

# Short Technical Reports

## Methods and Applications of Three-Color Confocal Imaging

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### ABSTRACT

A simple method for constructing two- and three-color merged images from grayscale confocal fluorescence images using Adobe Photoshop<sup>®</sup> is outlined. Various computer methods for manipulating and displaying the images are discussed in light of several recent biomedical applications of multi-label confocal microscopy.

### INTRODUCTION

The laser scanning confocal microscope (LSCM) is routinely used to produce digital images of single-, double- and triple-labeled fluorescent samples (14). The use of red, green and blue (RGB) color is most informative for displaying the distribution of up to three fluorescent probes labeling a cell, where any colocalization is observed as a different additive color when the images are colorized and merged into a single three-color image.

Here we describe a simplified version of our previously published method (18) for producing three-color confocal images using the popular image manipulation program, Adobe Photoshop<sup>™</sup> Version 3.0.5 (Adobe Systems, Mountain View, CA, USA). In addition, several applications of the three-color merging protocol for displaying confocal images are discussed. Note that these digital methods are not confined to images produced using the LSCM and can be applied to digital images imported into Photoshop from many different sources.

### METHODS

Images of fluorescently labeled specimens were collected using a Model MRC<sup>®</sup>-600 Confocal Laser Scanning System (Bio-Rad, Hercules, CA, USA) using previously described methods (18). Selected images were transferred from the host IBM<sup>®</sup> microcomputer of the confocal microscope to a Power Macintosh<sup>®</sup> 8500/150 (with 80

Mbytes of random-access memory [RAM]) microcomputer through Ethernet using Fetch Version 3.0.1 (Copyright © Trustees of Dartmouth College, 1996) as binary files. Images were imported directly into Adobe Photoshop (Version 3.0.5), either as a plain binary file (RAW) or as tag image file format (TIFF) files. In the case of RAW files, the actual dimensions of the Bio-Rad confocal image must be entered; for example, a typical image size is 768 × 512 pixels with a 76-byte header. The header is specific to the Bio-Rad proprietary "pic" file format and may vary with imaging systems from different companies. This information on the image files should be freely available from each confocal company. In contrast, TIFF files are opened directly into Photoshop. If necessary, confocal image files can be converted to TIFF files using such programs as Confocal Assistant (Todd Brelje, University of Minnesota Medical School, Minneapolis) or NIH Image Version 1.57 (Wayne Rashband at the NIH and available at no cost through file transfer protocol [FTP] from [zippy.nimh.nih.gov](http://zippy.nimh.nih.gov)). Both of these programs are useful for further manipulating confocal images.

Three-color images are produced in Photoshop by pasting each of the grayscale images from the confocal microscope into the red, the green and the blue channels of an RGB color image. In the following description of the process, the location of various commands within the Photoshop program is included (*in italics*) after each operation. More-detailed information on specific operations is available in the Photoshop User Guide.

Once the three grayscale images have been opened in Photoshop, a blank RGB image is constructed (*File, New*). The image must be the same size and pixel resolution as the three original grayscale images from the confocal microscope; in our case, 768 × 512 pixels and 72 pixels/inch. The size and resolution of an image can be determined and adjusted (*Image, Image Size*). This new RGB image should be black since the background in fluorescence images is usually very close to or at black (black = 0, white = 255). Another color can be chosen for the background for cosmetic purposes, but a black mask for the actu-

al region delineated by the image should be constructed so the new background color does not interfere with the information in the actual image.

If not already open, the Channels palette should be opened at this time (*Window, Palettes, Show Channels*). The three grayscale images are now ready to be pasted into the newly created RGB image. This is achieved by selecting the first of the grayscale images (*Select, All*) and copying it into the required red, green or blue channel of the new RGB image (*Edit, Copy*) by clicking on the newly created RGB image with the mouse and then on the required channel in the Channels palette column. For example, for the red channel, click on the red window in the column. Finally, the grayscale image is pasted into the RGB image (*Edit, Paste*). The image will now appear in the Channels palette column in both the red and the RGB channel. The second and third grayscale images are now selected, copied and pasted into the green and blue channels, respectively, of the RGB image using the same routine. The result of these manipulations is a single three-color merged image (Figure 1, a and b), which is displayed by double-clicking on the RGB image in the Channels palette column. There is no loss of bit-depth information from the three original source images since three 8-bit images are merged into a single 24-bit image.

The color levels in the resulting merged image can be changed using *Levels (Image, Adjust, Levels)* to independently adjust the red, green and the blue values of the image. Finally, for presentation purposes, the brightness and contrast (*Image, Adjust, Brightness/Contrast*) of the image is fine-tuned. Subtle changes to the image can be viewed and selected using variations (*Image/Adjust/Variations*). Many additional operations can be achieved at this time using the image manipulation features nested within Photoshop. Especially useful features allow addition of graphics and compilation of the images into multi-panel figures. Graphics are subsequently more easily edited if they are pasted into a separate layer in the image rather than permanently replacing pixels in the actual image itself.

Images are saved to the hard disk of

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the computer in Macintosh format by selecting Save As (*File, Save As*). Images are usually saved in the TIFF format and compressed using the Lempel-Ziv-Welch (LZW) scheme, which is entirely loss-less. We have found that other compression methods such as Joint Pictures Expert Group (JPEG) compression can introduce artifacts that may be compounded each time a file is resaved. Images are eventually archived to optical disk or CD-ROM.

Color hard copies of the images can be produced directly from Photoshop using a high-resolution digital color slide maker (e.g., Lasergraphics) or digital dye-sublimation printer (e.g., Tektronix Phaser XIISDX). These devices are controlled directly from Photoshop as plug-ins, which avoids the need to transfer the images to yet another program. Detailed aspects of mass storage and the preparation of hard copies have been reviewed recently (5). Single-col-

or images often lack contrast when printed on a dye-sublimation printer. The problem can be overcome, somewhat, by producing a single RGB image, as before, and adjusting the relative levels of the three channels so that there is information in all channels for printing (*Image, Adjust, Levels*).

For merging two images using this method, the third channel is left vacant. Earlier versions of Photoshop required the use of an empty or black, blank background image for the third channel and placing the red image in the red channel, the green image in the green channel and the blank image in the blue channel of an RGB image to produce a red/green double-label image. Using Version 3.0.5, two grayscale images can be pasted into a newly created black RGB image. For example, to produce a red/green image, separate images are pasted into the red and the green channels (Figure 1c).

## RESULTS AND DISCUSSION

The combination of colors within a three-color merged image is important for clearly conveying the biological information collected by the confocal microscope. The true emission colors of two of the most commonly used fluorophores, rhodamine and fluorescein, are conveniently red and green, respectively, and overlapping domains of expression are yellow. These are the colors observed by eye in a conventional epifluorescence microscope equipped with the appropriate filter sets for simultaneous double-label imaging, which is now available from most of the microscope manufacturers. Note that the third channel in a triple-label sample prepared for confocal analysis usually emits in the far red, e.g., Cyanine-5 (Cy5), which is conveniently shown as blue in digital images, whereas the real Cy5 emission is often extremely

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difficult to visualize by eye and not so easily depicted in a digital image (2).

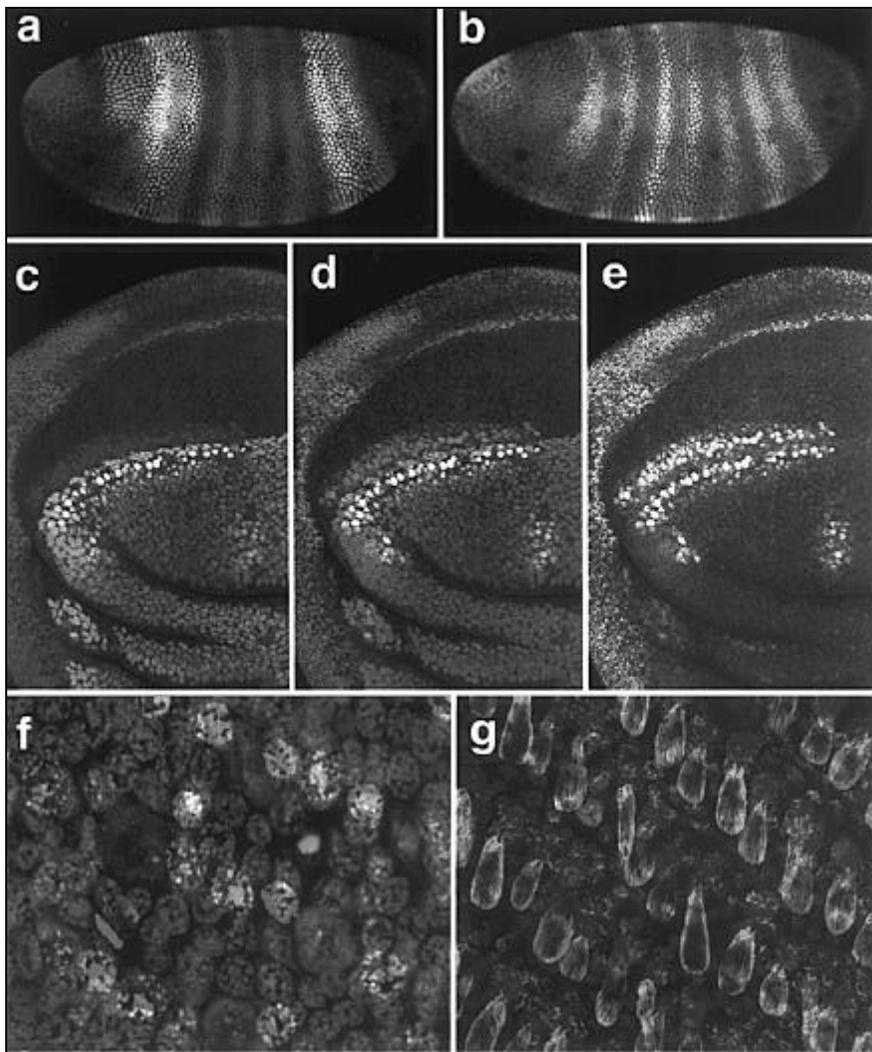
Using Photoshop, it is a relatively simple task to experiment with various color combinations by rearranging the images into different channels. For example, in the triple-labeled *Drosophila* embryo, the green *hairy* stripes appear light blue in the blue *Krüppel* domain (Figure 1a) and in a different color combination using the same images; the red *hairy* stripes appear yellow in the green *Krüppel* domain (Figure 1b). This is achieved by rearranging the component grayscale images of the three-color RGB image into different channels using Split Channels (*Window, Palettes, Show Channels, Split Channels*) and recombining the images using Merge Channels (*Mode, RGB Colour*). Using the Specify Channels option, the component grayscale images are assigned to different channels of the RGB image. The colors therefore do not always correspond to the actual colors of the specimen.

Extra colors can be included in double-label images by placing two versions of the same image into two of the three channels, with the second image of the merge in the third channel. For example, a purple and green image is produced by pasting the same image into the red and the blue channels to give purple, and the second image is placed into the green channel (Figure 1d). Additional color combinations are red and light blue, where light blue comprises the blue and green channels; or blue and yellow, where yellow comprises the red and the green channels (Figure 1e). Here, overlaps of expression invariably appear white in the image since all three channels now contribute to the overlapping signals (Figure 1, d and e). These additional color combinations can be useful when making a multi-panel figure of several double-label images where the expression patterns of more than three proteins are displayed in separate panels so that different colors are assigned to each protein (3).

This method of merging confocal images is used extensively for mapping the distribution of macromolecules in double- and triple-labeled specimens (6, 9, 15, 21). Because of the exquisite specificity of immunofluorescence, many

of the images are rather abstract when viewed as single grayscale images and, in many instances, can only be interpreted when a second or third image of a known landmark within the tissue is displayed at the same time. This is typical of nuclei labeled by fluorescence in situ hybridization (FISH). These im-

ages are often made up of relatively few bright pixels on a mostly black background, and the images can only be interpreted when a second, counterstained image of the entire nucleus or chromosome spread is collected and merged with the brightly stained centers of hybridization (1, 10, 12). The dimeric



**Figure 1. Applications of three-color imaging.** Panels a and b: three images are collected at three different excitation and emission wavelengths (18) and subsequently merged and displayed as a single three-color merged image using Photoshop. The image is a *Drosophila* embryo at the cellular blastoderm stage triple-labeled for three segmentation proteins: *hairy* in green (a) or red (b), *Krüppel* in blue (a) or green (b) and *giant* in red (a) or blue (b). Panels c, d and e: two images collected at different excitation and emission wavelengths are merged and displayed using Photoshop. Different color combinations are demonstrated in a *Drosophila* third instar wing imaginal disk double-labeled for *apterous* in red (c), green (d) or blue (e), and *achaete* in green (c), purple (d) or yellow (e). Note the nuclei that coexpress *apterous* and *achaete* are colored yellow in (c) and white in (d) and (e). Panel f: three images from a Z-series projection are merged and colorized so that color is now coded to depth in the specimen. Epithelial nuclei are shown in green with the nuclei of the scale-forming cells shown in blue and red (at different levels in the epithelium). The specimen is a whole mount butterfly pupal wing epithelium stained with propidium iodide. Panel g: different structures are mapped to depth in a double-label image in a whole mount of a butterfly wing epithelium at a slightly later stage of development than Panel f. Here nuclei are labeled with TOTO-3, and the emerging wing scales at a more dorsal position in the epithelium to the nuclei are labeled with Texas Red<sup>®</sup> phalloidin.

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nucleic acid stains such as YOYO-1 (Excitation maximum 491 nm) or TOTO-3 (Excitation maximum 642 nm) are excellent counterstains for these purposes (8). Recently, more than three probes have been localized on the same chromosome using combinatorial labeling strategies. Here, more sophisticated computer methods of image merging and measurement have been developed to display the biological information in the images (19,20).

Multi-label confocal imaging is a perfect tool for developmental biologists studying cell lineages (4) and analyzing clones (3,11) where the distribution of one, or sometimes two, proteins can be used as landmarks to map a third protein of interest within a developing tissue. Furthermore, fluorescently labeled phalloidin is a convenient probe to include in a triple-labeling strategy for imaging cell outlines (7). Specific combinations of dyes, for example: rhodamine phalloidin to label cell outlines, TOTO-3 to label nuclei (on the Cy5 channel) and an antibody to the protein-of-interest (on the fluorescein channel) can be used in a triple-labeling strategy for mapping the location of a protein to the nucleus, to the cytoplasm or to the extracellular matrix.

In addition to displaying the relative distribution of up to three different macromolecules within cells, this method of combining three images can be used as an alternative to three-dimensional (3-D) reconstruction for displaying depth information within a specimen (13). Using the LSCM, a series of images from different focal planes within the specimen is collected into a single file or Z-series. These images maintain the X, Y and Z registration from the specimen and are the same size and pixel resolution. Three images are chosen from such a Z-series, exported as single-image files into Photoshop and then merged as before, so that the colors red, green and blue are assigned to structures at different depths within the specimen (Figure 1, f and g). In a similar way, single images of three different time points can be extracted from a time-lapse movie sequence file collected from the confocal microscope and three-color merged. These files are similar to Z-series files except that time has replaced the Z-dimension. Here,

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color differences are used to summarize changes in the positions of structures over time in a single image (13).

Photoshop can also provide a bridge to further manipulating images since the files are compatible with many other programs. For example, sequences of confocal images of development have been manipulated using Photoshop and subsequently transferred to a commercially available morphing program such as Elastic Reality (Avid Technology, Tewksbury, MA, USA) and processed into short animated sequences of development (17). These sequences can be further edited and compiled using Adobe Premiere™ (Adobe Systems) and viewed as a digital movie using QuickTime™ (Apple Computer, Cupertino, CA, USA) software directly on the computer or exported to VHS video tape for presentation purposes. For more detailed information on the production of digital movies, refer to Eric Hazen's multimedia Web page: <http://bocklabs.wisc.edu/mbinfo/cnotes/multimedia/multimedia.html>.

Photoshop is available for many different computer platforms and can be run on the host computers of most confocal microscopes. Some of the more sophisticated confocal imaging systems are able to collect, merge and colorize three images simultaneously using a three-detector system, which may obviate the use of Photoshop. However, it is often necessary to use additional laboratory microcomputers to manipulate confocal images in order to liberate the confocal workstation for its primary purpose of collecting images (16). Moreover, for compiling final figures, it may be necessary to combine confocal images with images from other sources in the final plates, and in this situation the versatility of a single image manipulation program that can be used on many different laboratory computers is invaluable.

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