CONFOCAL AND FLUORESCENT IMAGING

In the world of fluorescent imaging, the need to colorize grayscale images can be an everyday task. Yet, when all too many of these colorized images go to publication, the results appear to contain undifferentiated blobs of a saturated color, rather than images that contain gradations of color.

While very small objects need to be as bright as possible in order to make these objects apparent (e.g. bacteria under low magnification), and while too many push the gain on photomultiplier tubes in order to top grayscale values off at 255 (on an 8-bit range) in order to show unquestionable colocalization, only the former can be defended on the basis of revealing lost features. The latter is done, it seems, because actual numeric measurements of colocalization often fall far short of what is both seen by eye and desired by the researcher, and it is far more beneficial to the researcher to show the saturated colors of red + green to yield large areas of yellow for the object of convincing both reviewers and readers (when most colocalization troubles concerning numeric measurements are best solved by reducing dark current noise with frame averaging versus yanking red and green values to saturated levels).

Add to this the cameras and confocal systems which collect grayscale images and then colorize these images red, green or blue, and again researchers are left with images that are colorized by unprintable colors. But these do look pretty on the computer screen.

Often these camera systems also show colorized images on a computer screen at video rates so that we can see the appropriate colors when acquiring fluorescent images. Yet, the eye does a much better job at seeing levels of gray (versus green or red or blue), and the eye can detect blobs of pure white much better than when images are colorized. With grayscale images, it's easier to see how adjustments to gain and and black level affect the image while the image is "live." Thus, because we often acquire from live, colorized images, it is no wonder that so many images end up saturated: we can't make the right decisions when we can't see in the first place.

Acquisition of images. To the seasoned microscopist it goes without saying that white levels and black levels need to stay within the dynamic range of the acquisition system. Put more simply, the blacks can't be too black and the whites too white. Remember that blacks can always be made blacker and whites whiter in post-processing programs. Nothing can be retrieved from areas that are too white or too black, and these areas are difficult to work with when going to publication.

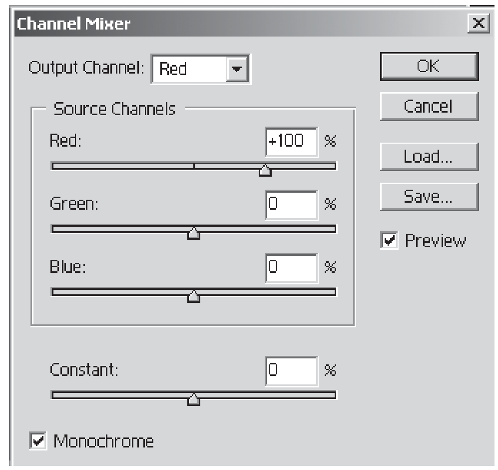
Often software acquisition programs contain a LUT (Look Up Table) overlay so that the user can visually discriminate areas that are saturated. These might show red, for example, where the whites are too white and green where blacks are too black. This overlay is not a part of the image, just something that covers the image to reveal saturation (based upon a mapping of saturated pixel values to a graphic overlay). The user is prompted, then, to adjust black level (or offset, or contrast) and white level (or gain, or brightness) to rid the image of all but 5 percent or so of these colors (assuming that these saturated pixels are a result of dark current noise and will be eliminated by using frame averaging).

The LUT overlay can be placed on grayscale images, or on colorized images when adjusting values during "live" acquisition. In that way, even though we cannot discriminate brightness well with colorized fluorescent images, we can overcome visual constraints with the LUT overlay. Also, monitor to monitor differences, or the contrast and brightness settings on the monitor used, will not affect gain and black level settings versus settings determined by eye.

Once the LUT overlay is used to bring values within the dynamic range of your imaging system at the beginning of your microscopy session, then these values can often be retained for subsequent samples.

Removing Color. It may seem counter-intuitive to believe that colors should be removed from fluorescent images after these have been acquired, but too many of these colors simply do not fit within the gamut (range of colors produced or "seen") of printers. Certainly, if the only destination for your image is for storage or for a PowerPoint presentation, then the colors may appear to your satisfaction and these do not need to be changed. If, however, the image needs to be printed or published, or if the image needs to be measured for optical intensities, then one should consider taking the image back to grayscale. Especially in light of measuring optical intensity and the dangers of doing that incorrectly, one must pay careful attention to removing color using correct methods. I have seen too many simply change the mode from color to grayscale and then wonder why images don't look as bright, and too many of those who do not measure luminosity in the correct channel (under Image>Histogram in Photoshop) and who wonder why values do not accurately reflect what is seen by eye.

A correct method for retrieving accurate grayscale values from colorized fluorescent images in Photoshop can be accomplished as follows:



1. Under Image, select Adjust(ments) then Channel Mixer. If Channel Mixer is grayed out and unavailable, then your image is more than likely saved as Indexed Color (common for confocals). Under Image>Mode change Indexed Color to RGB Color.

2. In the Channel Mixer dialogue box, select the Output Channel similar to the color of your image by clicking the arrowhead and revealing the drop down list (e.g. for a green image select Green from the drop down list).

3. Check the Monochrome box.

Note: The same end can also be accomplished by choosing Channels from Window on the menu, clicking the arrowhead in the Channels dialogue box, and then choosing Split Channels. Close all channels but the channel related to the color of your image.

Adding Color. In the case in which color has been removed from a colorized image to make it grayscale, or in the case in which the image was grayscale in the first place, color can be added. Again, the colorized image that has been made into grayscale has been done so that publishable colors can be added. The color green can still remain difficult to reproduce accurately for publishers, and I have been 90% successful. Occasional I get a green, "head-banger" image, and I can't put my finger on the source of this problem.

This procedure cannot be considered complete unless adjustments are also made to the white and black levels of the entire image, what is addressed at the first of this series of steps. The most effective method (though several can be used) uses the Curves dialogue box. This method colorizes all values evenly, including the brightest values.

1. Under Window, select Info or Show Info to reveal the Info dialogue box. Click on upper right hand arrowhead in the Info box to reveal drop down box. Be sure that the First Readout is set to Grayscale and the Second Readout is set to Actual Color. The Grayscale setting is shown as the K value in the dialogue box (K value is a percentage that reflects how much ink is placed upon the printed page). R, G, B values show pixel values under the eyedropper tool or cursor.

2. Under Image, select Mode to change the Mode from either Indexed Color or Grayscale to RGB Color.

3. Under Image on the menu, select Adjust(ments) then Curves. In the Curves dialogue box be sure that the gradient under the grid and line is black to the left and white to the right. If not, click the small arrowhead in the center of that gradient to swap position.

4. In the RGB Channel, adjust brightness by clicking at the top of the line and dragging to the left (to increase brightness) or change the gamma by clicking near the bottom of the line and bending the line upward. Iteratively make changes and move cursor over the brightest values to be sure that these values do not read at a K value of 0% or at 255 in green, red or blue. Click at the bottom of the line and drag upward along the left edge to raise the values of the blacks in the image so that these values read at a K value between 85 and 90% and a red, green or blue value of between 25 and 35. Note that the higher this reading, the better chance you will have at changing the mode to CMYK without sacrificing desired colors and contrast if you

need to do so in order to publish.

5. Eliminate the colors you do not desire according to the following method. In each instance, you drag the top of the line downward along the right edge until the output value reads as specified. Note that these output values are not set in stone, but the advantage of setting these in this manner lies in NOT completely eliminating unwanted colors: some of the other colors need to be included in order to get the best reproduction when printed.

To obtain Red colorized image:

Select Green Channel	Set to Output of	~65
Select Blue Channel	Set to Ouput of	~40

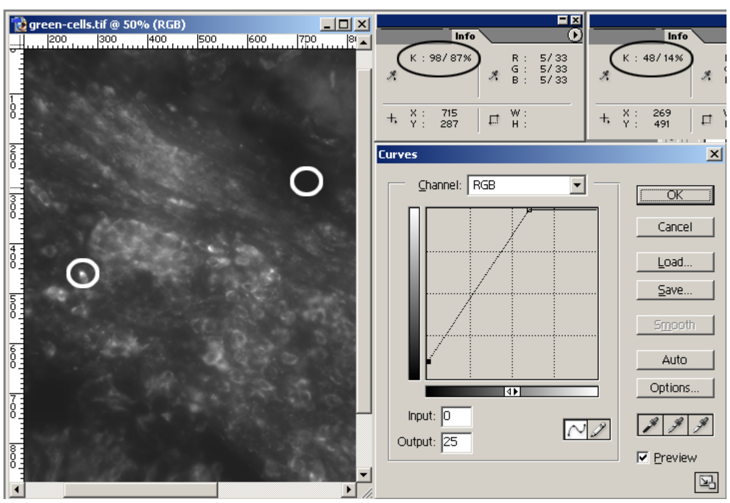
To obtain Green colorized image:

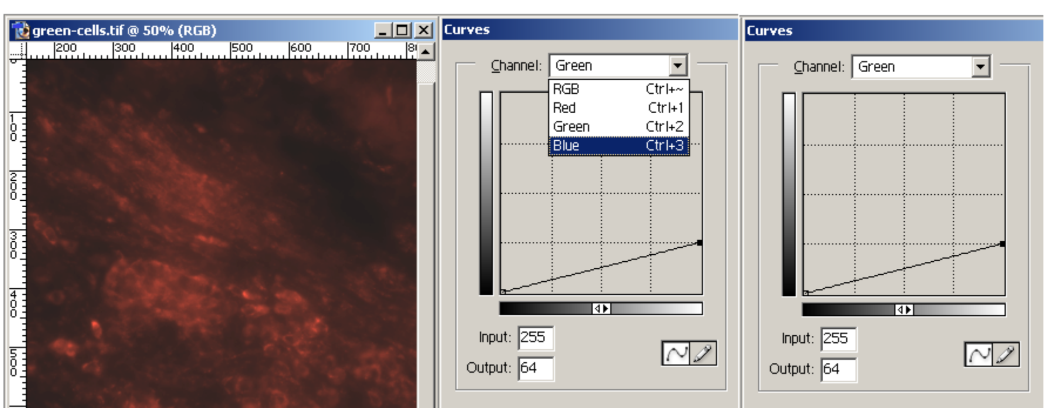
Select Red Channel	Set to Output of	~95
Select Blue Channel	Set to Output of	~105

To obtain Blue colorized image:

Select Red Channel	Set to Output of	~64
Select Green Channel	Set to Output of	~129

You will note that the blue colorized image is closer to cyan. That is because many printers cannot print purple-blue images as well as green-blue images, and that is especially true when these images are published.





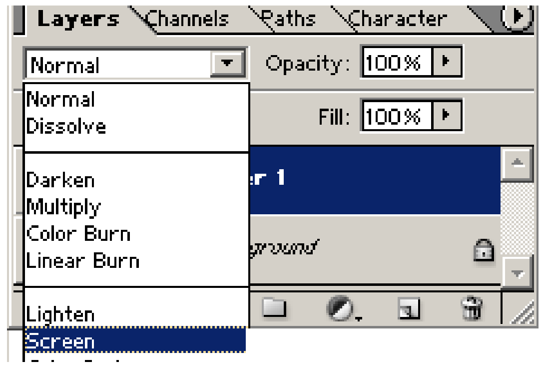
SHOWING COLOCALIZATION

When making an image which shows colocalization, it is best to start with the red colorized image. Often red is dim compared to the blue and green, and so the latter two may need adjustments to their opacity.

1. Open your first file, the red colored image. Then open the green colored image.

4. Under Select on the menu, choose All to select all of the green image. Under Edit choose Copy. Under File choose Close. Under Edit choose Paste to paste the green image onto the red image, thus creating a layer.

5. Open Layers dialogue box under Window on the menu, if this dialogue box is not already open. Click arrowhead for drop down list at top left (it reads Normal) to select Screen. Screen is a function normally used in the printing industry to give equal percentages of 4 layers for CMYK. Where you have colocalization, you should see yellow. If the green is too bright compared to the red (which often happens), then you can adjust the intensity of the green by changing the Opacity slider. Obviously, this should only be done to the degree that the relative intensities of both the red colored image and the green colored image appear to have the same brightness. Often the green image is never reduced by more than 15 percent.



6. If three or four images need to be colocalized, repeat what you did with the green colored image (Select All, Copy, Close, Paste, choose Screen from drop down in the Layers box).

You might also want to change contrast of each layer in case there is too much background color on any of the layers, or in case any of these layers are too dark AFTER running this method.