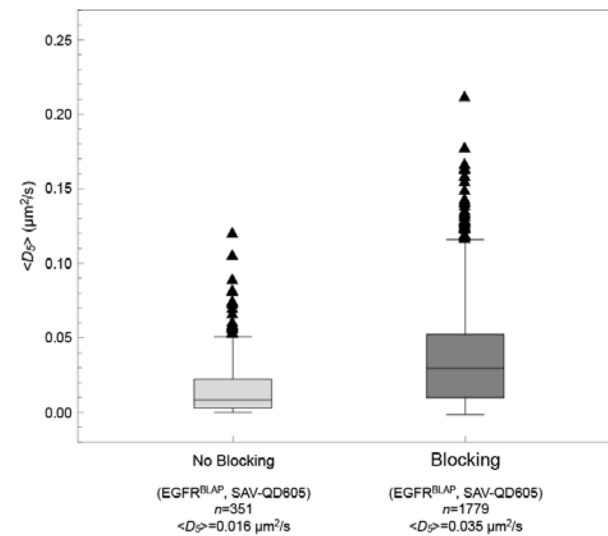
GM1 ACP–GPI BLAP–EGFR

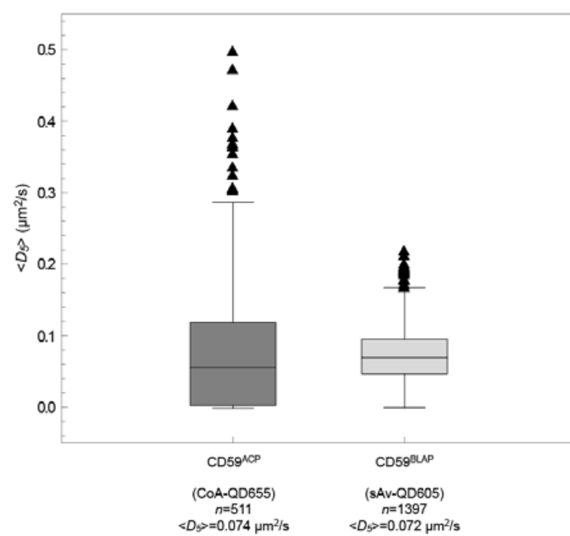
Differential effects of cholesterol depletion

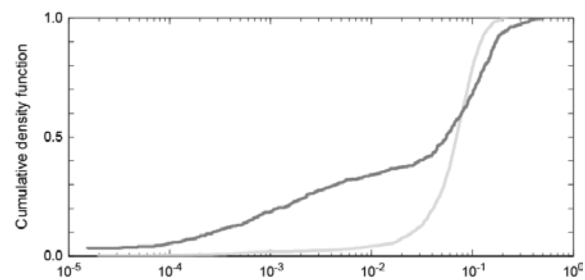
Free diffusion model, $1 \leq n_{lag} \leq 5$, corresponding to $40 \leq t_{lag} \leq 200$ ms

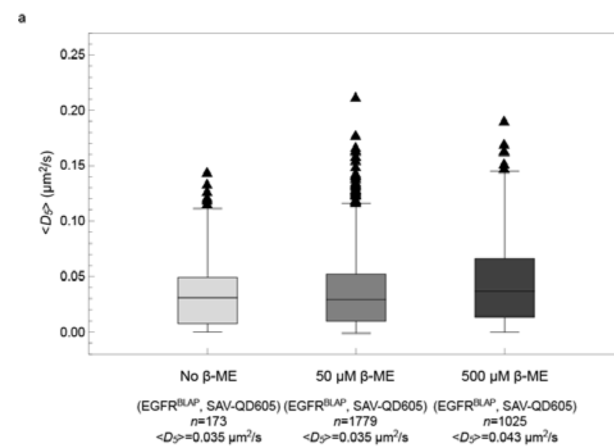


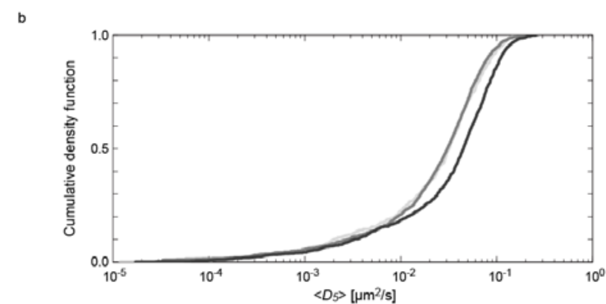
Controls











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, $40 \leq t_{lag} \leq 200$ 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

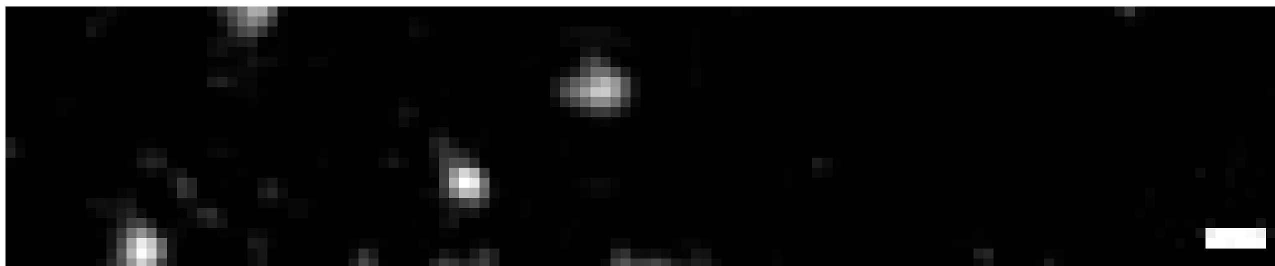
- Improved Qdot conjugates.

Study 2:

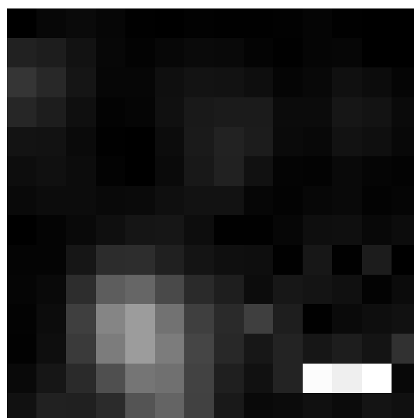
High-speed sequential single molecule imaging of a biotinylated lipid (DPPE), a biotinylated lipid anchored protein (BLAP-CD59), and a biotinylated transmembrane protein (BLAP-EGFR) with same preparation of sAv-QD655

Hop diffusion?

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



Scale bar = 1 μm



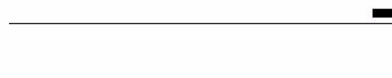
Scale bar = 0.5 μm

Biotin-cap-DPPE / sAv-QD655

Scale bar = 100 nm

Real time movie length 1.52 sec

(2412 detected pts out of 2699 frames)



Δt=1.52 sec

Biotin-cap-DPPE / sAv-QD655

Scale bar = 100 nm

Real time movie length 0.36 sec

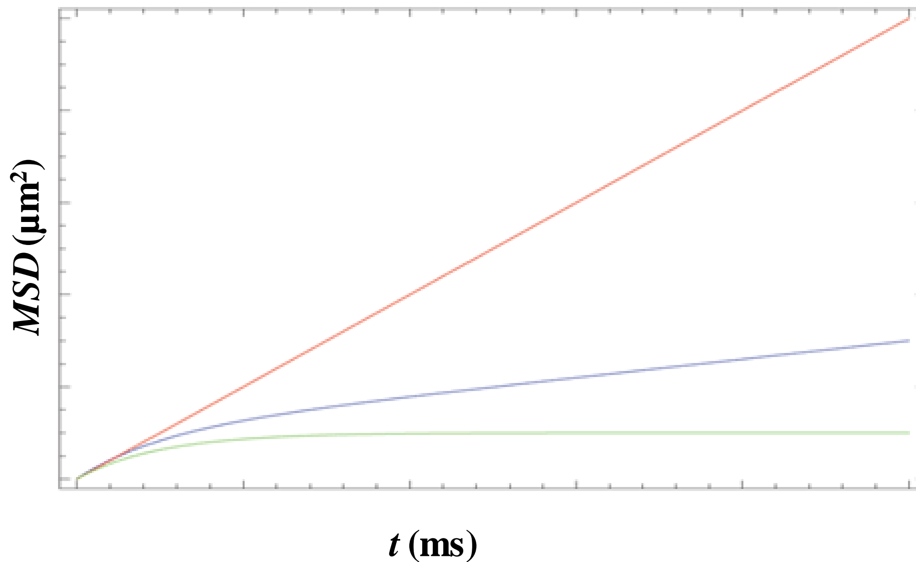
Δt=0.36 sec

Our SPT analysis

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

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

3. Curve fitting to three nested diffusion models:



$MSD = 4\,D\,t + c$
(free diffusion)

$MSD = 4\,D_\mu\,\tau\,(1\text{-}\exp[-t/\tau]\,) + 4\,D_{macro}\,t$
(mixed diffusion)

$MSD = 4\,D_\mu\,\tau\,(1\text{-}\exp[-t/\tau]\,) + c$
(confined diffusion)

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

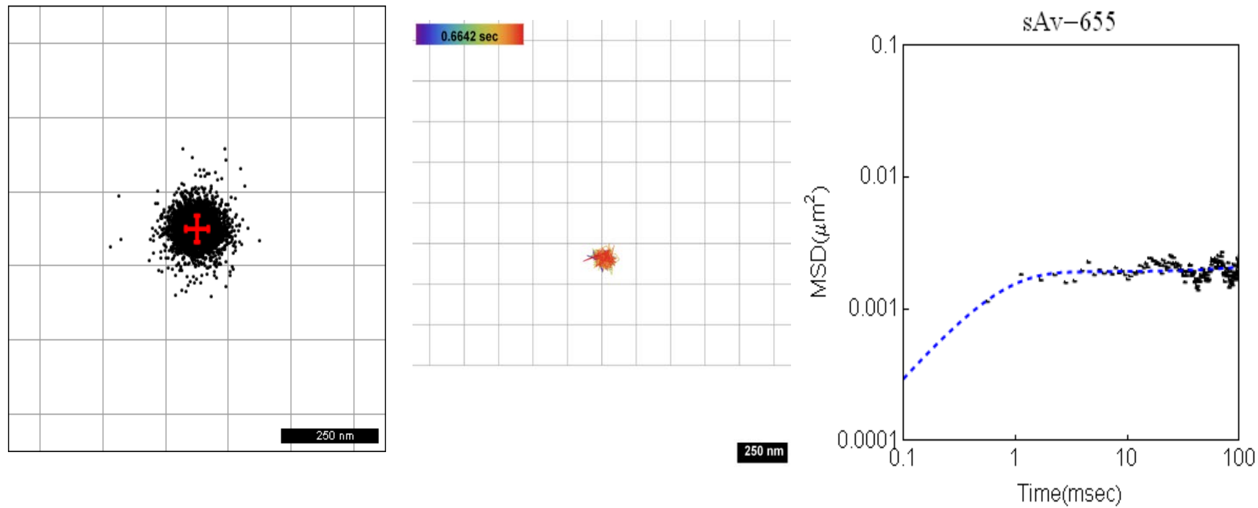
$L=\sqrt{(12\,D_\mu\,\tau)}$
(confinement size)

τ is the time constant at which the confinement boundary restricts free diffusion.
The lifetime of the confinement time zones, τ_{conf}, is given by:

$\tau_{conf} = L^2/\,D_{macro}$
(confinement 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

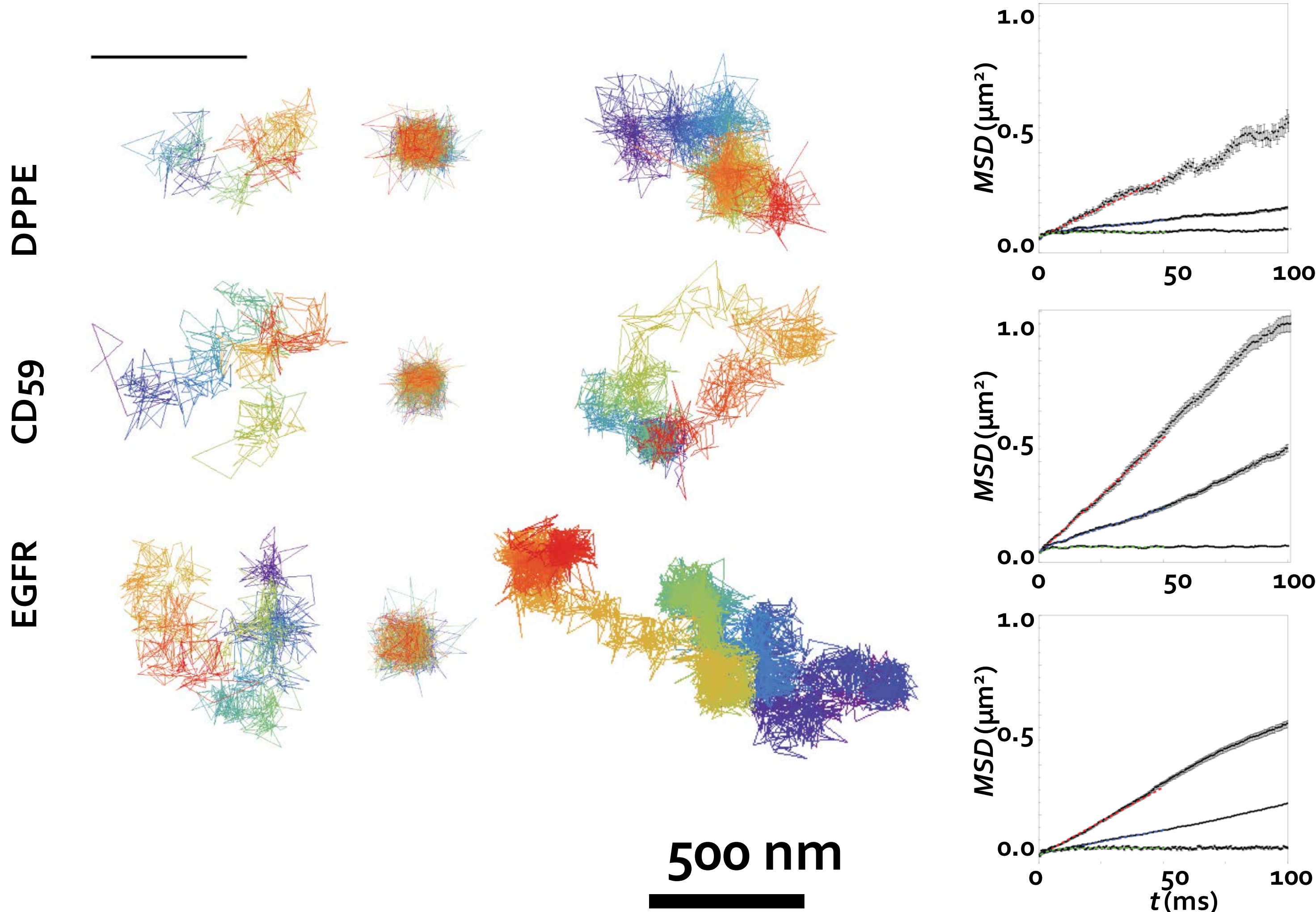


sAv-QD655	B. Immobilized
D_{macro} (μm²/sec)	0.0+/-0.0
τ_{Conf} (ms)	>10,000
D_{Micro} (μm²/sec)	0.9+/-0.2
L^2 (nm²)	$(80+/-7)^2$
τ (ms)	0.6+/-0.1

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, $\delta_{x,y}$, 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.

Trajectories: Representative examples of trajectories categorized according to the three different types of diffusion. The spatial precision of each position is ~30 nm.

Fits: Fits of the trajectories shown. Red: free diffusion. Green: Confined diffusion. Blue: mixed diffusion.



Summary of High-speed SPT data

Molecule	N_{total}	Diffusion model	N/N_{total} (%)	D_{macro} (µm²/s)	D_{μ} (µm²/s)	τ (ms)	L (nm)	τ_{conf} (ms)
DPPE	124	Free	10 %	0.23	-	-	-	-
		Confined	31 %	-	0.20	6.7	129	∞
		Mixed	59 %	0.047	0.38	2.5	131	96
CD59	444	Free	5 %	0.14	-	-	-	-
		Confined	27 %	-	0.21	4.8	109	∞
		Mixed	68 %	0.050	0.33	2.3	102	62
EGFR	272	Free	8 %	0.091	-	-	-	-
		Confined	28 %	-	0.14	6.9	99	∞
		Mixed	64 %	0.034	0.21	2.3	84	53

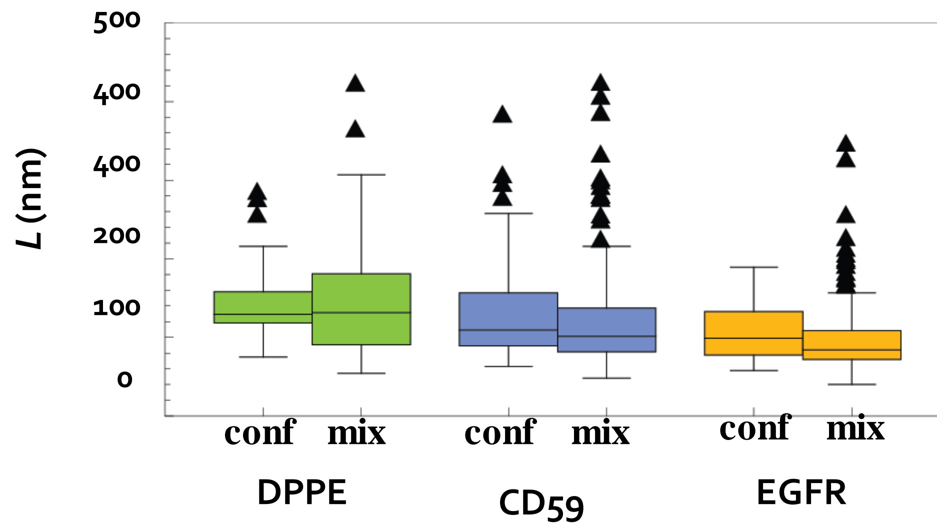
Distributions of D and L from High-speed SPT data

1.50
1.00
0.50
0.00
D (µm²)

D free | D_μ conf | D_μ mix | D_{macro} mix
DPPE

D free | D_μ conf | D_μ mix | D_{macro} mix
CD59

D free | D_μ conf | D_μ mix | D_{macro} mix
EGFR



Conclusions

- Demonstration that high-speed SPT is possible with Qdots at rates up to ~1700 Hz.
- The results show heterogeneous trajectories for all investigated molecules where ~10% of molecules are freely diffusing, ~20-30% of molecules are confined, and 60-70% of molecules are confined to domains of $L^2 \approx 100 nm^2$ for 50-100 ms. The diffusion coefficient within the domains, D_μ, is ~ 0.3-0.4 $\mu m^2/s$. The diffusion coefficient between domains, D_{macro}, is ~ 0.05 $\mu m^2/s$.

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