Single-image molecular analysis for accelerated fluorescence imaging

Yan Mei Wang

Department of Physics
Washington University in St. Louis

Washington University in St.Louis

Outline

1. Current single-molecule localization, separation, and dynamic measurement methods and challenges

2. Use single-image molecular analysis (SIMA) to determine

    a. Axial-localization precision

    b. Separations of unresolved molecules

    c. Diffusion coefficients of proteins in free solution

3. Applications

    a. Intraflagellar transport particle dynamics – BBSome and IFT

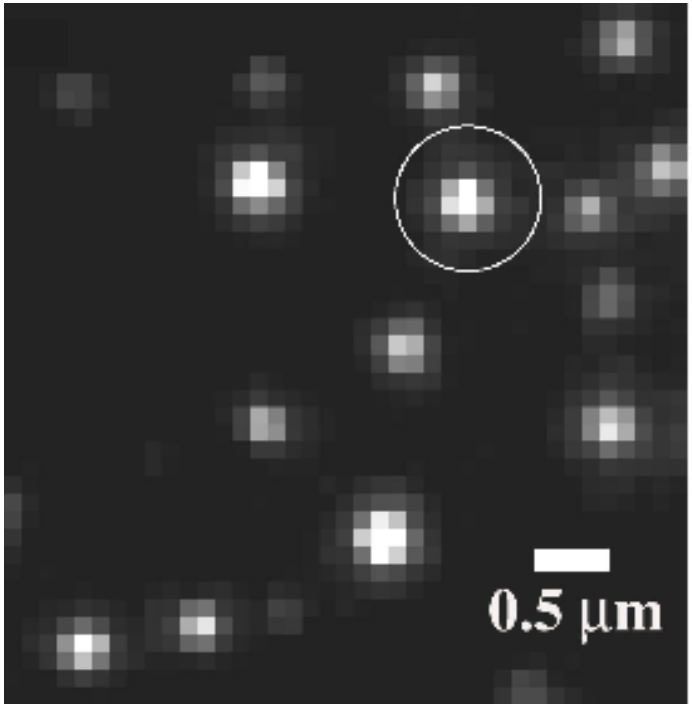
    b. Membrane-binding proteins –UgtP

    c. Photosynthesis - Phycobilisome

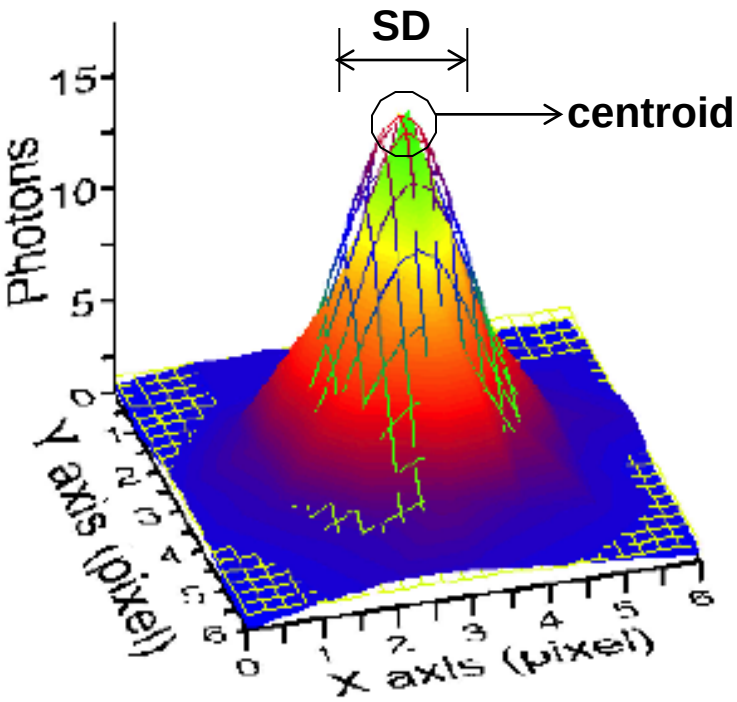
I. 3D Localization

Gaussian-approximated point spread function (PSF):

Centroid ➔ Lateral location, *x* and *y*
Standard deviation (SD) ➔ Axial location, *z*



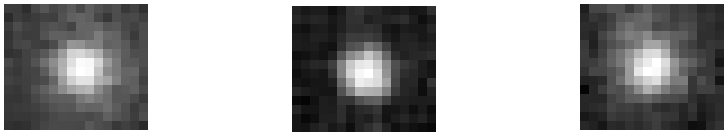
GFP, TIRF image



Y. M. Wang et. al., PNAS, 2005

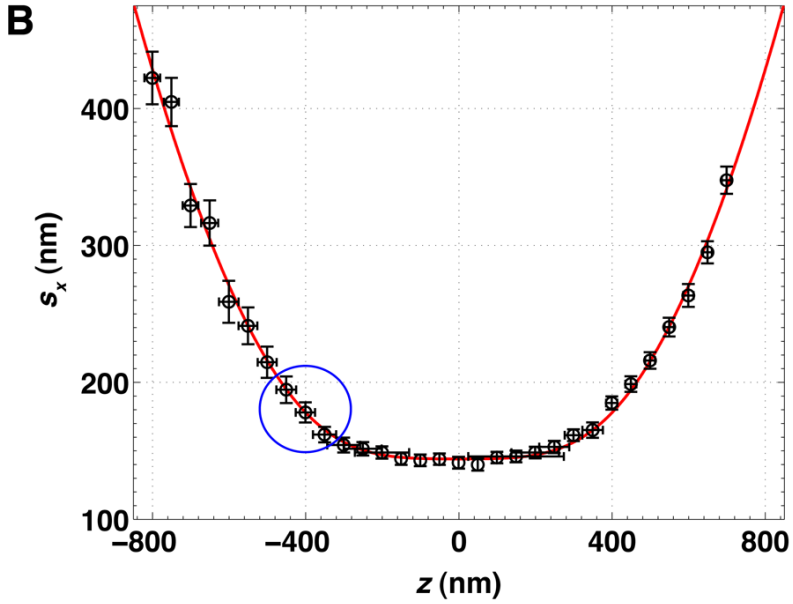
Axial location measurement, *z*

PSF Standard deviation, $s_{x,y}$, determines the axial location, *z*



- 400 nm **$z = 0$** **400 nm**

Phycobilisome protein axial location (relative to the focal plane)



$$s(z) = s_0\sqrt{1+\left(\frac{z}{d}\right)^2 + b\left(\frac{z}{d}\right)^4}$$

s_0 = SD at focus,
d = "imaging depth"
b = fitting parameter

Localization precision

Precision in *x*-direction:

$$\sqrt{\langle (\Delta x)^2 \rangle} = \sqrt{\frac{2(s_{0x}^2 + \frac{a^2}{12})}{N} + \frac{8\pi(s_{0x}^2 + \frac{a^2}{12})^{3/2}(s_{0y}^2 + \frac{a^2}{12})^{1/2}(\sigma_b^2 + \langle b \rangle)}{a^2 N^2}}$$

a **- pixel size**
$S_{0x/y}$ **- standard deviation (SD) in *x/y* direction**
N **- number of photons**
σ_b **- background noise standard deviation**
$\langle b \rangle$ **- background noise mean**

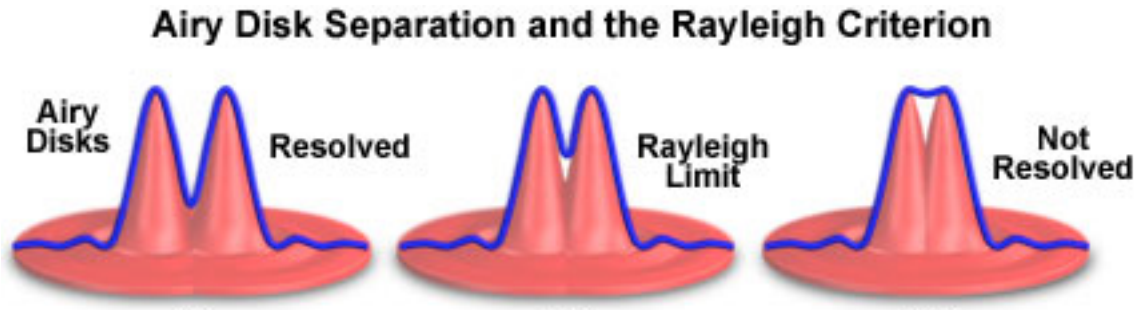
$\sqrt{\langle (\Delta x)^2 \rangle}$ **≈ 7 nm for 1000 photons**

- **Problem, there is no axial precision expression, Δz**

Repeated measurements are used to obtain Δz *(seconds of imaging)*

M. C. DeSantis, S. H. DeCenzo, J. L. Li, and Y. M. Wang, Optics Express, 2010
R. E. Thompson, D. R. Larson and W. W. Webb, Biophysical Journal, 2002

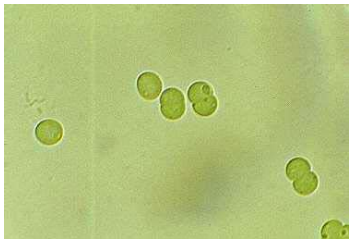
II: Separation measurements



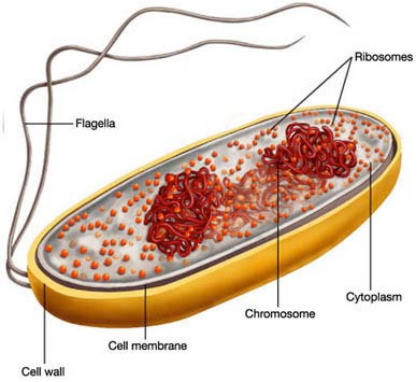
Rayleigh criterion separation = $0.61\lambda/NA \approx 240$ nm

λ = wavelength = 550 nm
***NA* = numerical aperture = 1.49**

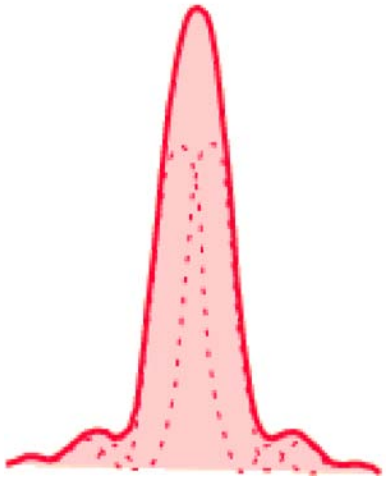
Synechocystis (3 μm cyanobacteria)



E. coli



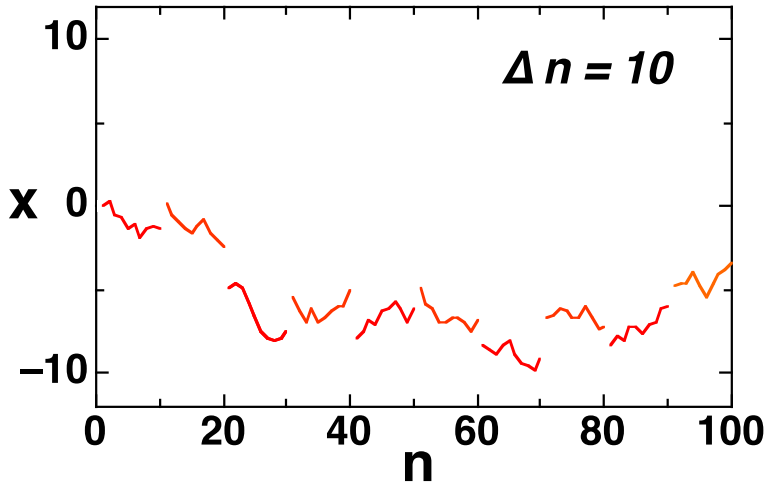
Disadvantages of current methods

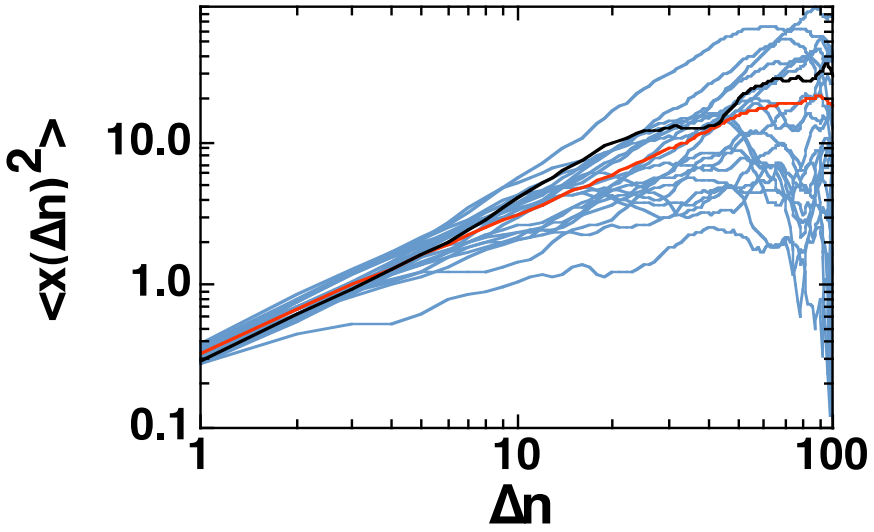


1. **Photobleaching of the molecule, SHRImP**
2. **Multiple color, SHREC**
3. **Photoswitchable fluorophores**
4. **Centroid measurements, long measurement time: > seconds**

III. Dynamics studies, single-molecule tracking

Simulation of individual Brownian trajectories





$\langle x(\Delta n)^2 \rangle = 2D_1 \Delta n$

$x(10)=(x_{11}-x_1), (x_{12}-x_2), \ldots (x_N-x_{N-10})$

$\langle x(\Delta n)^2 \rangle = 2D_1 \Delta n \pm \sigma(\Delta n, N)$

$\sigma(\Delta n, N) = 2D_1 \Delta n \, [(2\Delta n^2+1)/(3\Delta n(N-\Delta n+1)]^{1/2}$

$\Delta n = 1, 2, 3, \ldots N$

Qian et al., 1991

Limitation of current single-molecule D_3 measurements

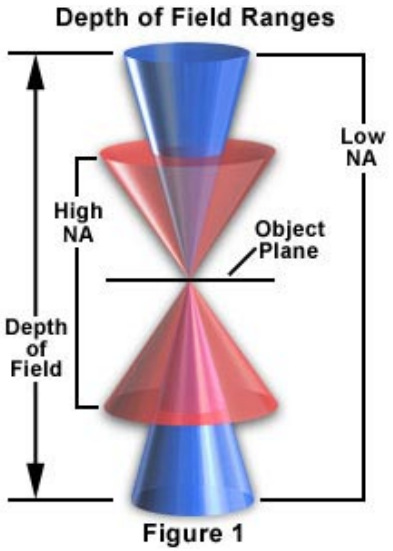


Figure 1

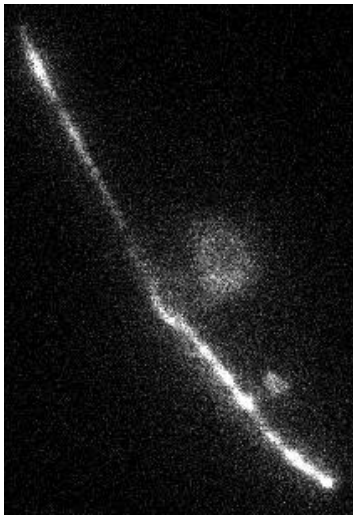
- Depth of imaging < *300* nm

- A *5* nm wide GFP, $D_3 \approx 10^8$ nm^2/s, moves out of the imaging depth in *1* ms
  - $\langle x^2 \rangle^{1/2} = 300\,nm = (2D_3t)^{1/2} \Rightarrow t = 1$ ms
  - With 100 Hz camera imaging rate, $D_{3,max} \approx 10^5$ nm^2/s

Recent $D_{3,max} \approx 2 \times 10^7$ nm^2/s measurements require two-color labeling
(*Stefan Semrau et al., BPJ, 2011)*

Biological systems need fast single-molecule investigations

Example: Intraflagellar transport - IFT



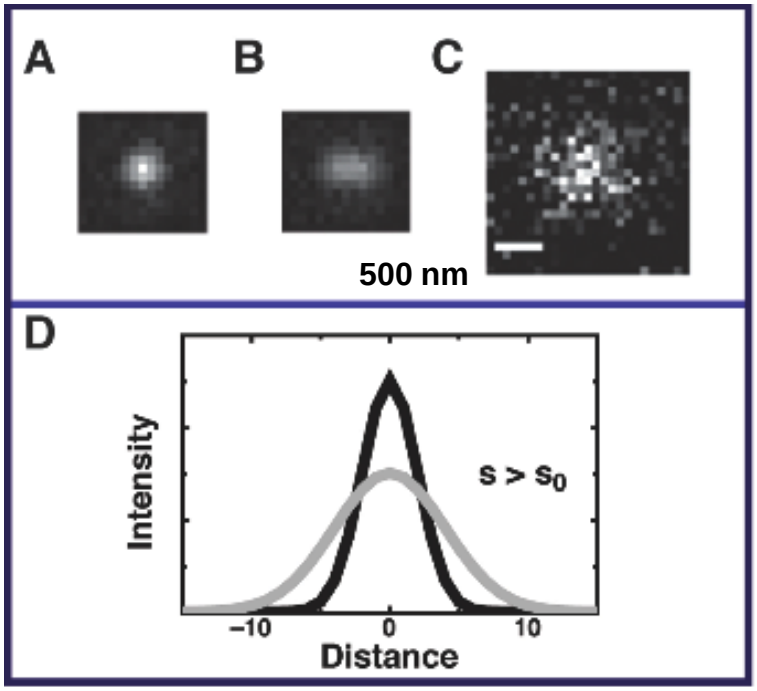
Kinesin-GFP, 2 μm/s

- **IFT particles travel to the flagellar tip and back**
- **Carry flagellar materials**
- **Carry signaling proteins**

Direct relevance to human disease:

such as, Bardet-Biedl syndrome & Polycystic Kidney Disease

Solution: analyze the spatial distribution of photons



Blurred moving car

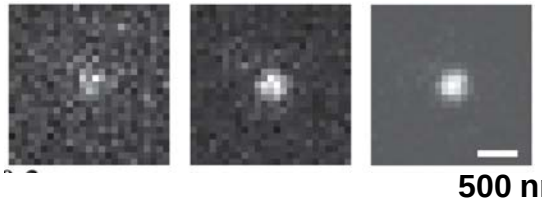
(A) A stationary molecule
(B) Two fluorophores separated by 237 nm
(C) A diffusing GFP in solution (1 ms exposure time)
(D) The SD difference.

Compromising the spatial resolution?

Temporal resolution = submillisecond-milliseconds = single-image exposure time

Spatial resolution = nanometers

N = 150 390 1,890 photons

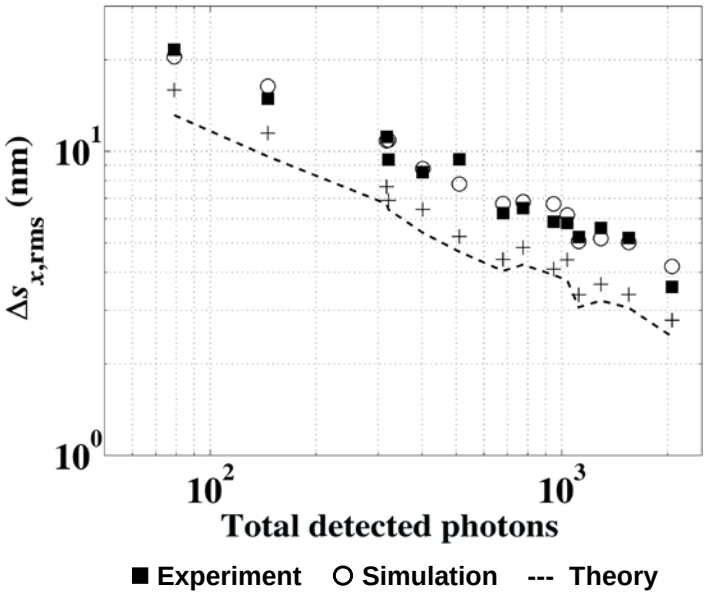


500 nm

Theoretical SD measurement error

$$\langle \Delta s_x \rangle = \sqrt{\frac{s_{0x}^2 + \frac{a^2}{12}}{2N} + \frac{16\pi (s_{0x}^2 + \frac{a^2}{12})^{3/2} (s_{0y}^2 + \frac{a^2}{12})^{1/2} (\sigma_b^2 + \langle b \rangle)^2}{3N^2 a^2}}$$

SD measurement error



Δs_x = SD measurement error

s_0 = theoretical PSF SD ≈ 120 nm for Cy3

a = pixel size ≈ 79 nm

N = number of photons in the PSF

σ_b = SD of background photon count

$\langle b \rangle$ = mean background photon count

M. DeSantis, S. DeCenzo, J. L. Li, and Y. M. Wang, Optics Express, 2010.

I. Δz, axial localization precision

Error propagation:

$$s(z) = s_0\sqrt{1+\left(\frac{z}{d}\right)^2 + b\left(\frac{z}{d}\right)^4} \Rightarrow \Delta z(s) \propto \Delta s$$

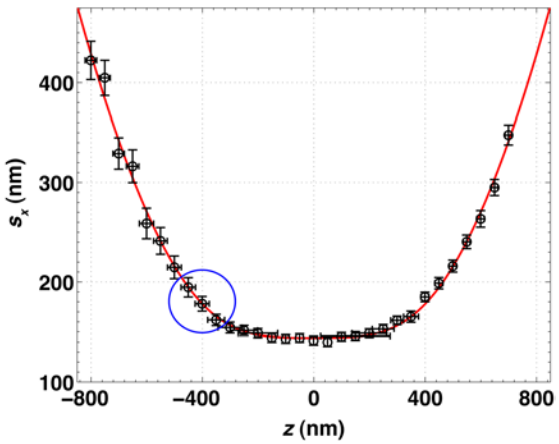
Δz is a function of SD, s

$$\Delta z = \frac{sd\Delta s}{\sqrt{2}s_0^2\sqrt{\left(\frac{s}{s_0}\right)^2 - 1 + \frac{1}{4b}}\left(2\sqrt{b}\sqrt{\left(\frac{s}{s_0}\right)^2 - 1 + \frac{1}{4b}} - 1\right)^{1/2}}$$

$$= \frac{sd\sqrt{\frac{s_0^2 + \frac{a^2}{12}}{N} + \frac{16\pi(s_{0x}^2 + \frac{a^2}{12})^{3/2}(s_{0y}^2 + \frac{a^2}{12})^{1/2}(b^2 + \langle bg \rangle)}{3a^2N^2}}}{\sqrt{2}s_0^2\sqrt{\left(\frac{s}{s_0}\right)^2 - 1 + \frac{1}{4b}}\left(2\sqrt{b}\sqrt{\left(\frac{s}{s_0}\right)^2 - 1 + \frac{1}{4b}} - 1\right)^{1/2}}$$

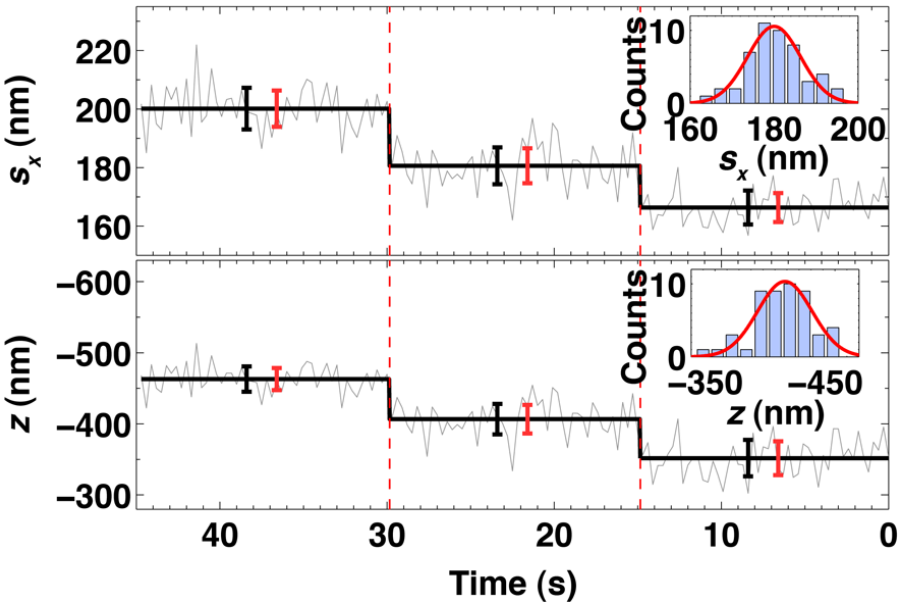
Experiments agree with the Δz expression

Phycobilisomes *s* vs. *z plot* (660 nm emission)



Error bars:
Δs (vertical) and Δz (horizontal) at 1000 photons/image;
Δz = 34 nm at z = 400 nm

s, z, Δs, and Δz for 20 nm Δz steps



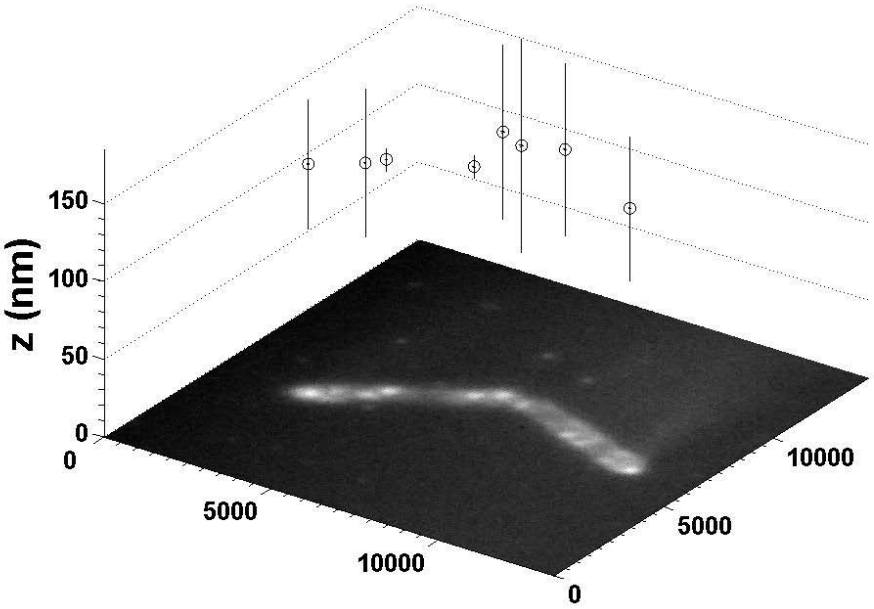
This study allows single PSF 3D-localization measurements with precision

M. DeSantis, S. Zareh, X.L. Li, R. Blankenship, and Y. M. Wang, in review in Optics Express, 2011

Application I: membrane glycolipid synthesis enzyme, UgtP-YFP, membrane interaction statics and dynamics

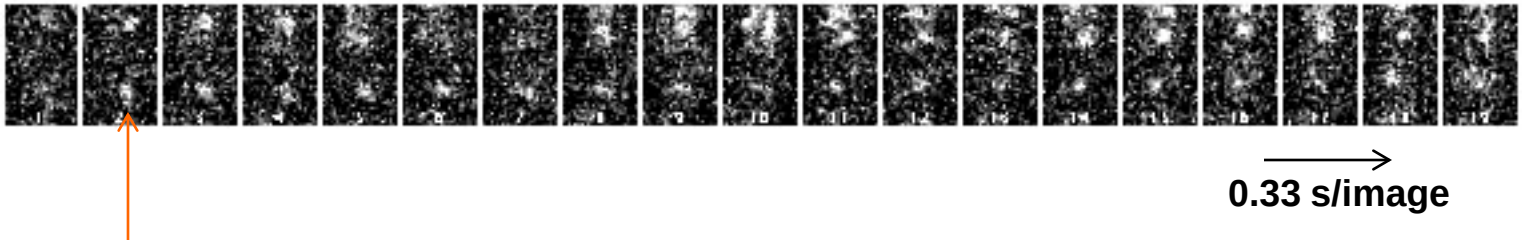
UgtP-YFP puncta in Bacilica *subtillis*

3D location and precision



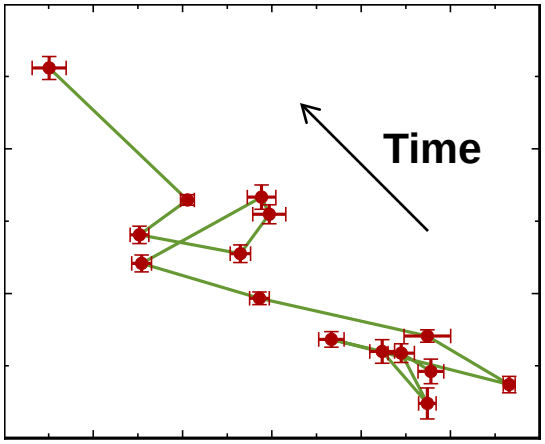
UgtP-YFP moves along a helical path

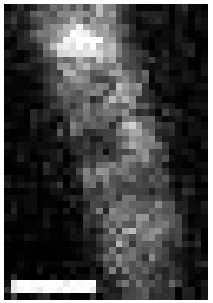
UgtP-YFP diffusing along the membrane?



0.33 s/image

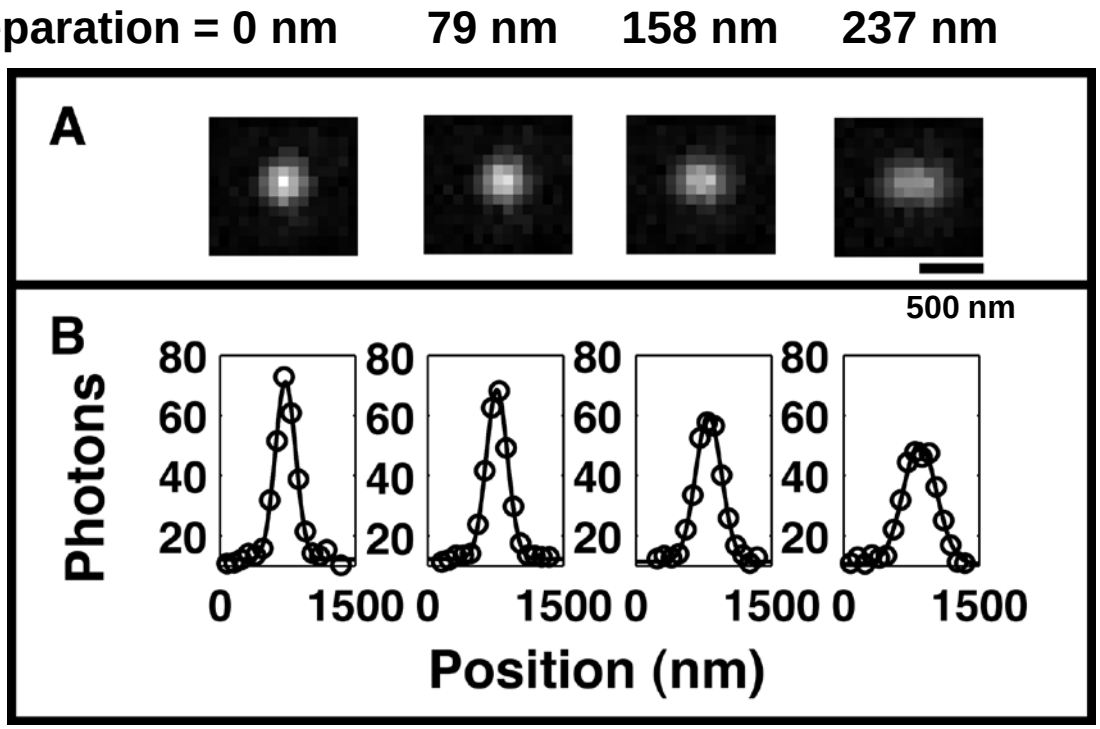
Helical path





1 μm

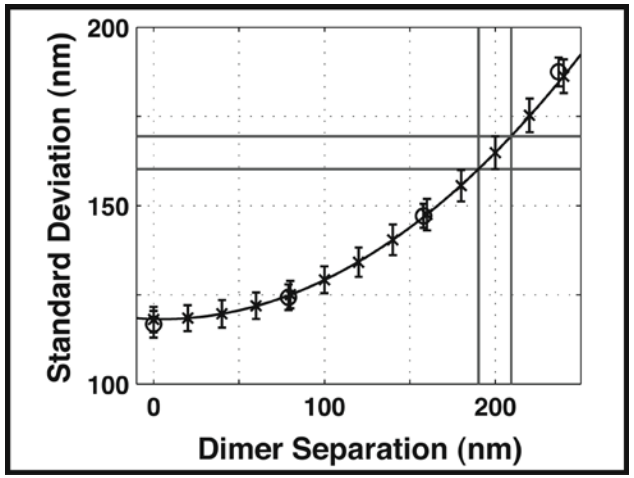
II. Unresolved identical fluorophores; dimer separation measurements



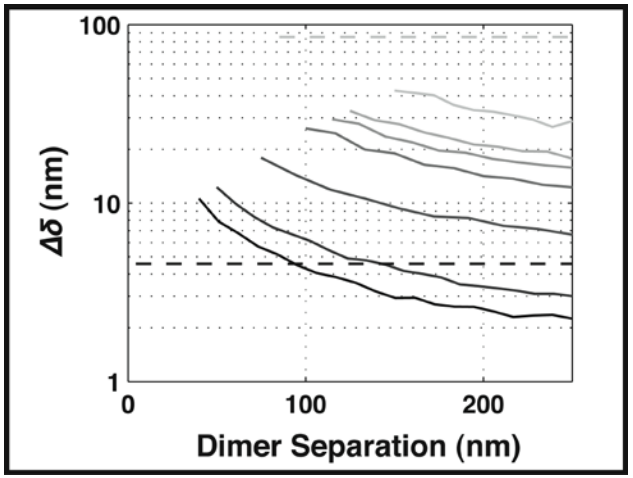
S. DeCenzo, M. DeSantis, and Y. M. Wang, Optics Express, 2010.

SD measurements of dimer separations

SD vs. separation

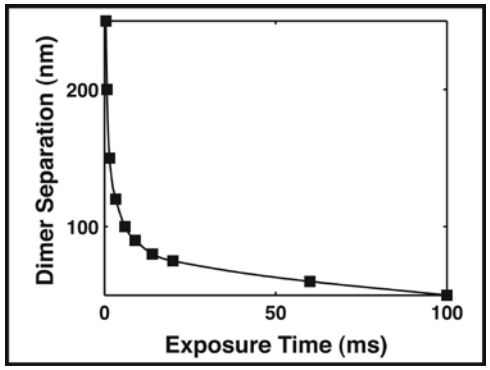


Error to separation measurements



Top down, 150 to 20,000 photons

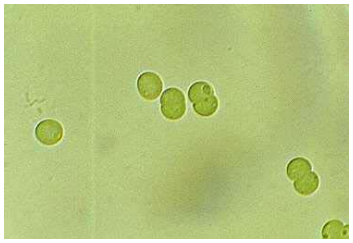
Milliseconds temporal resolution



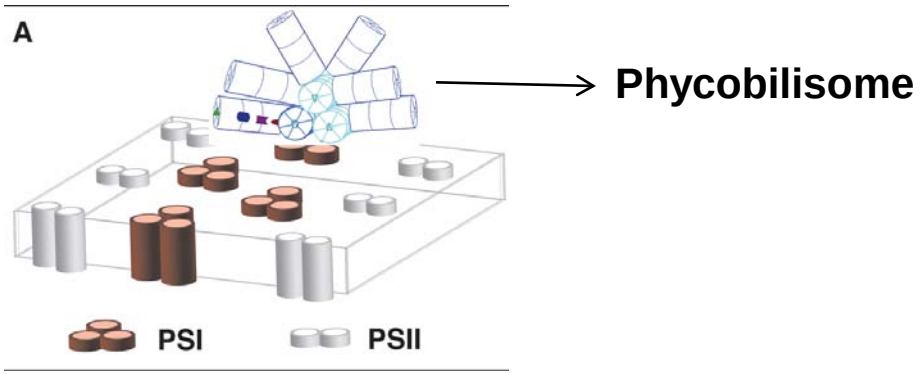
Application II: Photosynthesis

Cyanobacteria collect light for energy by photosynthesis; future substance for clean energy

Synechocystis (3 μm cells)

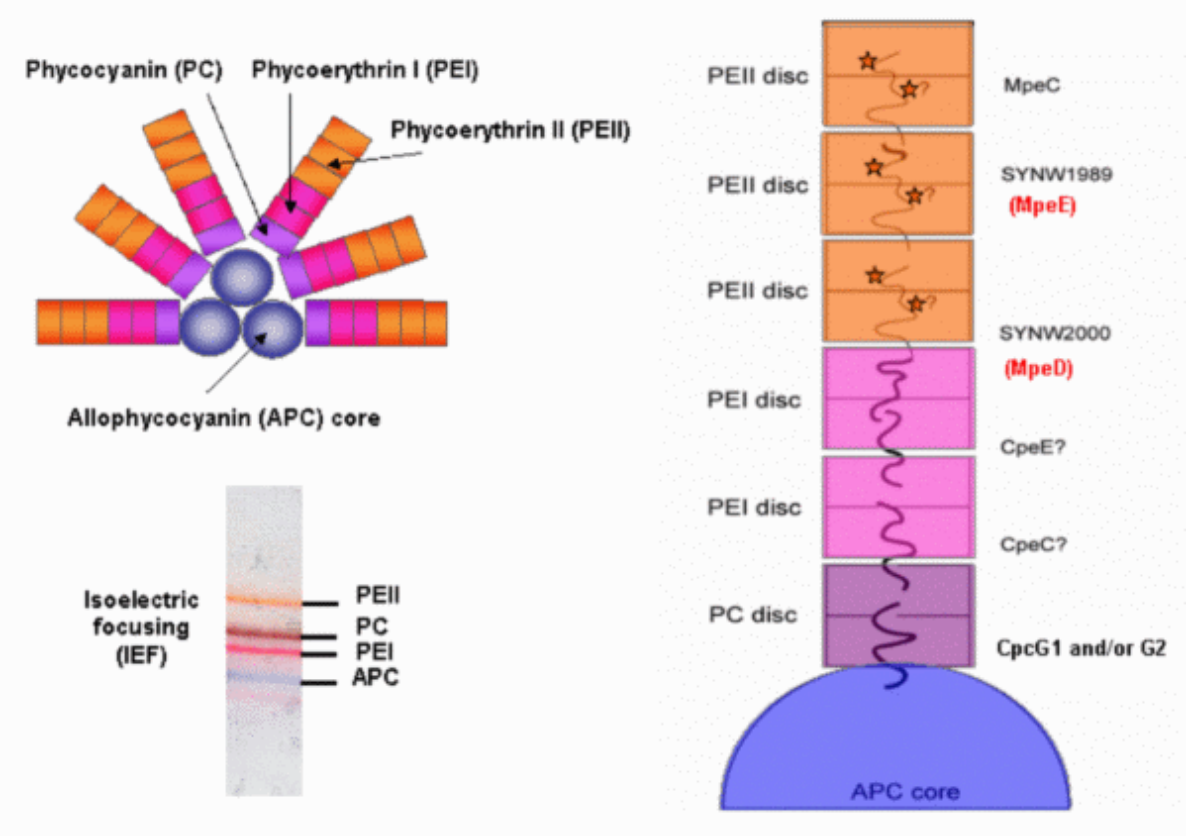


Phycobilisomes (60 nm across), protein on the cell thylakoid membrane that collects light energy



Phycobilisome light energy transfer mechanism

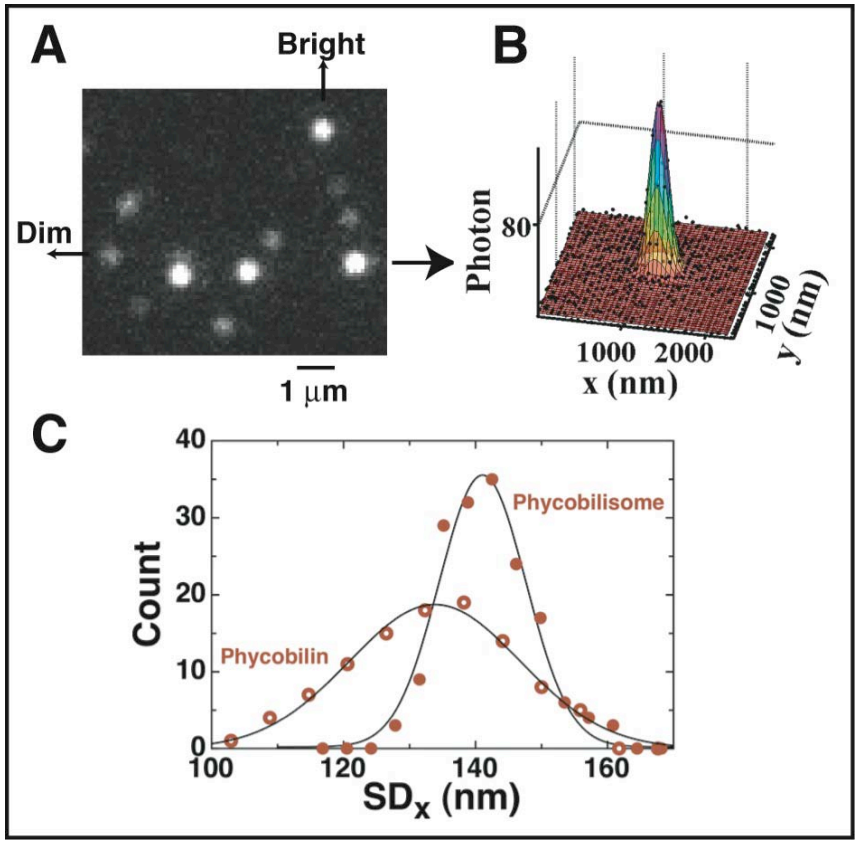
FRET through components to the terminal emitters in the core

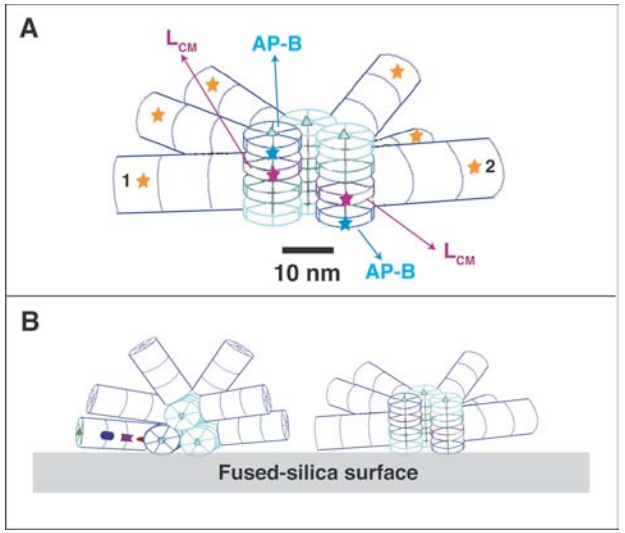


PC and APC emission peak at 650 nm and 660 nm, cannot differentiate using conventional spectroscopy

Energy transfer efficiency < 95%

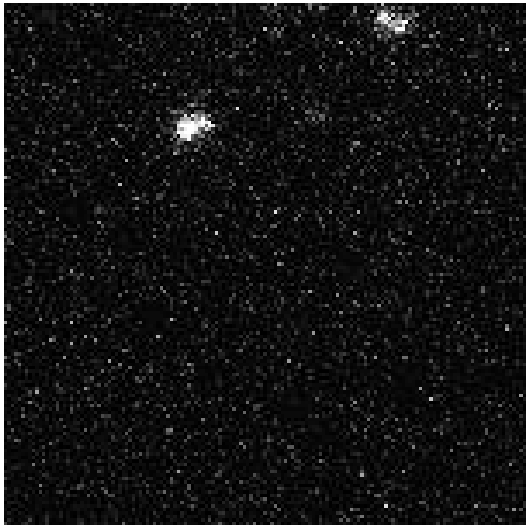
The SD of phycobilisomes is 6 nm larger than the expected value for 95% energy transfer efficiency





III. 3D diffusing GFP in free solution

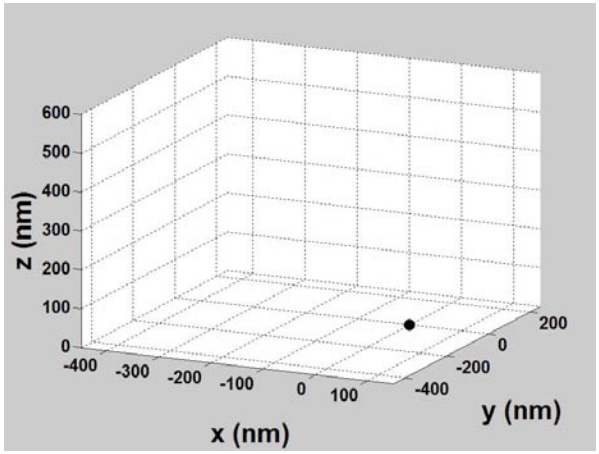
1 ms exposure time

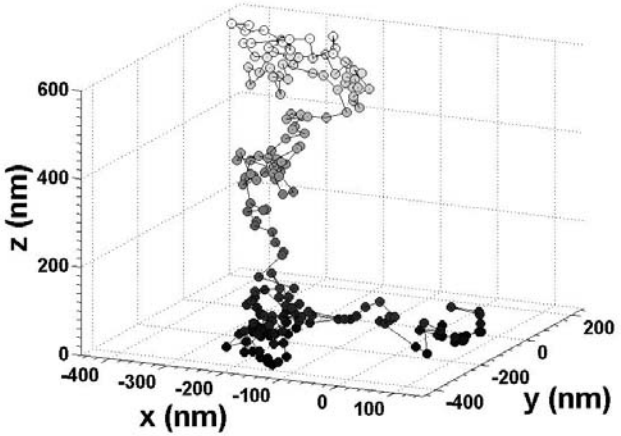


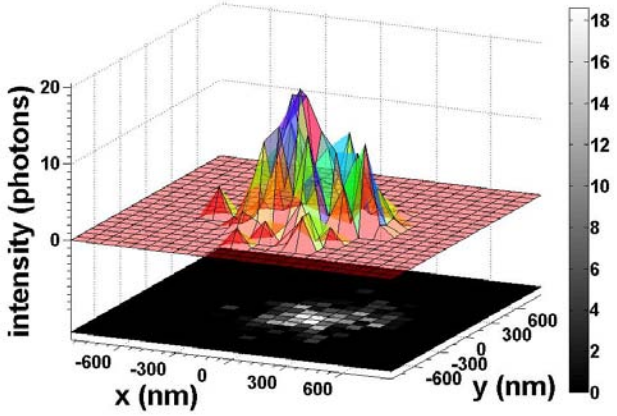
12 μm

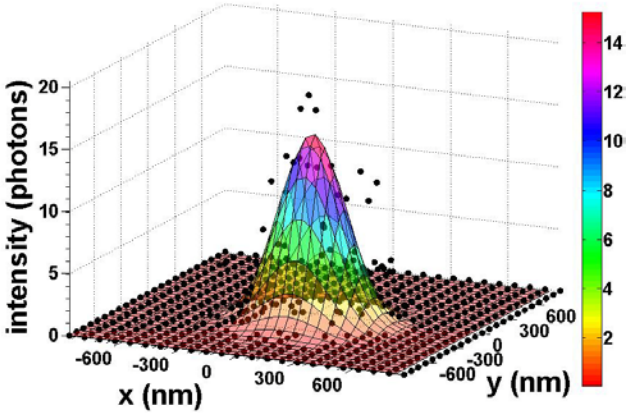
Only one image of a diffusing molecule can be obtained

Simulation


z (nm)
600
500
400
300
200
100
0
-400
-300
-200
-100
0
100
x (nm)
200
0
-200
-400
y (nm)


z (nm)
600
400
200
0
-400
-300
-200
-100
0
100
x (nm)
200
0
-200
-400
y (nm)


intensity (photons)
20
10
0
-600
-300
0
300
600
x (nm)
600
300
0
-300
-600
y (nm)
18
16
14
12
10
8
6
4
2
0


intensity (photons)
20
15
10
5
0
-600
-300
0
300
600
x (nm)
600
300
0
-300
-600
y (nm)
14
12
10
8
6
4
2

Theory: SD vs. exposure time *t* expression

I(x,y) = PSF(z-weighted) ⊗ PWDF$_{x,y}$

PWDF = pathway distribution function

$$\int_0^{\infty} \frac{1}{s_{x,z}s_{y,z}} \exp\left(-\left[\frac{x^2}{2s_{x,z}^2}+\frac{y^2}{2s_{y,z}^2}\right]\right)\exp\left(-\left[\frac{(z-\bar{z})^2}{2A_zD_3t}+\frac{z}{z_d}\right]\right)dz \otimes \exp\left(-\left[\frac{x^2+y^2}{2A_{x,y}D_3t}\right]\right)$$

Both PSF(*z*-weighted) and *PWDF$_{x,y}$* are Gaussian functions

SD vs. exposure time expression

I(x,y) = PSF(z-weighted) ⊗ PWDF$_{x,y}$

Since both PSF(*z*-weighted) and *PWDF$_{x,y}$* are Gaussian functions,

SD of the diffusing GFP image = (PSF variance + PWDF$_{xy}$ variance)$^{1/2}$

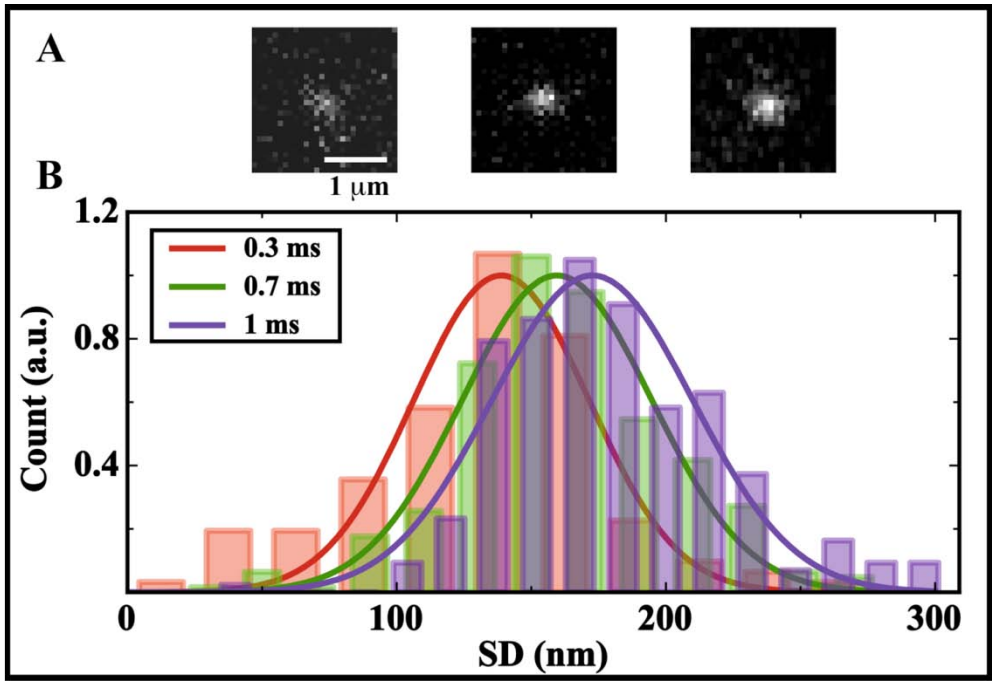
$$s_{x,y}(t) = \sqrt{s^2_{x0,y0}(t) + 2A_{x,y}D_3 t}$$

$$\Rightarrow D_3 = \frac{s^2_{x,y}(t) - s^2_{x0,y0}(t)}{2A_{x,y}t}$$

Single image D_3 error

$$\Delta D_3 = \frac{8s_{x,y}(t)}{t}\Delta s_{x,y}$$

Experimental: SD measurements

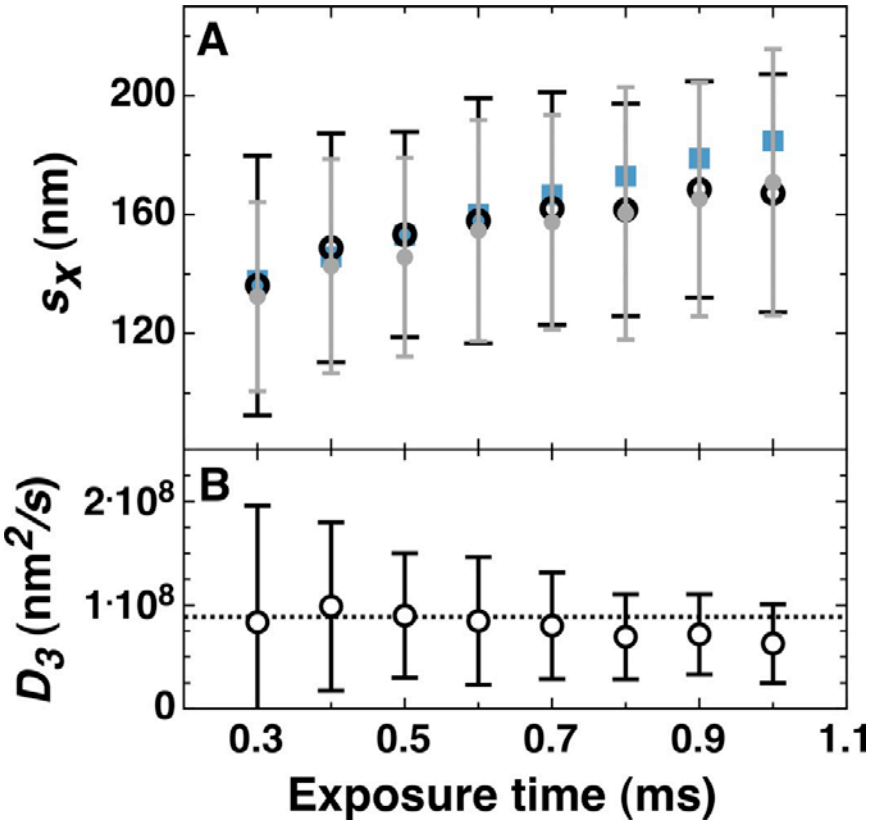


$t = 0.3$ ms $\Rightarrow$ SD $= 137 \pm 28$ nm

$t = 0.7$ ms $\Rightarrow$ SD $= 159 \pm 32$ nm

$t = 1.0$ ms $\Rightarrow$ SD $= 172 \pm 35$ nm

SD vs. exposure time

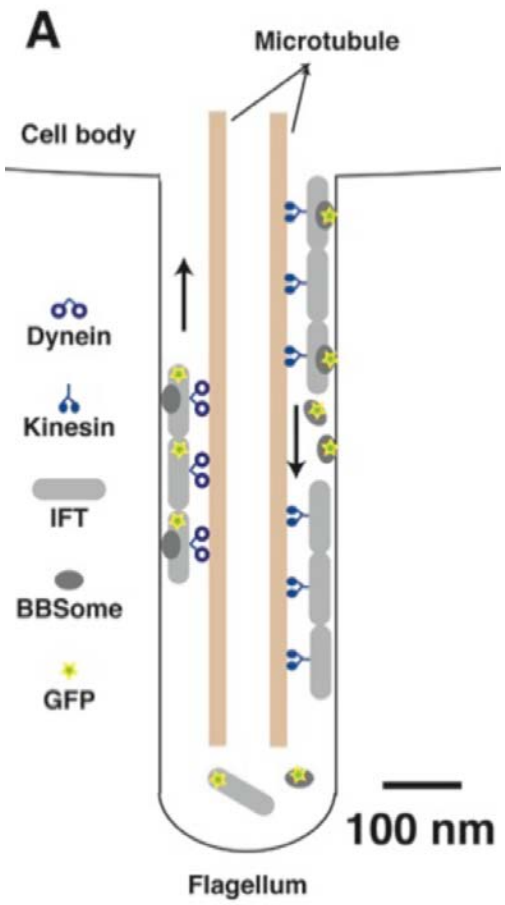


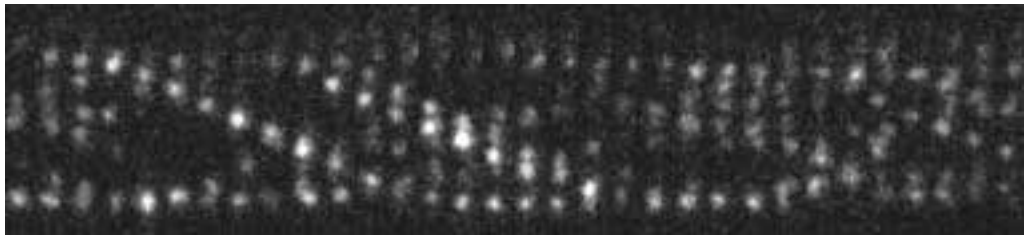
For 1 GFP image at 1 ms, $D_3 = 9 \times 10^7 \pm 5.2 \times 10^7$ nm^2/s

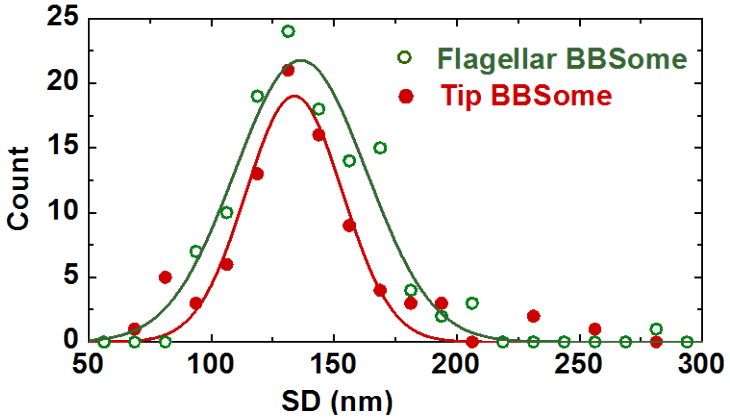
For 10 images, $D_3 = 9 \times 10^7 \pm 1.7 \times 10^7$ nm^2/s

S. Zareh, M. DeSantis, J. Kessler, J. L. Li, and Y. M. Wang, in review at PNAS, 2011

Application III: BBSome and IFT turnaround mechanism at the flagellar tip







- If IFT dissociates at the tip, SD will increase by 5 nm for 1 ms exposure.
- We observe no increase.

Summary

- **SIMA, single-image molecular analysis, can speed up single-molecule fluorescence studies to millisecond timescales.**

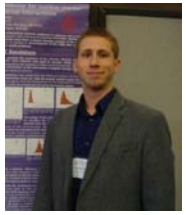
- **SMID, single-molecule image deconvolution analysis, provides localization, separation, and dynamic information of single molecules with nanometer precisions.**

- **Already show promise in biological systems**

Lab members

Shannon Zareh

Michael DeSantis



Jonathan Kessler

Anthony Kovacs

Collaborators

IFT dynamics: Susan Dutcher, Department of Genetics, WU medical school

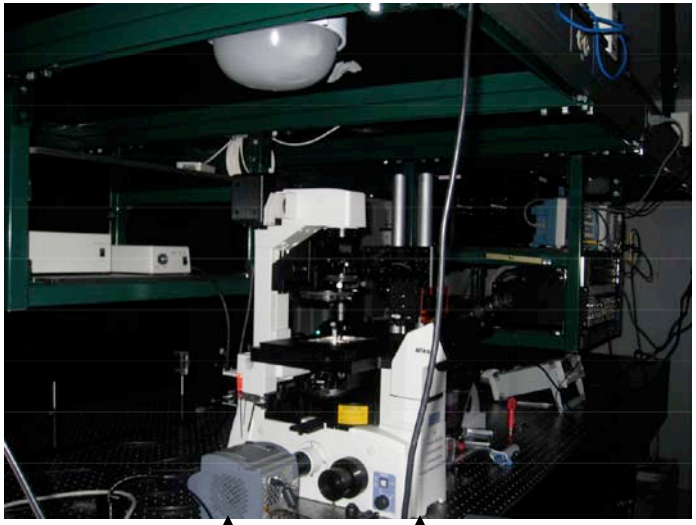
Photosynthesis: Bob Blankenship, Department of Biology, WU

UgtP dynamics: Petra Levin, Department of Biology, WU

Washington University in St. Louis

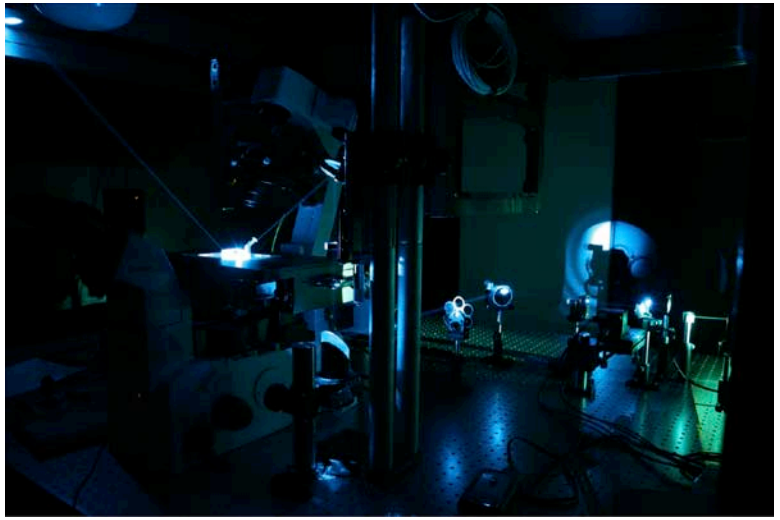
Imaging setup

Microscope imaging system

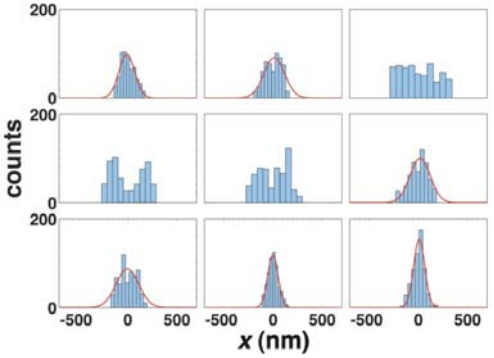


Camera **Microscope**

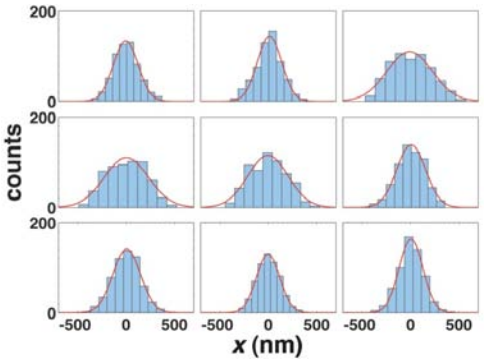
488 nm laser and prism TIRF



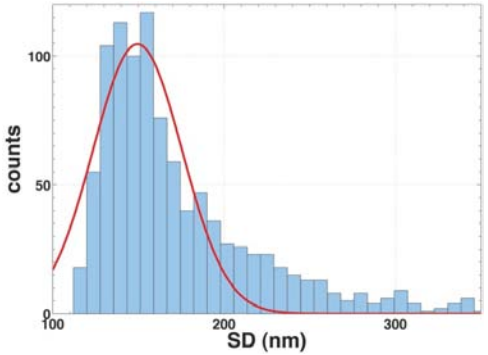
$PWDF_{xy}$ can be approximated by a Gaussian



Single trajectory position distributions at 0.6 ms, $PWDF_{xy}$



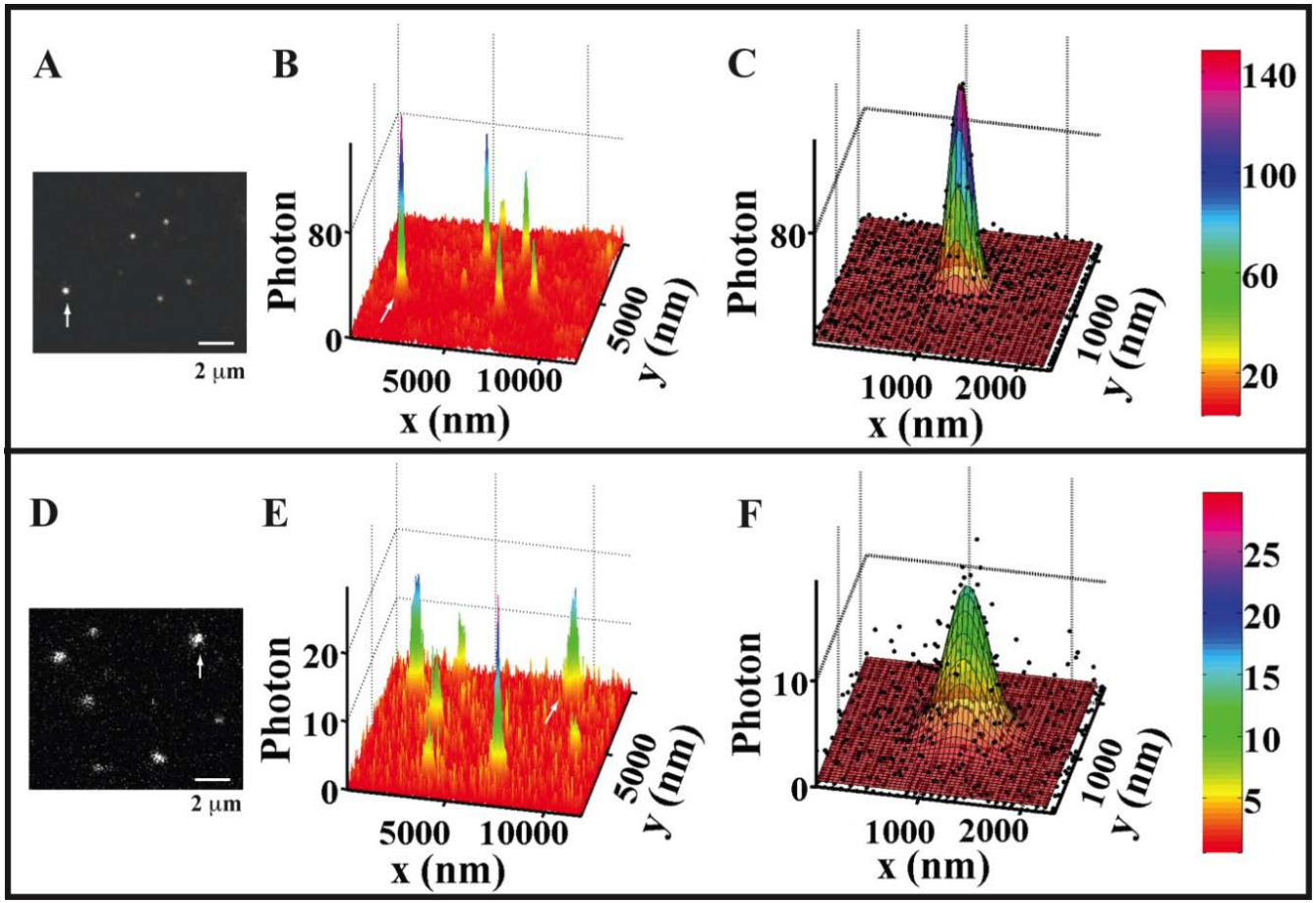
$PWDF_{xy}$ convolved with photon emission



Convolved $PWDF_{xy}$ SD distribution; the mean yields $A_{x,y} = 0.095$

$$PWDF_{x,y} = \frac{1}{2\pi A_{x,y} D_3 t} \exp\left(-\frac{x^2 + y^2}{2A_{x,y} D_3 t}\right)$$

Immobile vs. diffusing GFP



Questions:

1. Dynamics of BBSome in flagella to carry signaling proteins

2. BBSome, IFT, and Kinesin turnaround mechanisms at the flagellar tip