3D Microscopy: Confocal, Deconvolution or Both?

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Over the past 20 years great improvements have been made in the techniques available for doing 3D fluorescent microscopy on living cells. The first approach, generally referred to as image deconvolution, treats the stack of 2D widefield (WF) image data as merely the sum of a number of discrete point-spread functions (PSF) and uses the computer to find the array of emitters that, when blurred by the PSF, best fits the stored data. If the PSF is known, only presence of statistical and electronic noise in the data, prevents this best-fit set of emitters from being a perfect image of the dye distribution in the specimen.[1] The crucial role played by noise can be appreciated by comparing images from the Hubbell space telescope in its original condition, even after deconvolution, with images taken after the optics had been repaired.

The second approach to 3D microscopy requires the introduction of a confocal aperture in front of the photodetector of a scanning laser microscope so that only the fluorescent signal emitted from the plane-of-focus is recorded. As a result the image formed represents an "optical section" and a stack of such sections can be recorded at different focus heights to produce a 3D data set.[2]

The purpose of this paper is to list the differences between these two techniques with the idea of defining the types of studies best suited to each method. In these contexts, the word "best" is usually defined as that method which provides the largest amount of structural information (considering both resolution and contrast) from a given dose of light to the specimen. Even assuming that all methods employ the most appropriate optical components and use optimal sampling methods in all 3 dimensions, WF/deconvolution still differs from the confocal approach in a number of important respects:

- **Excitation Intensity:** The photon flux is ~10^5 - 10^7 x higher in a laser confocal than in WF.
- **Detector Quantum Efficiency:** Depending on wavelength, the CCD sensors used for WF have 3-10x more quantum efficiency (QE) than the photomultilplier tubes (PMT) used in confocal.
- **Signal Levels:** In fluorescent confocal images, the signal from bright areas often represents only 20-100 detected photons/pixels (dpp), while even in "low dose" WF images, bright areas represent 5,000 dpp and 30x this number is not uncommon. Although some of this increase results from the greater detector QE and the presence of out-of-focus light in the WF signal, it still seems that those using the WF method start with a signal representing 5-20x more light from the specimen.
- **Read-out Noise:** Although, at ±5 e-RMS/pixel, the CCD has more readout noise than the PMT, even this level becomes less than the Poisson or "shot" noise for signals above 25 electrons/pixel.
- **"Real-time" Imaging:** To date no deconvolution method has approached confocal for obtaining optical-section data at a high frame rate, especially where only a single plane is viewed over time.

The Intensity Problem

Can we assume that fluorescence efficiency and bleaching rate are the same for both confocal and WF image capture? Is it possible that the intensity of the confocal spot is high enough to drive a large fraction of the dye molecules in the focused spot into a triplet state during the first ~10% of a 1 µs pixel, thereby significantly reducing the "effective" dye concentration (and hence the signal level averaged over the 1 µs)? As triplet-state lifetimes are much longer (msec) than the µsec pixel times of confocal but shorter than CCD exposure times, such a mechanism would provide less signal/excitation-photon in the confocal case. It would also prevent the signal level from increasing linearly with laser intensity, an effect that should be less evident if the probe illuminates a given area for only a short time. To investigate this possibility we have collected quantitative fluorescence data using a wide variety of laser intensities (0.01-1.0 mW), zoom settings and scan rates (0.33 - 2 sec/frame) to vary spot intensities and pixel times over several decades. Although no large departure from linearity was noted, measurements have been limited to fluorescent plastics and liquids. Possibly the effect may still be important on aqueous or embedded specimens.

Although the light flux in the confocal microscope can be intense enough to cause singlet-state saturation of the fluorophor[2], we generally assume that it is insufficient to produce many 2-photon events unless fast, pulsed lasers are employed[3]. However, it is possible that, near siglet-state saturation, the absorption of a second photon by a molecule already in the excited state may occur. Though such events are probably too rare to effect the fluorescence efficiency significantly, they may cause bleaching of the fluorophor and thereby become another mechanism to explain the disparity in total light dose that the specimen evidently finds tolerable under the two types of observation.

The Detector QE vs. Out-of-focus-Signal-Noise Problem

Any comparison of the two methods depends on the extent to which the higher detector QE of the CCD used in WF provides enough extra signal to offset the effects of the Poisson noise associated with the presence of the photons from out-of-focus planes[4]. Does the detection of photons from out-of-focus planes provide "information": something that you wanted to know about the structure of the specimen, or simply "signal": that which comes from the detector? If light originating from planes more than, say 2 µm, above or below the plane of focus, is found to contribute only slightly to the final processed image, but adds significantly Poisson noise to the signal from the CCD, can it still be thought of as providing information? As a lot of time and money is being spent in this field of research, it would be good to know if one method is clearly superior, or even clearly-superior-for-some-subset-of-specimens. Clearly the answer to this question depends on how much out-of-focus light actually reaches the detector as well as how far out of focus it is: the Poisson noise associated with a small signal from a nearby plane, may add more information than noise but the converse is also true. Clearly, the analysis depends on the geometry of the stain distribution but unfortunately there are as yet few direct quantitative comparisons of the two techniques on a single specimen, perhaps because even viewing a

fluorescent specimen once causes bleaching and other damage. If we could make this measurement for a number of actual specimens, we might be able to find where those "missing" photons are going and, if we add S/N calculations, including Poisson noise in "real-photons", we might be able to define a "stain-density-thickness" product threshold above which mechanically excluding photons from out-of-focus planes would produce better data.

On the other hand, even using a confocal aperture diameter equal to the radius of the first Airy dark ring, only ~50% of light emerging from the plane of focus ever reaches the detector. It is hard to see this as an advantage, especially when combined with low detector QE. To address this latter point, I have been working with others to develop a device called the CCDiode. It is a single-channel silicon detector that is read out like a CCD but using 16x parallel readout channels to keep the noise level at ±3 e-RMS even at a 1 MHz readout rate.[5] A QE of a rear-illuminated detector of this type is the same as that of a CCD and using a more advanced version designed to readout from a 5x5 CCD-array at each pixel of the scanned raster, data could be effectively collected at several pinhole settings separately and simultaneously.

Is a compromise possible?

Although each method has been used to produce results of great interest and worth, aside from readout speed, no clear-cut rules to indicate the usefulness of either for a given study have yet emerged. It is certainly possible that two-photon fluorescence microscopy, in which the dye is not even excited unless it is near the plane of focus, may eventually be the best method of all.[3] Some have suggested that the best plan might be to do confocal[6] with a pinhole somewhat larger than the Airy disk and then deconvolving the results. Such an approach would make best use of data collected using the 5x5 CCDiode confocal detector mentioned above. However, this would require the development of new methods of deconvolution. ■

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5 Pawley, J.B., Blouke, M. and Janesick, J., The CCDiode: An optimal detector for laser confocal microscopes. Proc. SPEI, **2655**:41, 1996

6 Shaw, P. in #2 (above), chapter 23 and also, van der Voort HTM, and Strasters, K.C., Restoration of confocal images for quantitative image analysis, J. Microsc. **178**:165-181, 1995