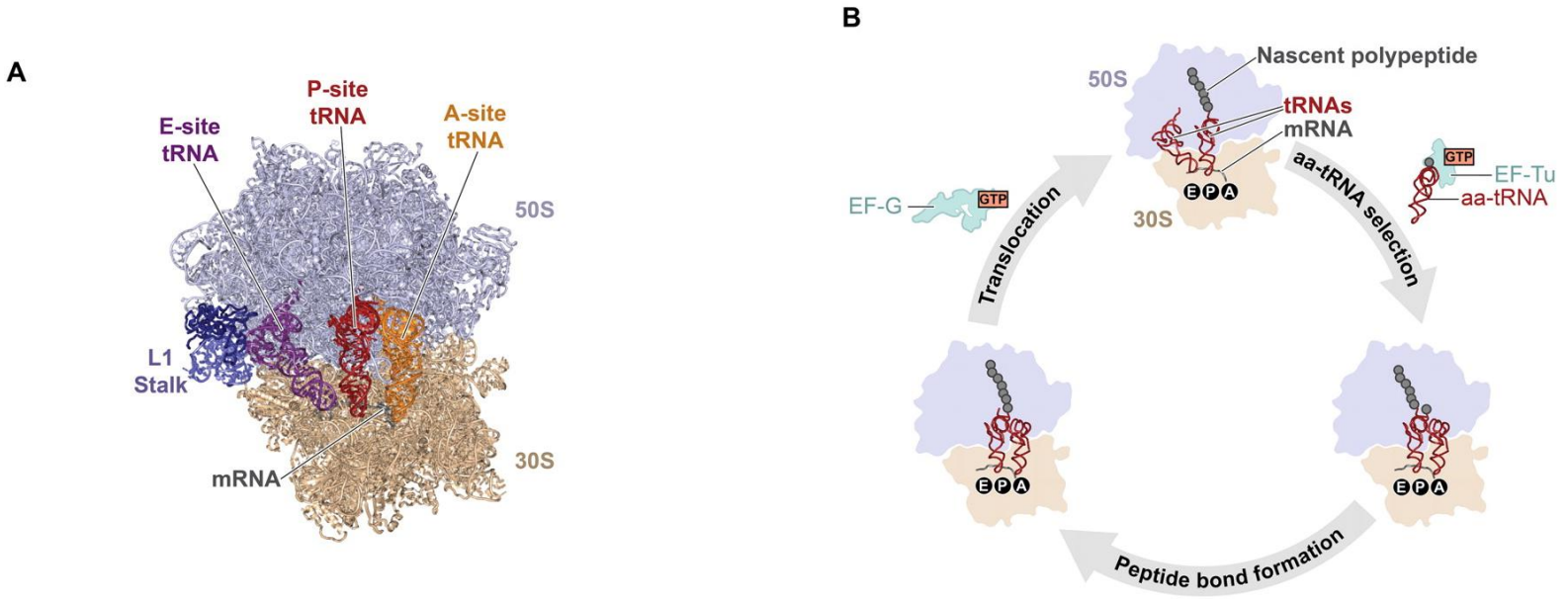
SINGLE MOLECULE TECHNIQUES

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

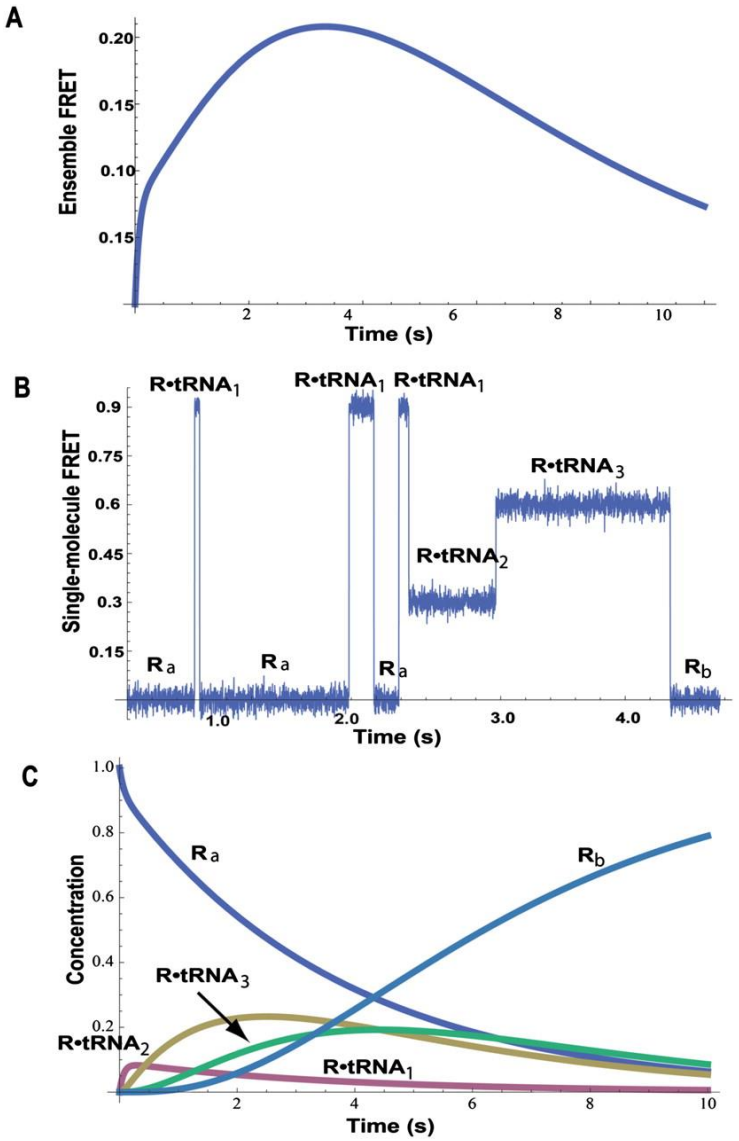
Watching the translation of a messenger RNA into its encoded protein by the ribosome, one molecule at a time.



Blanchard, SC; Gonzalez, RL; Kim, HD; Chu, S; Puglisi, JD. (2004) tRNA selection and kinetic proofreading in translation. (2004) Nature Structural & Molecular Biology 11: 1008-1014.

Tinoco I; Gonzalez RL. (2011) Biological mechanisms, one molecule at a time. Genes Dev. 25:1205-1231.

Comparison of ensemble measurements with single-molecule measurements.



Tinoco I , and Gonzalez R L Genes Dev. 2011;25:1205-1231

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

.

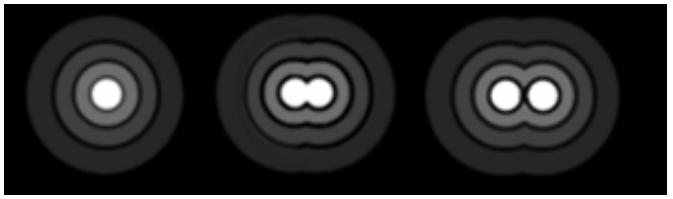
- NA ranges from <0.95 for air, <1.2 for water, <1.4 for oil
- For 100X magnification, 1.2 NA oil immersion objective and illumination with green light (500 nm = 0.5 μm)

$$r_{Airy} = \frac{(0.61)\,(0.5\ \mu m)}{1.2} = 0.25\ \mu m = 250\ nm$$

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 = 0.5 μm)

$$\text{Resolution} = \frac{(0.61)\,(0.5\ \mu\text{m})}{1.2} = 0.25\ \mu\text{m} = 250\ \text{nm}$$

Imaging Intracellular Fluorescent Proteins at Nanometer Resolution

Eric Betzig,[1,2]*† George H. Patterson,[3] Rachid Sougrat,[3] O. Wolf Lindwasser,[3] Scott Olenych,[4] Juan S. Bonifacino,[3] Michael W. Davidson,[4] Jennifer Lippincott-Schwartz,[3] Harald F. Hess[5]*

We introduce a method for optically imaging intracellular proteins at nanometer spatial resolution. Numerous sparse subsets of photoactivatable fluorescent protein molecules were activated, localized (to ~2 to 25 nanometers), and then bleached. The aggregate position information from all subsets was then assembled into a superresolution image. We used this method—termed photoactivated localization microscopy—to image specific target proteins in thin sections of lysosomes and mitochondria; in fixed whole cells, we imaged vinculin at focal adhesions, actin within a lamellipodium, and the distribution of the retroviral protein Gag at the plasma membrane.

Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM)

Michael J Rust[1,5], Mark Bates[2,5] & Xiaowei Zhuang[1,3,4]

We have developed a high-resolution fluorescence microscopy method based on high-accuracy localization of photoswitchable fluorophores. In each imaging cycle, only a fraction of the fluorophores were turned on, allowing their positions to be determined with nanometer accuracy. The fluorophore positions obtained from a series of imaging cycles were used to reconstruct the overall image. We demonstrated an imaging resolution of 20 nm. This technique can, in principle, reach molecular-scale resolution.

Signal Detection

- Single Particle (Molecule) Tracking relies on intensified video or CCD cameras both of 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

$$\text{Nyquist Sampling} = \frac{(0.61)\,(0.5\ \mu m)}{2\ 1.2} = 0.125\ \mu m = 125\ nm$$

- Hence for 100X magnification, 1.2 NA oil immersion objective and illumination with green light (500 nm = 0.5 μm)

- Proper sampling then requires 100 X 125 nm = 12.5 μm pixels

Localization Precision for Individual Fluorescent Probes

$$\delta^{x,y^2} = \frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\,\pi\, s_i^4 b^2}{a^2 N^2}$$

Si = STD of PSF

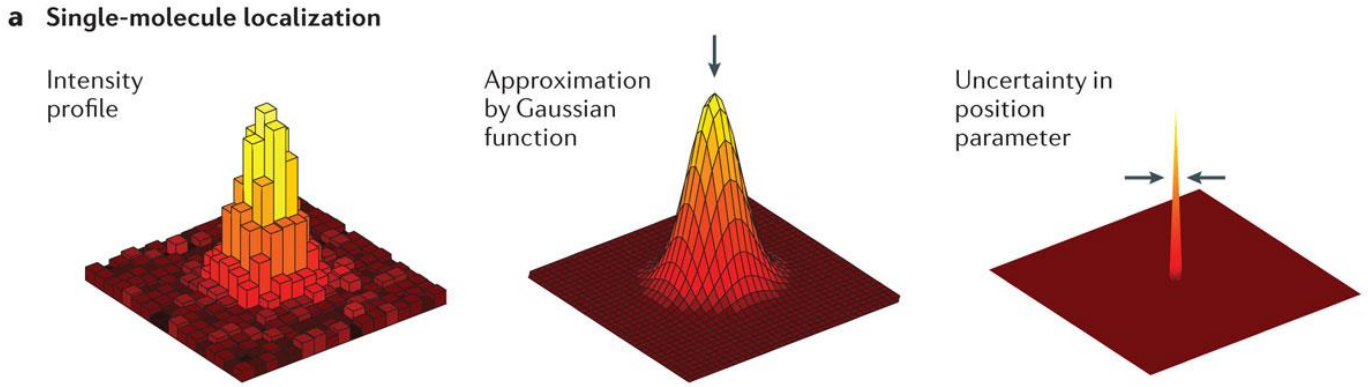
N = # of photons

a = size of pixels

b = STD of background

Thompson, RE; Larson, DR; Webb, WW. (2002) Precise Nanometer Localization Analysis for Individual Fluorescent Probes. Biophysical J. 82: 2775–2783.

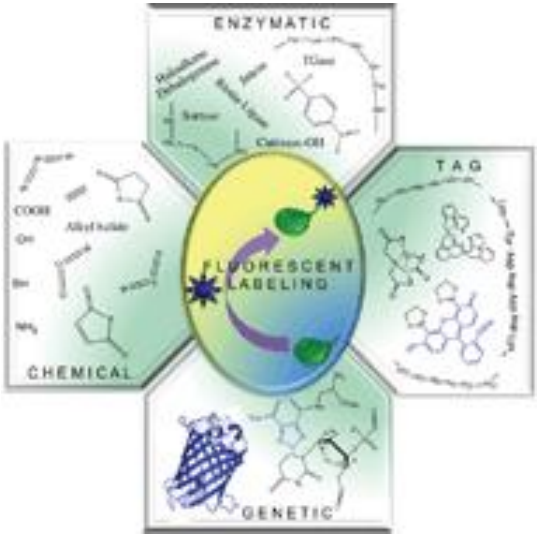
Principles of single-molecule tracking and imaging.



Gahlman, A; Moerner, WE. (2014) Exploring bacterial cell biology with single-molecule tracking and super-resolution imaging. *Nature Reviews Microbiology* 12: 9–22.

Probe Considerations

- Very bright and stable
- Small
- Monovalent (i.e. one probe per molecule of interest)
- Low non-specific binding


ENZYMATIC
TAG
FLUORESCENT LABELING
CHEMICAL
GENETIC

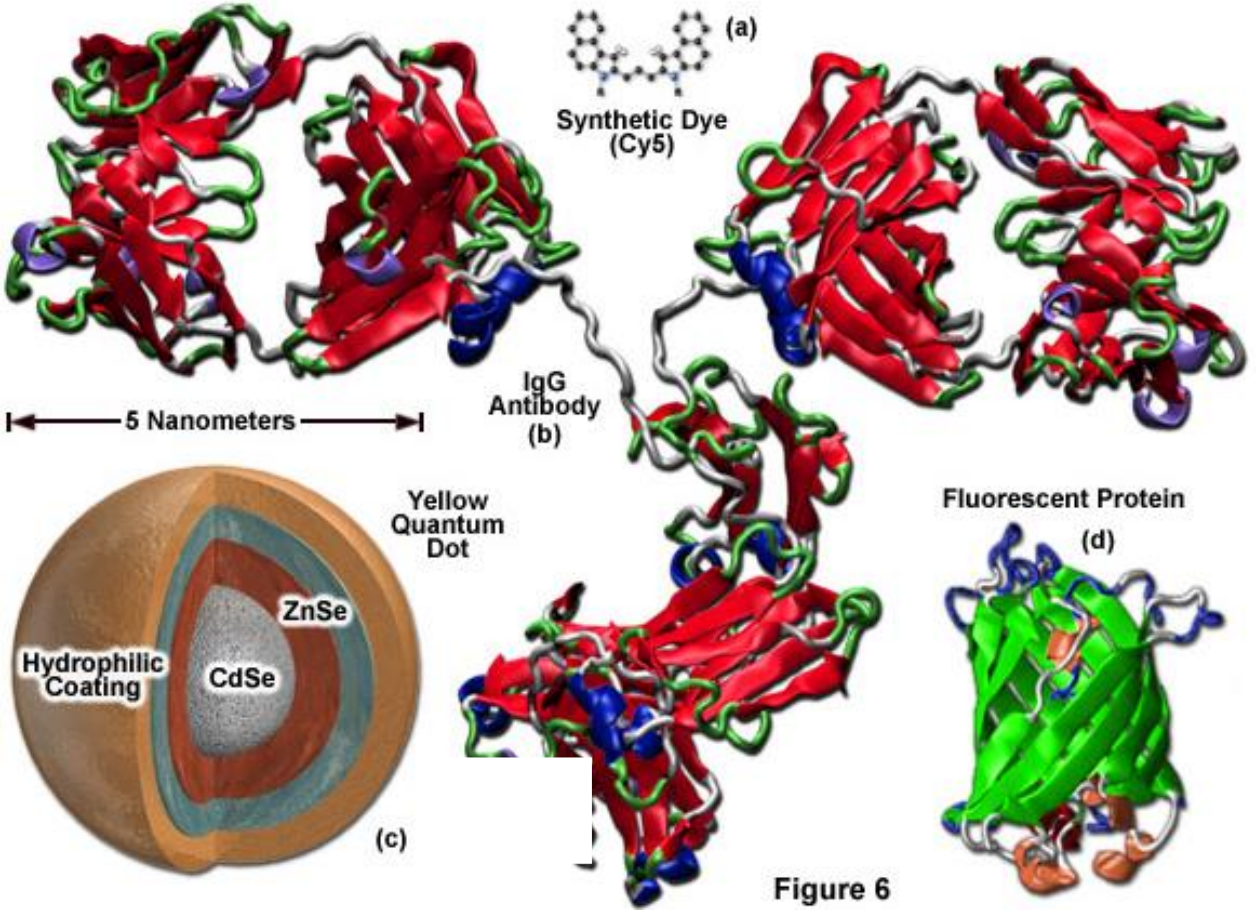
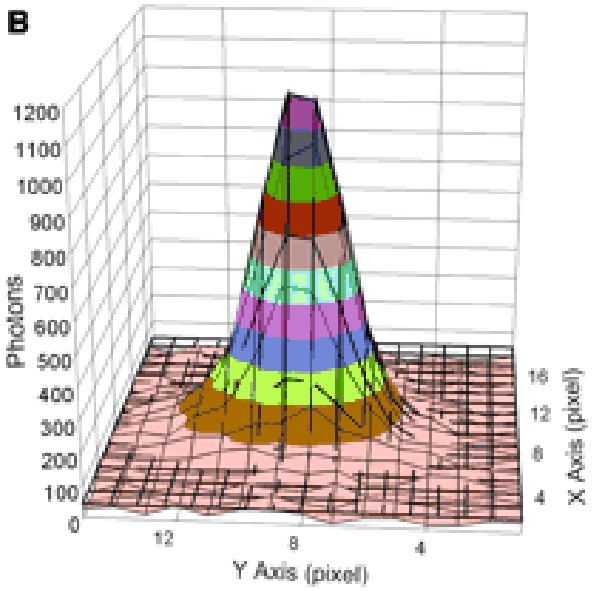

(a)
Synthetic Dye (Cy5)
5 Nanometers
IgG Antibody (b)
Yellow Quantum Dot
ZnSe
CdSe
Hydrophilic Coating
(c)
Fluorescent Protein
(d)

Figure 6

FIONA



Imaging single Cy3 molecules with 0.5 sec integration with 1.5 nm resolution

Imaging buffer: 0.4% Glucose, 1% β-mercaptoethanol, 10 mM MgCl2, 1% glucose oxidase, ~26000 units Catalase, 10 mM Tris pH 8.0, 50 mM NaCl

Yildiz, A; Forkey, JN; McKinney, SA; Ha, T; Goldman, YE; Selvin, PR. (2003) Myosin V Walks Hand-Over-Hand: Single Fluorophore Imaging with 1.5-nm Localization. Science 300: 2061-2065.

Localization Precision for Individual Fluorescent Probes

$$\delta^{x,y^2} = \frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\,\pi\, s_i^4 b^2}{a^2 N^2}$$

Si = STD of PSF

N = # of photons

a = size of pixels

b = STD of background

Thompson, RE; Larson, DR; Webb, WW. (2002) Precise Nanometer Localization Analysis for Individual Fluorescent Probes. Biophysical J. 82: 2775–2783.

One Common Approach - TIRF Microscopy

Total Internal Reflection Microscopy

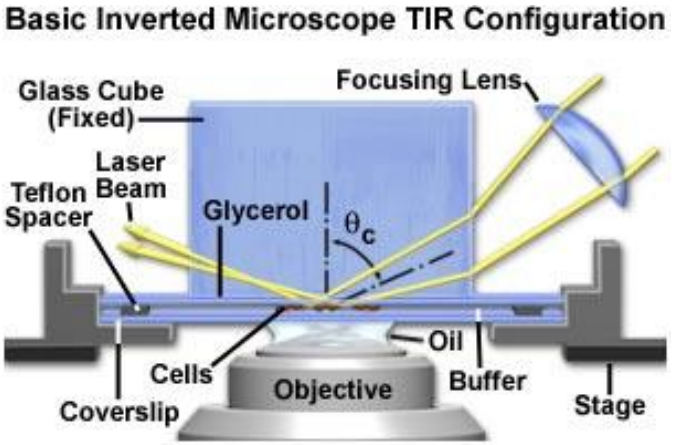
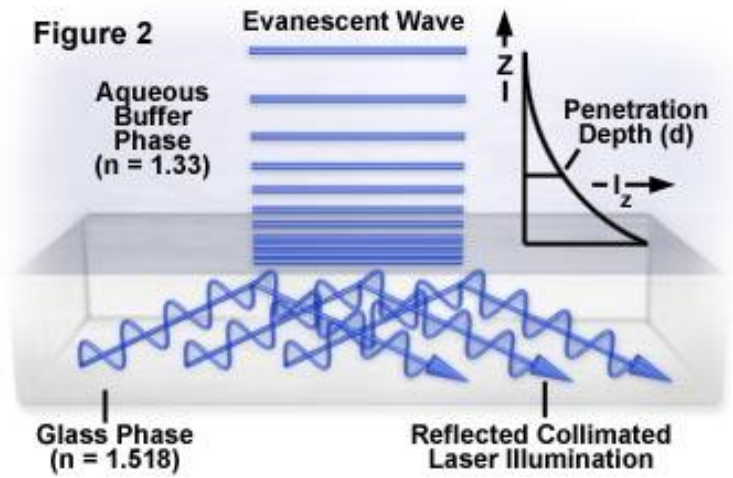


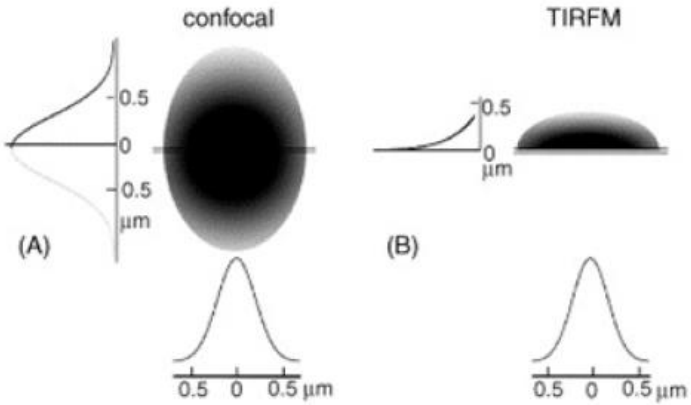
Figure 2

Advantages of the evanescent field

Evanescent Wave Exponential Intensity Decay



Point-spread function for confocal *versus* TIRF



FWHM of point-spread function:

Confocal 300 nm X 800 nm

TIRF 250 nm X ~100 nm (length constant)

Cell Focal Adhesions in Widefield and TIR Fluorescence

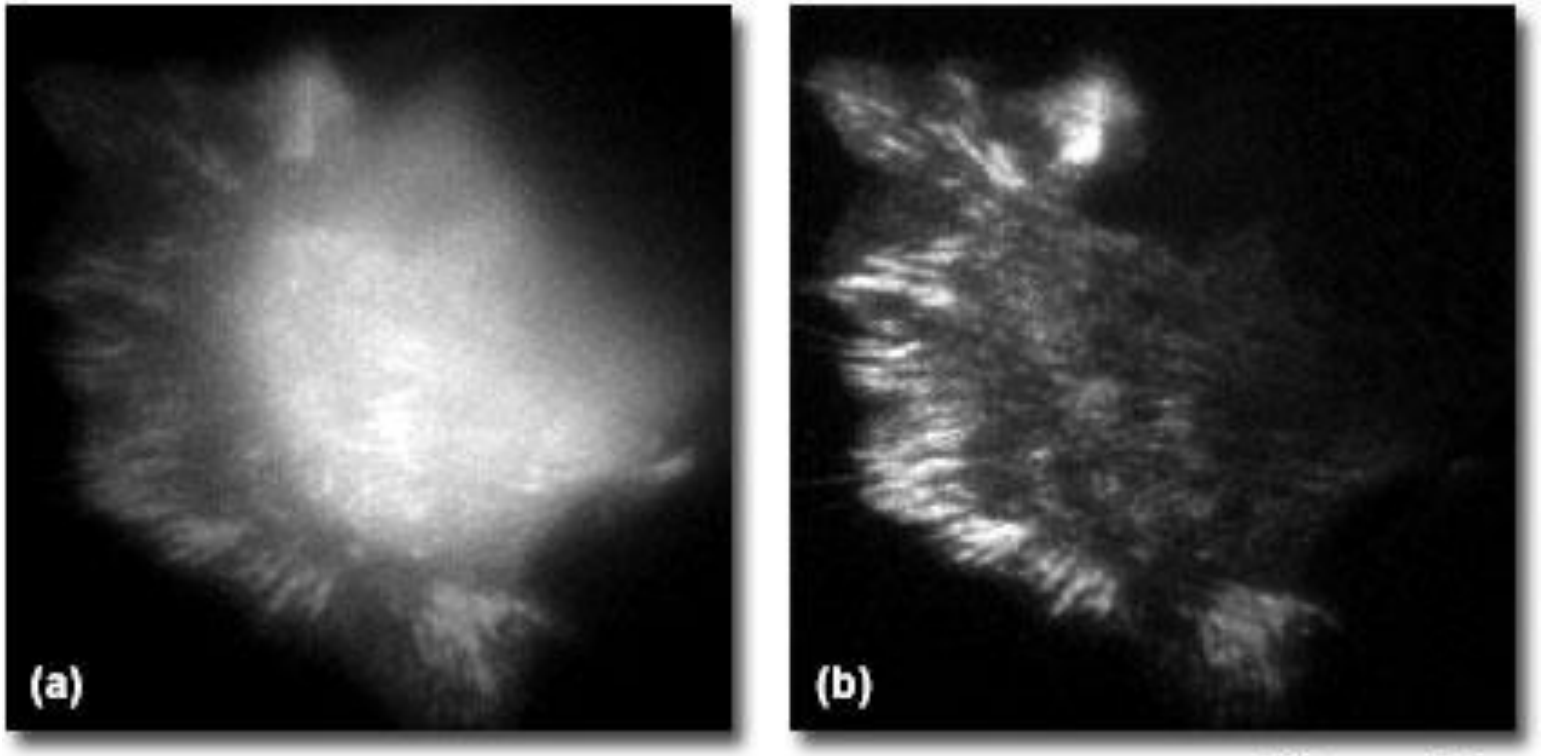
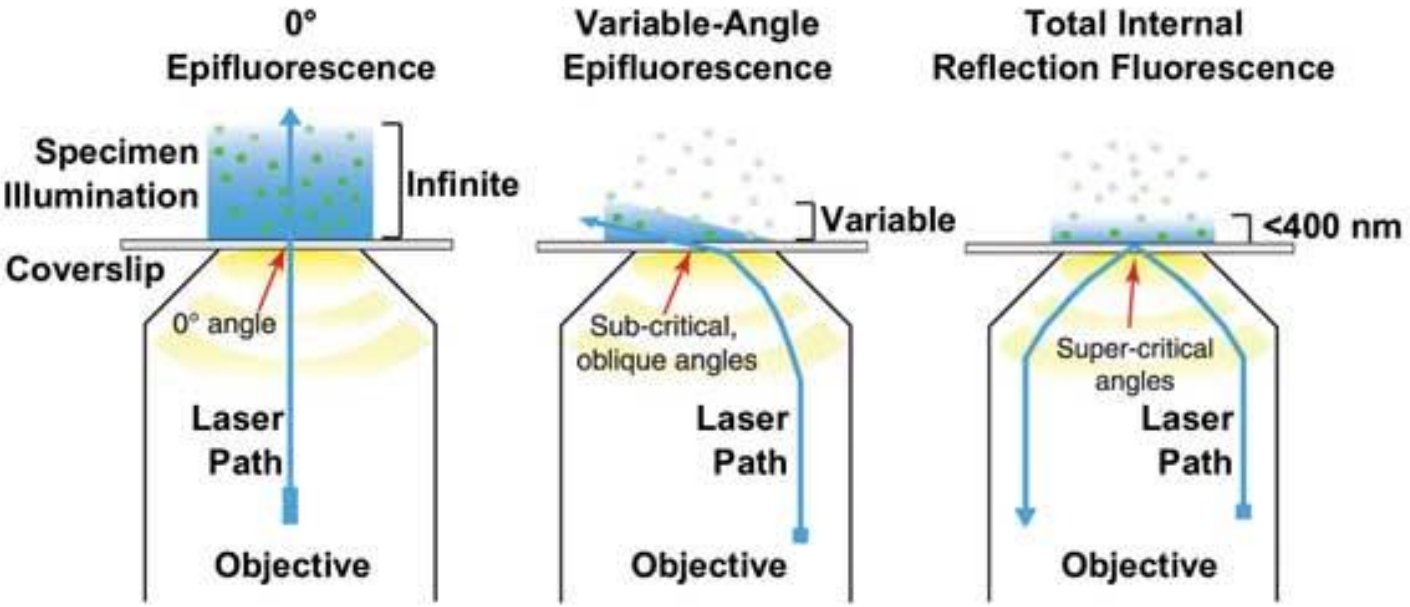


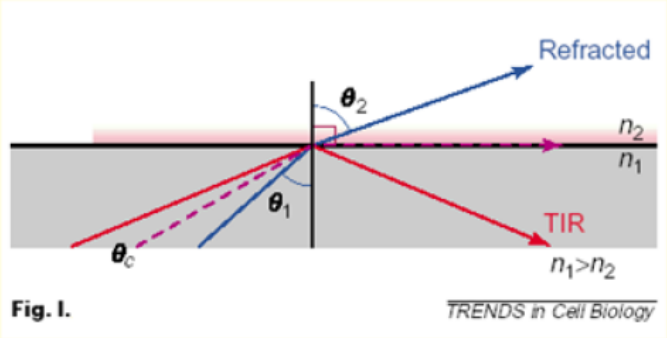
Figure 5

http://www.microscopyu.com/articles/fluorescence/tirf/tirfintro.html


0°
Epifluorescence
Specimen
Illumination
Infinite
Coverslip
0° angle
Laser
Path
Objective
Variable-Angle
Epifluorescence
Variable
Sub-critical,
oblique angles
Laser
Path
Objective
Total Internal
Reflection Fluorescence
<400 nm
Super-critical
angles
Laser
Path
Objective

Some key TIRF formulas

But electric and magnetic fields **cannot** be discontinuous at a boundary, therefore:



Evanescent field:

$$I_z = I_0 \exp^{-z/d_p}$$

$$d_p = \frac{\lambda}{4\pi\sqrt{n_1^2 \sin^2\theta_1 - n_2^2}}$$

I = intensity, z = distance, λ = wavelength, d_p = penetration depth

Snell's law:

$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$

θ = angle of incidence, n = refractive index

Critical angle:

At the critical angle, θ_c, $\theta_2 = 90°$; $\sin 90° = 1$;

$$n_1 \sin\theta_c = n_2$$

$$\theta_c = \sin^{-1}(n_2/n_1)$$

If $n_1 = 1.515$ and $n_2 = 1.36$, $\theta_c = \sin^{-1}\left(\frac{1.36}{1.515}\right) = 63.85°$

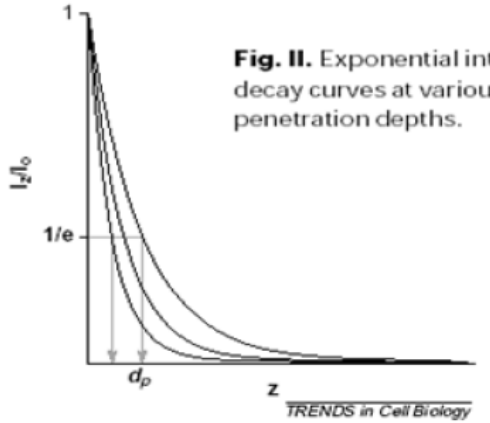


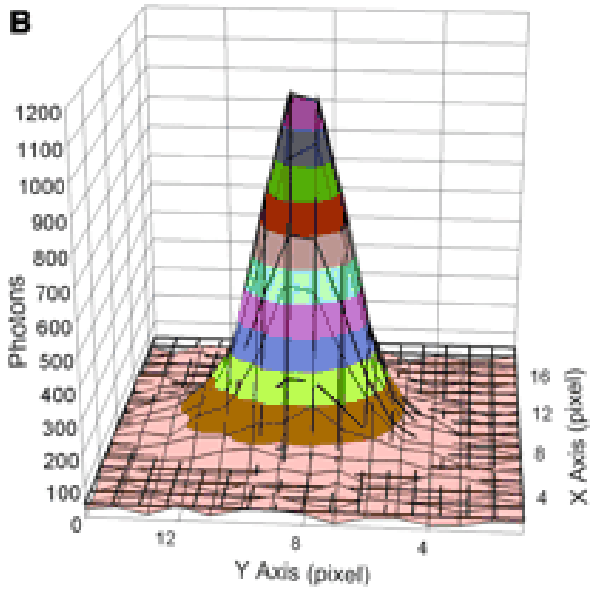
Fig. II. Exponential intensity decay curves at various penetration depths.

Toomre and Manstein (2001) Trends in Cell Biol 11:298


OLYMPUS
Apo N
60x/1.49 Oil
∞/0.13–0.19/FN22
23℃
OLYMPUS
UApo N
100x/1.49 Oil
∞/0.13–0.19/FN22
23℃
OLYMPUS
UApo N
150x/1.45 Oil
∞/0.13–0.19/FN22
23℃

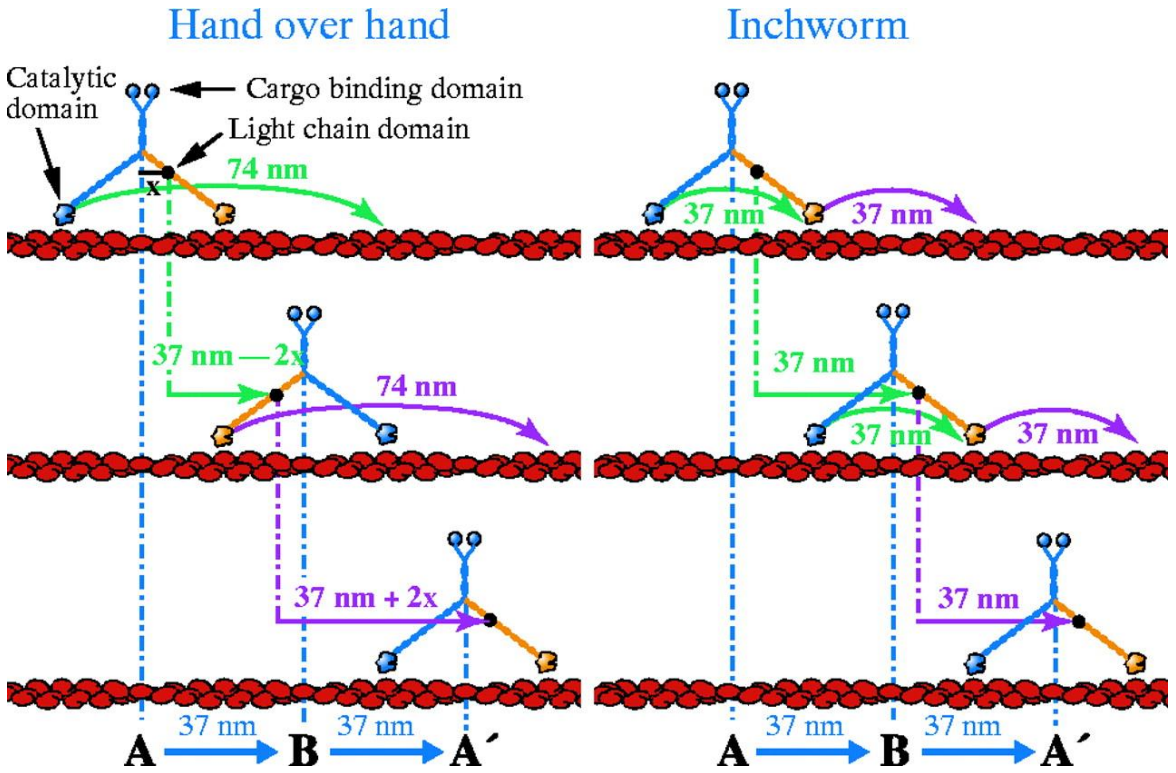
Single molecule imaging in vitro

Myosin V Walks Hand-Over-Hand: Single Fluorophore Imaging with 1.5-nm Localization.



Yildiz, A; Forkey, JN; McKinney, SA; Ha, T; Goldman, YE; Selvin, PR. (2003) Myosin V Walks Hand-Over-Hand: Single Fluorophore Imaging with 1.5-nm Localization. Science 300: 2061-2065.

FIONA: Myosin V Walks Hand-Over-Hand: Single Fluorophore Imaging with 1.5-nm Localization.



Yildiz, A; Forkey, JN; McKinney, SA; Ha, T; Goldman, YE; Selvin, PR. (2003) Myosin V Walks Hand-Over-Hand: Single Fluorophore Imaging with 1.5-nm Localization. Science 300: 2061-2065.

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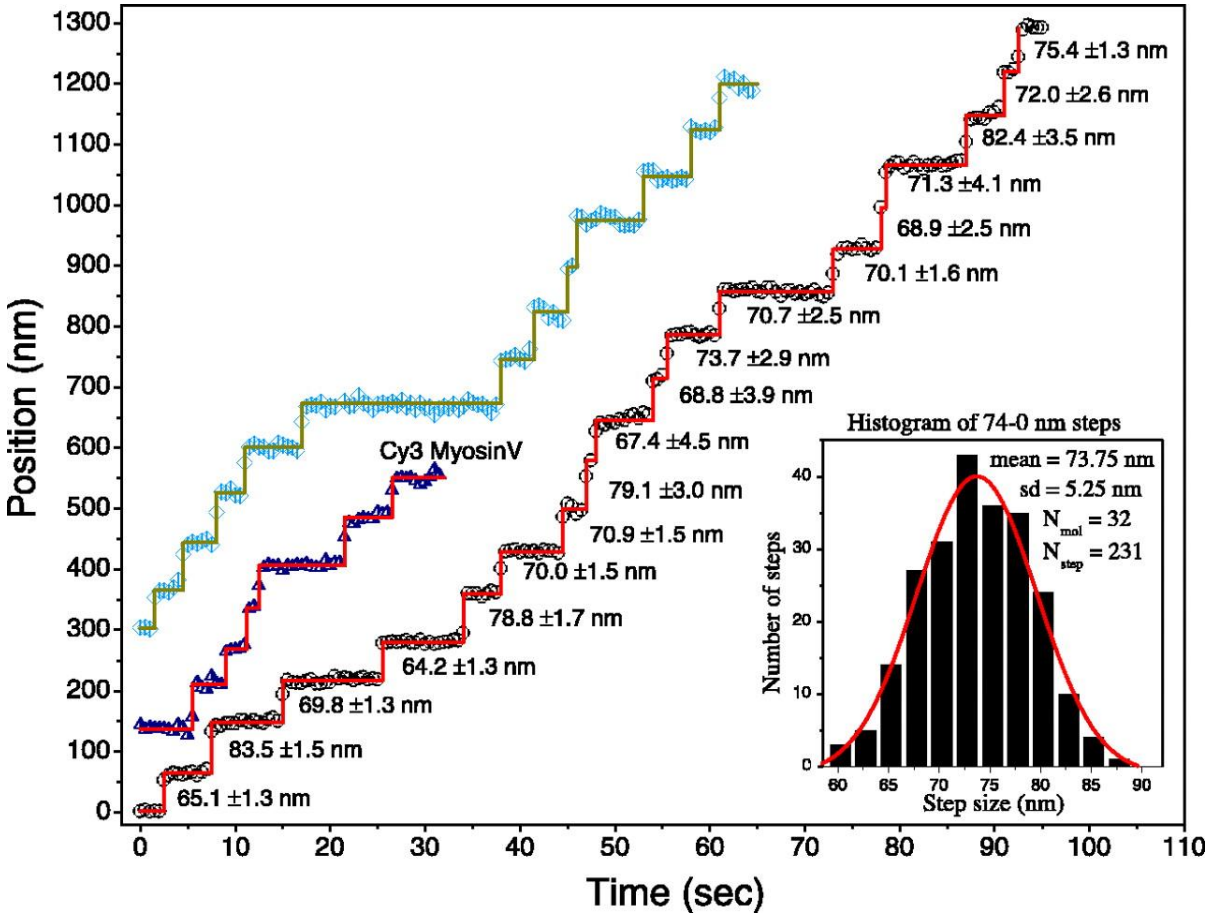
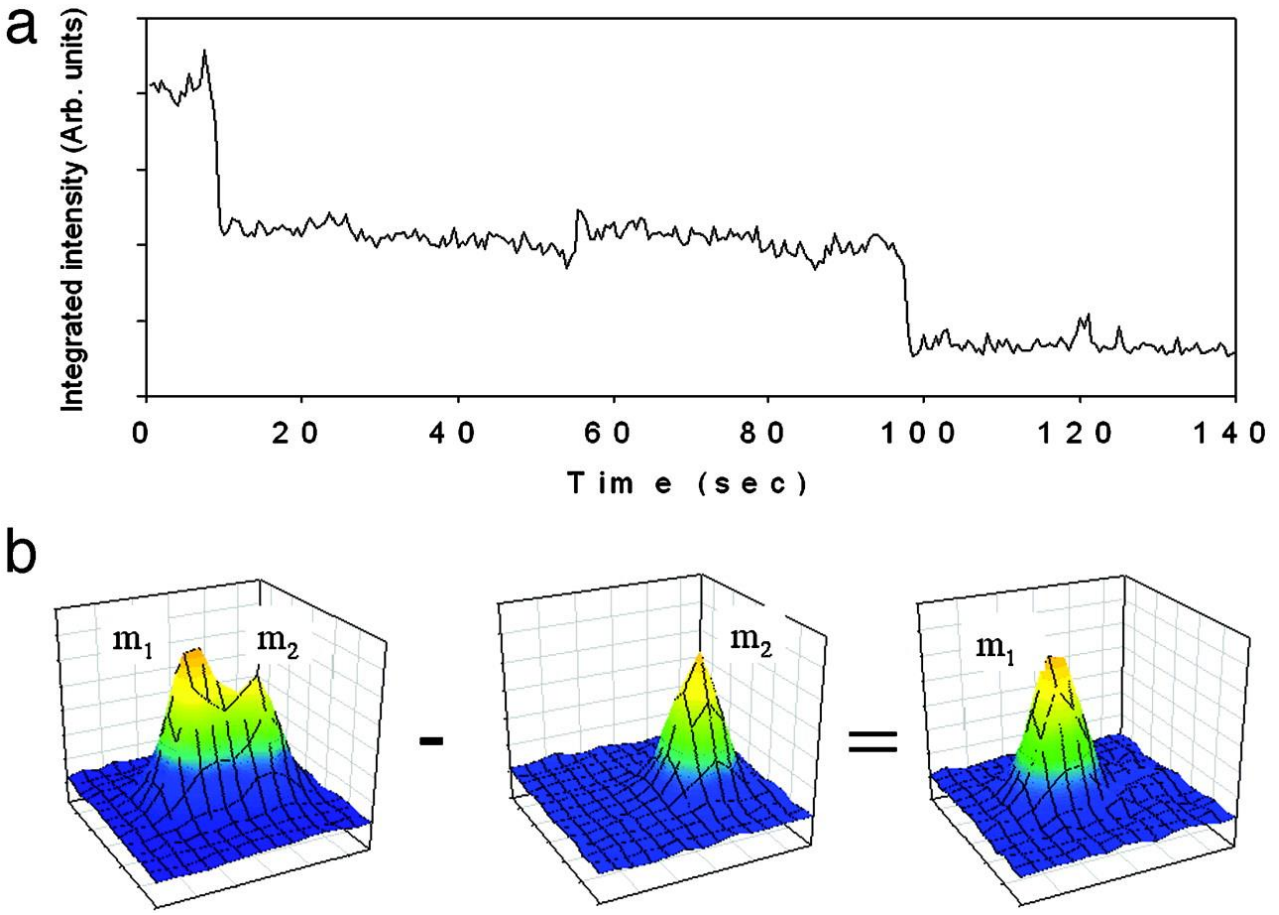


Yildiz, A; Forkey, JN; McKinney, SA; Ha, T; Goldman, YE; Selvin, PR. (2003) Myosin V Walks Hand-Over-Hand: Single Fluorophore Imaging with 1.5-nm Localization. Science 300: 2061-2065.

Single-molecule high-resolution imaging with photobleaching

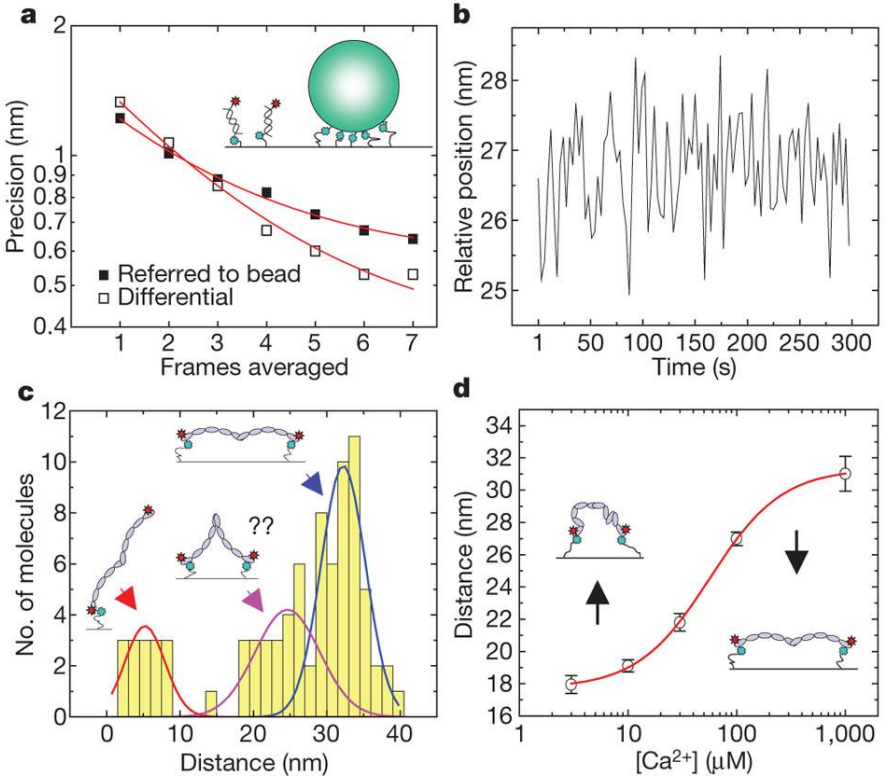
A plot of total integrated intensity versus time for two closely spaced Cy3 molecules, showing a two-step photobleaching behavior.



Gordon, MP; Ha, T; Selvin, PR. (2004) Single-molecule high-resolution imaging with photobleaching. PNAS 101: 6462-6465.

PNAS

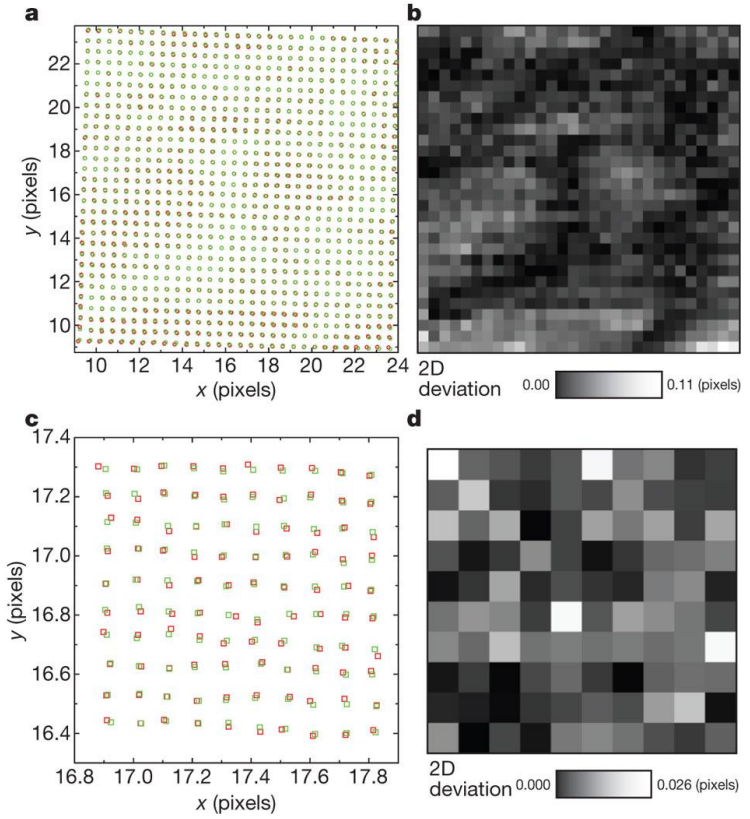
Measurements of molecular-scale structures using same-colour probes with active feedback control.



Pertsinidis, A; Zhang, Y; Chu, S. (2010) Subnanometre single-molecule localization, registration and distance measurements. Nature 466, 647–651.

nature

Mapping function calibrations.

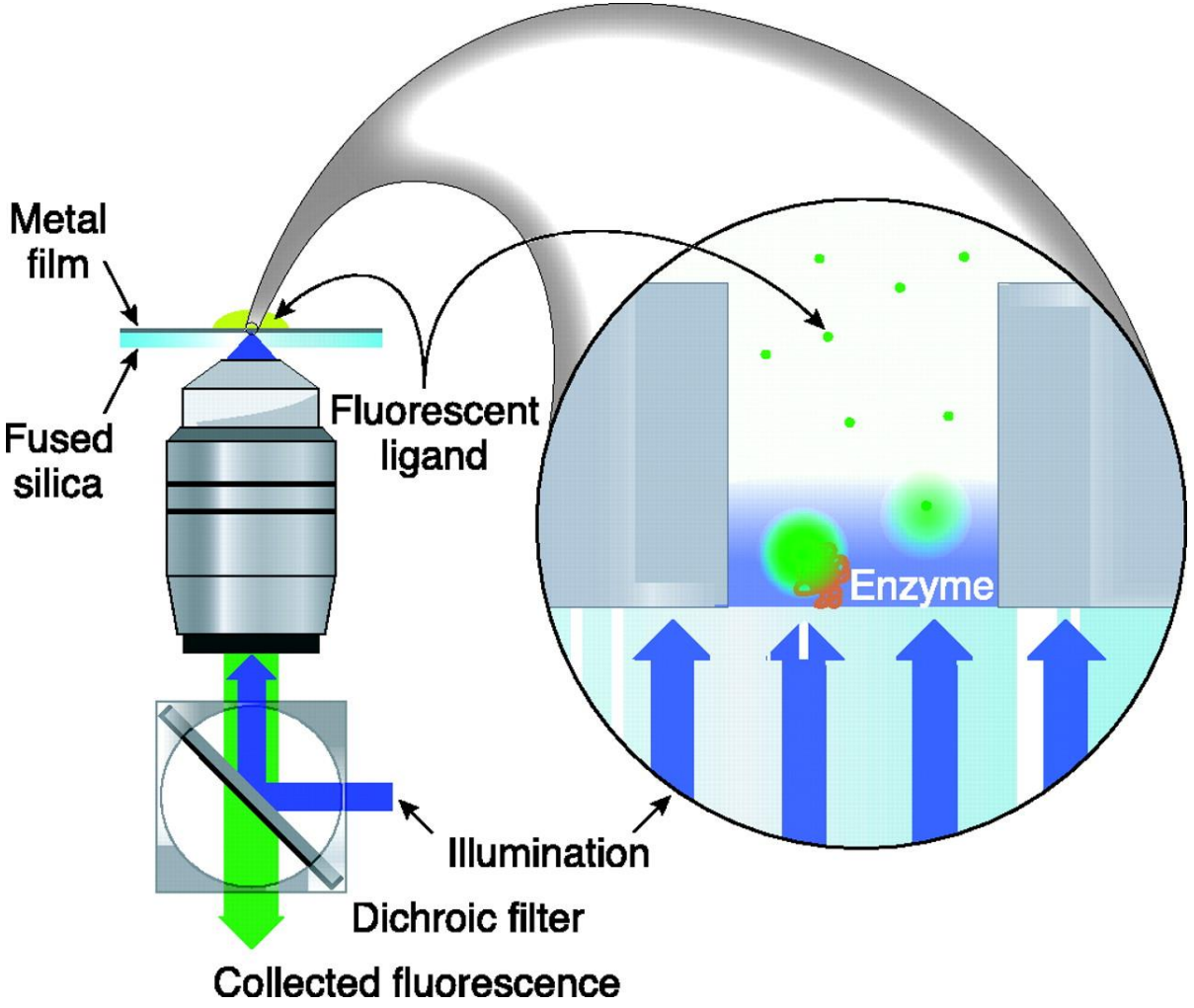


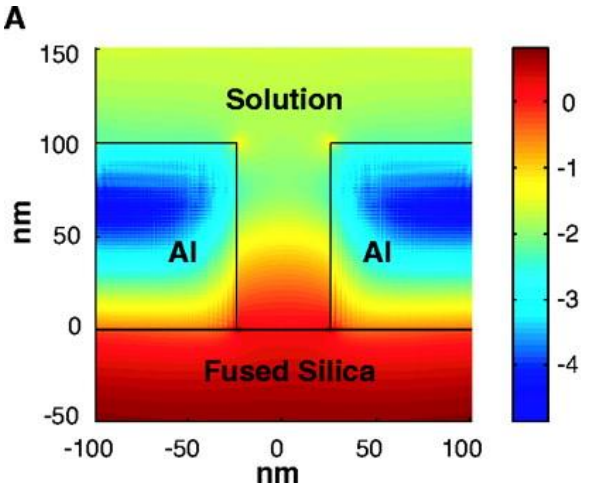
Pertsinidis, A; Zhang, Y; Chu, S. (2010) Subnanometre single-molecule localization, registration and distance measurements. Nature 466, 647–651.

nature

Another Approach – Zero mode waveguides

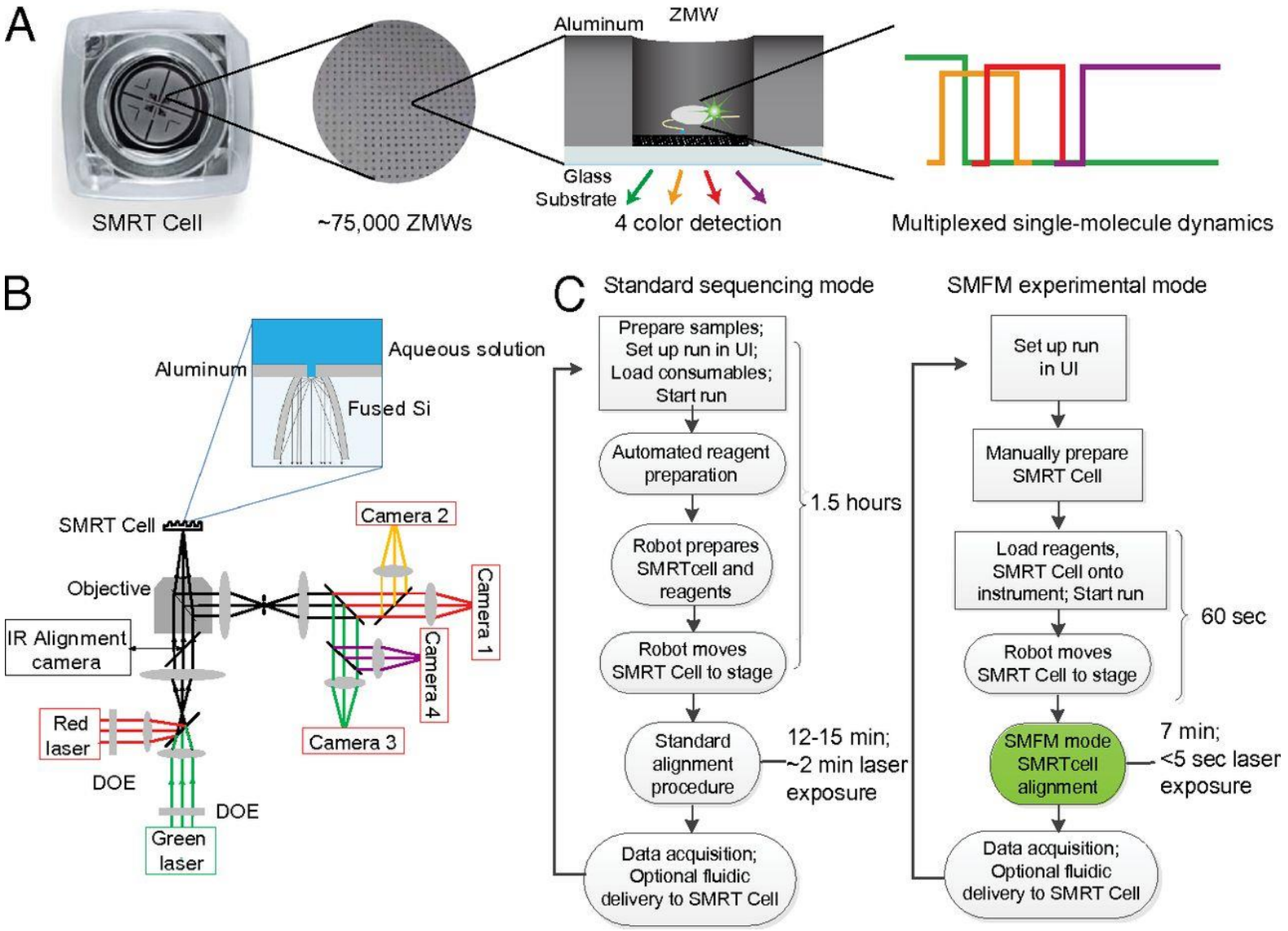
Zero-Mode Waveguides for Single-Molecule Analysis at High





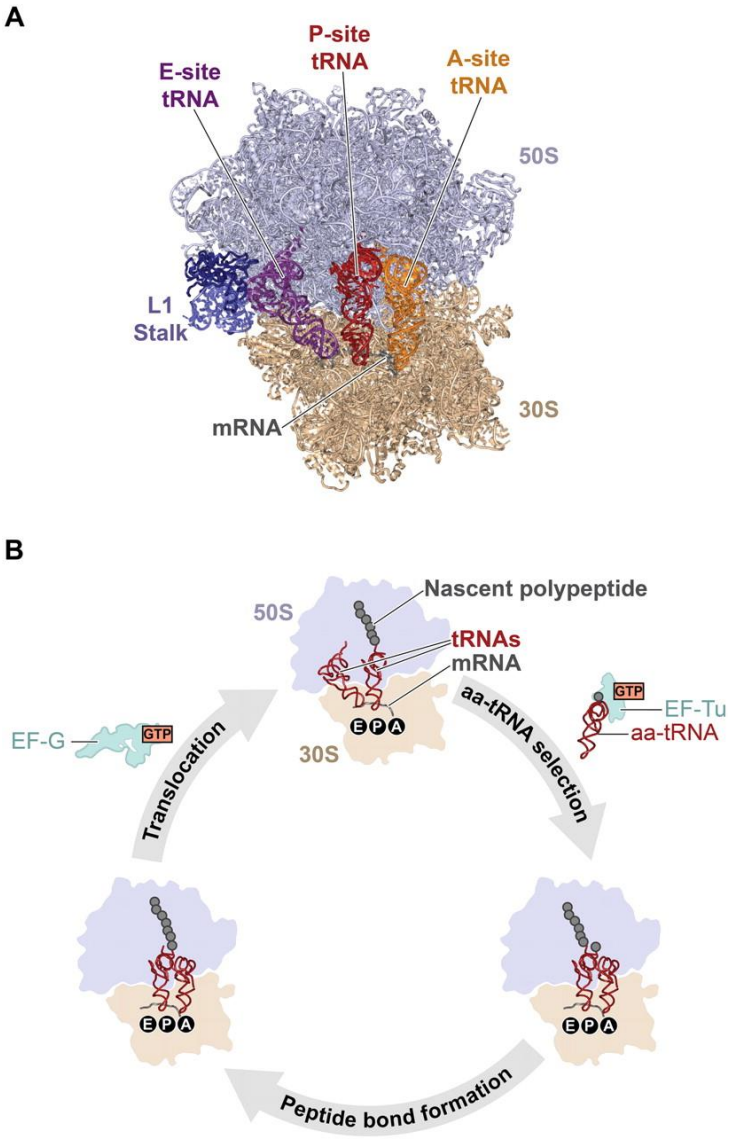
Levene, MJ; Korlach, J; Turner, SW; Foquet, M; Craighead, HG; Webb, WW. (2003) Zero-Mode Waveguides for Single-Molecule Analysis at High Concentrations. Science, 299:682-686

Overview of the customized RS instrument.



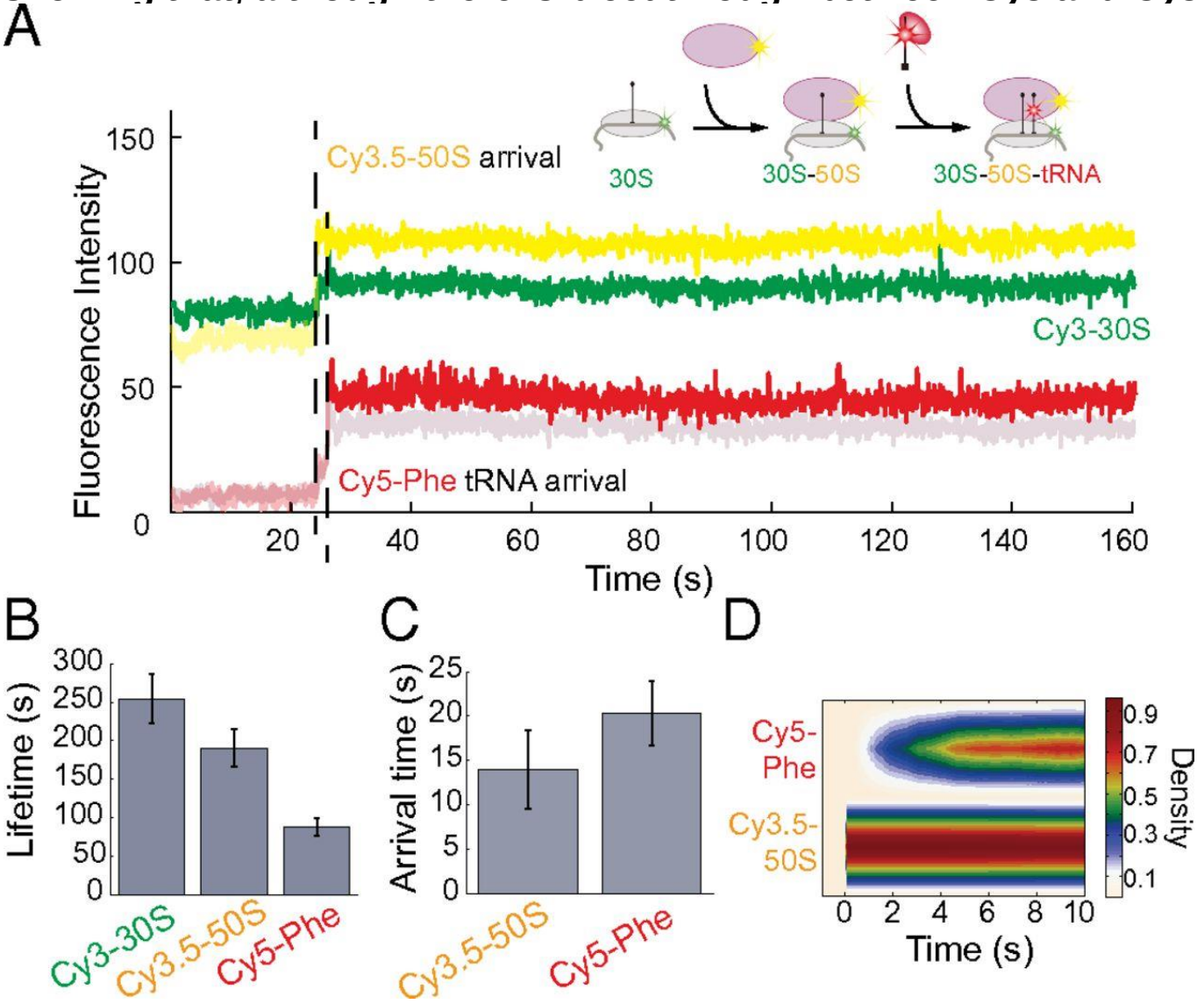
Chen J et al. PNAS 2014;111:664-669

(A) Structure of the ribosome.



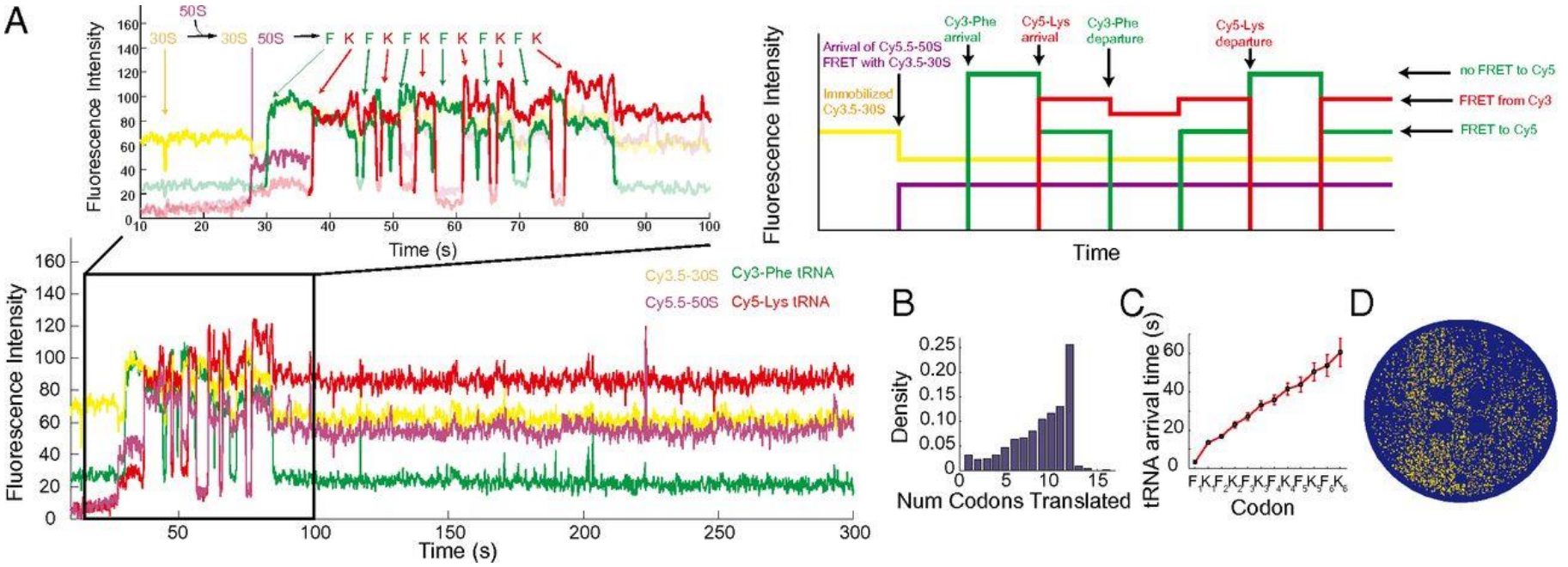
Tinoco I , and Gonzalez R L Genes Dev. 2011;25:1205-1231

Compositional dynamics of fluorescent ligands on the custom RS. (A) Schematic of the expected signal sequence and example trace of ribosome compositional dynamics during late initiation showing that, although there is bleedthrough between Cy3 and Cy3.5 chan...



Chen J et al. PNAS 2014;111:664-669

Multiplexed four-color dynamic experiment on the custom RS. (A) Sample trace and schematic showcasing the power of the custom RS to follow simultaneously the composition and conformation of four components.



Chen J et al. PNAS 2014;111:664-669

Dynamic Single Molecule Imaging – Single Particle Tracking

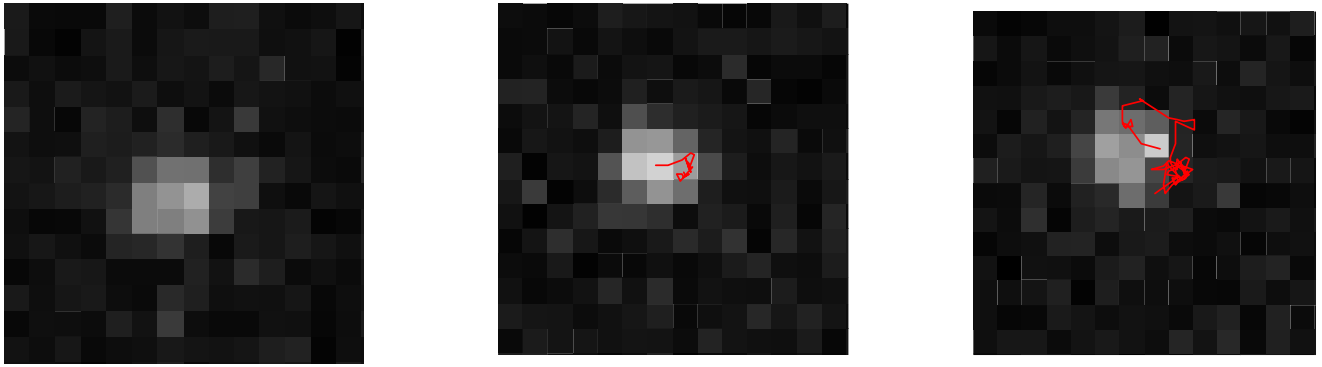
Data Analysis

- 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 (~250 nm)



Gold

- Detectable by brightfield microscopy

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

- Particles > 30 nm diameter are detectable

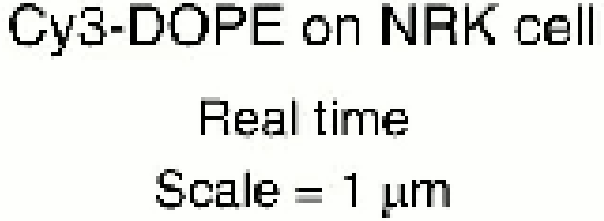
- Conjugation of protein to gold particles depends upon
  (a) ionic attraction between negatively charged gold and positively charged protein
  (b) hydrophobic attraction between the antibody and the gold surface;
  (c) dative binding between the gold conducting electrons and sulphur atoms which may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

Phospholipids undergo hop diffusion in compartmentalized cell membrane

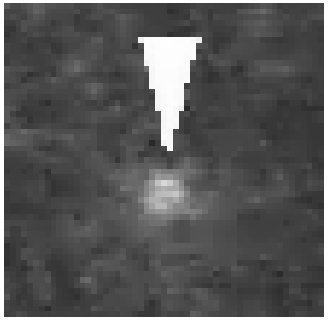
Takahiro Fujiwara,[1] Ken Ritchie,[1,2] Hideji Murakoshi,[2] Ken Jacobson,[3] and Akihiro Kusumi[1,2]

[1]Kusumi Membrane Organizer Project, Exploratory Research for Advanced Technology Organization (ERATO), Japan Science and Technology Corporation, Nagoya 460-0012, Japan
[2]Department of Biological Science, Nagoya University, Nagoya 464-8602, Japan
[3]Department of Cell and Developmental Biology and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599

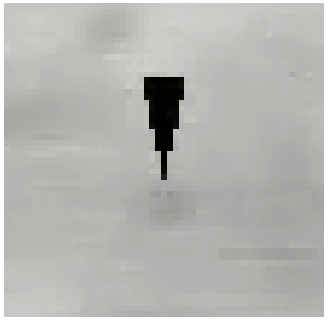
Supplementary Movie I



Single Cy3-DOPE



Single Gold-DOPE



Movement of single Cy3-DOPE molecules and a gold-tagged DOPE molecule on the NRK cell surface recorded at the video rate. Arrowheads in the first sequence indicate some of the DOPE molecules (with its fluorescence signal lasting longer than the average). Fluorescent spots are photobleached in single steps, indicating that these represent single Cy3-DOPE molecules.

Phospholipids undergo hop diffusion in compartmentalized cell membrane

Takahiro Fujiwara,[1] Ken Ritchie,[1,2] Hideji Murakoshi,[2] Ken Jacobson,[3] and Akihiro Kusumi[1,2]

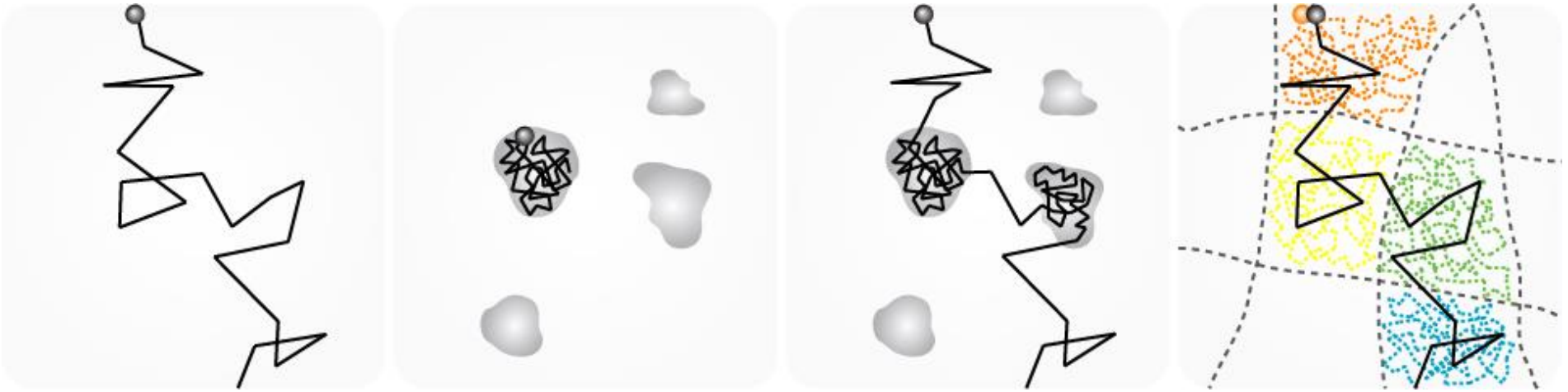
[1]Kusumi Membrane Organizer Project, Exploratory Research for Advanced Technology Organization (ERATO), Japan Science and Technology Corporation, Nagoya 460-0012, Japan
[2]Department of Biological Science, Nagoya University, Nagoya 464-8602, Japan
[3]Department of Cell and Developmental Biology and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599

The Journal of Cell Biology, Volume 157, Number 6, June 10, 2002 1071–1081
http://www.jcb.org/cgi/doi/10.1083/jcb.200202050

Supplementary Movie 2

Shows the hop diffusion of gold-tagged DOPE over 230-nm compartments observed at a 25-μs resolution for 62 ms (x270 slowed). In the second sequence, their trajectories are superimposed. In the trajectories, plausible compartments are shown in different colors, based on quantitative analysis. 750-nm compartments cannot be detected in the 62-ms period.

Diffusion in cell membranes is very heterogeneous



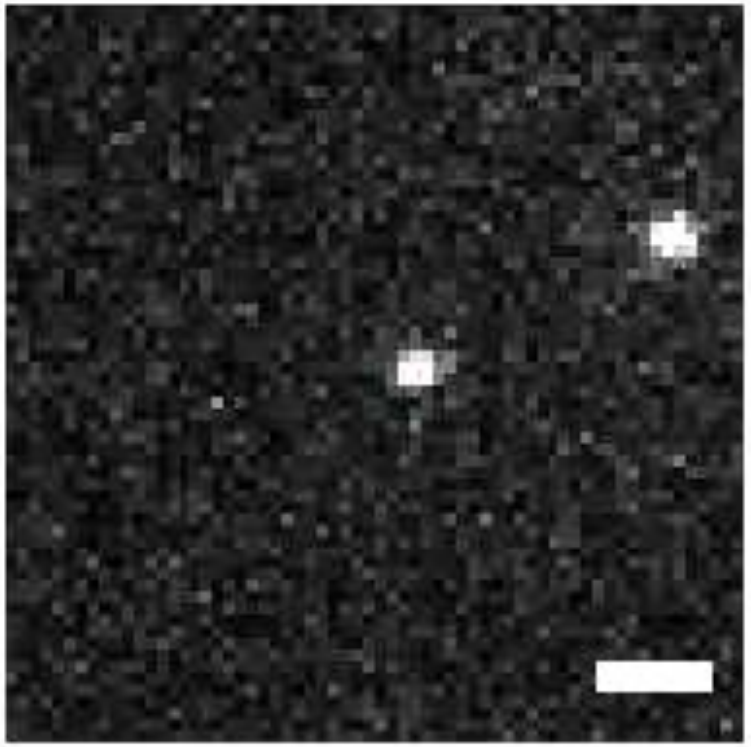
Free diffusion **Confined diffusion** **Mixed diffusion** **Hop diffusion**

From Clausen, M.P. & Lagerholm, B.C., *Curr Protein Pept Sci*, 2011)

Quantum Dot single molecule imaging

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

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



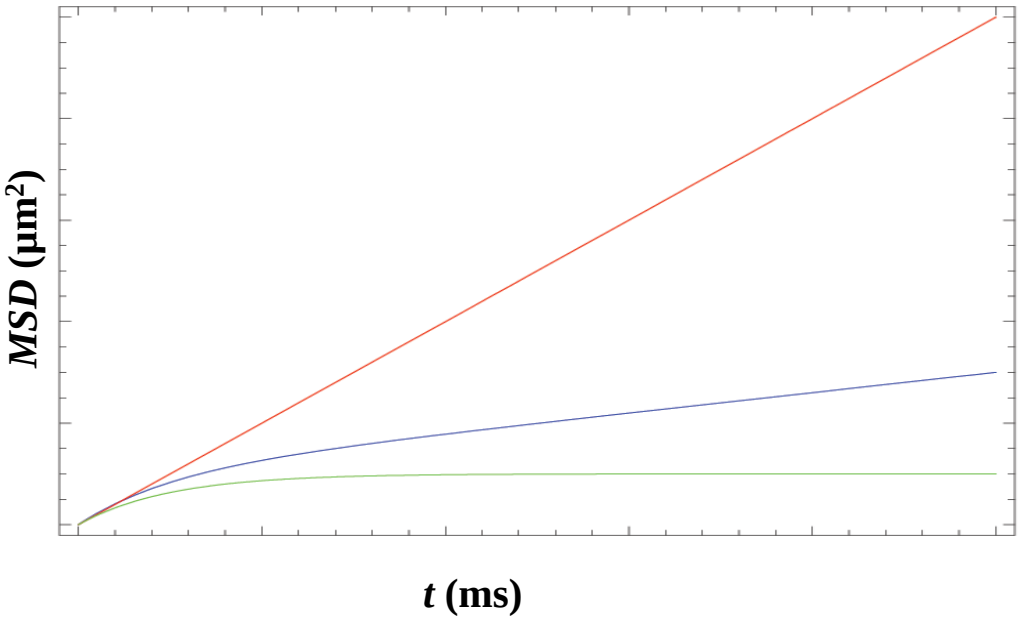
Playback 100 Hz, 2000 frames
Scale bar = 1mm

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:

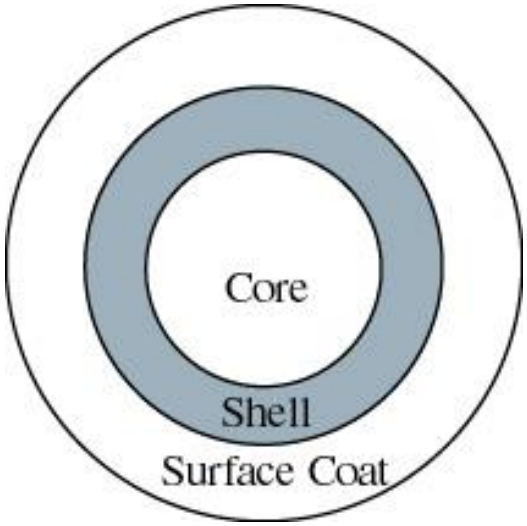
$L=\sqrt{(12\,D_\mu\,\tau)}$
(**confinement size**)

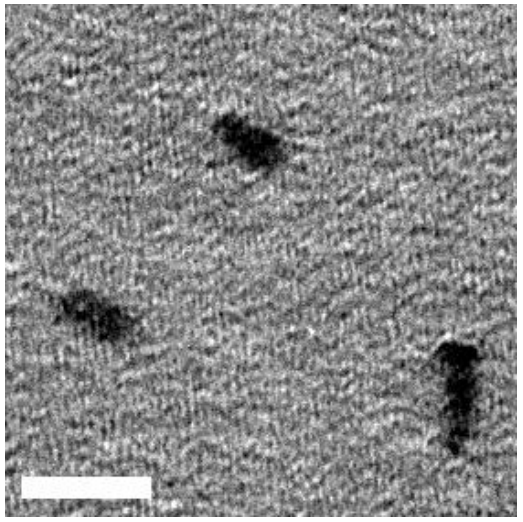
τ is the time constant at which the confinement boundary restricts free diffusion. The lifetime of the confinement time zones, τ_{conf}, is given by:

$\tau_{conf} = L^2 / D_{macro}$
(**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.

Quantum dot basics





Qdots are inorganic nanocrystals
(~10^2-10^3 atoms)

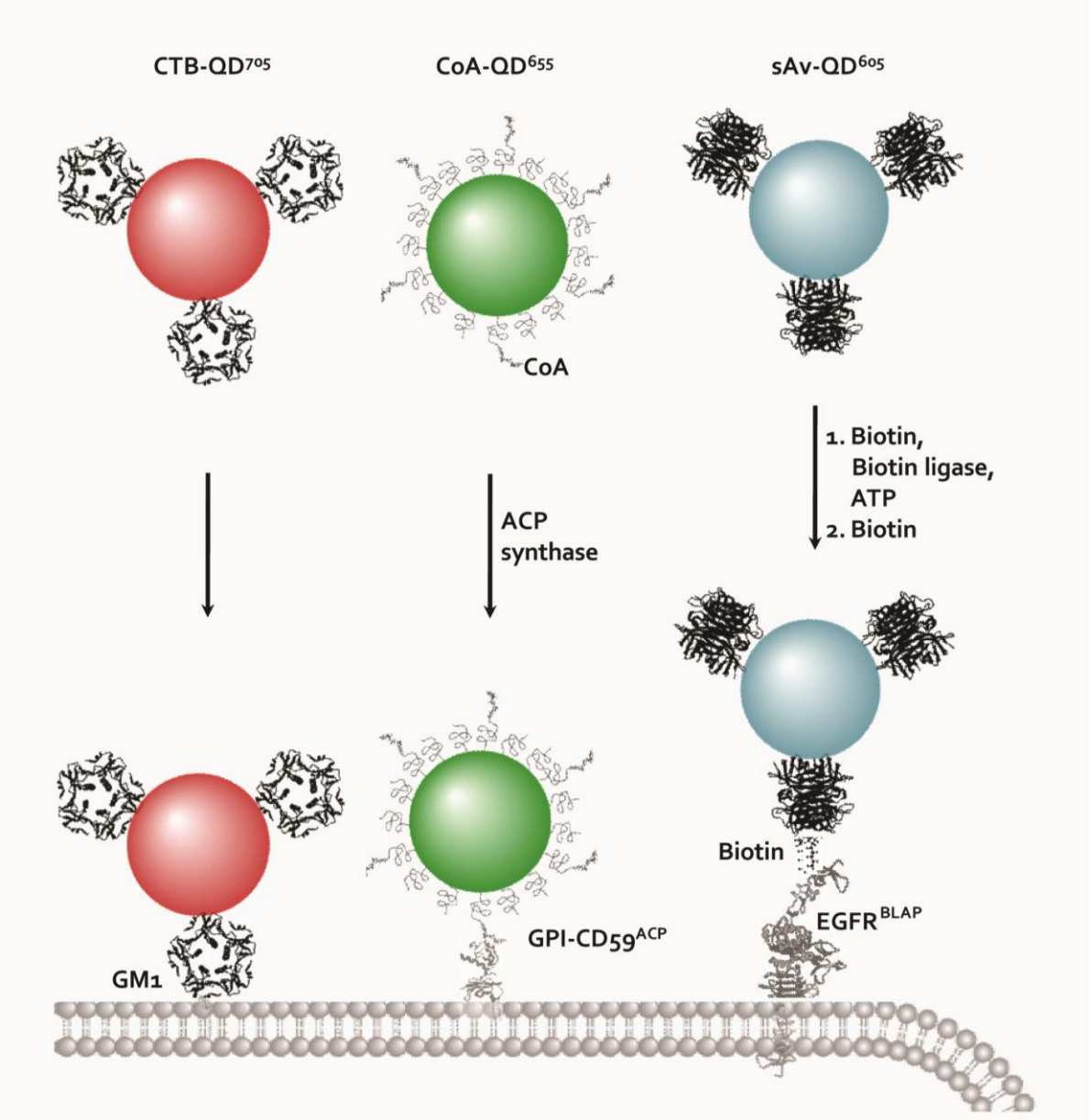
Core/Shell: Material determines wavelength range
CdSe/ZnS (visible)

Size determines exact wavelength
3 nm CdSe -> 520 nm emission
5.5 nm CdSe -> 630 nm emission

Surface Coat: Renders water solubility
Facilitates bioconjugation

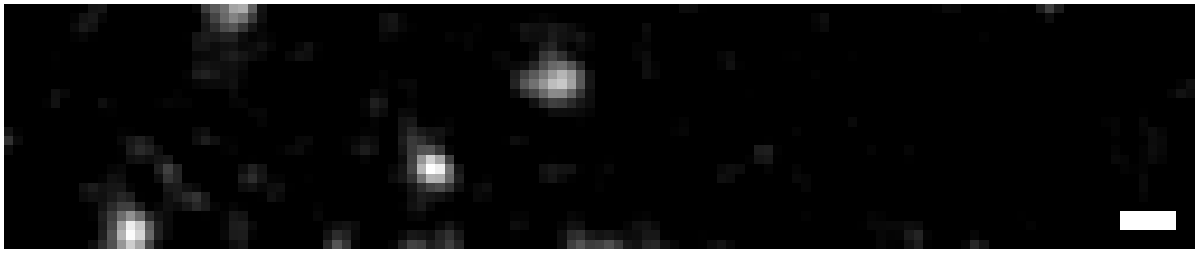
Overall size of 10 nm – 20 nm diameter

Parallel Targeting Schematic with Qdots

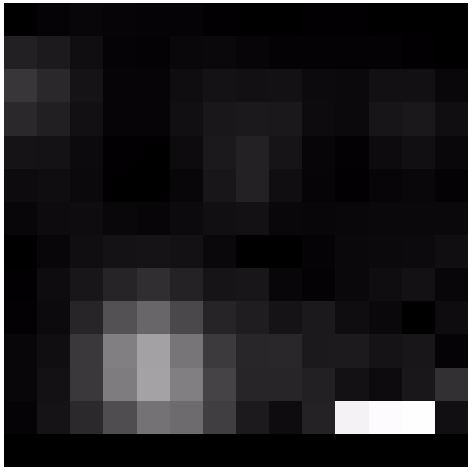


Hop diffusion?

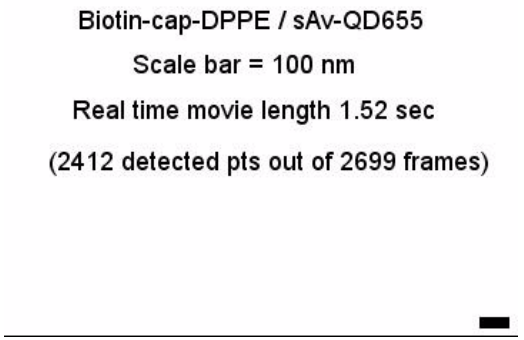
Super fast, 1700 Hz, 0.5 msec integration,
sAv-QD655 attached to Biotin-cap-DPPE in MEF

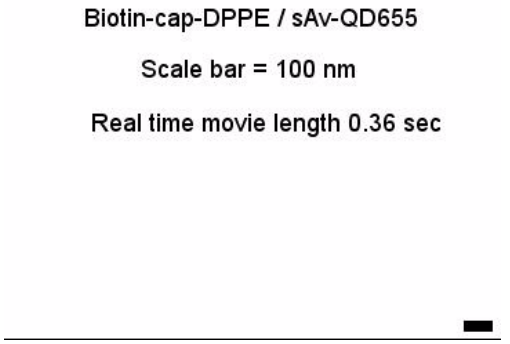


Scale bar = 1 μm



Scale bar = 0.5 μm



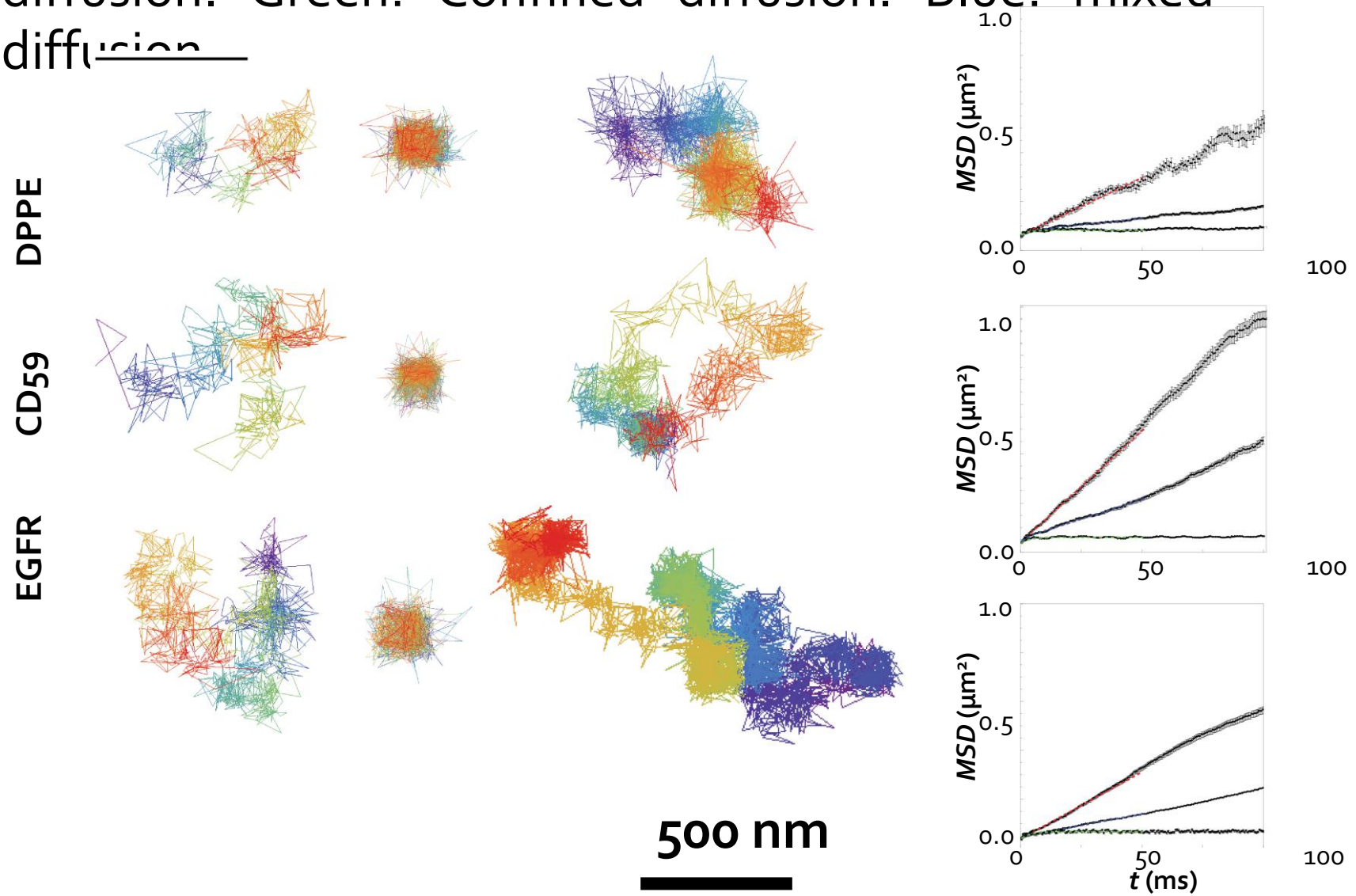


Δt=1.52 sec

Δt=0.36 sec

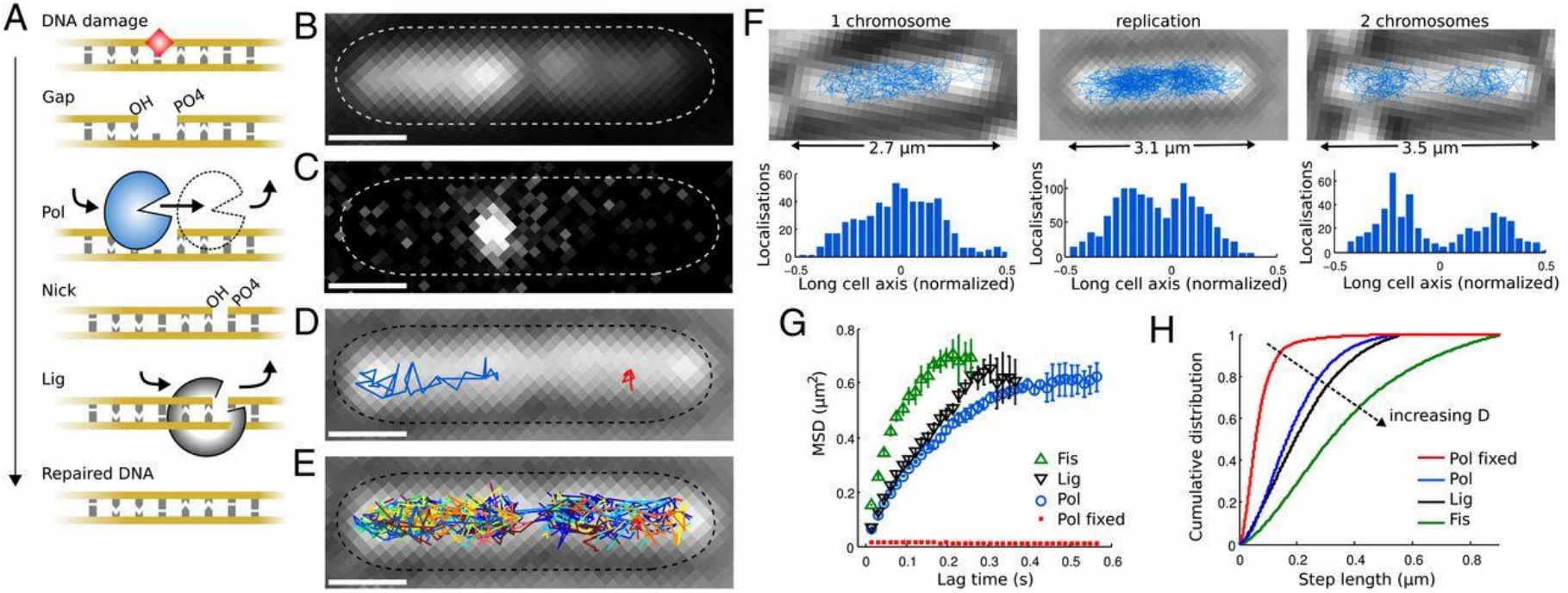
Trajectories: Representative examples of trajectories categorized according to the three different types of diffusion. The spatial precision of each position is ~30 nm.

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



SPT-PALM in living bacteria

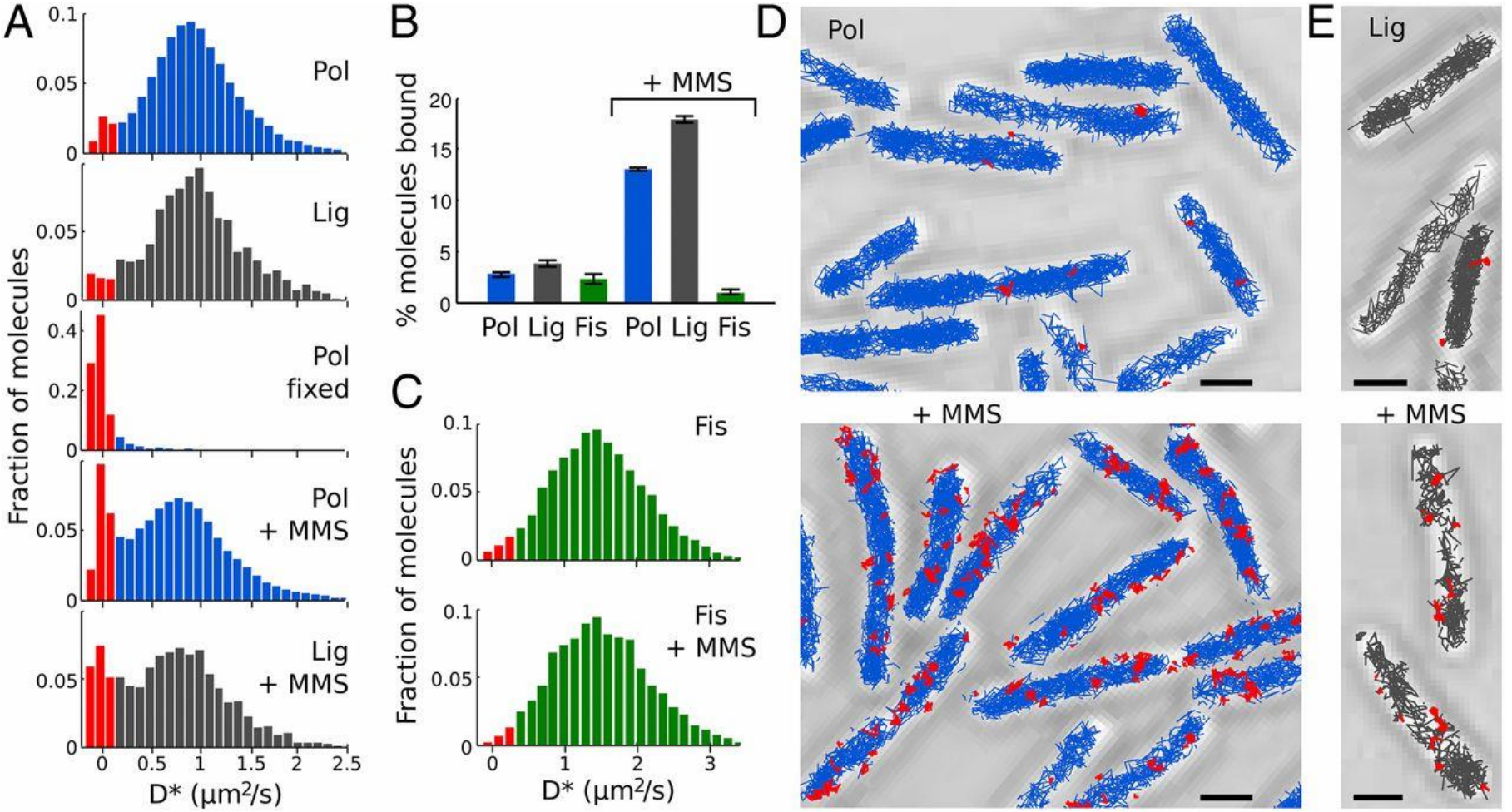
PALM and tracking of DNA-binding proteins in live E. coli.



Uphoff S; Reyes-Lamothe, R; de Leon, FG; Sherratt, D; Kapanidis, AN. (2013) Single-molecule DNA repair in live bacteria. PNAS 110:8063-8068

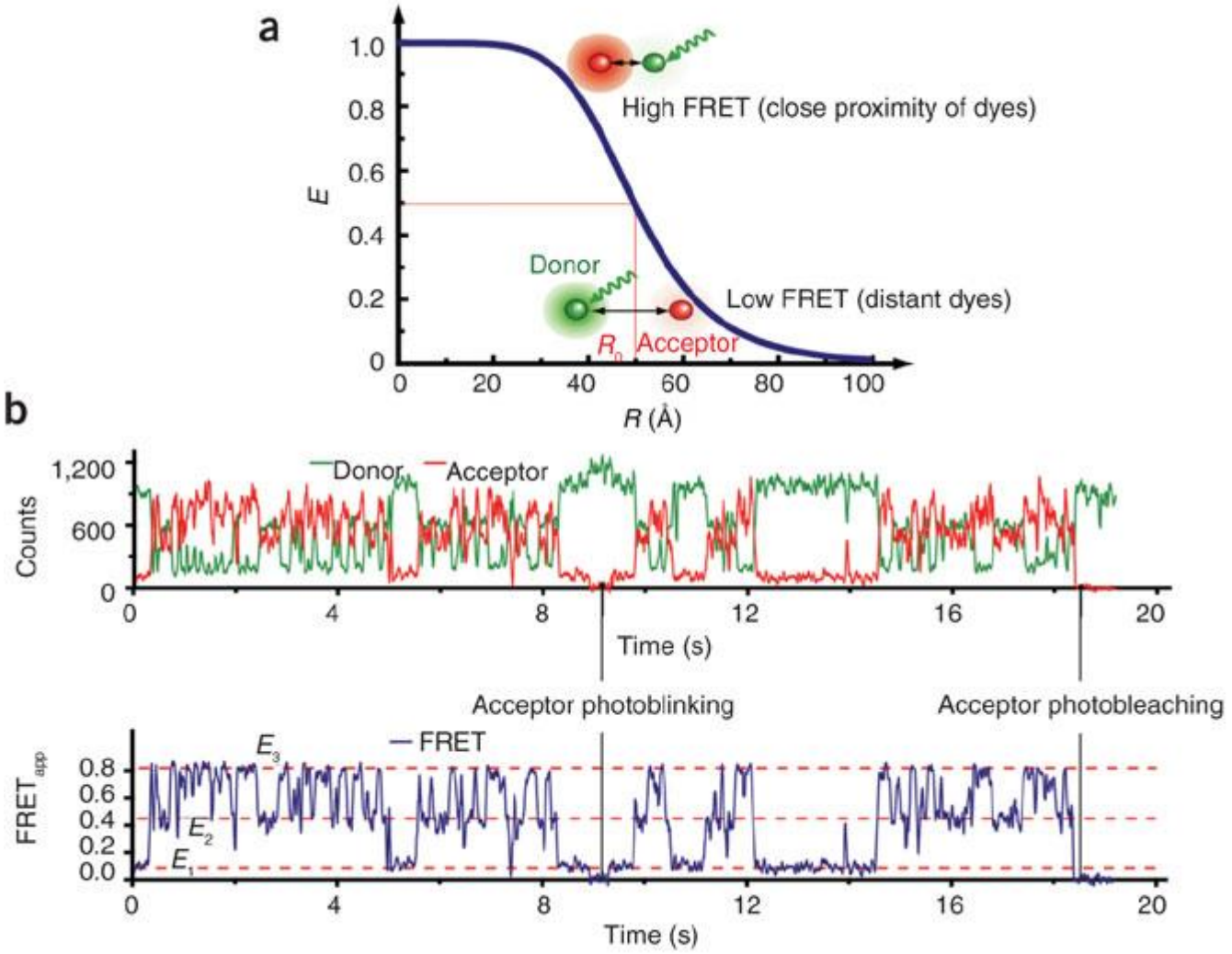
PNAS

Direct observation of DNA repair in live E. coli.



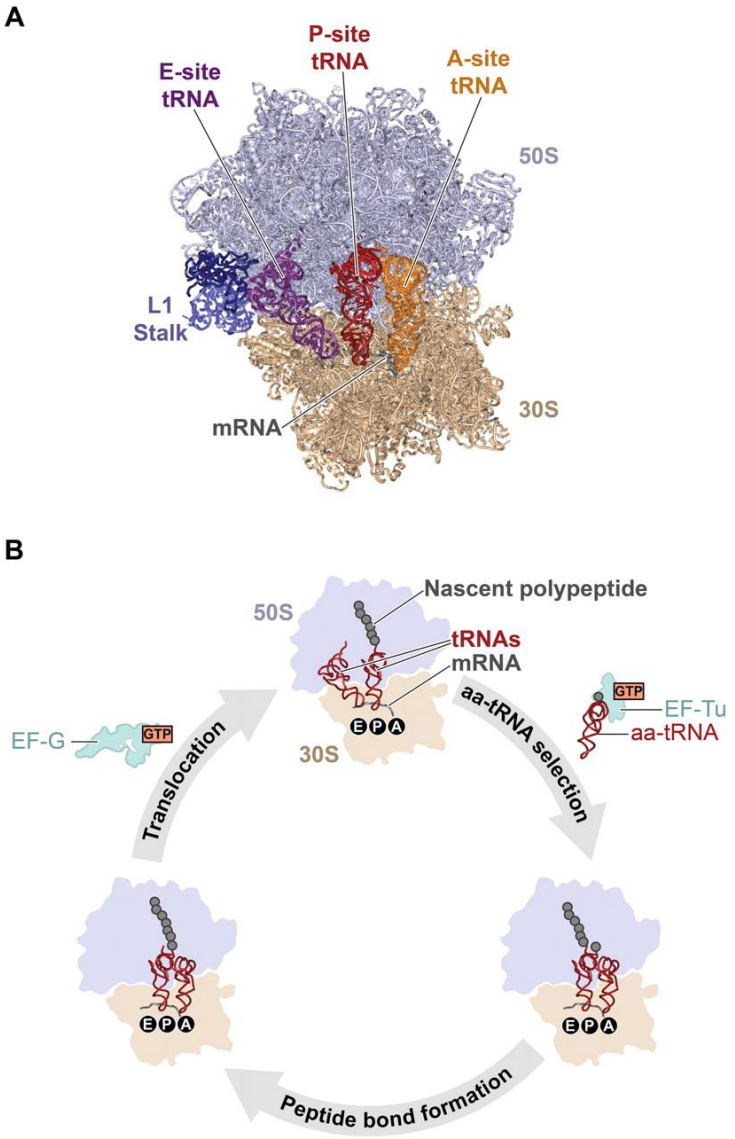
Uphoff S; Reyes-Lamothe, R; de Leon, FG; Sherratt, D; Kapanidis, AN. (2013) Single-molecule DNA repair in live bacteria. PNAS 110:8063-8068

Single Molecule FRET



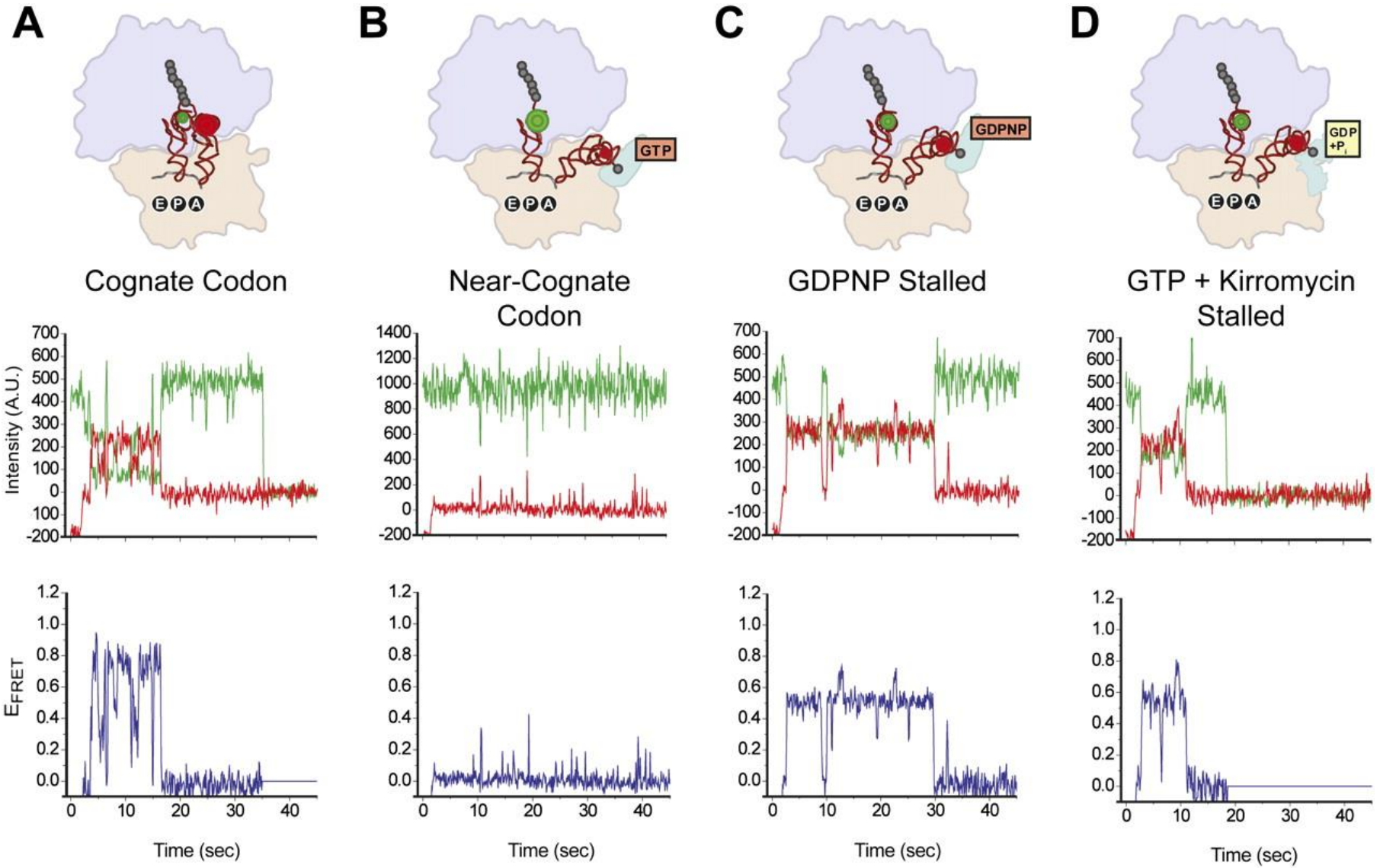
Rahul Roy, Sungchul Hohng & Taekjip Ha (2008) A practical guide to single-molecule FRET. Nature Methods - 5, 507 - 516

(A) Structure of the ribosome.



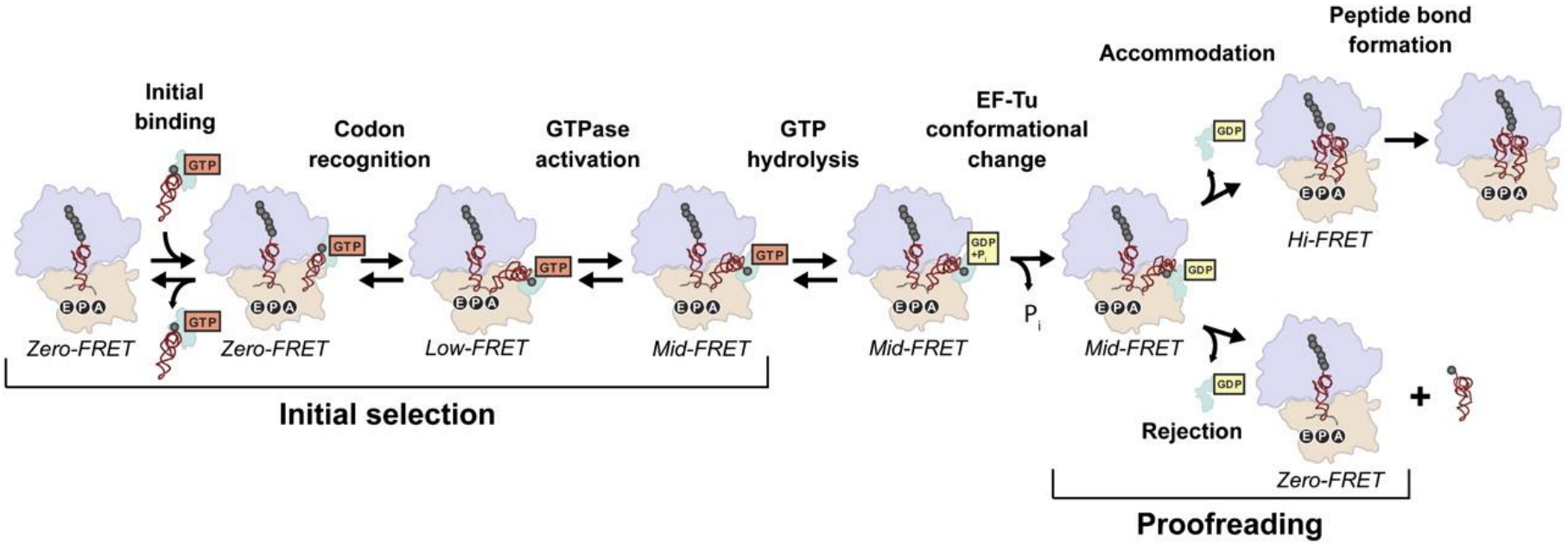
Tinoco I , and Gonzalez R L Genes Dev. 2011;25:1205-1231

Pre-steady-state EFRET versus time trajectories obtained using TIRF microscopy of INI complexes undergoing aa-tRNA selection under various experimental conditions.



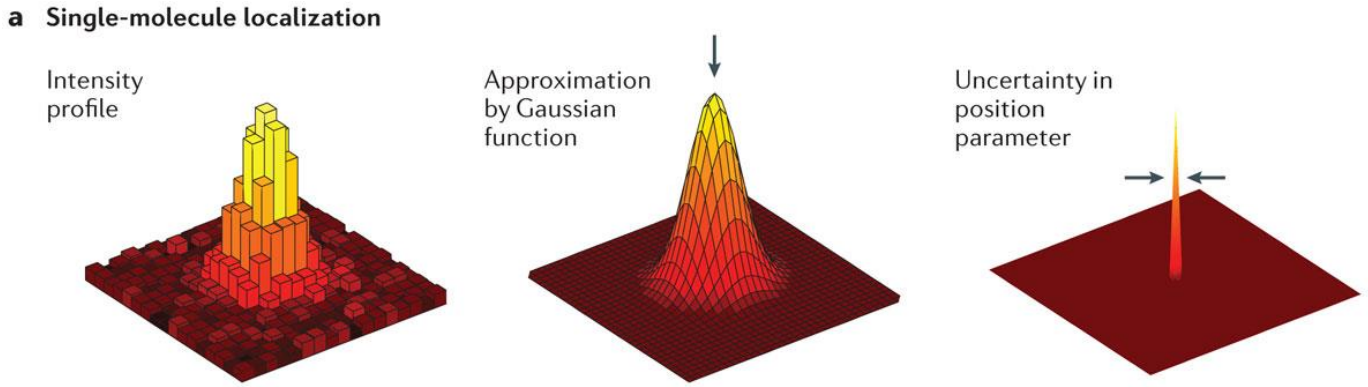
Tinoco I , and Gonzalez R L Genes Dev. 2011;25:1205-1231

The kinetic mechanism of aa-tRNA selection.



Tinoco I , and Gonzalez R L Genes Dev. 2011;25:1205-1231

Principles of single-molecule tracking and imaging.



Gahlman, A; Moerner, WE. (2014) Exploring bacterial cell biology with single-molecule tracking and super-resolution imaging. *Nature Reviews Microbiology* 12: 9–22.

Localization Precision for Individual Fluorescent Probes

$$\delta^{x,y^2} = \frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\,\pi\, s_i^4 b^2}{a^2 N^2}$$

Si = STD of PSF

N = # of photons

a = size of pixels

b = STD of background

Thompson, RE; Larson, DR; Webb, WW. (2002) Precise Nanometer Localization Analysis for Individual Fluorescent Probes. Biophysical J. 82: 2775–2783.