Using photon statistics to boost microscopy resolution

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The magnifying power of light microscopy exerts a universal fascination that lasts a lifetime. Soon enough, however, users of optical microscopes learn (or should learn) that this power comes with limitations due to diffraction, as explained by Ernst Abbe (1) more than a century ago: any object, no matter how small, will be imaged by a conventional optical system as a finite-sized spot, with a minimum dimension obtained for point-like objects (such as single molecules) approximately equal to the wavelength of light, λ, multiplied by the optical magnification, M, and divided by the numerical aperture (N.A.) (Fig. 1*A*). The radius of this so-called point-spread function (PSF) can be used as a convenient criterion to define an upper limit to the minimum distance below which two nearby objects in the object plane cannot be distinguished (Fig. 1*B*). It has been known for some time that this Rayleigh criterion (2) is a bit too conservative, and that objects significantly closer can still be resolved with careful image analysis or clever illumination and detection schemes. In an article published in a recent issue of PNAS, Ram *et al.* (3) revisited this question and demonstrated that there is really no limit to how close two identical point-like objects can be and still have distances measurable with almost arbitrary precision by using conventional microscopy.

Before discussing Ram *et al.*'s (3) approach, it is worth putting the question of optical resolution in a broader context. As mentioned previously, conventional optical systems are limited in their ability to provide images of details smaller than a fraction of the wavelength due to diffraction. This imaging-resolution issue has been tackled and somewhat overturned by different means: (*i*) using nonconventional optics [near-field optics (4) or negative refraction index lenses (5, 6)], it is possible to obtain images with finer details but at the expense of considerable complexity and limited to local surface observations; (*ii*) deconvolution of fluorescence images using the knowledge of the PSF (or not) allows superresolution to be attained (7); and (*iii*) structured illumination (8), nonlinear effects in fluorescence (9, 10), or both (11) can also be used to obtain higher imaging resolution. These approaches yield exquisite images but are still rather complex to implement and oftentimes require a very photostable sample to collect enough photons.

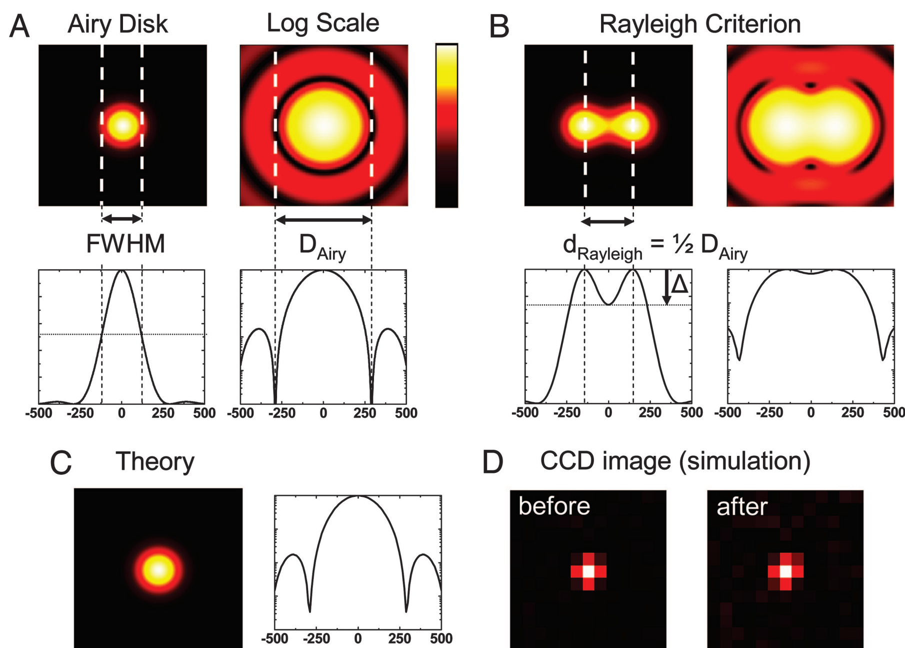

Fig. 1. Images of point-like sources in the optical microscope. (*A*) Image of the intensity profile of a microscope (Airy Disk). (*Upper Left*) The normalized color-coded profile for λ = 690 nm, N.A. = 1.45, M = 1, with its logarithm on the right. (Scale, 4 decades; image size, 1 μm.) (*Lower*) Graphs showing the median cross sections (in nm). The distances defined are: D_{Airy} = 1.22 λ/N.A., full width at half maximum (FWHM) = 0.29 D_{Airy}, and $d_{Rayleigh}$ = 1/2 D_{Airy}. (*B*) Intensity profile corresponding to two point-like sources $d_{Rayleigh}$ apart. They are easily resolved, because the dip in the intensity profile is Δ = 26.3% of the maximum. (*C*) The case of two Cy5 dyes attached to two ends of a 12-nm DNA duplex studied by Ram *et al.* (3) leads to a theoretical profile that is practically indistinguishable from the pure Airy disk shown above. (*D*) To give the feel of Ram *et al.*'s result, we show two simulated charge-coupled device images corresponding to the experimental conditions used in this work: pixel size, 129 nm; 13 × 13 pixels, 1-s integration; background, 80 counts/pixel per s. The first image (*Left*, before) shows the two molecules, each emitting ≈4,000 photons for 1 s. The second image (*Right*, after) shows the remaining molecule after the photobleaching of its partner.

A related but different problem is that of measuring the distance between individual nanoobjects, which we will call the distance-resolution problem. Here what matters is the ability to precisely pinpoint the location of each individual object to be able to measure its distance. There are, of course, quite a few subtleties even in this simpler problem, but, in practice, the diffraction-limited size of the PSF is not really the issue: what matters is its sampling (pixel size versus PSF size) and contrast [signal-to-noise ratio (SNR) and signal-to-background ratio (SBR)] (12–14).

To illustrate the difference between these two resolution definitions, imagine an isolated pair of two nearby (nanometer-scale-distance apart) identical fluorescent emitters, whereas other similar pairs are separated by distances larger than the diffraction limit (low dilution limit). The task at hand is to measure the interdistances of all pairs; the distance-resolution limit provides an estimator for how well those distances could be determined. On the other hand, when the concentration of identical fluorescent emitters within the diffraction limit spot is high, the task at hand is to derive the spatial distribution of these emitters; the estimation of this distribution is governed by the imaging resolution.

The results presented by Ram *et al.* (3) definitely pertain to the second category of problem. Using a simple mathematical description of the image-collection process in which the image is formed by individ-

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ual photons accumulated in pixels with diverse sources of noise contamination, they have recently shown how to use a statistical quantity, called the Fisher information matrix, to compute a lower bound on the accuracy of the position of individual fluorescent objects (14). Here they extend their approach to compute a lower bound on the accuracy with which the position and distance between two nearby identical fluorescent emitters can be determined, by computing the Fisher information matrix for the position of one emitter (x_0, y_0) and the distance and angle to the second emitter (d, ϕ). A lower bound on the distance-measurement accuracy is then obtained as the square root of the diagonal element corresponding to the distance parameter in the inverse Fisher information matrix. The remarkable result of this analysis is that nanometer distances can, in principle, be measured with arbitrary precision, provided enough photons can be accumulated from each object. For instance, the distance between two GFP molecules separated by 10 nm should be measurable with a resolution of a few nanometers, provided 10,000 photons can be detected per molecule. Moreover, the precision of the measurement can be significantly improved (or fewer photons are required) if one molecule bleaches and the remaining isolated molecule is included in the analysis, as had already been demonstrated by others (15).

Can this theoretical limit be approached practically? Although tests of the method with simulated data are lacking, Ram *et al.* (3) present a few examples of distance measurements between individual Cy5 molecules obtained with a standard epifluorescence illumination setup that result in standard deviation approaching the theoretical limit they have derived. Whereas excellent relative precision can be obtained for dyes 200 and 300 nm apart, the rapid bleaching of Cy5 molecules attached at both ends of a 30-bp-long DNA duplex (12 nm) resulted in uncertainties of 50% of the measured distance in the two examples shown here. This was nevertheless the predicted uncertainty, and the estimation of the distance itself by the maximum likelihood method used here by Ram *et al.* turned out to be close to the expected value.

Although this result may not sound new to practitioners of the field who have become used to measuring nanometer distances between individual (nanometer-sized) objects of distinguishable characteristics by using conventional optics [e.g., different absorption (16) or emission wavelength (17), different fluorescence lifetimes (18), or different time points (19)], Ram *et al.*'s work (3) shows that similar performances can be obtained for identical nanoobjects. This has a significant practical advantage, because it dispenses with the use of different dyes (and therefore performing separate labeling and purification of the targeted molecules) to measure the respective location of single molecules. Of course, a lot of photons are needed to attain nanometer resolution, which practically means integrating for a long period (1–3 sec in the observations described here), currently limiting this type of analysis to relatively static samples. But brighter fluorophores (such as quantum dots) or controlled *in vitro* conditions limiting photobleaching of the dyes may permit obtaining a higher photon count rate.

This new work provides single-molecule microscopists with a convenient way to estimate the best distance resolution that can be expected in their specific experimental conditions. It would certainly be convenient to reformulate this limit in terms of the ratio between the pixel size and the PSF dimension, as well as a function of SNR and SBR. Additionally, the lower bound obtained in this work should be interpreted with caution in the case of small distances and large uncertainties, because the probability distribution of the distance may become asymmetric in this case, as has been discussed in the literature (12, 20). Finally, although it is always interesting to have a theoretical limit on the attainable precision of a measurement, it is even more important to have a reliable estimate of the actual uncertainty of a single measurement. This actual uncertainty will, of course, depend on the method used to estimate the value of the measured parameter (here the distance between two molecules) but will in particular depend on the precise distribution of photons in the image. It would be interesting in this respect to systematically compare different estimation methods (nonlinear least-square fitting and maximum-likelihood estimation are the two that first come to mind) with the theoretical limit. These methods usually provide a value of the uncertainty of the estimated parameter that is valid only in specific conditions of independence between the parameters, which may or may not be satisfied in this particular case. Bootstrap estimation of this uncertainty may be a possible alternative (12, 21).

Interestingly, there seems to be no fundamental obstacle to extending this analysis to more than two identical single emitters within the diffraction-limited spot, therefore blurring the distinction between imaging resolution and distance resolution stated above. This approach could in principle "deconvolve" the distribution of emitters within the diffraction limit spot and therefore provide a novel superresolution imaging method. This would require knowledge of the total number of molecules in the spot, which could, for instance, be determined by a complete recording of the bleaching steps of all single molecules. Another fascinating possibility would be to use this method for measuring the stoichiometry of multimers (such as membrane proteins, like ion channels and receptors), where individual monomers are tagged with a single fluorophore (e.g., GFP), a long-standing problem in biology. Finally, that the work of Ram *et al.* (3) considers an image to be comprised of photons with stochastic individual positions and arrival times, information eventually lost by the integration process of the charge-coupled device camera, suggests even more efficient measurements could be obtained with a detector that would preserve this information.

It is quite amazing to see that, more than 130 years after Abbe's pioneering statement (1), we apparently still have not unleashed all the potential of the optical microscope.

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