The 39 Steps: A Cautionary Tale about "quantatative" 3D Fluorescence Microscopy

(stolen from BioTechniques **28-5:**2-4)
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We all "know" that fluorescent micrographs tell one the location of the labeled molecules in a tissue. Don't we? Well, maybe not. In fact, all you can be really confident in measuring with most laser-scanning, fluorescence confocal microscopes is some measure of the number of photons collected at a particular time. We fondly hope that this is an accurate measure of one of two 'interesting" parameters: the local analyte concentration or the local ion concentration. In fact, many other factors may affect the number actually stored in the computer memory at any moment. Over the years, students participating in the course on the 3D Microscopy of Living Cells, held each June at the University of British Columbia, compile a list of these extraneous factors. The first year the final list had 39 entries and so we borrowed the name from the Hitchcock film. Since then the list has grown.

Although there is not room in this short article to fully describe each term, a brief, and I hope useful, explanation is included in brackets. That the terms have been ordered under headings, should not obscure the fact that many interact with factors found under other headings. You may note that many of these variables are usually thought of in terms of their effect on spatial resolution. They are listed here because reduced resolution translates to putting the same number of exciting photons into a larger spot. This lowers the excitation intensity in th e spot and hence the number of photons produced by a given molecule. It also reduces the fraction of these that are detected. It is often forgotten that "normal" signal levels in fluorescence confocal correspond to only 10-20 photons/pixel in the brightest areas. Under these conditions, statistical noise is a more important limitation on spatial resolution than that defined by the Abbé equation.

The Laser Unit: This is the illumination source for the confocal microscopeand in general, the fluorescence mesured is proportional to the laser power level. Although total laser output power is usually regulated, the amount of power in each line of a multi-line laser may not be and may vary widely with time.)

Power output instability (Usually noise and instability is usually < 1% but lasers can become much more unstable as they age.)

Efficiency of the optical coupling to the connecting fiber (if used. Dust, misalignment or mechanical instability here can be the source of random changes of 10-30%)

Alignment and reflection characteristics of laser mirrors (Can be the source of long-term drift in laser output)

Beam-pointing error/alignment. (The location from which the laser light appears to emanate is determined by the laser mirrors. Instability here will show up as changes in brightness because changes in the apparent source position will change the efficiency of the optics coupling the laser light into the single-mode optical fibre used in most instruments.

Objective:

Numerical aperture (Effects fraction of light emitted by specimen that can be collected. Ditto for light from laser.)

Objective magnification (Magnification is inversely related to the diameter of the objective lens entrance pupil. The objective will only function properly if the entire entrance pupil is filled with exciting laser light. Underfilling will reduce spatial resolution and hence peak intensity. Overfilling will cause some laser light to strike the metal mounting of the objective and be lost, also reducing the intensity in the spot.)

Cleanliness (Dirty optics produce much larger, dimmer spots.)

Transmission (The fraction of light incident on the objective that can be focussed into a spot on the other side. Varies with wavelength. Beware using older optics in IR or UV)

Chromatic and spherical aberration (Both make the spot bigger and vary with wavelength. Spherical also varies strongly with coverglass thickness and the refractive index of the immersion and embedding media).

Diffraction (Diffraction is the unavoidable limit to optical resolution. It effectively enlarges the image of objects smaller than the diffraction limit, making them appear dimmer than they should be.)

Scanning system:

Zoom Magnification (This control determines the size of a pixel at the specimen. For Nyquist sampling, the pixel should be at least 2x smaller than the smallest features that you expect to see in your specimen. Assuming a Rayleigh Criterion resolution of 200 nm, the pixels should be <100nm. Larger ones produce undersampling, reducing the recorded brightness of small features.)

Affects the bleaching rate (Bleach rate is proportional to the square of the zoom magnification.)

Scan speed (The longer the dwell time on a particular pixel, the more signal will be detected and the less it will be distorted by Poisson Noise. At high scan speeds [<100ns/pixel] signal from dyes with fluorescent decay constants that are longer than this dwell time can be reduced.)

Raster size (Together with the zoom magnification, the number of pixels along the edges of your raster will determine the pixel size. More pixels [1024x1024 vs. 512x512] makes undersampling less likely but means that one must either spend less time on each pixel [reducing the number of photons collected and increasing Poisson Noise] or take more time to scan the larger image [possibly causing more bleaching])

Geometrical distortion: (Can be introduced by the optics or the scanning mirrors. Can result in a discordance between the shape of the object and the image.)

Environment: (Vibration and stray EM fields caused by cooling fans etc. can cause improper mirror deflections, resulting in distortions that may vary with time.)

Other optics:

Transmission (Measure of the absence of absorption and reflectance losses in optical components, particularly: ND and/or bandpass filters, beamsplitters, objectives.)

Reflections from air/glass interfaces (Usually represent lost signal but may appear as bright spots, unrelated to specimen structure.)

Mirror reflectivity (May be strong function of wavelength in the IR and UV and degrades with exposure to humidity and dust)

Coverslip thickness (The least expensive optical component and the most likely to be carelessly chosen. Should be 170 ±5 µm.)

Immersion oil (Its refractive index must be exactly matched to the objective used. This may only occur over a small temperature range. Alternatively, it can be especially mixed.)

Focus-plane position (A feature slightly above of below the plane of focus will appear dimmer. When collecting 3D data, Niquist sampling must also be practiced in the spacing of z planes.)

Mechanical drift of stage (Causes the plane of the object actually imaged to change with time.)

Dyes:

Concentration (The Good News! What you are usually trying to measure.)

Penetration into (or steric exclusion from) the specimen, (often a function of ionic strength and pH)

Absorption crossection (measures the fraction of the exciting photon flux that will be absorbed by a dye molecule. Affected by method of conjugation to probe, pH, specific ion concentration and ionic strength.)

Quantum efficiency (The chance that energy absorbed from an exciting photon will be re-radiated as a fluorescent photon. A strong function of wavelength and also effected by pH, specific ion concentration and ionic strength. Also affected by dye-protein interactions.)

Singlet state saturation (When >1mW. of laser power is used with a high-NA objective, the light is intense enough to put most of the dye molecules near the crossover into an excited state, reducing the effective dye-QE)

Loading (the amount of dye you manage to get into your cell. Important variables include: number of dye molecules/antibody or other protein marker and fixation/permeablization protocol used.

Quenching (Absorption of the fluorescence from one dye molecule by others nearby.)

"Unloading," (Dye that was in cell but has now been pumped out or otherwise inactivated.)

Substrate-reaction rate (For dyes whose fluorescent properties are related to their interaction with ions or other molecules in the cell: The pixel-dwell of ~microseconds may not be enough time to reach equilibrium.)

Pre-bleaching (Bleaching of the dye before the present measurement was made)

Compartmentalization (Redistribution of the dye by intracellular processes.)

Dye/dye interactions (Not only the quenching mentioned above but also Fluorescence Resonance Energy Transfer (FRET). The latter occurs when the emission spectrum of one dye overlaps with the absorption spectrum of a second dye molecule, a few nm away. FRET can result in the emission of light only at the emission wavelength of the second dye. If, as probably often happens, the second molecule isn't fluorescent, the fluorescent light is lost or quenched.)

Effects of dye on the specimen (For instance, killing it? May effect many environmental variables.),

Specimen:

Embedding medium (The RI of the specimen AND its embedding medium will determine the severity of the spherical aberration present. Even for "aqueous" biological specimens viewed using a "water" objective, this match is unlikely to be perfect, or even close. Spherical aberration of this type is THE major cause of signal loss with increasing penetration depth.)

Immersion oil (It is essential to use an oil with the correct RI and dispersion [change of RI with wavelength]. It is also important to make sure that there isn't an air bubble to act as a "lens" in the middle of it! [Check by focusing up and down with the Bertrand lens used for aligning your phase rings.])

Vital state (Dying cells, have different shapes, sizes and ionic environments than living ones.)

Autofluorescence, (Presence of endogenous fluorescent compounds. The efficiency of these dyes may vary with pH, ionic concentration, and metabolic state. Additional autofluorescence can be produced by improper fixation: in particular, using glutaraldehyde.)

Refractile structures or organelles between the objective and the plane of focus (For instance, spherical globules of lipid may refocus the beam to a totally unknown location. Smaller features may have lesser effects but any refractile features that scatters light out of the beam, reduces the intensity of the exciting spot and hence the detected signal. Their cumulative effect also adds up to cells having an RI much higher than that of water.)

Presence or absence of highly stained structure above or below the focus plane. (Z-resolution is finite and also large structures may absorb substantial exciting light if highly stained.)

Pinhole:

Size (Signal proportional to square of diameter. Usually set equal to the diameter of the Airy Disk at the plane of the pinhole.)

Alignment (The image of the laser that is focused onto the specimen and then refocused back through the optical system, should coincide with the center of the pinhole.)

Detector: (assumed to be a photomultiplier tube, PMT)

Quantum efficiency (The detected signal is directly proportional to QE. The effective QE of the PMTs used in most confocals drops from ~15% in the blue to ~ 4% in the red end of the spectrum.)

Response time (Most fluorescent signals can be amplified rapidly but detectors for others, such as trans-membrane currents, respond only slowly, making slow scanning speeds necessary)

PMT voltage (Determines the amplification of the PMT. An increase of 50 volts corresponds to a factor of ~2 more gain.

PMT black level or Brightness. (Beware! This control permits the addition or subtraction of an arbitrary amount from the signal that is presented to the digitizer. Set so signal level in the darkest parts of the image is 5-10 digital units.)

Noise (There are many possible sources of noise. All will distort the recorded value. Usually, PMTs produce dark current noise that is small compared to the signal level, however, this is less true for red-sensitive PMTs that are permitted to become warm or when viewing very poorly stained specimens. Apart from any stray light that may inadvertently reach the PMT, the main remaining noise source is Poisson or statistical noise. This is equal to the square root of number of photons recorded in a given pixel. As a result, it becomes larger at higher signal levels, although the ratio of signal to noise improves.)

Digitization:

Linearity (The electronic signals presented to the digitizer of "8-bit" microscopes must be of a size to be recorded between 1 and 255. Because of statistical noise, >10 and < 220 is safer.)

Digital conversion factor (The ratio between the number of photons detected and the number stored. Depends on PMT voltage and other electronic gain, but usually about 30 for "normal' specimens recorded on 8-bit instruments.)

Remember, to measure one of these factors accurately, one must hold the other "38" constant.

Good luck!

Jim and all the Gang!