Benchmarking Image Analysis Algorithms for Superresolution Fluorescence Microscopy

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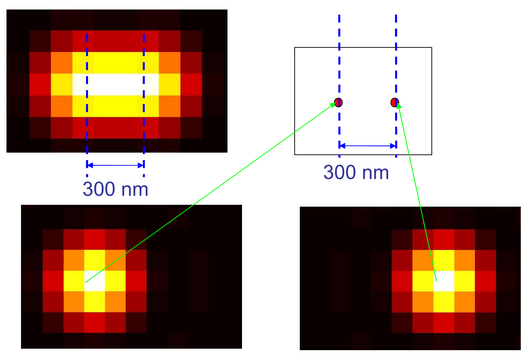
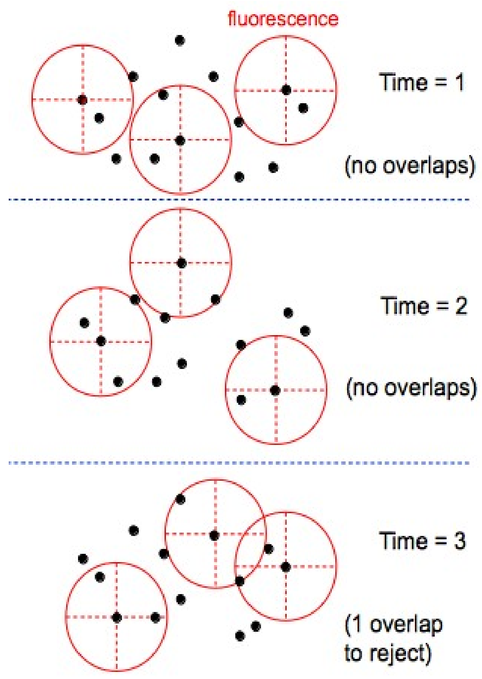
•Abstract:

Emerging super-resolution fluorescence microscopy techniques (e.g. PALM and STORM) are of growing significance in biophysical research as they enable high resolution imaging of live cells. Key structures imaged by these techniques include the cytoskeleton, membranes, and mitochondria. Recent theoretical work confirms that the experimentally achievable image acquisition rate and resolution of these techniques is limited by the performance of both the physical imaging system and the rejection algorithm used to distinguish single-fluorophore images from multi-fluorophore (overlap) images. Better rejection algorithms may therefore yield faster and more accurate experiments in addition to faster image analysis.

An effective and representative method of benchmarking rejection algorithm performance is needed to determine the "optimal" rejection algorithm. To benchmark a rejection technique, a set of simulated images is generated and then analyzed by the rejection algorithm. Previously, photon counts per molecule were arbitrary simulation parameters; however, selecting photon counts from an exponential distribution is more realistic (the time an activated molecule spends in its excited state is exponentially distributed). The constraints (or lack thereof) placed on exponentially distributed photon counts greatly effect rejection algorithm performance. We found that imposing a minimum photon count of approximately half the mean photon count per molecule was necessary to obtain realistic and meaningful rejection algorithm performance characteristics. Our benchmarking results show that rejection algorithms (ellipticity tests) based on principal components perform better than rejection algorithms based on curve fits. (Ellipticity is used to infer the presence of multiple closely-spaced activated fluorophores.) Since rejection performance is a key factor in super-resolution microscopy techniques, improved rejection algorithm characterization is an important step towards robust and powerful STORM/PALM image processing tools for widespread use in biophysical research.

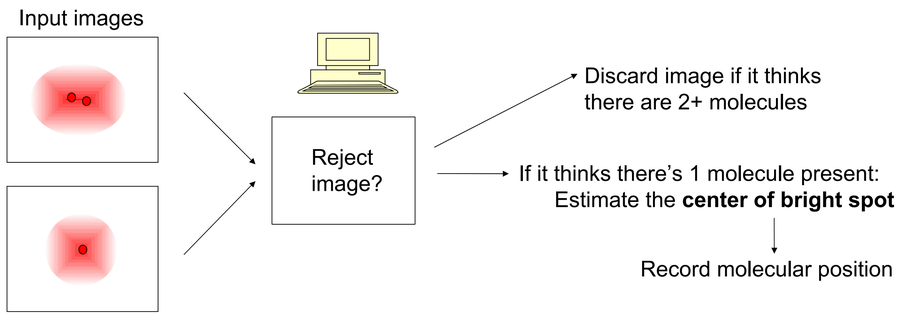
•PALM/STORM Process





- Not all the molecules in a crowded image are "on" at the same time.
- When blurs don't overlap you can localize molecules with sub-pixel resolution
- If 2 close molecules are "on" at the same time, PALM/STORM fail: so discard that image.
- Repeat this process, randomly switching different molecules on and off. Build up image.

•Rejection Algorithms

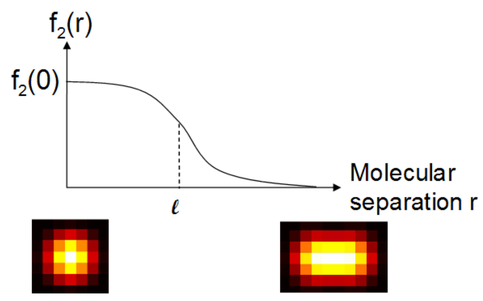


- Given an image with *m* activated molecules in a bright spot:
  f_m = fraction of *m* molecule images accepted by filter.
- We want $f_1 = 1$, and other f_m to be small
- A good filter can spare you the hassle of doing position estimates on overlap data (2+ molecules)

•Rejection Algorithms (cont.)

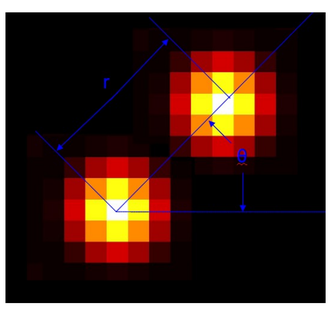
Assume a general separation-dependent acceptance probability, f_2

$f_2(0) = 1$, if we don't use overall intensity in rejection criteria



- A rejection algorithm's f_1 value and $f_2(r)$ curve can be used to compare its performance to other rejection algorithms.
- To determine f_1 and $f_2(r)$, the rejection algorithm was applied to simulated diffraction-limited images of 2 molecules

•Simulation Method



Generate images with:
$I = I_0 * PSF(r) + \text{Poisson Noise}$

- Effective pixel size: 72nm, 120nm (scaled to object plane)
- 16 subpixel displacements
- r: 0 to λ/2
- θ(r): 0° to 45°
- # photons/molecule : Drawn from an exponential distribution with mean (<N>) of 1000 photons or 3000 photons
- Only analyze bright spots with a minimum photon count of 700 or 2000
- About 38000 cases, 300 images per case to average over noise

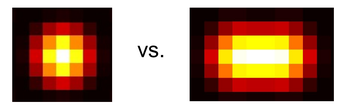
•Ellipticity Rejection Algorithm: least squares curve fitting

Fit bright spot intensity profile (via Levenberg-Marquadt) to:

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

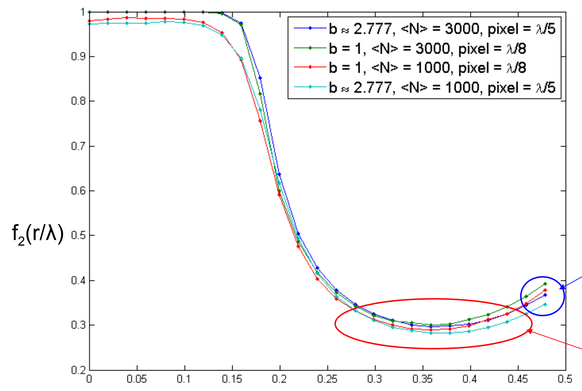
Infer ellipticity from a, b, c.

Reject bright spot if ellipticity exceeds 15%, i.e. if:



vs.

$$\frac{\text{semi-major axis} - \text{semi-minor axis}}{0.5*(\text{semi-major axis} + \text{semi-minor axis})} > 0.15$$

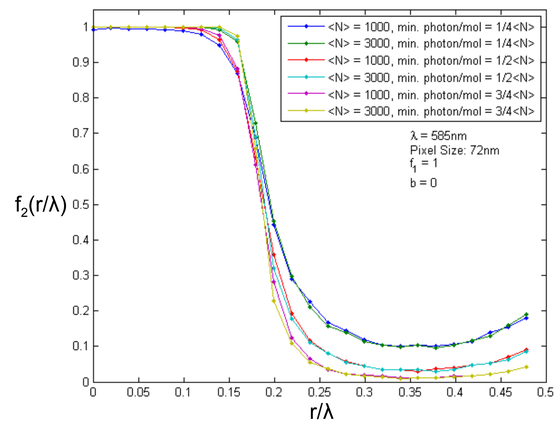


Simulation results vs. Ideal curve: Non-ideal effects?

f_2 increases with increasing molecular separation

f_2 doesn't go to zero at large molecular separations

•What's in the tail?



- We removed some images from analysis based on photon count/molecule to see which images comprise the tail
- Width of curve nearly independent of photon count threshold and pixel size

•Ellipticity Rejection Algorithm: Principal Components

Compute 2nd moments of bright spot: I_{xx}, I_{yy} and I_{xy}

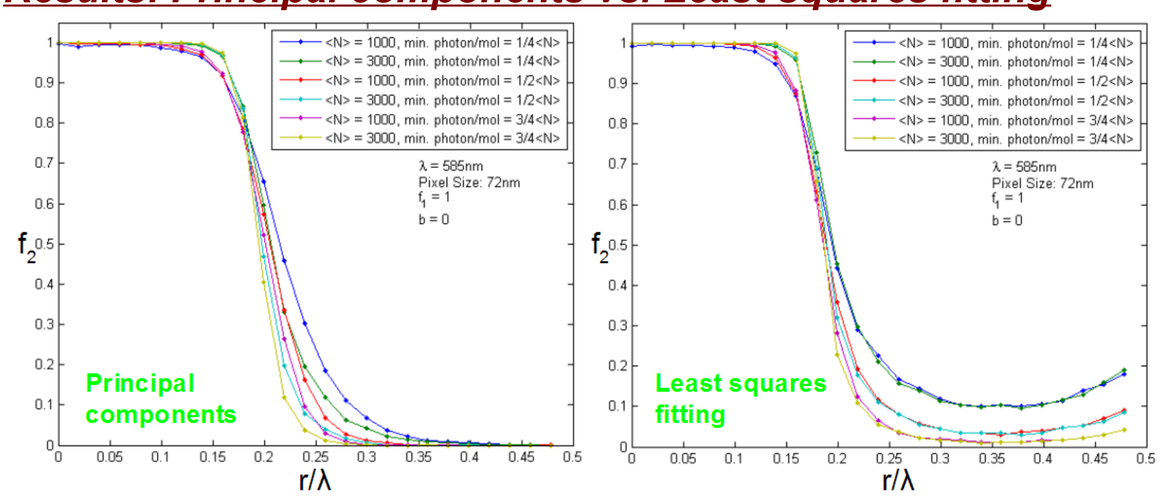
$$I_{ij} = \sum_{pixels} (i - cm_i)(j - cm_j) * img(x,y)$$

Infer ellipticity from $\dfrac{\sqrt{(I_{xx} - I_{yy})^2 + 4 * I_{xy}^2}}{I_{xx} + I_{yy}}$

Reject bright spot if ellipticity exceeds 10%

Much faster than nonlinear fitting!

•Results: Principal components vs. Least squares fitting



- Both have similar widths, but principal components rejects more at large separations
- Principal Components is less sensitive to background noise

•Conclusions and acknowledgments

✓We have established a methodology that enables meaningful benchmarks and comparisons among very different approaches to rejection

✓Rejection algorithms based on principal components are better than rejection algorithms based on curve fits

✓The "tail" of the f_2 curve, and its implications for localization precision, need to be studied.

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Some of the theory can be found in:

1) Small, A. R. Theoretical Limits on Errors and Acquisition Rates in Microscopy of Switchable Fluorophores. Biophysical Journal 2009, 96, L16-L18

1) Shore, E.W., Small, A.R., Optimal acquisition scheme for subwavelength localization microscopy of bleachable fluorophores, Optics Letters 2011, 36, 289-291.