Theoretical Limits on Speed, Errors, and Resolution in Microscopy with Switchable Fluorophores

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Abstract: We consider the theoretical limits to microscopy techniques that involve activation of a sparse subset of the fluorescent molecules in the sample until each molecule has been imaged and localized (e.g. STOchastic Reconstruction Microscopy). The number of activation cycles required can be reduced by the intelligent use of image processing techniques to remove bad data from the analysis. We will also show that the performance of the image processing algorithm determines the trade-offs between speed (reducing the number of activation cycles) and resolution.

OCIS codes: (100.6640) Image Processing-Superresolution; (180.2520) Microscopy-Fluorescence Microscopy

Recent experimental work has shown that it is possible to obtain images with subwavelength resolution in fluorescence microscopy. Promising techniques, with the potential for truly single-molecule resolution, use fluorophores that can switch between a non-fluorescent "dark" state and a fluorescent "activated" switch, e.g. photoactivatable fluorescent proteins[1-3], quantum dots[4-6], and pairs of cyanine dyes[7-10]. In all of these methods, only a sparse subset of the fluorophores is activated at any given time, and so the probability of any two fluorophores being separated by less than ~λ (the resolution limit of a microscope lens) is low. One can thus treat each bright spot in the image as representing a single molecule, determine the center of the bright spot (with resolution limited by the number of photons detected, due to shot noise[11]). After enough cycles of activation and imaging, all of the fluorophores in the sample will have been localized.

However, the key assumption in this procedure is that only 1 fluorophore is activated in any diffraction-limited region of area λ^2 (in 2D microscopy) or λ^3 (in 3D confocal microscopy). It therefore follows that the average number of fluorophores activated in a region of size λ^2 or λ^3 must be *at most* 1. With reported resolutions of $\lambda/10$ or better, the number of activation cycles required could exceed 100 in 2D and 1000 in 3D confocal microscopy. The need to avoid significant numbers of images with overlapping bright spots means that the average number of fluorophores activated in a diffraction-limited spot should actually be significantly smaller than 1, introducing many frames with no activated fluorophores in a diffraction-limited region and increasing the image acquisition time still further. There is thus a trade-off between speed and errors.

One way to avoid errors, while activating more fluorophores to speed up the imaging process, is to use an algorithm that can analyze a bright spot and determine whether the spot results from 1 activated fluorophore or multiple activated fluorophores. If 1 fluorophore is activated, the algorithm should accept the bright spot and find its center. If, on the other hand, the bright spot contains $m \geq 2$ activated fluorophores, the algorithm should reject the spot. In reality, the algorithm will be imperfect, and will accept single-fluorophore spots with probability $f_1<1$ while accepting m-fluorophore spots with probability $f_m>0$. The performance of the algorithm can be completely specified by the set of parameters $\{f_m\}$. This concept is illustrated in Fig. 1.

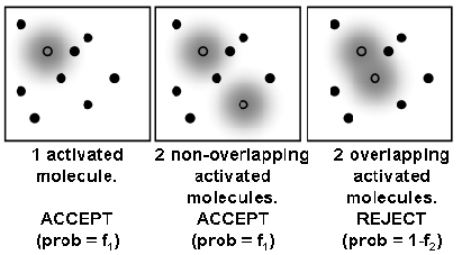

Fig. 1: Concept of a rejection algorithm. Open and dark circles represent activated and dark molecules. The algorithm should reject (with probability $1-f_2$) any image in which spots from 2 or more molecules overlap and accept (with probability f_1) images with non-overlapping spots.

In this work, we will show that the non-ideal performance of the rejection algorithm can introduce artifacts that limit the resolution of the image when working with high activation probabilities (and fast image acquisition), and

show that the performance of the algorithm must exceed a certain threshold to get noise-limited resolution while working at the maximum acquisition rate.

We will assume that the probability of activating a single molecule is some number p, which the experimenter determines, and that the number of fluorophores in a diffraction-limited spot is n>>1. The probability that a spot contains m activated fluorophores is then $p_m = [n!/m!(n-m)!]p^m(1-p)^{n-m}$. To ensure that each fluorophore is activated at least once, we would want to activate it T>>1 times, where the precise value of T depends on the experimenters tolerances. In recently published work[12] we showed that the number of activation cycles should then be $N = nT/f_1p_1$., and that the maximum value of p is $1/n$. Setting p larger than $1/n$ actually increases N by replacing single fluorophore spots with 2-fluorophore spots, decreasing p_1. One result of this limit on p is that our errors are dominated by 2-fluorophore spots, since p_m is a decreasing function of m if $p<1/n$ [12].

Now, consider 2 fluorophores separated by a distance $d<<\lambda$, as shown in Fig. 2a. If only 1 of them is activated, it is possible to localize the fluorophore with a resolution $\sim\lambda/\sqrt{N_p}$ where N_p is the number of photons collected from the fluorophore. If the fluorophore is localized several successive times, the estimated positions will vary, but they will cluster in a region of size $\sim\lambda/\sqrt{N_p}$. As long as only 1 fluorophore is activated at a time, the fluorophores will be distinguishable in the image built up by successive activation cycles (provided that $d> \lambda/\sqrt{N_p}$) (Fig. 2b). However, if both fluorophores are activated at once in a frame, and if the algorithm does not reject that frame, then the apparent center of the bright spot will be intermediate between the true positions of the 2 fluorophores. (Fig. 2c).

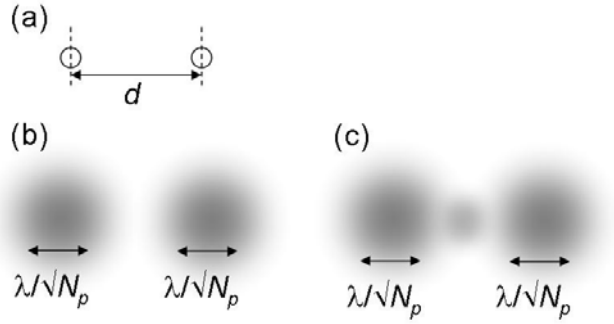

Fig. 2: (a) Schematic of fluorophore positions. (b) Image built up from successive localizations of the fluorophores with noise-limited resolution. (c) Image that includes erroneous spot between fluorophores due to simultaneous activation of both fluorophores. (Central spot is drawn smaller only for schematic puroposes, to show that it is dimmer and less prominent.)

Suppose that the rejection algorithm utilizes information on the shape of the bright spot (as fluorophores with larger separations will give rise to ellipsoidal bright spots), leading to a separation dependent rejection probability as shown in Fig. 3. The intensity of the erroneous central bright spot will be proportional to the probability that 2 fluorophores are activated and that the fluorophores are separated by a distance less than ℓ, the effective range of the separation-dependent rejection probability in Fig. 3. This gives the following for the intensity of the central spot:

$$I_2 \propto 2 f_2(0)(n-1)p(1-p)^{n-2}(\ell/\lambda)^2 \quad (1)$$

where f_2 may depend on d if the rejection algorithm makes use of the bright spot's shape (e.g. the curve-fitting procedure in [10]) and the factor of 2 comes from having 2 activated fluorophores emitting photons. The intensity of the spots on the left and right in Fig. 2c will be given by the probability that the fluorophore is active while all others are dark and the probability that the image is accepted:

$$I_1 \propto f_1(1-p)^{n-1} \quad (2)$$

The central bright spot will only impede our ability to resolve the fluorophores on the left and right if it is sufficiently bright, i.e. $I_1/I_2<C$ where C is some target contrast ratio.

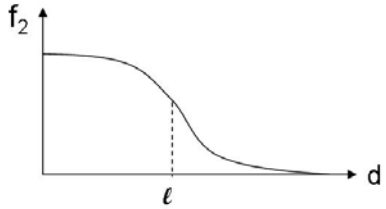

Fig. 3: Characteristic form of a separation-dependent rejection probability.

If we suppose that $p=1/n<<1$ (i.e. working at maximum activation rate with a sample containing a high density of fluorophores) and set $I_1/I_2 \geq C$, we get the following condition on the performance of the algorithm:

$$\ell \leq \lambda \sqrt{\frac{f_1}{2 f_2(0) C}} \tag{3}$$

It is difficult to make the parameter ℓ small, as it requires being able to infer the presence of multiple fluorophores from slight asymmetry in the shape of the bright spot. However, $f_2(0)$ can be made less than 1 (increasing our target value of ℓ) by rejecting spots with intensities significantly above average. Since tests of the overall intensity of a spot require significantly less computation time than, say, nonlinear curve fitting to detect asymmetry in a spot's shape, this result suggests that tests of intensity combined with simple shape tests (e.g. moments about principle axes) may yield very rapid post-processing while still rejecting multi-fluorophore images with enough reliability to ensure noise-limited resolution in a rapid experiment.

It is also interesting to note that one's ability to obtain noise-limited resolution while using the minimum number of activation cycles is constrained not just by the detectors and their noise characteristics, but also by the performance of the algorithms used to analyze the series of images obtained. This suggests a new concept that we term "algorithm-limited resolution" to distinguish this limitation from the noise limit on a detector's resolution and the diffraction limit to a lens's resolution. We are currently working to quantify the performance of image-processing algorithms used by experimental groups in this field, and compare the performance of their algorithms with other candidate algorithms for distinguishing single-fluorophore images from multi-fluorophore images.

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