Fluorescence microscopy – avoiding the pitfalls

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On p. 1704, in the section 'Image acquisition', line 6 should read 'all four pixels in a 2×2 square are summed into a single pixel'.

For clarification, 2×2 binned pixels are twice the height and twice the width of the original pixel, but the pixel area is four times larger; similarly, for a 4×4 binned image the pixel height and width are four times larger, resulting in a pixel that has 16 times the area of the original pixels.

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Fluorescence microscopy – avoiding the pitfalls

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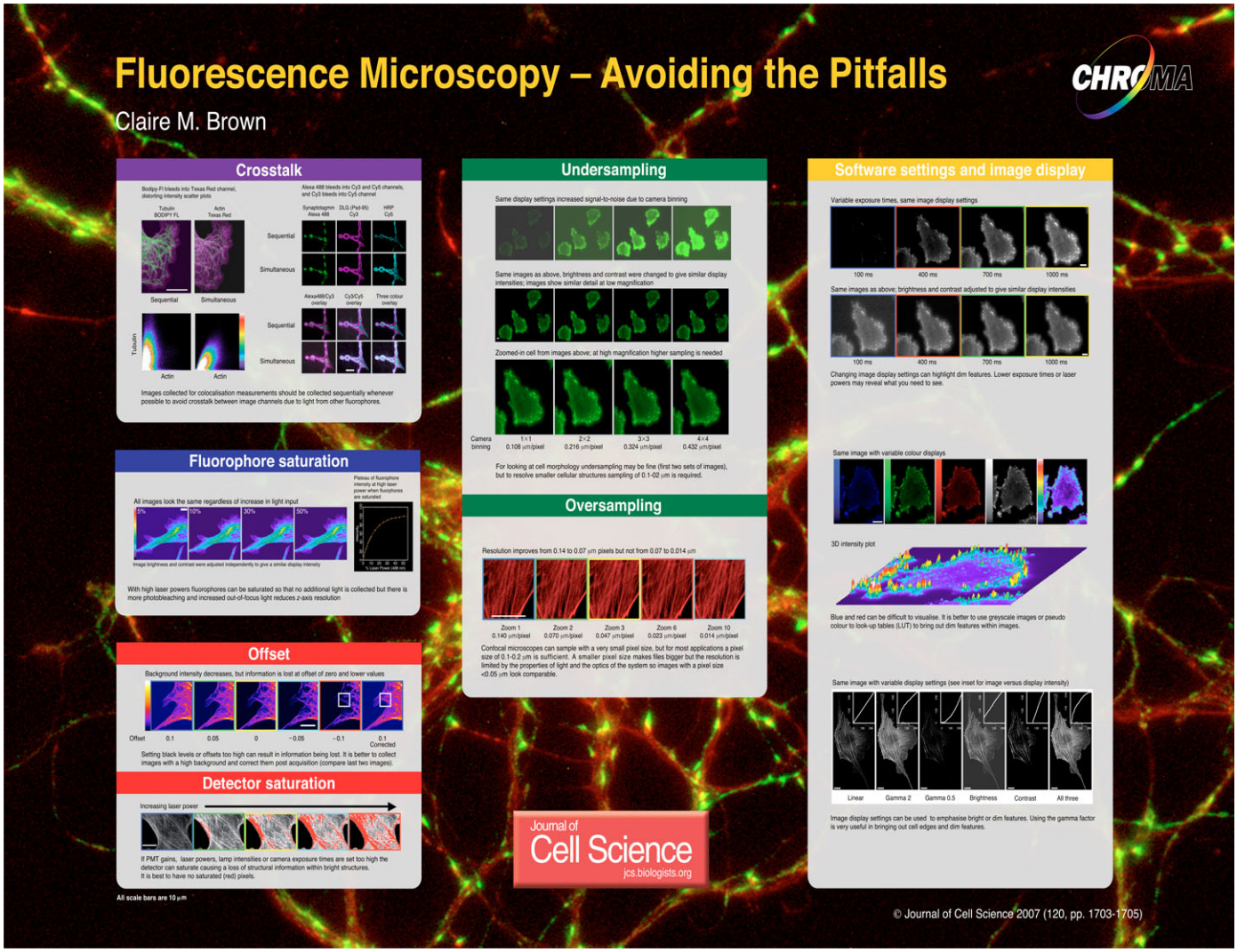
The advent of fluorescent proteins and the continued development of novel fluorescent probes have put fluorescence microscopy at the center of life science research. Fluorescence microscopes range from relatively straight-forward wide-field microscopes to highly specialised spectral-imaging confocal microscopes. Confocal laser scanning microscopes (LSM) are used to improve fluorescence image quality by eliminating out-of-focus fluorescence and for 3D imaging using software reconstruction. Spinning disk confocal microscopes offer the advantage of video rate (30 frames per second) imaging with charge-coupled device (CCD) cameras. For live cell imaging at these speeds, 3D structures can be imaged on the sub-second time scale with the added benefit of reduced photobleaching/phototoxicity (Graf et al., 2005). Programmable array microscopes (Hanley et al., 1999) and line scanning microscopes are available and offer similar advantages to spinning-disk confocals. Finally, multi-photon microscopes use infrared light, which readily penetrates up to 600 μm, allowing deep tissue imaging in living animals (Helmchen and Denk, 2005).

With the complexity of modern fluorescence microscopes there is an endless number of possible set-ups and image acquisition settings. Here, I highlight common pitfalls encountered when performing fluorescence microscopy and discuss how to avoid them. Interested readers can consult in-depth technical reviews and books on various aspects of fluorescence microscopy for more information (Conchello and Lichtman, 2005; Goldman and Spector, 2005; Herman and Tanke, 1998; Hibbs, 2004; Lichtman and Conchello, 2005; Muller, 2005; Murphy, 2001; North, 2006; Pawley, 2006). Numerous interactive web-based resources are also available; Molecular Expressions (http://micro.magnet.fsu.edu/), Nikon Microscopy U (http://www.microscopyu.com/), and Olympus Microscopy Resource Center (http://www.olympusmicro.com/).



(See poster insert)

Experimental set up

Crosstalk between fluorophores

When performing colocalization experiments to determine whether two fluorescently labelled molecules are in very close spatial proximity, it is important that fluorescence intensities of different proteins or dyes are expressed at similar levels. Confocal slice sections should be of similar and small *z* thickness to avoid artifacts due to labelling from different image planes appearing coincident. Collect images for different fluorophores sequentially whenever possible to avoid crosstalk between image channels when multiple fluorophores are excited simultaneously. When imaging fast dynamic processes in live cells, delays between images can be reduced by using fast sequential line scanning, available on many confocal systems using acousto-optic tunable filters (AOTF) that can rapidly turn different laser lines on and off. Only one fluorophore is excited at a time, eliminating crosstalk, and the delay between image lines is only a few milliseconds. Control images taken with the same settings and using samples labelled individually for each fluorophore are essential for all colocalisation experiments. These images provide an estimate of crosstalk between the fluorophores and can be used to correct images post acquisition. Crosstalk appears perfectly correlated and changes colocalisation scatter plots significantly. It is important also to quantify colocalization by using standard techniques, such as the Pearson's coefficient (see Pawley 2006; Bolte and Cordelieres, 2006). However, these methods do have drawbacks and more sophisticated correlation-based techniques can provide more accurate information, especially with moderate to high labelling densities (Comeau et al., 2006). To ensure overlay images are clear to people with red-green colour blindness (10% of men), display them as green and magenta, with white representing colocalisation in overlay images.

Fluorophore saturation

The general belief is that more light produces higher-contrast images. This is true to a point but if laser powers are excessively high essentially all of the fluorophores within the focal volume will be excited, leading to excited-state saturation. Adding additional light does not increase signal intensity in the plane of focus, but more out-of-focus fluorophores will be excited, resulting in poorer *z*-axis resolution and increased photobleaching/phototoxicity. In general, it is advisable to start at the lowest laser power possible, with a higher photo-multiplier tube (PMT) voltage (>600 V) and gradually increase the laser power as required. Line averaging using faster scan speeds, for example sampling a pixel four times for one-quarter of the time and averaging, generates images with a better signal-to-noise ratio because signal builds while noise averages out.

Image acquisition

Sampling frequency: undersampling and oversampling

Imaging with a CCD camera yields a fixed number of pixels. To increase the signal-to-noise ratio the camera pixels can be averaged or 'binned'. For example, if 512×512 pixels are binned 2×2, each square of 2×2 pixels is averaged into a single pixel of twice the size and a 256×256 pixel image is generated with half the resolution. Depending on the situation lower resolution may be fine – for example if cell shape is important – then even 4×4 binning (each block of 4×4 pixels is averaged into one pixel four times larger) can define cell shape. Binned image files are smaller; so cameras can perform more efficiently. Binning is particularly useful for large data sets or live-cell imaging, where binning and shorter exposure times can be used to reduce phototoxicity. However, for sub-cellular structures such as focal adhesions, binned images can be blurry because of undersampling (pixels are too large relative to the structures of interest).

Confocal images can have very small pixel sizes (~10 times smaller than CCD cameras), but using high numerical aperture objectives, making pixels smaller does not always add resolution because objects that are smaller than the wavelength of light (e.g. 488 nm) cannot be resolved. At higher zoom settings, pixels are smaller and more data points are collected, making image files larger, but the specimen is oversampled and additional structural information not attained. For visible light and high numerical aperture objectives (>0.8) a pixel size of ~0.1-0.2 μm is ideal.

Offsets and detector saturation

Confocal microscopes have a software offset setting and users are trained to set the background of images to 'black' (i.e. zero). However, if the background intensity is set too dark, low intensity cellular details can be lost. These features are maintained when correcting high intensity background images post acquisition. Offsets can lead to grave quantitative errors. For example, if there are two points in an image with 200 and 100 intensity units, then one is 100% brighter. However, if the offset is set at 50 then the two points are now at 150 and 50 intensity units and one is 300% brighter. Offset errors will propagate when comparing intensities of multi-labelled samples or calculating image ratios. When imaging no pixel should measure zero intensity; however, it is critical to subtract the average background intensity before performing quantitative analysis.

Similarly, if laser powers, lamps, PMT gains or camera exposures are set too high, detectors can be saturated and features within images can be lost. Most software programs offer a high/low or range finder look-up table (LUT) where blue pixels read zero intensity and red pixels are saturated. The image acquisition parameters should be set so that no detection channel shows pixels reading zero or saturated levels.

Software settings and image display

In general it is best to use the full dynamic range of the detector. This means for an 8-bit detector use all 256 intensity levels (use 4096 for a 12-bit detector). Increasing the brightness of the excitation light (lamp or laser), the detector sensitivity (gain), or the camera exposure time (decreasing scan speeds for LSM) can ensure that the fluorescence signal is bright enough to nearly saturate the detector. However, less excitation light and shorter exposure times (or faster scan speeds) are better for imaging live cells or dim samples because phototoxicity/photobleaching is reduced. A common problem with imaging software is the image display

settings default to displaying the full detector dynamic range with a linear relationship between the image and display intensities. For example, using an 8-bit detector there are 256 intensity levels but when imaging a dim sample maybe only 64 of these levels will be used. The data will only occupy the lowest 25% of the display intensities; the rest of the levels will be empty and the image will appear dark. The information required may be present in the image and visualised simply by adjusting the display settings. Other programs default to a 'min/max' or 'autolevels' display so that dim images appear bright on the screen. This is fine for visualisation but the sample may not be as bright as it appears and samples of very different brightness will appear similar. There is often an 'autoexposure' setting in the software that automatically adjusts the exposure time to use the full detector dynamic range. This setting is most useful for fixed cells or as a starting point for live cells. In general it is important to know how the software is displaying the images and to look at the numeric values of the pixel intensities.

Most sensitive and quantitative scientific detectors are monochromatic and multi-colour images are generated using filters to separate various colours. Monochromatic images are then pseudo-coloured by the imaging or post-acquisition software. The colour coding of images is defined by a LUT that defines what display colour a given intensity will correspond to. Grey-scale images are displayed from black to white over 256 (8-bit display) grey levels. In turn, images could be displayed from black to green (or any colour) with 256 green levels. It is best to display images in grey scale whenever possible, but if colour is used keep in mind that the human eye is most sensitive to green light; so green images reveal more detail than blue or red. In addition, the eye only distinguishes up to about 100 grey levels even if 256 levels are displayed. Rainbow or spectrum look up tables show even higher contrast by using multiple colours to represent different intensity values (0-25 intensity units could be shades of purple, then 26-50 intensity unit shades of blue etc.). 3D plots of images are available in many software packages and can reveal more subtle differences in intensity, but can be difficult to fully interpret. For colocalisation figures it is best to show each image in grey scale with indicators pointing out common features and only show overlays in colour.

Dim features, such as the lamellipodia or cell edge, can be enhanced by modifying the display to a non-linear LUT using the gamma factor. Changing image brightness or contrast alone is not as effective because bright structures will be saturated when trying to emphasise dim ones. Display settings do not change the underlying data, however, these manipulations should be mentioned in figure captions. Finally, as always, care must be taken with any image manipulation that the data are not being misrepresented (Rossner and Yamada, 2004) and see the JCS instructions for authors (http://www.biologists.com/web/submissions/jcs_information.html).

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