

# Guidance for alignment of images to generate multiplex images - Immunofluorescence

**This memo summarizes a process by which sequential singleplex images can be aligned and overlaid to generate multiplex images.**

While there are a variety of methods for aligning imaging, this workflow specifically describes the Fiji image processing package distribution of ImageJ, available as a free download from <https://fiji.sc/>.

The Fiji package requires individual images to be stained with a red or a green dye. During image acquisition, the same color of positive staining (dye) may be used for each singleplex image, and the final multiplexed image can be false-colored. DAPI staining is recommended to assist the alignment process and to help find identical locations on the tissue during subsequent imaging steps. The Fiji package supports a maximum of 7 total channels of color which are usually assigned to DAPI and 6 primary antibody staining patterns.

Acquiring images at multiple locations on the tissue is recommended to ensure that positive staining of the markers being detected is captured. Maintaining a wide field of view for each image will ensure that small differences in alignment of each singleplex image will not significantly limit the size of the multiplexed image.

The Fiji package requires that images be in .jpg format. (Images in other formats can be loaded into Fiji and saved as .jpg.) The final image will be a compilation: one DAPI image taken from a reference image, and each of the positive staining images taken from the other images. The intensity of each channel can be adjusted, and the false color to be used can be assigned before the singleplex images are merged into a multiplex image.

1. Download Fiji and install the program.
2. Convert images to .jpg if necessary by uploading the files into Fiji and exporting them in this file format.
3. Each single plex image needs to be in .jpg format and have DAPI staining. The program will use the DAPI signals for the alignment. You will be saving up to 7 total images comprised of 1 DAPI only image and 6 positive staining only images.

4. Alignment will be conducted with 1 image at a time beginning with a reference image. Select one of the images to be the reference image for alignment, usually the image that appears most centered on the region of interest. Save this file as "Location 1\_Reference".
5. Using only the "Location 1\_Reference" file, we will create the first two images that will go into the final image. First, split the image: Image -> Color -> Split Channels (see Figure 1). This will separate the single image into 3 different channels of color: red, green, and blue. You will notice the split images are now in black and white. Save the blue channel (DAPI) image and the positive staining which will be in either the green or red channels depending on which dye was used. We recommend a strong structure to the naming of the files to avoid confusion ("Location 1\_DAPI" and "Location 1\_CD3" as examples). After saving, close all the images. You just created 2 of the 7 total images you will need for merging. One was the DAPI channel and the other was your first positive staining image (CD3 in the example).

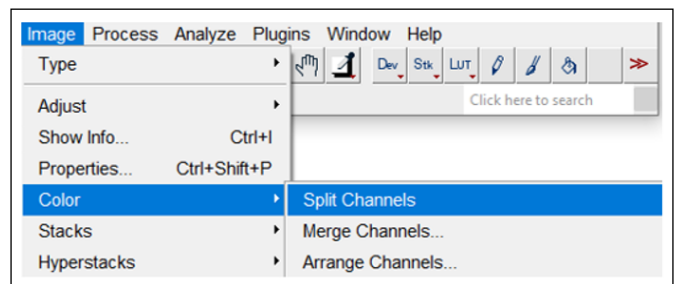


Figure 1: Image -> Color -> Split Channels

6. To generate subsequent images that are aligned to your reference file, you will open two images to align in Fiji, one of which is your reference image (Location 1\_Reference) that was saved in step 2. Open a second image of positive staining that you wish to be aligned and merged in the final image.
7. Select the reference image first and split the color channels: Image -> Color -> Split Channels (see Figure 1). It is critical to split the reference image first so that the images are in the correct order for alignment in subsequent steps. This will separate the single image into 3 different channels of color, red, green, and blue just like before.

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Continued from page 1.

8. Click on the image you wish to align to the reference image and split the color channels: Image -> Color -> Split Channels (see Figure 1). This will again separate the single image into 3 different channels of color, red, green, and blue. There should be 6 images total open.
9. Next, form a Stack out of the 6 images: Image -> Stacks -> Images to Stack (see Figure 2). This will now pile all 6 images into a single stack. You can view all 6 images using the mouse wheel or the scroll bar at the bottom of the image. Make sure the set of reference images (red, green and blue channels) are the first 3 images in the stack (1 of 6, 2 of 6, and 3 of 6) as the program will adjust the back images to fit the images in front. This aspect is most critical for subsequent alignments.

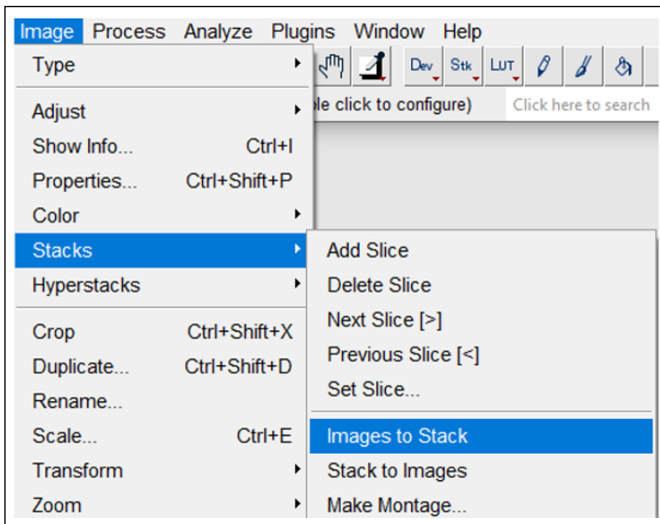


Figure 2: Image -> Stacks -> Image to Stacks

10. Now run the alignment tool SIFT: Plugins -> Registration -> Linear Stack Alignment with SIFT (see Figure 3). You can adjust the parameters in the pop-up window but most of the initial parameters work well. Make sure the maximum image size (4th field from the top) is sufficiently high enough to encompass the starting image size. You can see how many pixels are in your starting image in the upper left corner of the image (see Figure 4A). Also, for simple alignments, you can select “Rigid” as the expected transformation (dropdown menu near the bottom of the popup). Under the Output, “interpolate” should be checked (see Figure 4B). Click OK. This may take a couple of minutes to run and you can monitor the Fiji log to see when it is done.

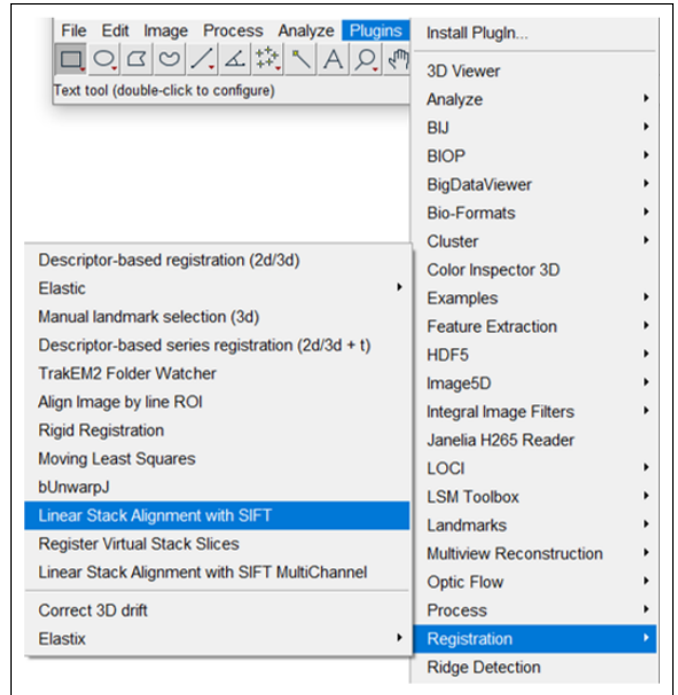


Figure 3: SIFT: Plugins -> Registration -> Linear Stack Alignment with SIFT

11. A new window will pop that is called “Aligned” where you can now see all 6 images and the back 3 have been shifted to align with the front 3 images. Scroll through to make sure this occurred. If not, something was not done correctly or was performed out of order. It is also useful to try and have awareness of what the different images and channels are as the identity or name of the specific image gets lost in the next step.

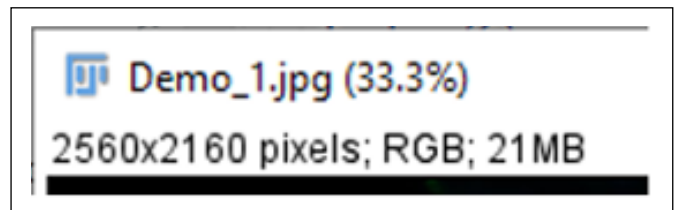


Figure 4A: Pixel size in header

Continued from page 1.

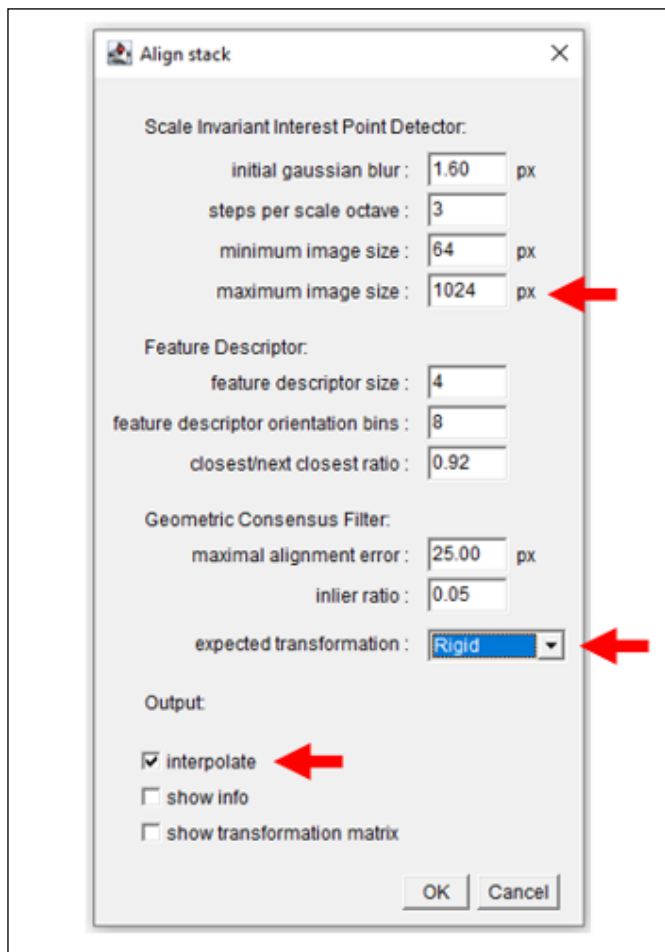


Figure 4B: SIFT pop-up

12. Now unstack the images by clicking on the Aligned stack: Image -> Stacks -> Stack to Images (see Figure 5). This will now split out the 6 images. Scroll through each image and determine if you will save it or close it. Make sure any image you wish to keep is saved before closing as there is no warning or recovery of closed, unsaved images. Since the images now have generic names (Aligned 0001, Aligned 0002, etc) you can use the original stack (it should still be open in a back window) to help identify which image is which. Find and save the positive staining of the newly aligned image (example: "Location 1\_CD20\_aligned"). There may be some additional black borders on some sides as the image has been shifted to match the reference image. After saving the desired image, close all the images.

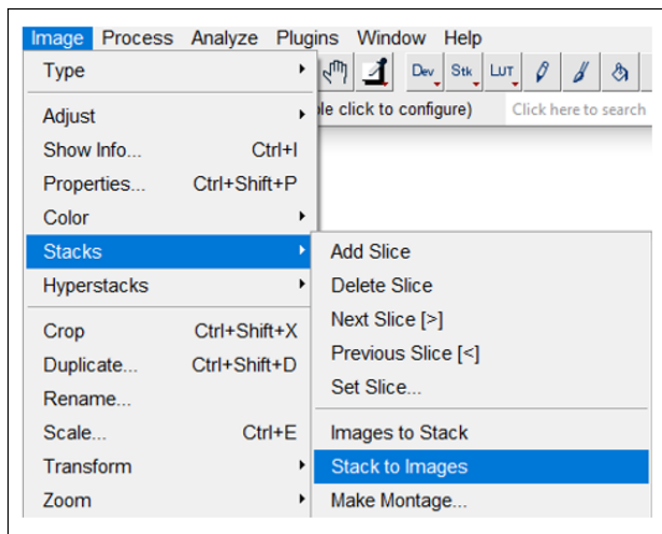


Figure 5: Image -> Stack -> Stack to Image

13. Next, align the other images to the reference image following the same steps 3-9. In the end you should have one image of each positive staining and one DAPI image.
14. Once all the images have been aligned you can adjust the intensity of each channel before merging into a single image. Starting with all the images closed, open the adjustment tool: Image -> Adjust -> Brightness/Contrast. Now open the first aligned image. Adjust the image as needed, predominantly using the maximum and minimum sliders. Your images should all still be in black and white. Save the adjusted aligned image (example: "Location 1\_CD20\_aligned\_adjusted"). Continue opening all the images you would like in the overlaid/merged image and adjusting the intensity. Adjust according to standard imaging practices but in general, no feature should be clipping or above the maximum intensity and you can adjust the minimum parameter to remove very faint features. You can leave the other images open and just make sure the image you want to adjust has been selected.

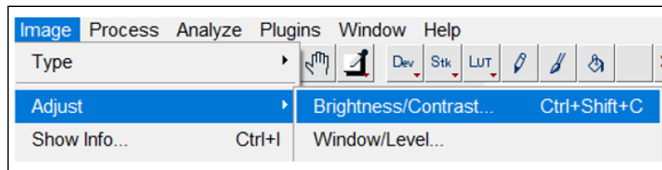


Figure 6: Image -> Adjust -> Brightness/Contrast

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15. To merge all the adjusted and aligned images into a single image, make sure you have all the individual images open and combine them: Image -> Color -> Merge Channels...(see Figure 7). Within the popup window, assign which image you would like to go with which color. DAP I is usually presented as blue. Make sure "Create composite" is checked (see Figure 8). If you merge the images and are not happy with the colors you assigned, you can close all the images, reopen all the adjusted images and repeat the merging process.

16. Once the merged image has been created you can save it. You will also notice that around the edges, not all the images overlaid precisely. You will need to crop the image such that none of the compromised edges are captured.

17. To crop the image, select the area you wish to keep by clicking and dragging on the merged image. Then crop by: Image -> Crop. Save the newly cropped image.

18. There are many Fiji, SIFT, and other imaging tutorials available on the web that can also be useful.

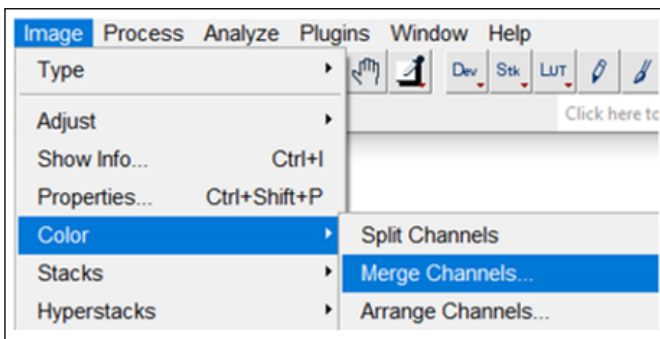


Figure 7: Image -> Color -> Merge Channels

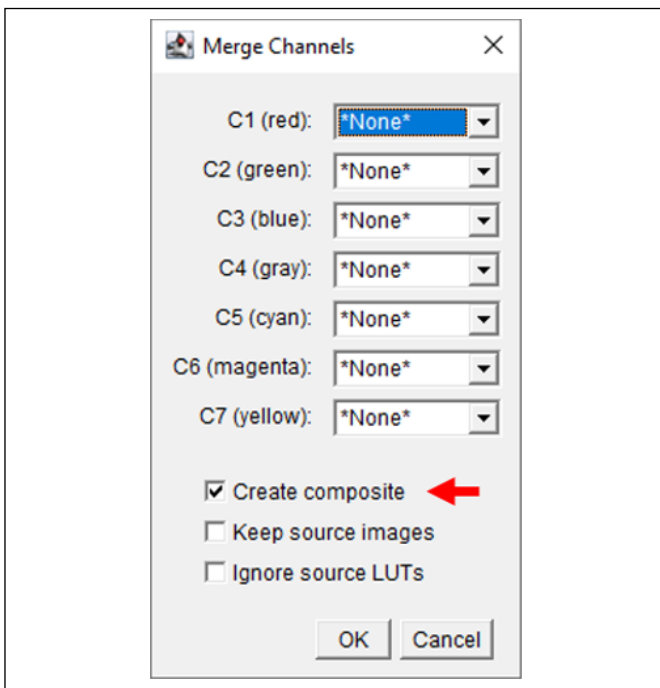


Figure 8: SIFT: Merge Channels pop-up