Making montages of cell images for publication

Basic points

Micromanager collects 12 bit (ie 1-4096 grey levels) images with the ER camera. To make the images more informative, MM does not by default show the complete 1-4096 range on the onscreen images, but sets lower and upper ranges. Thus images which are different in terms of fluorescent intensity may have different lower and upper ranges which makes direct on screen comparison unreliable.

A second point is that many programs only work with 8 bit images and information can be lost or distorted in transferring a 12 bit image from imageJ to e.g. photoshop if you are not careful. In the protocols suggested here it is suggested that the manipulation of the image is done in Image J, and photoshop (or powerpoint etc) is just used for making montages, labelling etc.

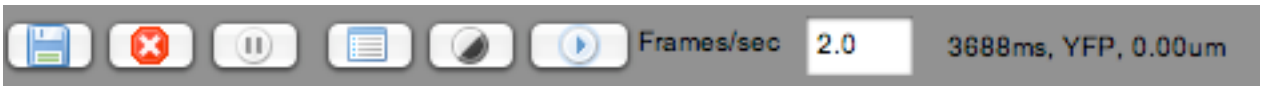
A final point is that some file saving formats such as JPEG involve lossy compression which means that original image quality/information is lost (to achieve a smaller file size). *Always use TIF format for saving (or another lossless format).*

A. Collecting and processing FP images

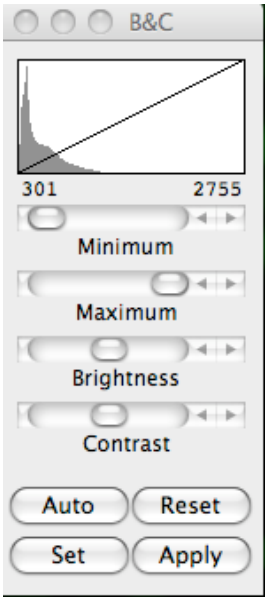
It is important to have comparability between images collected for a particular experiment. Obviously the exposure, microscope settings etc must be the same and it is important not to introduce differences by processing the image in different ways (eg by changing the brightness and contrast to different extents). Note also that repeatedly taking pictures of the same part of the slide will bleach the image, and that images collected on different days may differ as the intensity of the lamp will decrease with its age. Finally, using a high DAPI concentration can introduce a bleed through artefact on other channels (particularly on CFP and GFP). This can be minimized by reducing the DAPI concentration but also by imaging first with CFP/GFP and then with DAPI etc for a particular part of the slide.

Collecting the image

1. Ensure that for a particular experiment you use the same exposure. It is a good idea to check with samples that you expect to have the most intense fluoresence to set an exposure that does not saturate the camera. To check on saturation, click on the right hand check box and on the contrast symbol



this should bring up a histogram window



which shows the range of pixel intensities displayed (here 301 to 2755) and the frequency of pixels with particular intensities within this range. If the range of pixel intensities goes up to 4096 AND the shape of the grey histogram shows that there are pixels with an intensity of 4096 then clearly the image is saturated and a shorter exposure must be used. Obviously it is pointless trying to do image comparisons with saturated images.

It is also a good idea to use this histogram to check that the pixel values collected are spread across a reasonable range of the available 12 bit intensity scale. If all your data are in the first 100 values the image will be of poorer quality than if 0-1000 were used. So in this case increase the exposure.

B. Making a montage of several images collected on the FP channel.

1. Open Micromanager
2. Open relevant image using

file>open acquisition data as image 5D> (choose relevant “metadata.tex file”)

3. Convert image to stack using

image 5D>copy to stack (channels)

4. Decide on the image size and aspect ratio you will want in your final figure. Using the slide bar at the bottom move to the FP image (usually in the middle).

5. Use
Image>Adjust>Brightness/Contrast

and choose SET, lower 0, upper 4095

6. Using rectangular selection tool select appropriate part of image, then Edit>Copy, then FIle>New>internal clipboard.

This should open a new window with the selected region copied. Save this image as a TIFF file using
File>SaveAs>Tif. Note at this stage you may also want to process a phase/dapi image - see D below.

7. Repeat this for all the FP images that will be in the final figure.

8. *Changing the range of levels displayed and converting to 8 bit image.* If you use your FP image using the full 0-4095 pixel intensity rate you may find that the contrast is poor and the localization of the protein is not clear. It is perfectly ok to change the range of levels display provided that (i) a linear range is used (ii) the same range is used for all images in a particular experiment. A second problem is that many applications such as powerpoint or adobe photoshop may not deal with 12 bit images, so we use Image J to convert to 8 bit images AFTER the final adjustment of the range of levels used.

If you want a large histogram to look at the range of pixel values before doing the next steps use

Analyze>Histogram

Then carry out these steps in this order for each image:

- Open relevant image then go to image>adjust>brightness/contrast
- Use SET and put in min and max values (you will use the same values for all FP images in this sequence
- then change to 8 bit using

Image>Type>8-bit

-than save as File>Save as>Tif

Repeat this sequence for each image required in the montage and finally assemble them using photoshop (see E). Do not use Photoshop to change the levels/brightness/contrast etc, ONLY to put them together and label them.

C. Converting a B&W image to colour

This is not generally necessary but can add clarity for talks particularly if you want to display several channels for the same image.

Having carried out all necessary manipulations in step 8 (changing range of levels and converting to 8 bit image)

go to IMAGE>Look up table
and choose the colour of choice.

If you save (TIF) and open again in photoshop, the Image J colour should be retained (file format is “Indexed colour”). Note that if want to conbine images with different colours in photoshop you will have to open, then convert to RGB format using
Image >Mode > RGB Color

D. Processing DAPI and phase images for comparison with FP image

It is often useful to show an image of cells and DAPI stained nuclei alongside the fluorescent protein image. With this image we are usually only interested in qualitative information - where is the nucleus? - so it is not necessary to be so careful in processing the images in the same way. Obviously if we were going to say something about nuclear intensity/ morphology etc then all the steps in part B should be observed. This section provides a quick way of obtaining a combined Phase/DAPI image for comparison to the FP image.

You will probably want to do this at the same time as processing the FP image so that the same subsection of the image is selected.

Follow steps B1-6 to process the FP image

then

1. select phase image in stack by moving bottom slider
2. Use image>adjust>brightness/contrast to make the images of the cells clear
3. copy then select File>New>Internal clipboard. Convert image to 8 bit using Image>Type>8 bit. Save image as TIF
4. Go back to main stack and move slide to DAPI channel. If you accentally lose the region of interest (ROI) selection it can be recovered by SHIFT-e

5. Repeat steps 2 and 3 (you need good contrast for the nuclei to be clear in the final image. Save image as TIF
6. This makes two images, one phase one DAPI. These will now be combined to show DAPI and phase in one image.
7. To combine images, open both.
8. Using Image>Color>Merge Channels, select
   Red, Green, Blue - DAPI image
   Gray - Phase image

alternatively if you want a colour image
Red OR Green OR Blue - DAPI image (two colours leave as None)
Gray - Phase image
>OK

This generates a stack with the merged images. To convert the stack to a B&W image, select
Image>Type>RGB color
then
Image>type>8 bit
(this will discard colour information)

Otherwise
just convert to RGB color
Image>Type>RGB color

Making montages of cell images for publication

Save as TIF format.

Note that it also possible to combine phase and dapi images using photoshop. Open the phase and dapi images, copy the phase image and paste it on top of the DAPI image. Using the layers window change the phase transparency to screen, or just reduce the opacity. In photoshop it is possible to subsequently alter the brightness and contrast of each image. Finally merge the two image (Layer>Flattern image) and save as TIF.

E. Making final figures in Photoshop.

1. Open set of images to be put together. **Include a scale bar before resizing.**
2. Using canvas resize canvas for one image (Image>Canvas Size) so that images can be assembled into one file (e.g if you are combining 4 images in row, width must be 400-410%).
3. Copy and past images and align (use of guides recommended).
4. When all images are assembled, decide on final dimensions (if for a journal usually one or two columns, about 8 or 16 cm width). Using Image>Image Size set width, check scale styles, contrain proportions, resample image, and set resolution to at least 300 pixels/inch. If the image size goes down in this operation resolution is being lost. If you will need to label outside the image, resize to less than final desired size, then use Image>Canvas Size to generate blank space around figure.
5. Save as TIFF format.

Useful references on pitfalls in image manipulation.

What's in a picture? The temptation of image manipulation.
Rossner M, Yamada KM.
J Cell Biol. 2004 Jul 5;166(1):11-5. No abstract available.

Seeing is believing? A beginners' guide to practical pitfalls in image acquisition. North AJ. J Cell Biol. 2006 Jan 2;172(1):9-18. Review.

This site is for astronomers and might seem irrelevant but it has helpful information on how to use imageJ
http://www.uni-sw.gwdg.de/~hessman/ImageJ/Book/Image%20Processing%20with%20ImageJ/index.html