

# Colour combining multiple epi-fluorescence images using Photoshop CS.

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Many confocal and mercury lamp epi-fluorescence users will want to colour combine images from say DAPI, FITC and Rhodamine B&W 8-bit (256 grey levels) images, to provide a three colour 24-bit RGB image showing co-localisation. Generally this done easiest in dedicated imaging software programs like the freeware ImageJ ([rsb.info.nih.gov/ij](http://rsb.info.nih.gov/ij)) or expensive packages like MetaMorph ([www.moleculardevices.com](http://www.moleculardevices.com)) and ImagePro Plus ([www.mediacy.com](http://www.mediacy.com)). These image analysis packages can also work with Z stacks, and handle multi-tiff or unusual formats like Bio-Rad's PIC files.

However you can easily combine two or three fluorescent B&W images using Photoshop if you wish. I will assume that you wish to combine three fluorochrome images (any more and the resulting RGB image is often too complex for useful information anyway).

Run Photoshop CS. Open the three images to colour combine (see Figure 1). Note that there are freeware Bio-Rad Photoshop plug-ins available to read Bio-Rad confocal PIC files and even to colour combine Z stacks – giving you File, Import : Bio-Rad PIC Import, Merge, Multiple Input, & Multiple Merge [I can supply these to anyone interested]. Most other microscope systems will supply the images as an export in standard TIFF form. The images should be 8-bit [256 greys], not 12-bit or the image contrast info is lost and the images appear black (and there's no point messing about with Photoshop's contrast controls to bring the image back – besides we can only distinguish 191 grey levels anyway).

So export your images from the microscope software at 8-bit to get a 24-bit colour combined image. In Photoshop, ensure the 'Channels' palette window is open (Window, Channels is ticked – don't confuse this palette with 'layers'). Check the images are 8-bit greyscale (Image, Mode, Greyscale). Note that Photoshop CS doesn't seem to read multi-tiff z stack files – you just get the first image in the sequence, likewise the B&W tiff file may or may not display a LUT colour.

Left click the Channels palette small right hand arrow tab, and from the drop down menu select 'Merge Channels' (see Figure 2). It doesn't matter which image is selected. When the Merge channels window opens, select Mode to 'RGB color' and OK. Then simply specify which of the three image files gets what colour. In this case the DAPI will be the blue channel, the FITC green and the Rhodamine red. Click OK and the colour combined image will appear (see figure 3) and the three original B&W images will vanish.

As a 24-bit colour image, you can adjust colour balance (the intensities of each fluorochrome), curves etc. The Image, Adjustments, Levels provides intensity histograms and adjustments for each fluorochrome channel (see figure 3). Now save the file as a tif, or use File, Save for Web to export as a smaller file size using jpg

compression (reducing the image size as required, e.g. from 1024 x 1024 to 512 x 512).

Note that you can move any channel to re-align it with the other images. Use 'select all' under the rectangular Marquee tool on the chosen image to be re-aligned (say the blue channel, which will then have dotted lines around it when selected). You then can use Edit, Free Transform. You can now move the selected image to re-align it anywhere with the other images. Ensure that 'Color Picker' is set to black background so that any areas no longer having any image will appear blank in the combined colour image (as a white background will colour this region bright blue). Click the rectangular Marquee tool button again to go out off free transform. You then get prompted to apply the transformation move, select OK. The DAPI region in this case will now appear in it's new location on the RGB image.

If you want to add a fluorescent channel or two to a B&W transmission image, I would only do this using ImageJ or similar software geared up this sort of thing. In ImageJ you simply use the 'Gray RGB Stack Merge' plugin available from the ImageJ color plug-ins website. In ImageJ select Plug-ins, Color, Grey RGB Stack Merge. Change the stack channels as required to select the correct image files. There will be four channels to merge labelled Gray [trans] stack, red stack, green stack and blue stack. Input the appropriate loaded 8-bit tif file names and press OK to give the fluorescent areas overlaid on the phase/DIC transmission image (see figure 4 – FITC, Rhodamine and phase contrast transmission). You can do this in Photoshop using things like Image, Apply Image and by using masks. It's not an easy technique though and so I think it's far simpler to stick with the likes of ImageJ and it's RGB Grey Stack Merge plug-in for this.

Often image capture software that came with the microscope, like Leica's confocal software (LCS and freeware LCS Lite, see [www.leica.com](http://www.leica.com)) or Improvision's Openlabs ([www.improvision.co.uk](http://www.improvision.co.uk)) can also do this multiple image merging quite simply. However, if you are happy with Photoshop and wish to do further image editing for say publication or talks, Photoshop CS can colour combine images quite successfully.

## Acknowledgements

Thanks to Clifford Todd (Dow Chemicals) and John Ross (North Carolina University) for suggesting this simple Photoshop method of colour combining via the microscopy.com list-server. There are similar ways to do colour combine in Photoshop CS, such as opening one image as RGB and then cutting and pasting the other two images into the other colour channels – and you can re-align the images as you do it (a method I have used occasionally).

## Figures

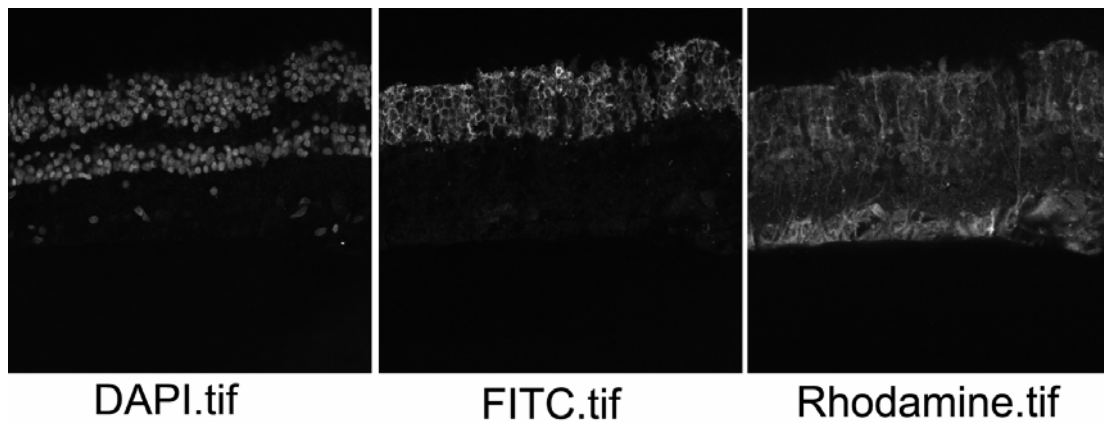


Figure one. The three Bio-Rad Radiance 2000 confocal fluorescence images used to colour combine in Photoshop.

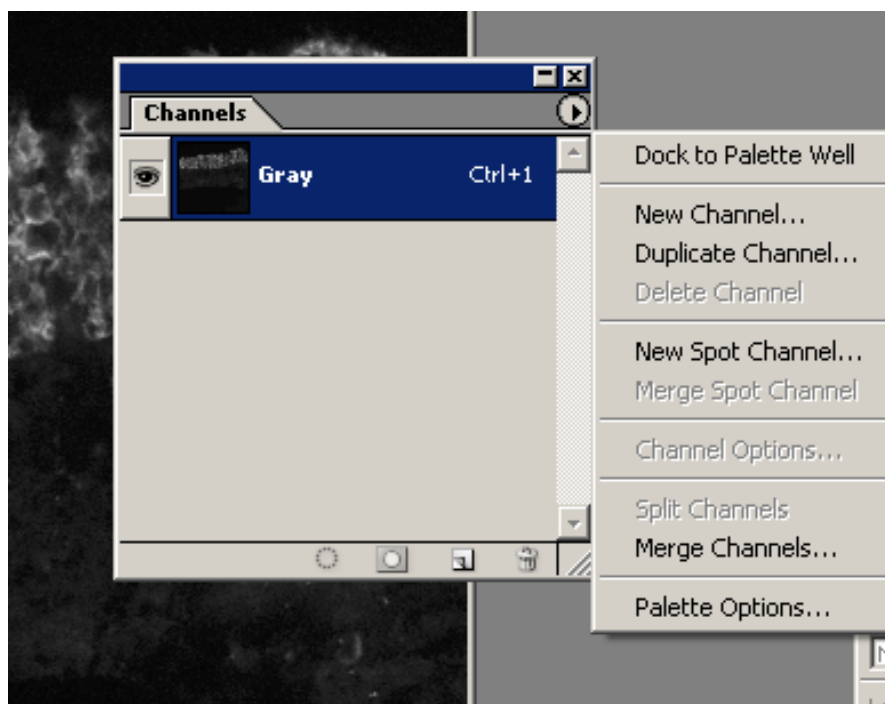


Figure two. The Photoshop CS Channels palette, and drop down 'Merge Channels' menu.

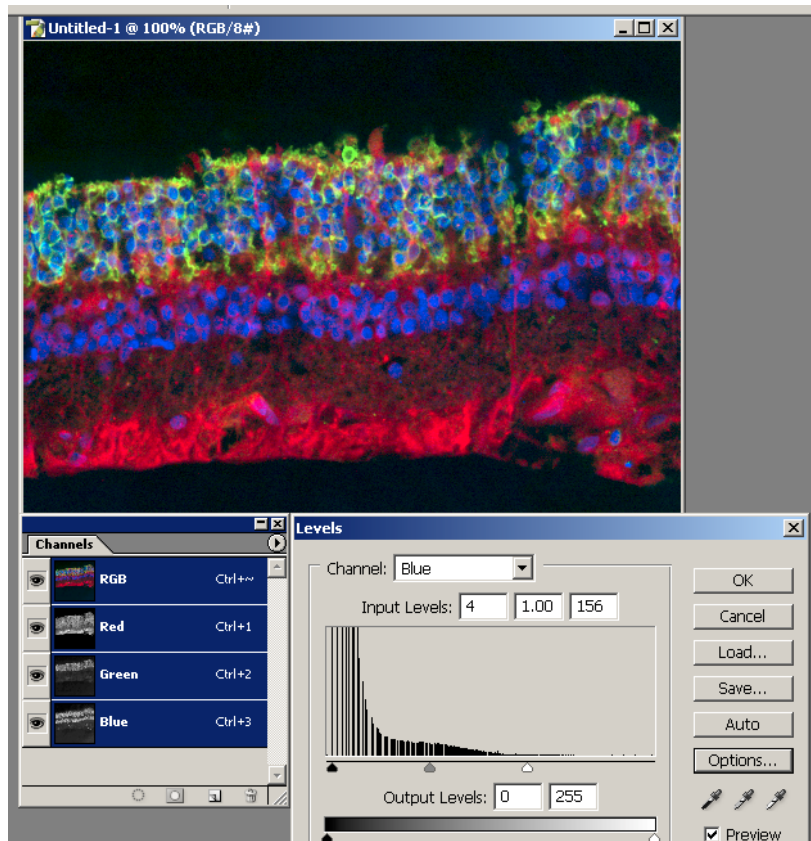


Figure three. The colour combined image of those in figure 1, plus the Photoshop CS Image, Adjustments, Levels palette, and the Channels palette.

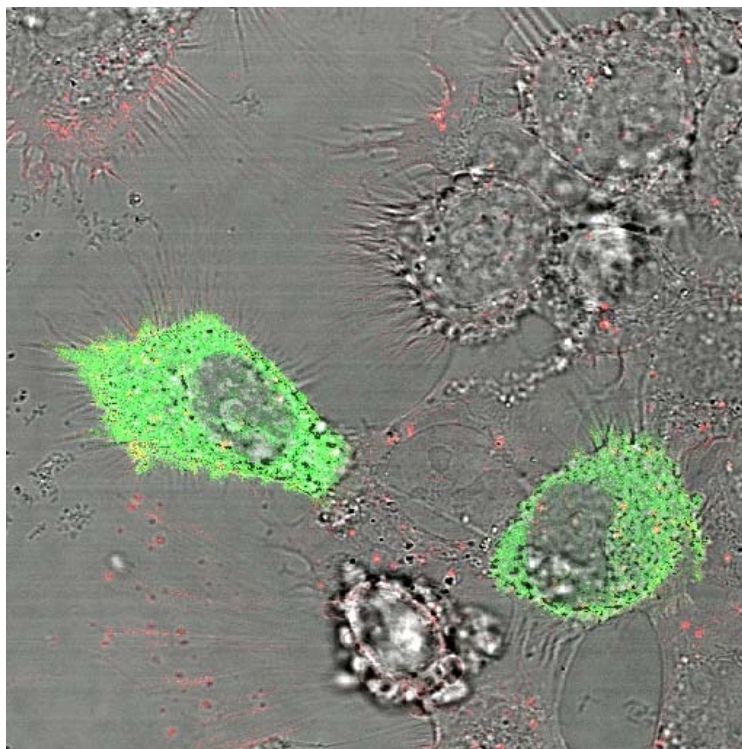


Figure four. A phase contrast Bio-Rad Radiance confocal transmission image combined with the FITC (green) and Rhodamine (red) fluorescence images of the same region. This image was created using ImageJ and the Grey RGB Stack Merge color plug-in.