CAL POLY POMONA

Optics Beyond The Diffraction Limit, or: Don't Believe Everything You Hear in Physics 4!

Alex Small

Department of Physics

California State Polytechnic University, Pomona

Students:

Edward Shore
Michael Mansell
Adeline Kornelus
Triet Nguyen
Forrest Requarth

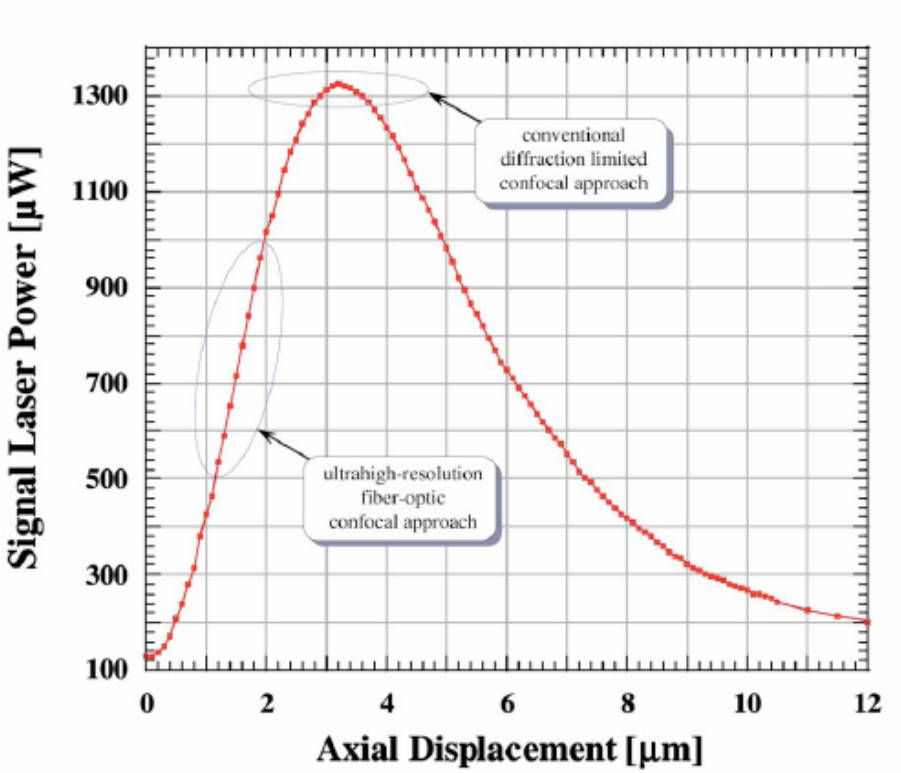
Support:

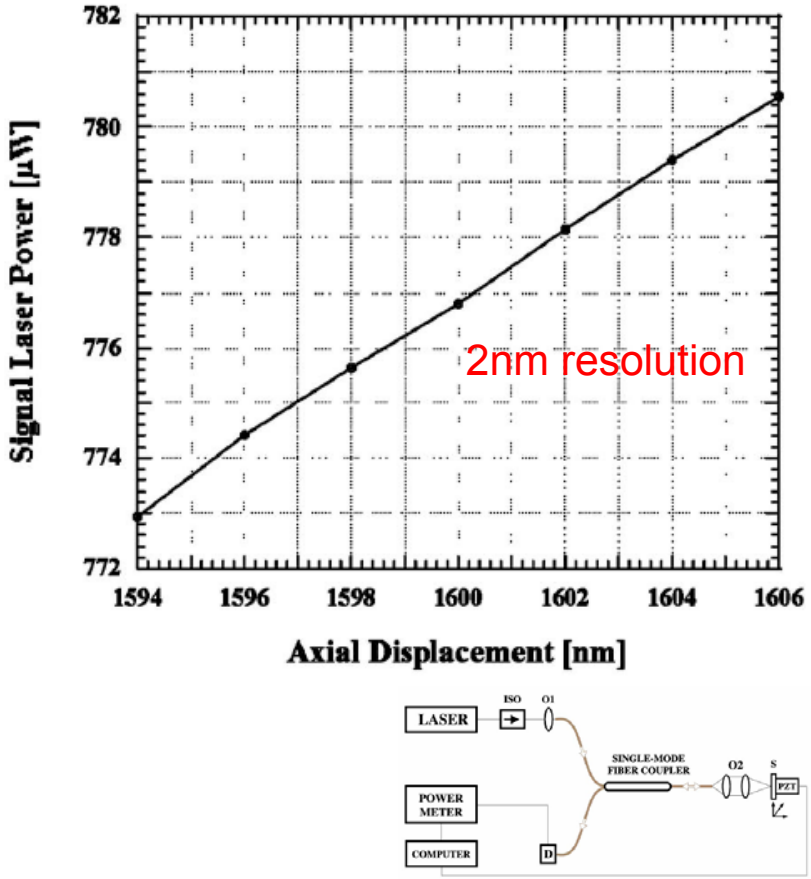
-Cal Poly (startup funds)
-Kellogg Honors College (student support)
-Research Corporation
-CCRAA
-NICHD

Outline

- Diffraction limit
- STORM Imaging
- Theory of STORM
- Nanolithography Idea

I saw something nice when I arrived at NIH

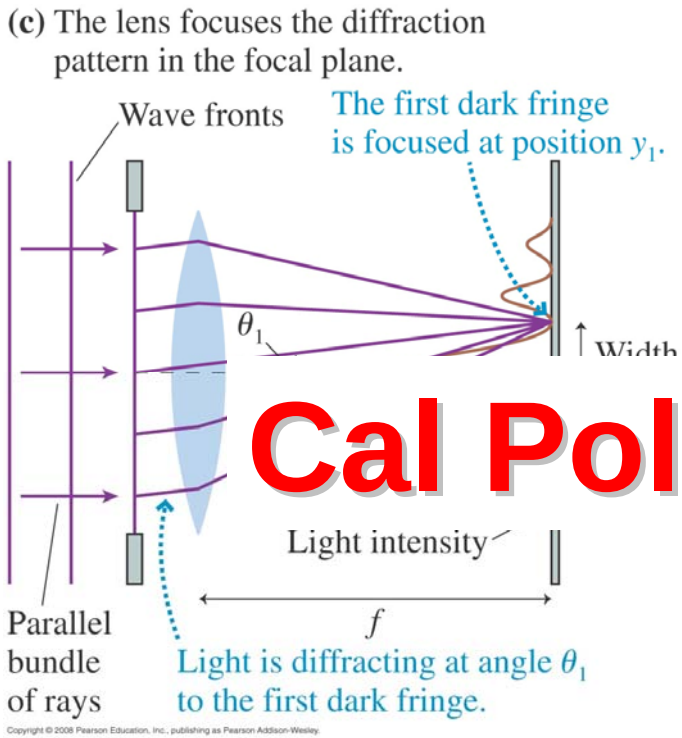


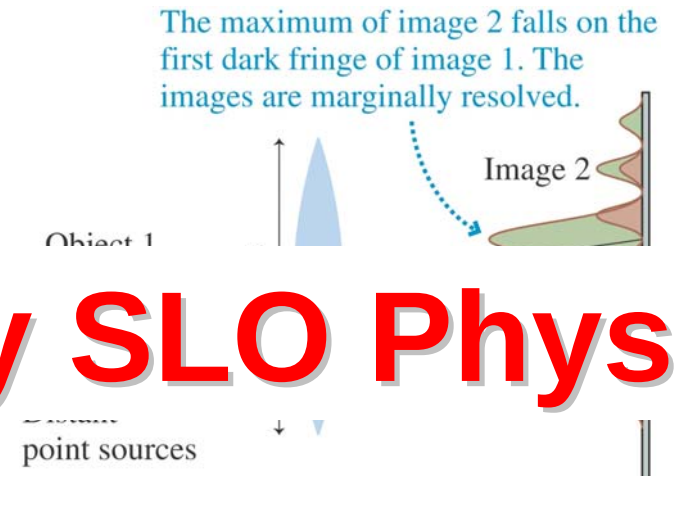


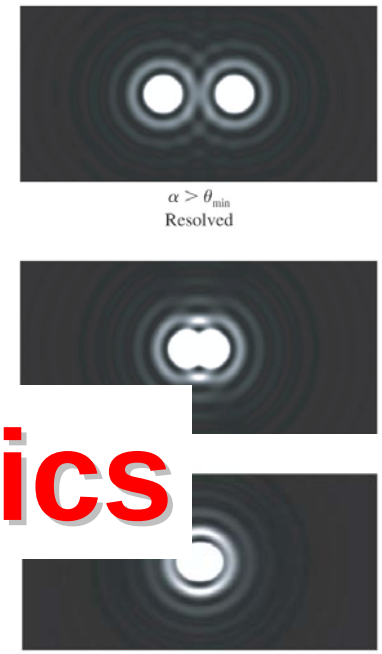
- My mind was blown: Optical microscope obtaining information on nanometer scales. Didn't they tell me that this was impossible?
- This works for axial resolution, but I got interested in using these ideas for **lateral resolution**.

Figs from Ilev, Waynant, Gannot, & Gandjbakhche, Rev. Sci. Inst. 2007

Small things get blurred







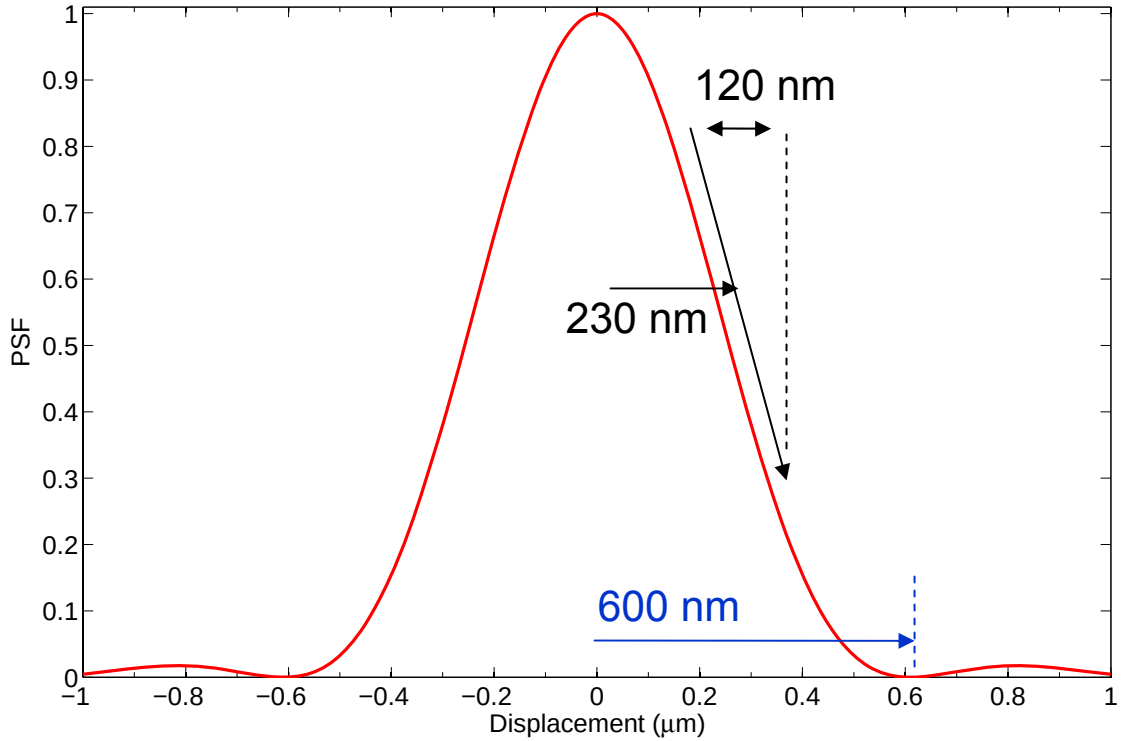
Cal Poly SLO Physics

- Even the best lenses face this problem!
- Troublesome for biologists and electrical engineers!

Light	Best Possible Resolution
Infrared	500 nm to 1 micron
Red	300 nm
Green	250 nm
Blue	200 nm
UV	<200 nm, needs special materials

Cal Poly Pomona Physics

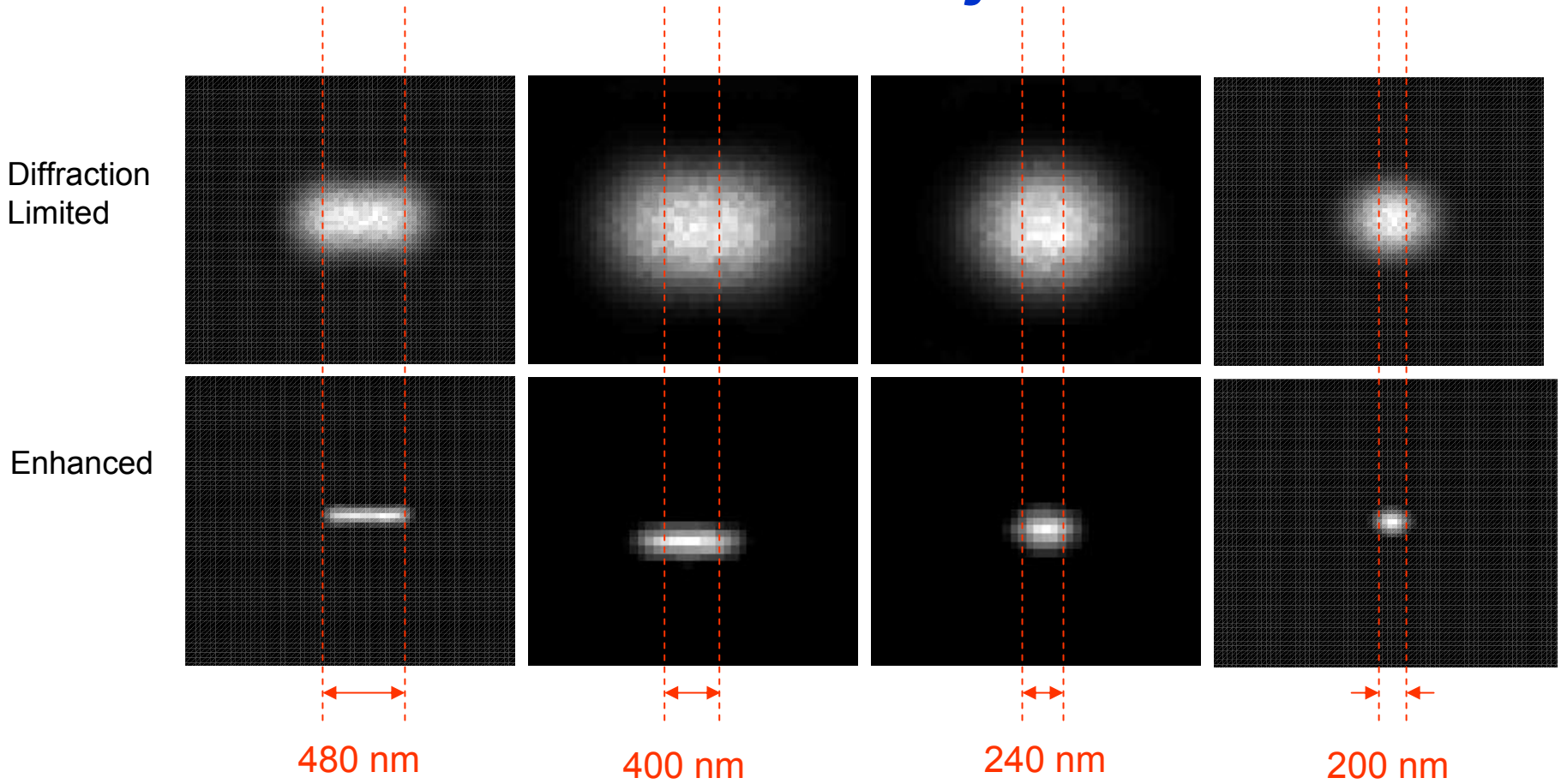
λ = 500 nm
NA = 0.5



- **Zeros:** Light detected there uncorrelated with center, represents *background*, other objects
- **Steep region:** Maximum sensitivity to small displacements
- Steep slope has been used to improve axial resolution in differential confocal microscopy

Goal: Use the same ideas to improve lateral resolution.

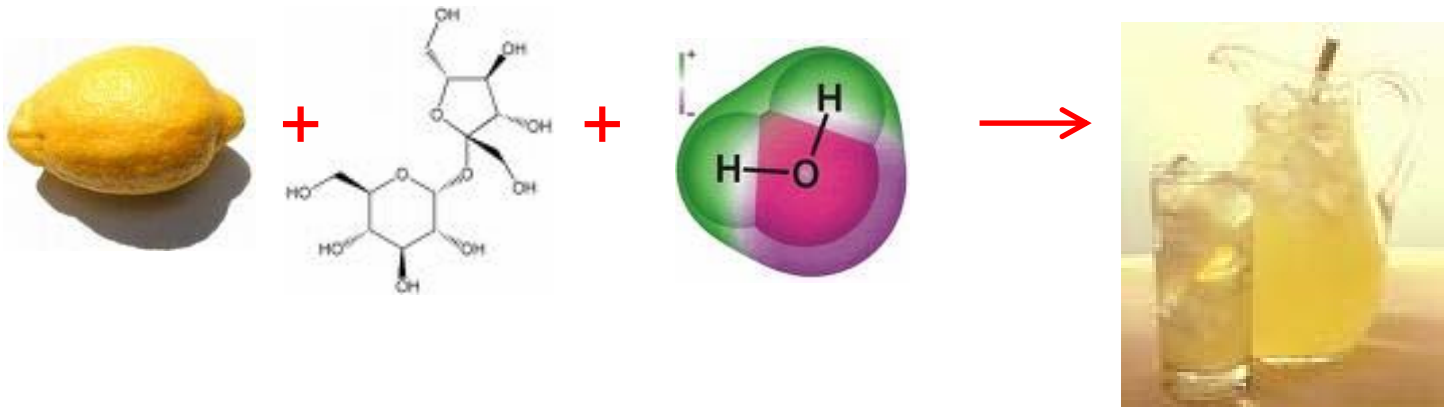
Close Point Objects



- Poor resolution of centers, but enhanced aspect ratio.
- Aspect ratio can distinguish single probe vs. multiple close probes
- Aspect ratio robust against higher noise levels.

Kind of a Lemon

This method fails for anything other than sparse, discrete point sources…

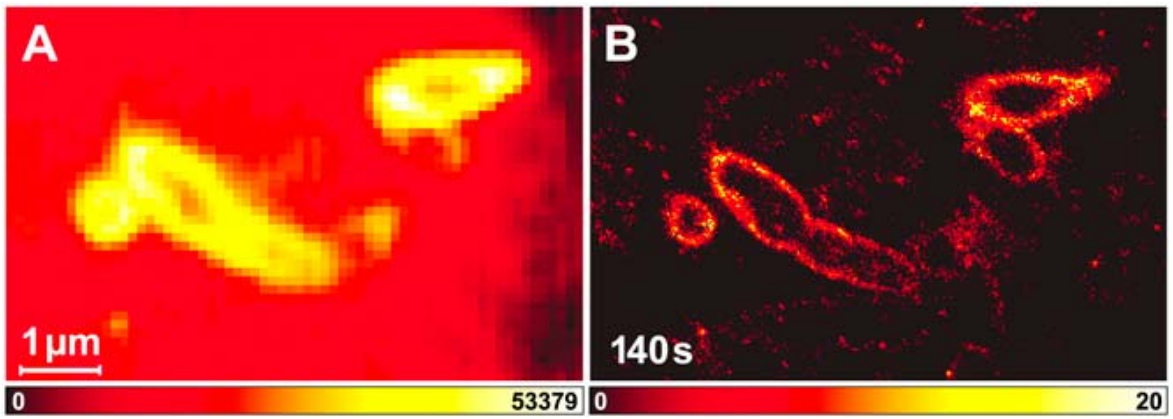


Let's make Lemonade

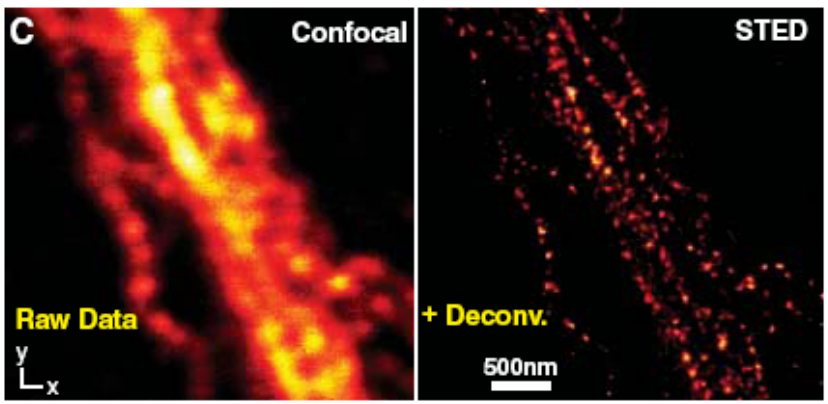
People are doing useful and fun things with sparse, discrete point sources.

Beating the Diffraction Limit

- There are some other nice tricks for getting around diffraction:



Fluorescently labeled membranes in E. Coli., imaged with “blinking” molecules



Let’s start with this one

Neurofilaments imaged by controlled fluorescence technique called STimulated Emission Depletion (STED)

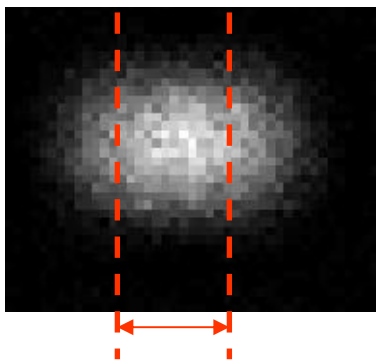
Pictures from Stefan Hell’s group (Biophys J. 2007)

PALM/STORM/etc.

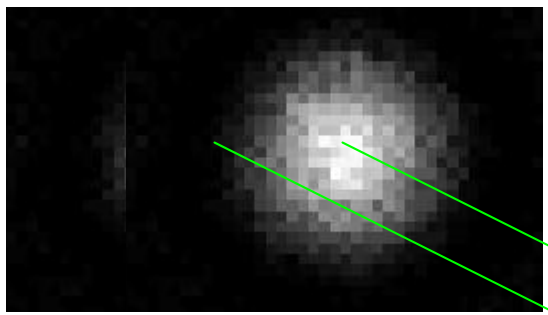
- Photo
- Activation
- Localization
- Microscopy

- STOchastic
- Reconstruction
- Microscopy

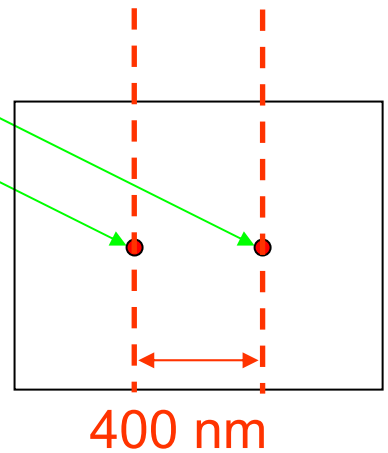
•2 fluorescent molecules close together would look like this under microscope
•(simulation, λ = 500 nm)



What if only one at a time is shining?



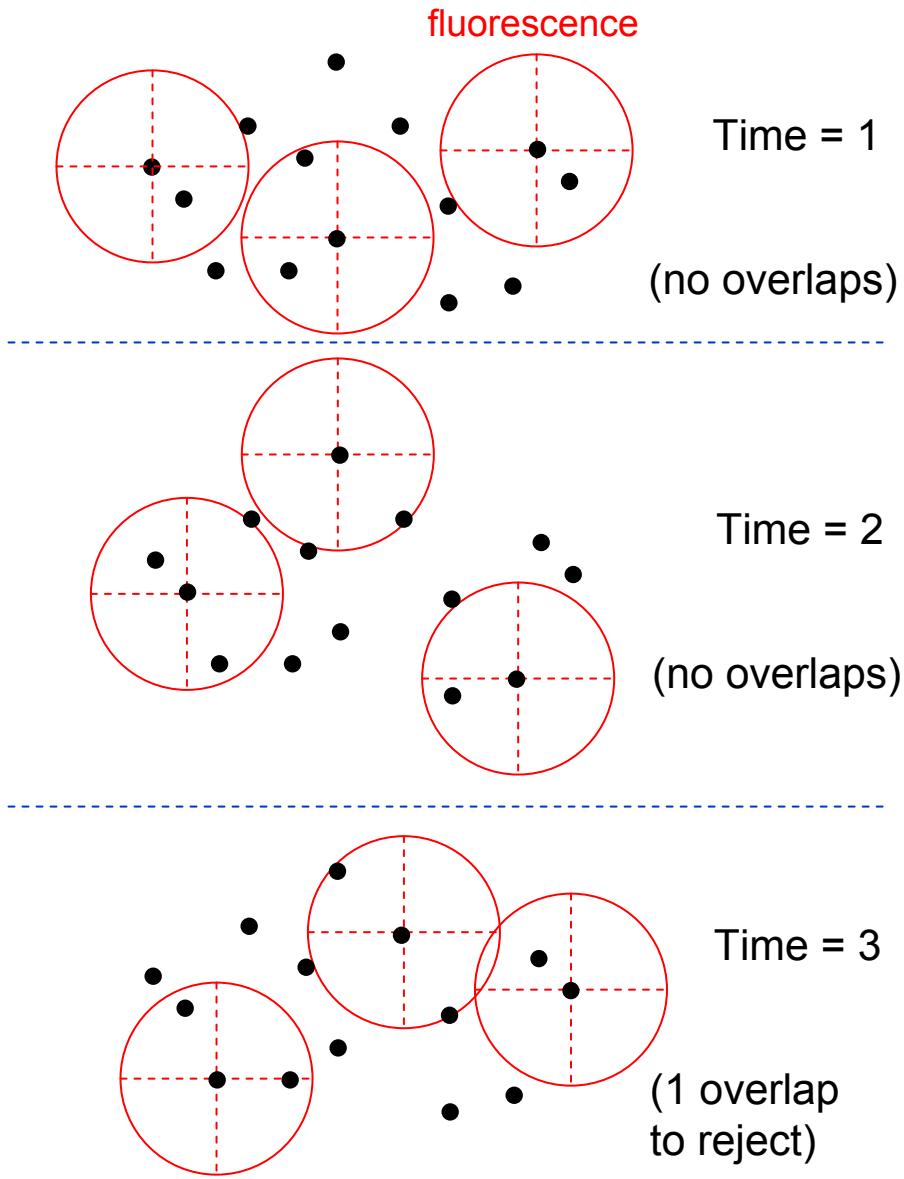
Find centers and infer molecule locations



Do this enough times, and eventually you know where every molecule is!

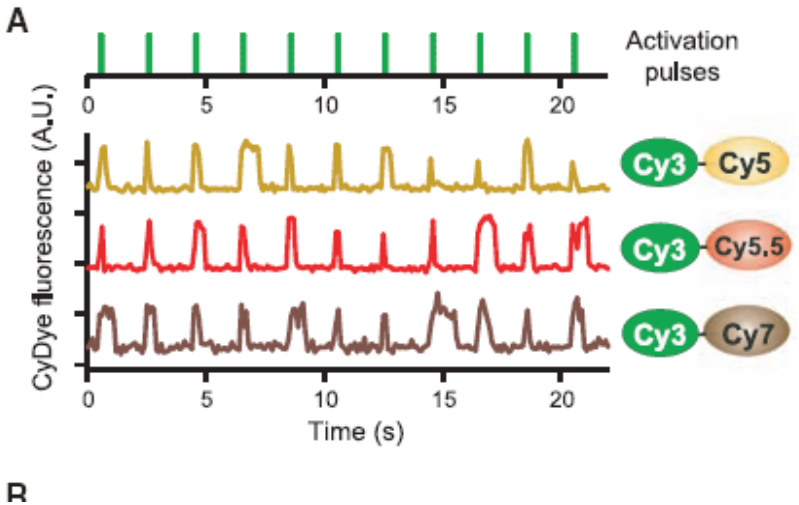
In the blink of a molecule

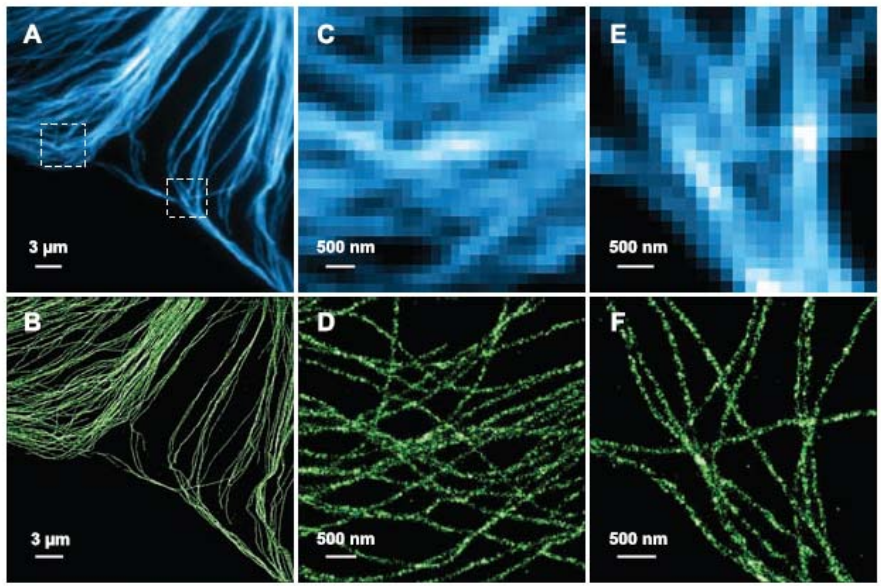
- Not all the molecules in a crowded image are “on” at the same time.
- With enough frames you can localize molecules with sub-pixel resolution
- Depends on density of “on” molecules
- 2 close molecules are “on” at the same time: Need to discard that image.



More Pictures

- Switch molecules on and off with light pulses, detect fluorescence.

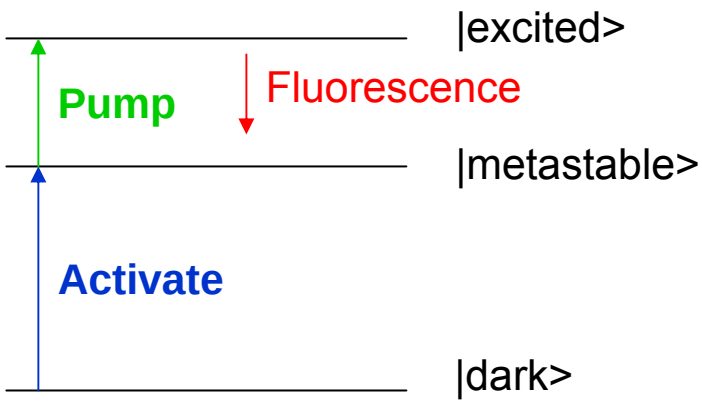




Microtubules

From Zhuang's group at Harvard (Science, 2008)

Energetics and Kinetics



- Rate of return to dark state ✪ I(pump)
- Rate of fluorescence ✪ I(pump)

ॐAvg. # of photons collected independent of I(pump)

ॐPump as hard as you can!

What can theory do for microscopes?

Ernst Abbe

"Already as a young scientist, Abbe placed his knowledge at the disposal of Carl Zeiss. In 1866 he became a member of Zeiss' scientific staff. From 1870, Abbe was a professor at the University of Jena. His theory of image formation in the microscope made him the founder of scientific optics and gave Carl Zeiss an important technological lead: ***while microscopes had been previously built on a trial-and-error basis, they were constructed on a sound mathematical foundation from 1872 onwards and therefore displayed considerably better optical properties****. This led to pioneering research in biology and medicine, e.g. by Robert Koch and Paul Ehrlich."*

http://www.zeiss.com/C12567A100537AB9/Contents-Frame/389DC7D126A463B7C1257141002E7C77

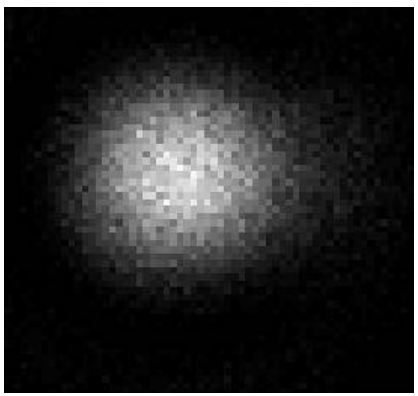
Problem

- Only works if you are pretty sure that adjacent molecules are never “on” at the same time.

- They play the odds. Works most of the time.

- High density of molecules?
- Excite more molecules to get data faster?
- Do these things matter?

Q: Are there 2 molecules on in this (simulated) image below?



A: Of course.

But how do we teach a computer to recognize that fact?

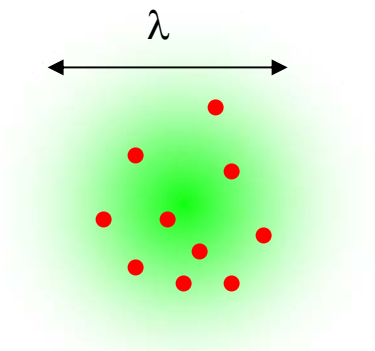
Activation Probability

- n fluorescent molecules per diffraction-limited spot.
- Density ~n/λ^2
- p = probability of exciting any particular molecule
- Set by activation pulse dosage
- p_1 = probability of exciting exactly 1 of the n molecules in a spot

$$p_1 = np(1-p)^{n-1}$$

- p_m = probability of *m* molecules being activated.
  m>1 →Bad

$$p_m = \frac{n!}{m!(n-m)!} p^m (1-p)^{n-m}$$



● = fluorophore (n of them)

30 nm resolution → n~100

Minimum Number of Detections

- Image each molecule an average of *T* times (if no bleaching)
- *T* depends on signal/noise, targets for subpixel resolution and intensity accuracy
- So we need *N* activation cycles:

$$N = nT / p_1 = \frac{T}{p(1-p)^{n-1}}$$

- N is a min. when

$p = p_{max} = 1/n$ $\quad N_{\min} = \frac{nT}{\left(1-\frac{1}{n}\right)^{n-1}} \rightarrow nTe$ (n>>1)

If you activate too many molecules, you're actually slowing yourself down!

Overlap Errors

- Frames with overlapping spots are errors.

- Define an error rate:
  Ratio of overlap images to single molecule images

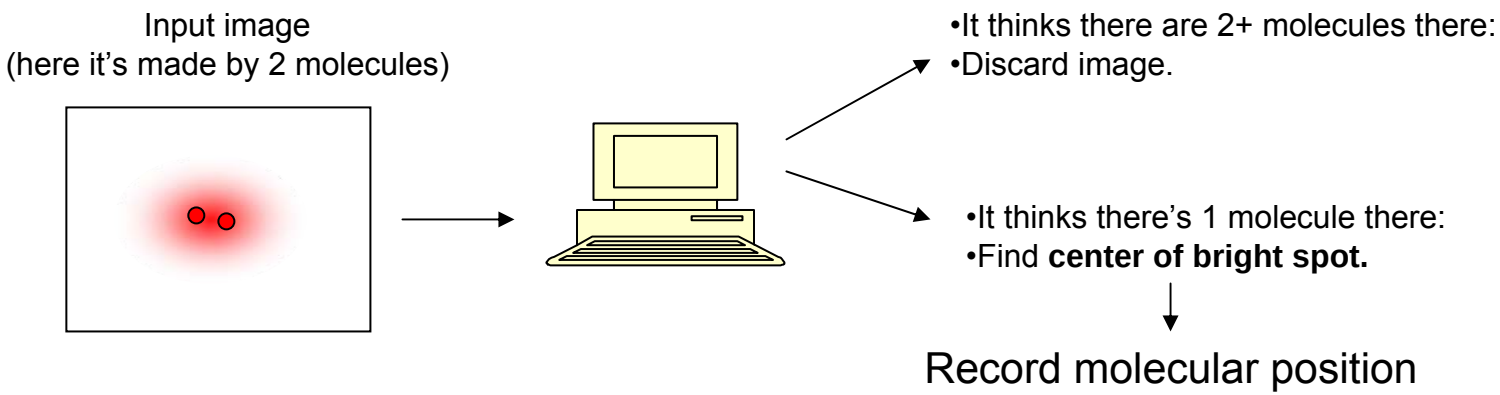
$$E = \frac{\sum_{m=2}^{n} p_m}{p_1} = \frac{1 - p_0 - p_1}{p_1} \rightarrow e - 2 = 0.7$$

- ***Error almost as large as signal!*** Two solutions:

1) Keep *p* low, take lots of images, accept that you get a lot of images with nothing. →Slow Experiment

2) Find a way to reject overlaps.
   →Fast Experiment

Rejection Algorithm



- Given an image with *m* activated molecules in a spot:

f_m = fraction of *m* molecule images accepted by filter.

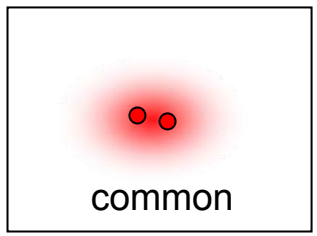
- Average over all possible sets of *m* molecules with all possible locations.
- We want large f_1, all the rest small
- Common filters: Ellipticity, intensity quantization

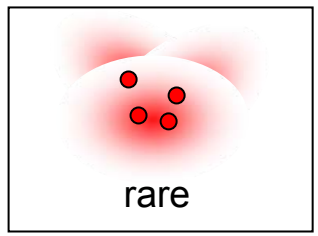
Algorithm Error Rates

- Error rate of algorithm excludes rejections:

$$E = \frac{\sum_{m=2}^{n} f_m p_m}{f_1 p_1}$$

Second order approximation:
Most overlaps are 2-molecule events





- Justification: p_m is decreasing factorially:

$$\frac{p_m}{p_{m-1}} = \frac{1}{m}\frac{n-m+1}{n-1}$$

- **f_m is decreasing:** More molecules means larger, brighter spot

$$E \approx \frac{f_2 p_2}{f_1 p_1}$$

→Easier to identify!

Maximum Error Rate

- E depends on p as well as f_1, f_2:

$$E = \frac{f_2 np}{2 f_1 (1 - p)}$$

- Maximum p is 1/n, so:

$$E \leq E_{\max} = \frac{f_2}{2 f_1}$$

- Scaling behavior is expected.

- What does error imply for data acquisition time?

Minimum Number of Activations

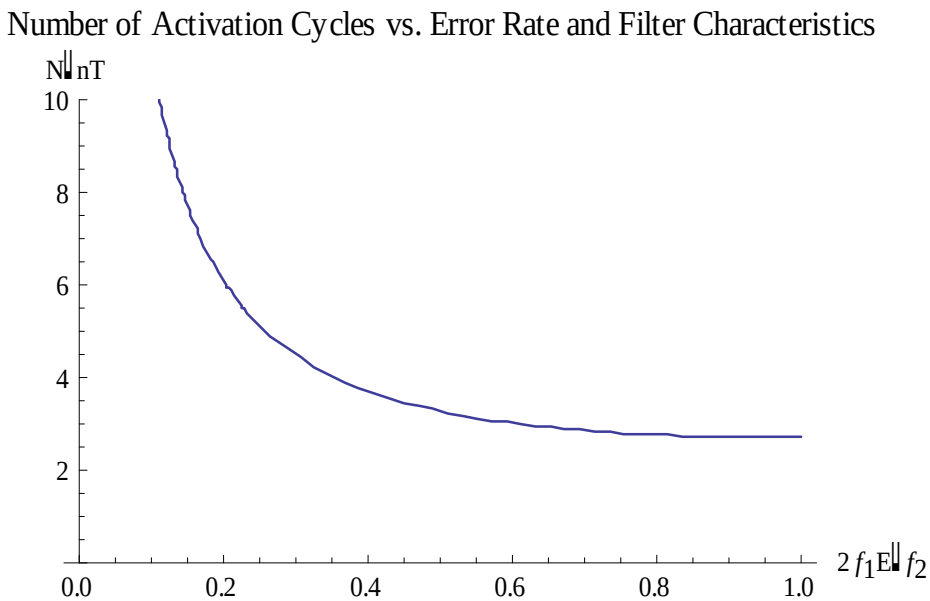
- Given an error rate, find *p*

(i.e. how many should you activate in expt.?)

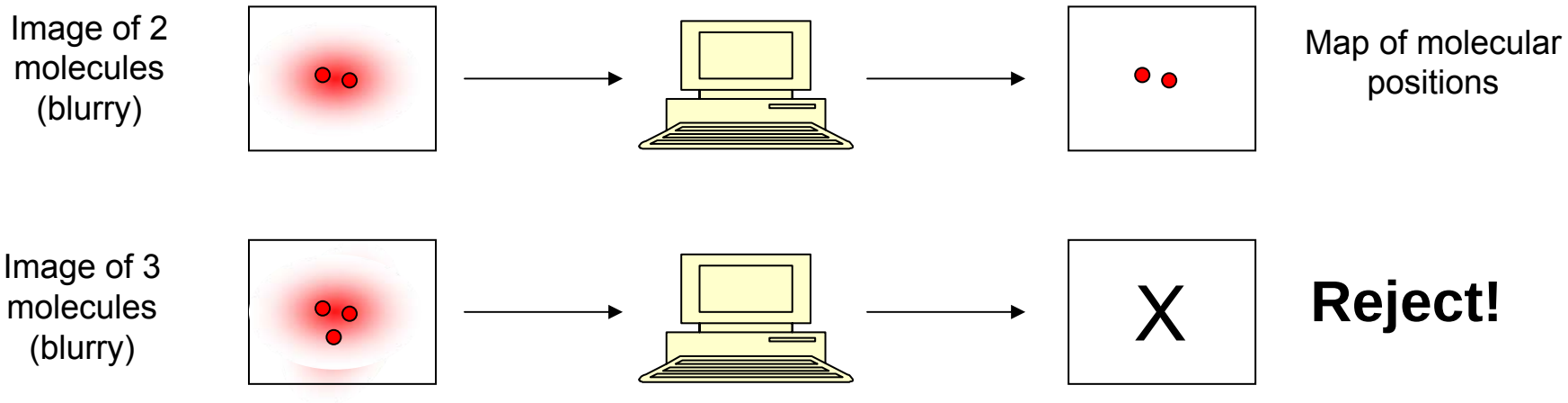
$$p = \frac{2 f_1 E / f_2}{2 f_1 E / f_2 + n - 1} \approx \frac{2 f_1 E}{f_2 n}$$

- Given *p*, what is *N*?

$$N = \left(f_2 \frac{n-1}{2 f_1 E} + 1 \right) T e^{2 f_1 E / f_2}$$



Higher Order Rejection Algorithm



- Suppose we can look at an image of 2 molecules and solve the inverse problem to find their positions.
- If it could do this for arbitrary numbers of molecules in the image, we wouldn't need to switch molecules on and off.
- Suppose it works for up to $m<<n$ molecules, and rejects images with $m+1$ or more molecules activated
- What is the minimum acquisition time?

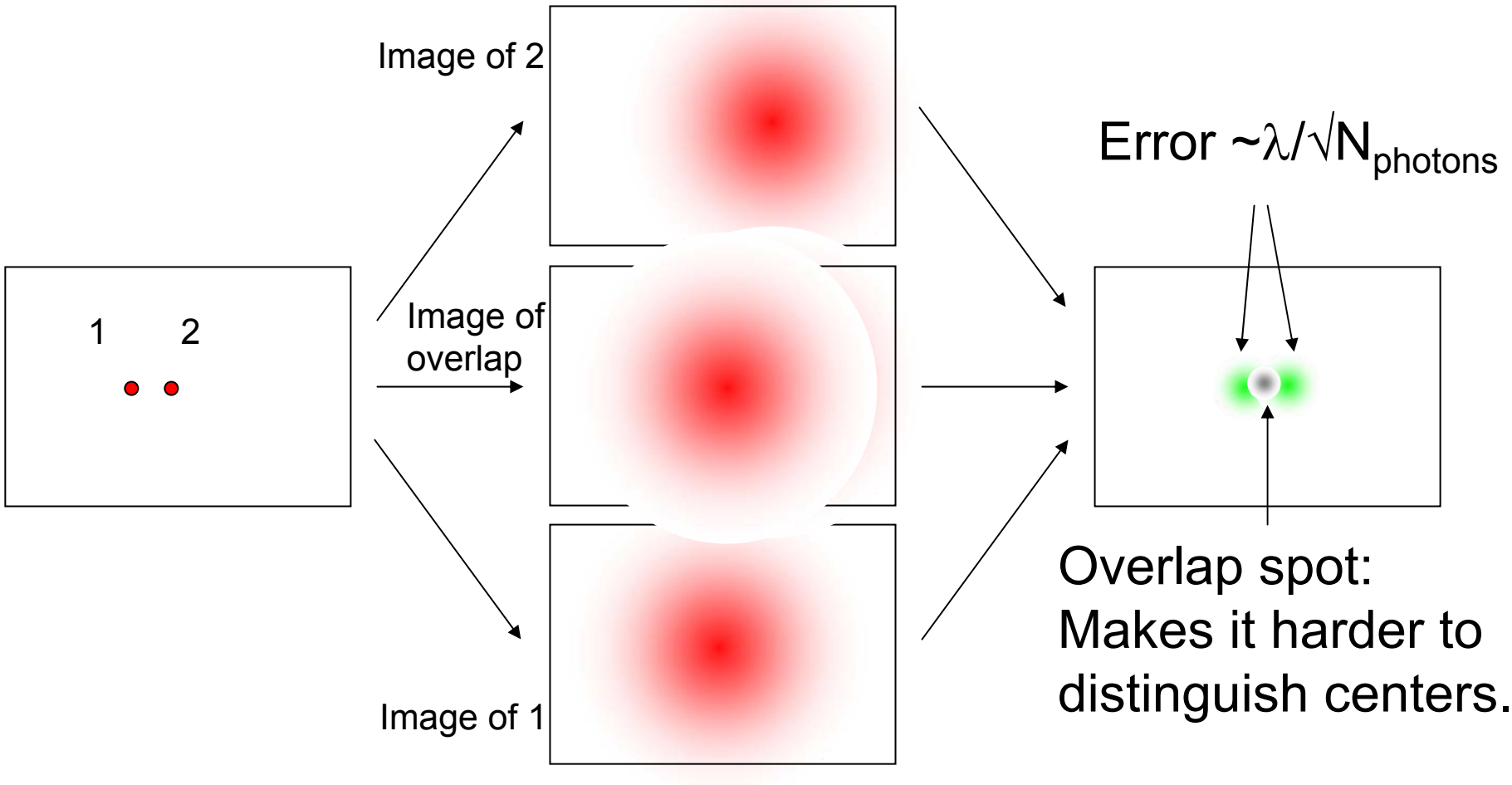
How smart does an algorithm need to be?

- Being able to distinguish 2 closely-spaced molecules with perfect accuracy drops the time 50%.
- But not an easy task!

m (# of molecules filter can discern)	*N/nT* (normalized acquisition time)
1	e=2.71828
2	1.19
3	0.73
4	0.51
5	0.39
6	0.32

Resolution

- Repeated localizations give different center positions

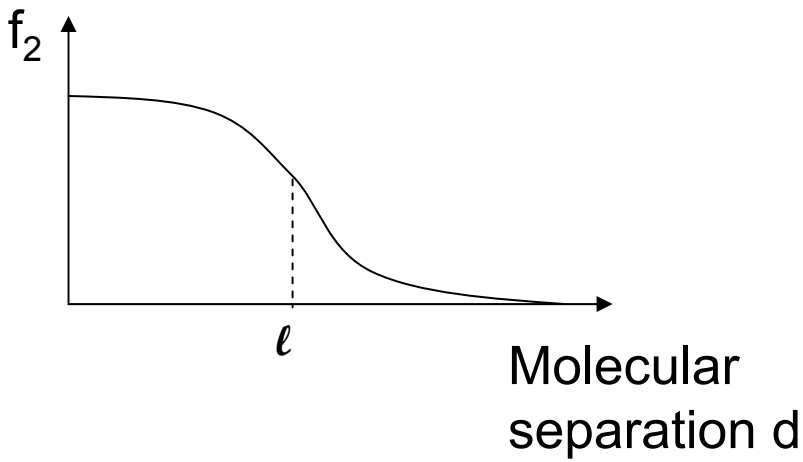


Rejection and Resolution

- Intensity of single molecule images: $I_1 \star p_1 f_1$
- Intensity of overlap image: $I_2 \star 2p_2 f_2$
- Resolution criterion: $I_1/I_2 > C$ (contrast)

Resolution

- Assume a general separation-dependent acceptance probability



I_2: Only depends on molecules closer than ℓ

I_2 ✪ $2*f_2(0)*p(2 \text{ activated})*p(2^{nd} \text{ closer than } \ell)$

✪$2*f_2(0)*(n-1)p(1-p)^{n-2}*(\ell/\lambda)^2$

I_1 ✪ $f_1*(1-p)^{n-1}$

Algorithm-Limited Resolution

- $I_1/I_2 \geq (f_1/2f_2(0))/(\ell/\lambda)^2$ →Since average # per spot always less than 1!

- If I_1/I_2 exceeds target contrast C, resolution is limited by the number of photons detected.

- Otherwise, your resolution is limited to ℓ.

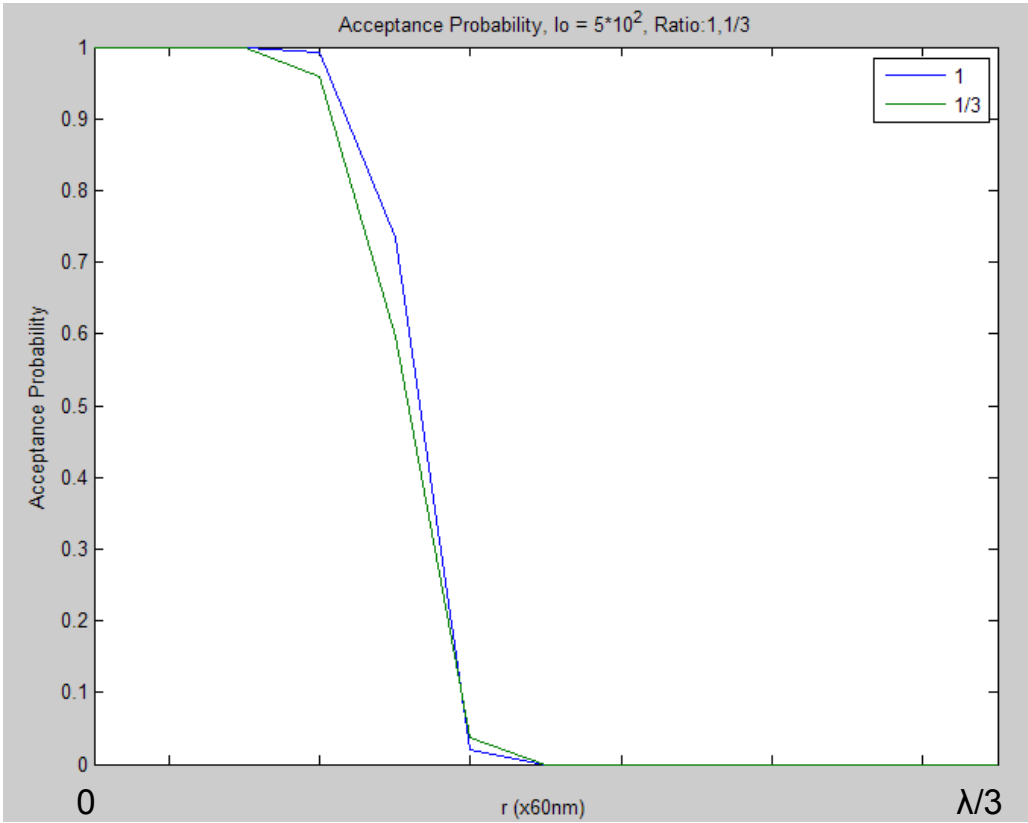
- Criterion:

$$\ell \leq \lambda \sqrt{\frac{f_1}{2 f_2(0) C}}$$

Make this less than f_1 by using tests of integrated intensity.

Computationally efficient!

Preliminary Results: Nonlinear curve fitting

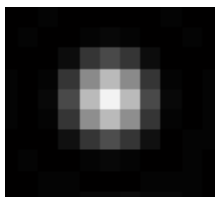


Fit to:

$$I = A + I_0 e^{-a(x-x_0)^2 - b(y-y_0)^2 - c(x-x_0)(y-y_0)}$$

Infer ellipticity from a, b, c.

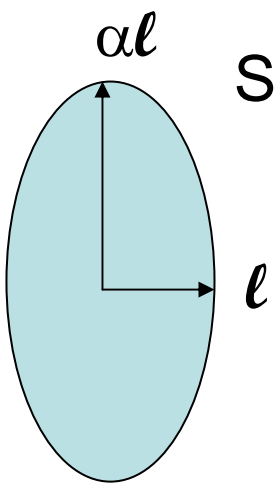
Reject if more than 15% elliptical.



3D Criterion

- Very similar:

$$\ell \le \lambda \left(\frac{f_1}{2 f_2(0) \alpha C} \right)^{1/3}$$



Surface of constant f_2

To Do:

1) Curve-fitting can take a hundred iterations or more. Are there simpler rejection algorithms that give similar performance curves?

   -My student has lots of good ideas.

2) Center-finding takes many iterations, evaluations of transcendental functions.

   -Working on ways to speed that up.

3) Effects of bleaching: Loss of information is a distinct type of error from bad information. Need to include it in models.

   -Reducing *n* over time increases $p_{max} = 1/n$.

Goal:

Real time STORM!!!!

- Faster rejection algorithms
- Faster center-finders
- Make post-processing as fast as acquisition!

- Acquisition currently ~minutes, so we need to be able to analyze hundreds of cycles in a minute.