

Supplemental File 2 — Protocol for Combining Red and Green Fluorescent Images of Cleared-and-Stained Vertebrates to Highlight the Relative Placement of Bones and Soft Tissues.

This procedure is a generalized protocol for combining independent fluorescent red and green images of the same photograph under different lighting regimes to emphasize the relative placement of bony and soft tissues in a cleared-and-stained vertebrate. This protocol was specifically completed using Adobe Photoshop Release 19.1.0, but these steps could be completed in other imaging software. For example, Pixelmator 3.7 can be used for this technique, but instead of choosing the independent channels described below, a researcher would have to make three separate layers and take advantage of the *Channel* component of the “Effects Browser: Color Adjustments” panel. The researcher would then need to reduce two of the channels to 0% in each layer to make one color dominate in each layer. This would be done for each color resulting in a red layer (green and blue set to 0%), a green layer (red and blue set to 0%), and a blue layer (red and green set to 0%). Finally, we want to highlight that this is one of many possible techniques that can be used to combine fluorescent images, and it was specifically used to make the images in Figure. 2A, C, E, G. Researchers are strongly encouraged to be creative and explore other possible uses and composition for fluorescence imagery.

Step 1.—Take independent images of the specimen under traditional white light (transmitted and reflected light as desired), red fluorescence (under green light), and green fluorescence (under blue light). It is critical that the focus and composition of each image are completely unchanged among the three images.

Step 2.—Correct the exposure and contrast of each individual image. Typically, this involves the automatic or manual manipulation of the *Tone*, *Contrast*, *Color*, and *Levels* (these are all found under IMAGE: ADJUSTMENT in the menu bar). Begin by trying the automatic correction settings for each adjustment. If the automatic correction is satisfactory, continue to the next adjustment. If the automatic correction is undesirable, experiment with variations in the manual controls until you are pleased with the results of each particular adjustment. Pay particular attention to the *Levels* setting in the images taken under fluorescence to ensure that the majority of the black (or near black) regions of the image are truly black (RGB #000000). If the background is not truly black, this will introduce irregularities or imperfections into the final composite image.

Step 3.—Create a new file that is in the RGB color space that will be called “combo.tif” that has the identical dimensions and resolution (i.e., dots per inch/cm) as the source files. Fill this file with pure black (RGB #000000) using the “paint bucket tool.”

Step 4.—Open the green fluorescent image (image taken under blue light), select all, and copy the image.

Step 5.—Return to the “combo.tif” file. Select *Channels* from the WINDOW heading in the menu bar. In the “channels panel,” select only the green channel and paste the green fluorescent image into this channel.

Step 6.—Open the red fluorescent image (image taken under green light), select all, and copy the image.

Step 7.—Return to the “combo.tif” file. Select *Channels* from the WINDOW heading in the menu bar. In the “channels panel,” select only the red channel and paste the red fluorescent image into this channel.

Step 8.—In the “combo.tif” file, select *Channels* from the WINDOW heading in the menu bar. In the “channels panel,” select the RGB channel and examine the composite image with both the red and green images combined. Often, the composite image will need modifications to improve its clarity. The modifications are done individually in either the red or green channel. If the image needs manipulation, continue to *Step 9* and/or *Step 10*; otherwise, proceed to *Step 11*.

Step 9 (If the red channel needs correction).—In the “combo.tif” file, select *Channels* from the WINDOW heading in the menu bar. In the “channels panel,” select the red channel. Often, the red channel needs correction to the lighting using the same techniques discussed in *Step 2*. In our experience, modifications to *Levels* from the IMAGE: ADJUSTMENT heading in the menu bar are the most important. Typically, we simply need to slide the gray or “midtones” slider in the “levels panel” to the left to brighten the image in the red channel.

Step 10 (If the green channel needs correction).—The green channel can often overwhelm the red channel. If this is the case, you will need to return to the original green fluorescent image to reduce its opacity. In the green fluorescent image file, select *Channels* from the WINDOW heading in the menu bar. In the “channels panel,” select the RGB channel. Because you cannot alter the opacity of the background while retaining the black as pure black (RGB #000000), you will need

to place the image into a second layer above the background. To accomplish this, select and copy the image. Use the “paint bucket tool” to fill the entire image with pure black (RGB #000000). Next, select *Layers* from the WINDOW heading in the menu bar. In the “layers panel,” use the button on the bottom to create a new layer. Paste the copied green image from the clipboard into this new layer. In the “layers panel,” reduce the opacity of this new layer. This opacity often has to be dropped to 40–60% of the original image. Next, select *Flatten Image* from the LAYER heading in the menu bar to return the fluorescent image to the background. Next, select all, and copy the image. Return to the “combo.tif” file and select *Channels* from the WINDOW heading in the menu bar. In the “channels panel,” select only the green channel and paste the revised green fluorescent image into this channel. After this opacity change, the green channel often still needs corrections to the lighting using the same techniques as emphasized in *Step 2*. In our experience, modifications to *Levels* from the IMAGE: ADJUSTMENT heading in the menu bar are the most important. Typically, we only need to slide the white or “highlights” and/or gray or “midtones” slider to the left in the “levels panel” to brighten the image in the green channel.

Step 11.—Finally, select *Channels* from the WINDOW heading in the menu bar. In the “channels panel,” select the RGB channel. Make any final corrections to the image, and crop the image to remove any vignetting around the margins of the image that are introduced by imaging through a microscope.