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Contrast, Resolution, Bleaching and Statistics in Confocal Microscopy

James B. Pawley
Department of Zoology, University of Wisconsin, Madison, 53706, USA

Although those using the transmission electron microscope (TEM) have long been accustomed to thinking of the resolution of their instrument as being usefully defined only in terms of contrast, until quite recently it was common for those involved in biological light microscopy to speak of the resolution of their instruments in some sort of absolute sense. In part, this due to the fact that, as spherical aberration cannot be corrected in the TEM, the contrast transfer function (CTF) of the TEM varies with defocus in a complex manner: oscillating in sign across the zero-contrast line as one proceeds to higher spatial frequencies. In practice, the absolute TEM information-limit is set not by diffraction but by the limited coherence of the source and by electro-magnetic instabilities which combine to cause the "envelope function" of the CTF to decline steadily at higher spatial frequencies. This complexity forces the user to explicitly consider the CTF in order to interpret high-resolution TEM images.

In contrast, when operated with absorption or epi-fluorescence contrast, the CTF of a diffraction-limited light microscope goes obediently to zero in a fairly simple manner that holds few surprises and, for most purposes, is adequately summarized by the Rayleigh Criterion. At the other extreme, the many biological applications of high-resolution LM imaging often involve interference effects such as differential-interference contrast (DIC) and are used in combination with video-based image enhancement techniques. The CTF of such a complex system is perhaps too complicated to encourage theoretical analysis.

Unfortunately, this general lack of attention to the relationship between contrast and resolution in LM has tended to obscure a simple but important fact: that the contrast produced by small features is transmitted to the detector much less efficiently than that of larger features. (i.e. that small features inevitably appear in the image with low contrast.) The purpose of this paper is to focus on the way this fact interacts with signal level and photobleaching to place a practical limit on resolution in fluorescence confocal imaging.

Although contrast is an essential ingredient in our ability to "see" a feature, the signal-to-noise ratio (S/N) of the data is equally important. These two factors were first combined to produce a measure of "visibility" by Rose (1948) who quantified the relationship between the minimum size that a feature in a television image must have in order to be seen as different from its surroundings, δ, the contrast and S/N. A similar analysis was later applied to TEM images by Glaeser (1971, 1975) who was trying to image molecules that were damaged by the electron beam illuminating the specimen. He wanted to calculate the minimum dose of illumination necessary to produce a useable image and derived the equation, $\delta = A/C_i(f \bullet n)^{1/2}$ where n is the average of number quanta/unit-area (q/pix), at the specimen and these are utilized to form the image with a fractional efficiency, f. C_i is the contrast (in %) intrinsic to the interaction between the specimen and the impinging radiation, as transmitted by the microscope. This formulation assumes that the only source of noise is the statistical variation in **n** as described by Poisson statistics. A is a constant that must be ~ 5 for a single pixel to be "seen" above the local background by a human observer but which is smaller for larger image features.

In practice, this equation shows that in an image with only 2 gray levels (feature and background), the intensity of a feature one pixel in size must be >25q/pix in order to be dis-tinguishable from a background of 1 q/pixel. In other words, the staining ratio would have to be at least 25:1 even if the microscope did not reduce the contrast of the feature at all. However, if the contrast be-

tween two adjacent point features were reduced to that of the Rayleigh Criterion (~25% darker at the midpoint between the features), the peak brightness would have to be about 400 q/pix to separate the 75% signal from the 100% peaks by 5x the statistical noise level. This represents a 16x increase in signal that must be acquired and, hence, in the dose of illumination applied to the specimen. Dropping the contrast to 10% (making the dip in the intensity between the peaks only 90% of the peak value) implies a 100x increase in dose.

Now that confocal-fluorescence microscopy is beginning to approach diffraction-limited resolution, it is easy to demonstrate the importance of this relationship. Fig. 1 shows a series of confocal images made with only a few photons/ pixel, of part of a test specimen consisting of a metal-film-on-glass pattern over a "sea" of fluorescent oil (Centonze and Pawley 1995). As the average peak signal level varies from (a): 2, (b): 4, (c): 8 to (d): 16 counts/pixel, the visibility of the bars in the smaller patches improves. This happens not because the optical resolution changes but because by we are now counting enough particles to permit such low-contrast features to be recognized above the noise level. Unfortunately, in confocal fluorescence microscopy, both fluorescence saturation and photobleaching prevent an arbitrary increase in the signal level.

This analysis emphasizes again the importance of increasing the quantum efficiency (QE) of the detector in the confocal microscope above its present level of 3-20%. It also points to the crucial importance of S/N in any effort to compare the relative photon efficiency for 3D-microscopical imaging of confocal/fluorescence microscopy vs. the widefield/deconvolution technique. While the CCD detector used with the latter technique has a clear edge in QE, the contrast produced in the recorded data is greatly reduced if highly stained features are located above or below the

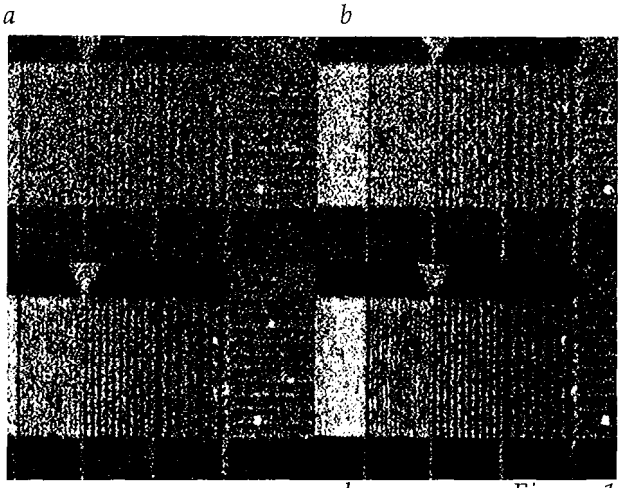

Figure 1

plane of focus. It is as yet unclear where in the range of staining sparcity, what is gained by the widefield method in terms of QE (and hence improved S/N) becomes more important than that which is lost by the reduced contrast of the recorded data. (i.e. the stain contrast as modified by the point-spread function of the microscope). What is clear is that it is most important to increase the "use factor," f, as much as possible and, in the case of confocal-fluorescence microscopy, this means increasing detector quantum efficiency.

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