

## diSPIM image processing tutorial Ryan Christensen

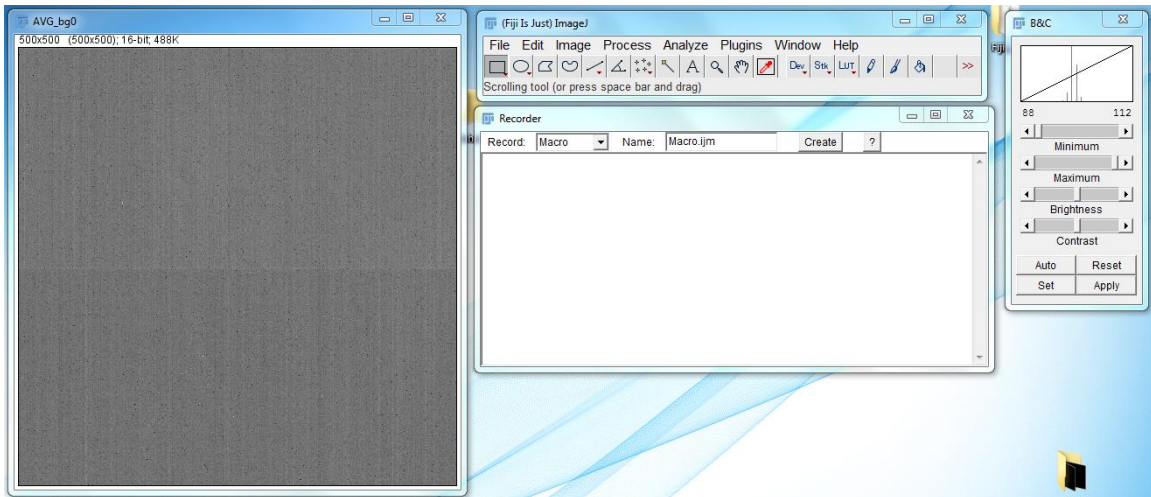
diSPIM image processing involves several steps, including background subtraction, cropping, transformation, fusion, and deconvolution. The first two steps in this process, background subtraction and cropping, are carried out in ImageJ or FIJI, while the remaining steps are carried out in MIPAV. Before describing the image processing, a couple notes on file naming and organization are in order.

- diSPIM images are originally written as image sequences (with each volume being equivalent to one image sequence). Image sequences are saved in individual folders, and need to be converted into a single .tiff stack for MIPAV to be able to read them. Typically we use bgX( where X is a number) to name the folder containing the background image sequence, and bvX (where X is a number) to name the folders containing the actual data.
- The image sequences for each SPIM arm get their own separate folder. The naming of the SPIM arm folders depends on whether you are using Micromanager or Labview. For Labview-based systems, when facing the diSPIM the right-side camera is called SPIMB, and writes to a folder we name SPIMB\_unprocessed, while the left. Micromanager uses an opposite camera naming system, so the right-side camera is named SPIMA and writes to a SPIMA\_unprocessed folder, while the left-side camera is named SPIMB and writes to a SPIMB\_unprocessed folder. Keep track of which naming system you are using as this can affect the rotations done in MIPAV.

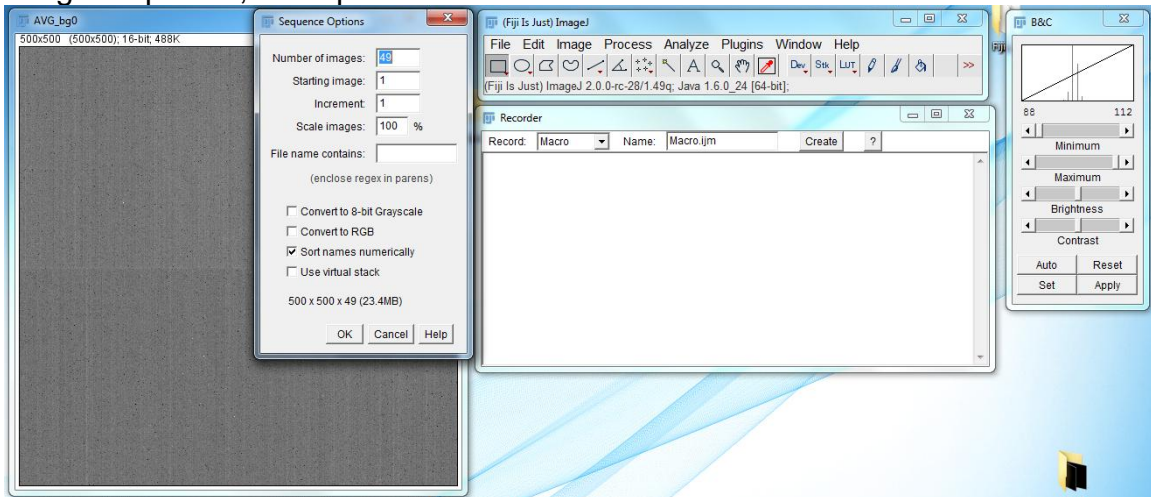
The SPIMA\_unprocessed and SPIMB\_unprocessed folders are used to contain individual image sequence folders. So the file path for an image sequence before processing is usually SPIMA\_unprocessed/SPIMB\_unprocessed -> bvX -> individual image files. -Processed images are saved in SPIMA and SPIMB folders. MIPAV assumes the input images are stored in folders named SPIMA and SPIMB, so it's easiest to just use the conventional naming formula when saving the processed images.

Image processing in ImageJ/FIJI:

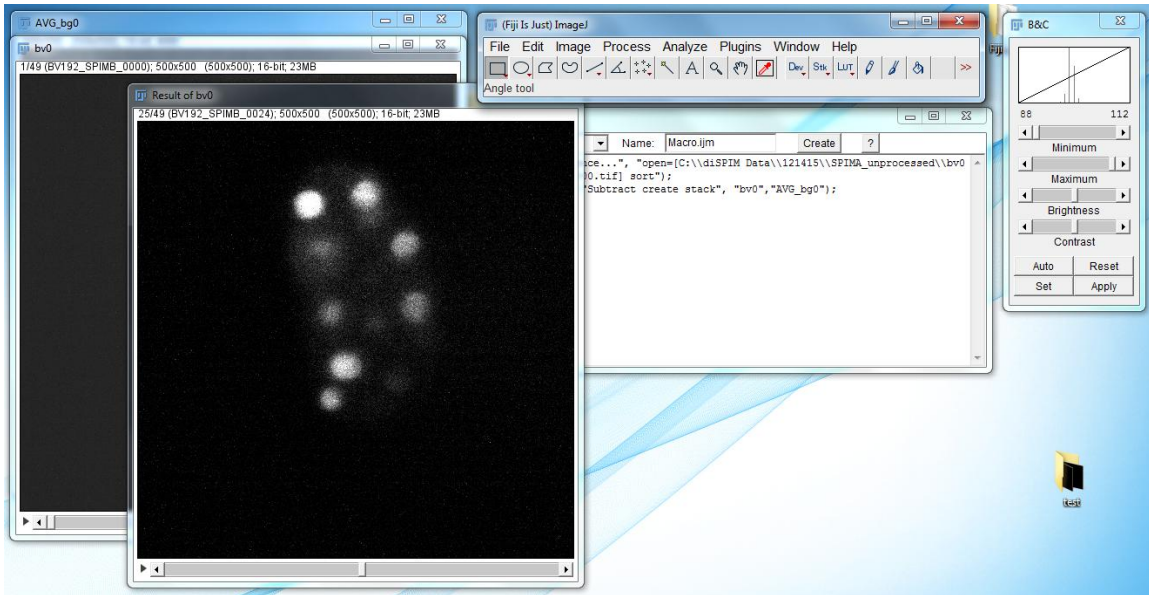
1. Open ImageJ.
2. Import the background image. Go to File -> Import -> Image Sequence, navigate to the folder where the background image sequence is stored, and import it. The settings for the import image sequence dialog box are as follows:
3. Go to Image -> Stacks -> Z Project and set Projection type to Average intensity. Click on the OK button. Then close the original bg0 window, leaving only AVG\_bg0.
4. Go to Plugins -> Macros -> Record. The screen should look like this:



5. Next, open the first image sequence for the diSPIM images. To do this, go to File -> Import -> Image Sequence, navigate to the folder containing the diSPIM image sequence, and open it.

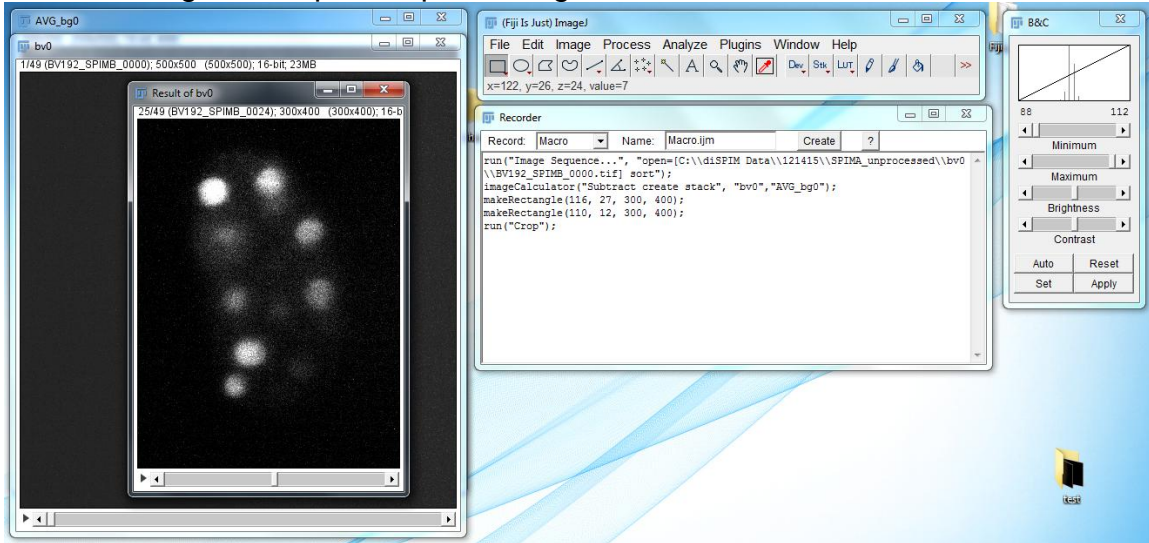


6. Subtract the image background by going to Process -> Image Calculator. Set Image1 to the image you are working with (in this case bv0), Operation to Subtract, and Image2 to AVG\_bg0. Press OK. Press Yes when the Process Stack dialog box appears. The result should appear as:



7. Use the Polygon select tool to draw a box around the sample. This box will be used to crop the image. While size will vary depending upon the sample, make sure that room is left (perhaps 25 pixels minimum) surrounding the sample in the cropped image in all directions. In this demo I have used a crop size of 300 x 400 pixels.

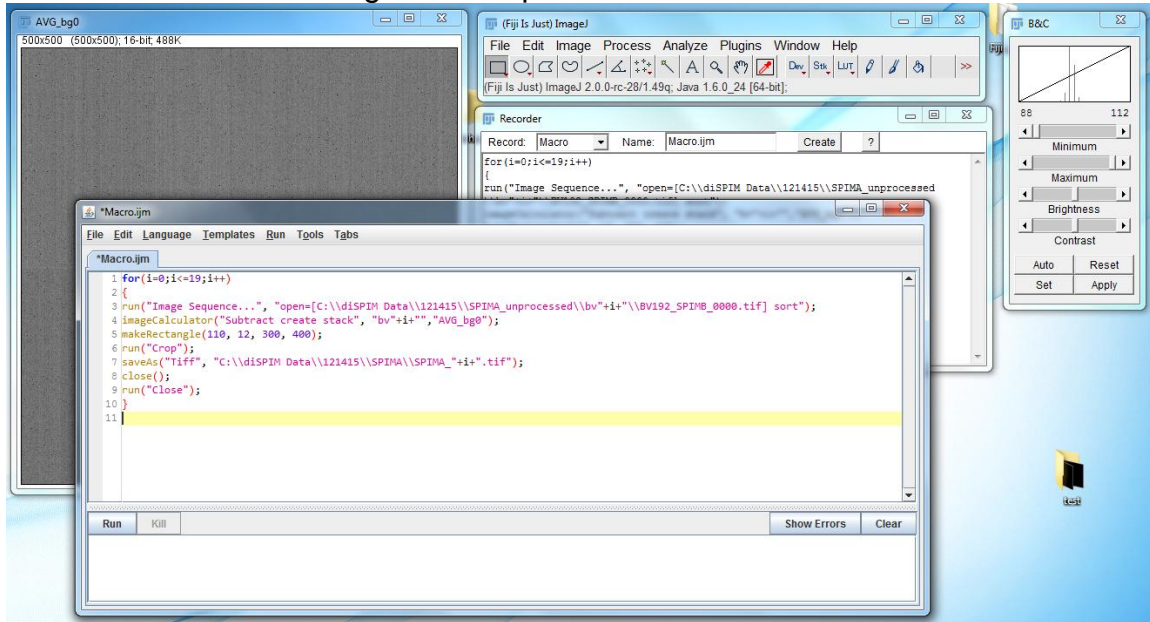
8. Go to Image -> Crop to crop the image.



9. Go to File -> Save As -> Tiff... to save the image. It should be saved in a new folder; conventionally I name this new folder SPIMA or SPIMB depending on which arm the images come from. The image itself is named SPIMA\_XX, where XX is the number of the image in the image sequence.

10. Close the SPIMA\_20 and bv20 images.

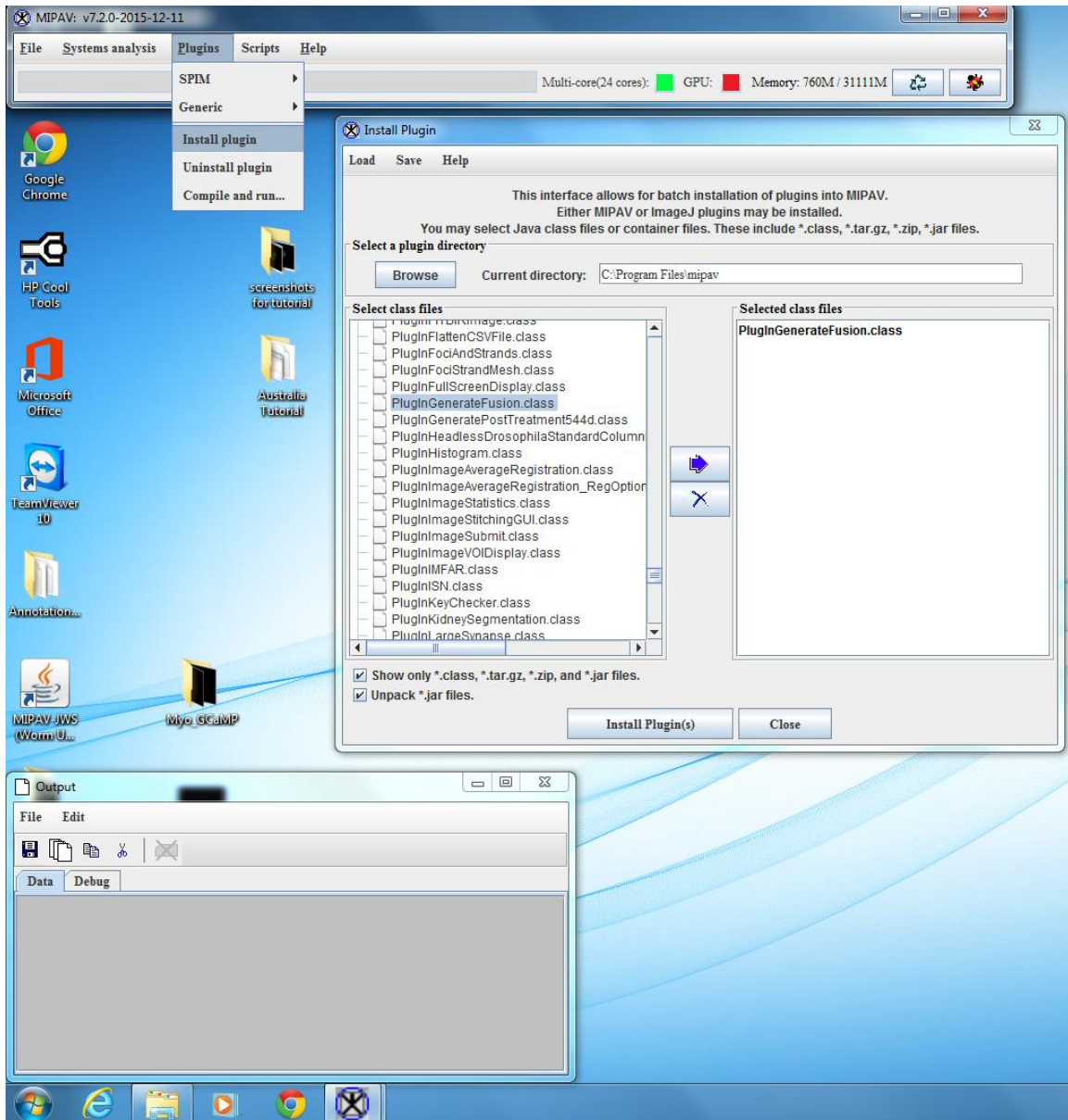
11. Edit the macro to run through as many images as required. An example of a finished macro can be seen below. We also attach a sample version of the macro edited to run through the sample data.



12. Repeat the process for the SPIMB images. Make sure that the size of the cropped images is the same between SPIMA and SPIMB images.

Once the images have been cropped and background subtracted, they can be registered and deconvolved in MIPAV.

1. Install the GenerateFusion plugin by going to Plugins -> Install plugin. In the Select class files window, select PlugInGenerateFusion.class and move it to the Selected class files window. Click on Install Plugin(s) to install the GenerateFusion plugin. The Install window should appear like:



2. Go to Plugins -> SPIM -> Generate Fusion.

There are four tabs in the Generate Fusion plugin: File, Options, Decon, and Image Rotations. Settings in the File and Options tab have to be changed, while settings in the Decon and Image Rotations tabs do not.

3. In the File tab, select the following options:

- Select "All times registration" in the registration options, as this is the most effective option for dealing with sample movement. The No registration requires a preexisting matrix to work from, while the One time registration option does one registration and then applies the matrix generated there to all of the images in the image sequence. These can be faster, but give less accurate results if there is any significant shift in sample position during the imaging session.

- Set the directory that will contain matrix files. Typically I use the SPIMB folder

for this.

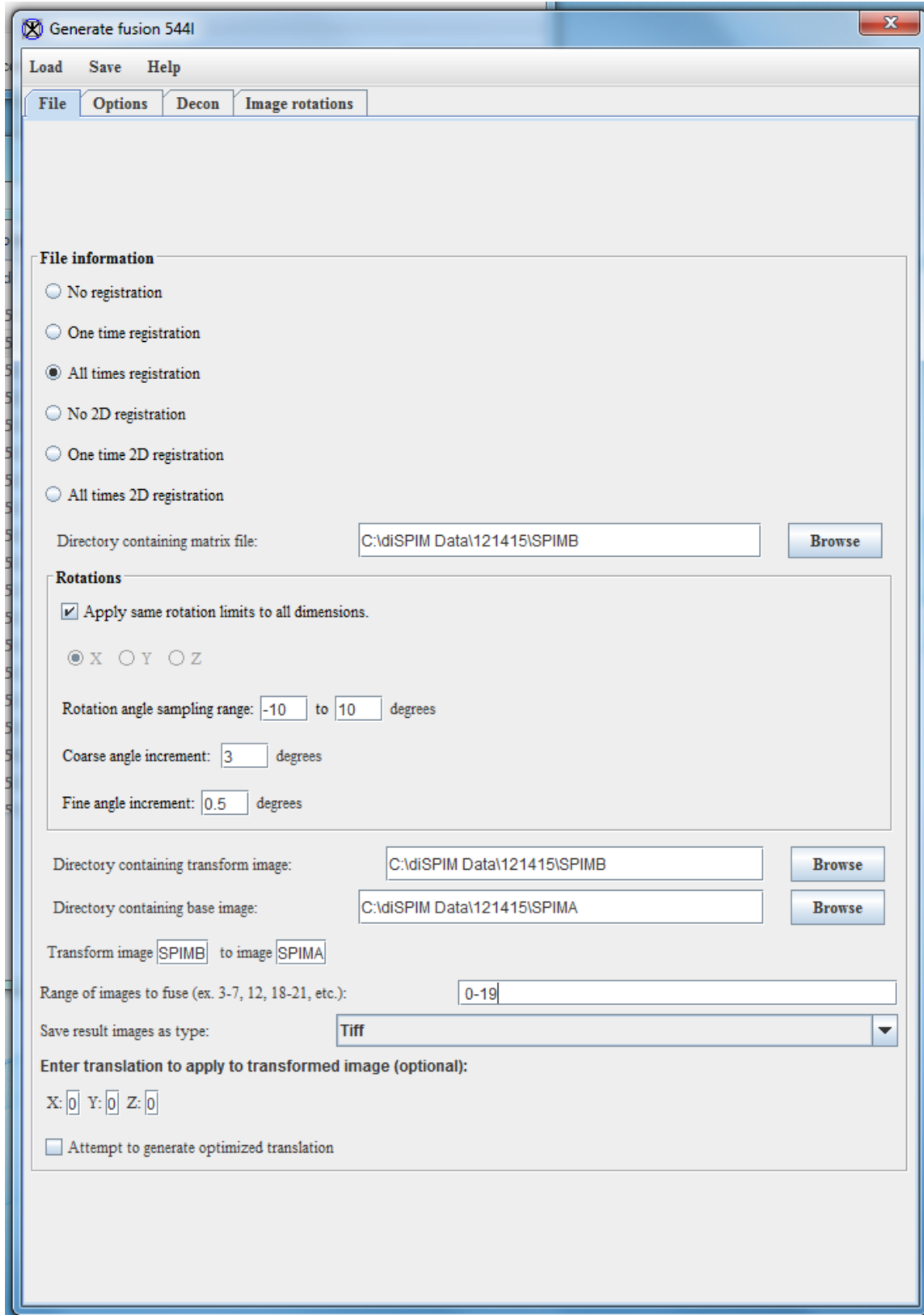
-For Rotations, check the Apply same rotation limits to all dimensions. Leave the Rotation angle sampling range and Coarse angle increment at their defaults (-10 to 10 degrees and 3 degrees, respectively). Set Fine angle increment to 0.5 degrees.

-Select the directory containing the transformed image. If you are using Labview this is the SPIMB directory, while if you are using Micromanager this is the SPIMA directory.

-Select the directory containing the base images. If you are using Labview this is the SPIMA directory, while if you are using Micromanager this is the SPIMB directory.

-Set the range of images to fuse. For this example the range of images is 0-3.

-The completed File tab will have the following settings:



4. In the Options tab, select the following options:

-Set the Initial resolutions. For diSPIM images the standard resolutions are 0.1625 for X and Y and 1.0 for Z. If you are using the stage-scanning mode, the Z-resolution is 0.65.

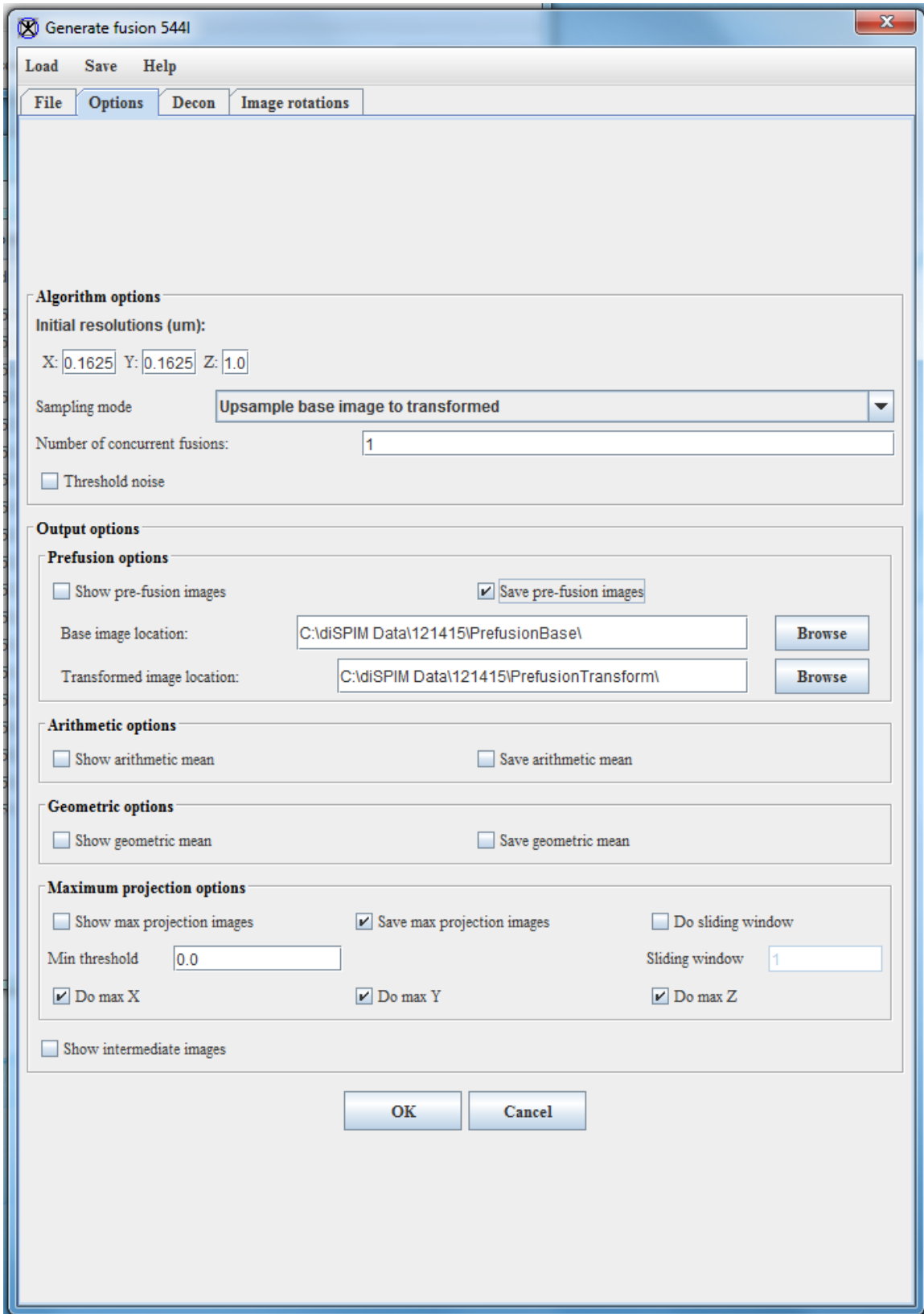
-Set the Sampling mode to Upsample base image to transformed.

-The number of concurrent fusions allows a user to devote multiple cores to the registration and deconvolution process. If the computer only has one core available, set this number to 1. If multiple cores are available, this number can be set higher (though for obvious reasons it can't exceed the number of cores present in the computer.) I typically use 15 cores out of my 24-core computer, though I note that this selection is fairly arbitrary on my part and could probably be increased.

-For Output options, check Save pre-fusion images and Save max projection images.

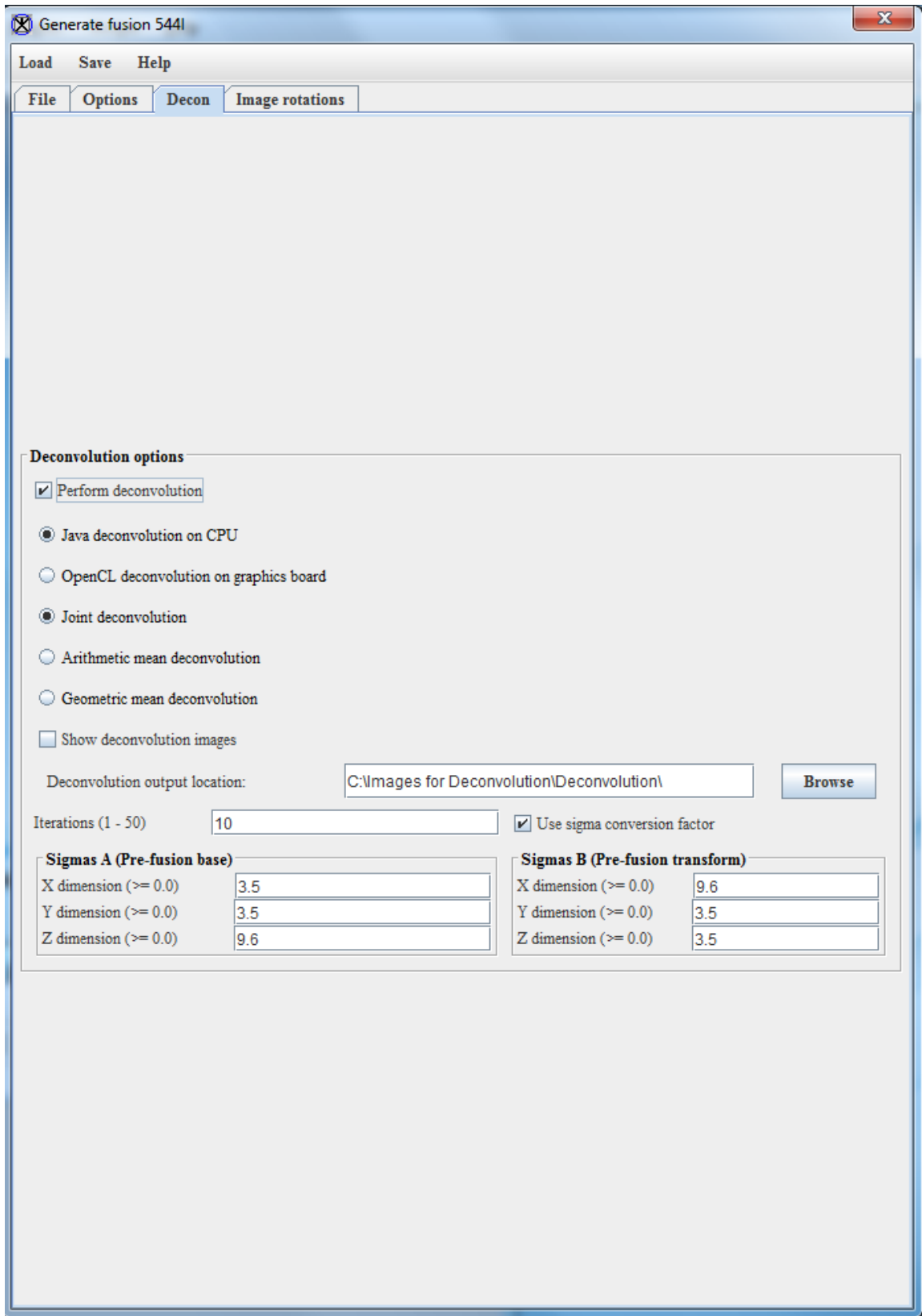
-The completed Options tab will have the following settings:

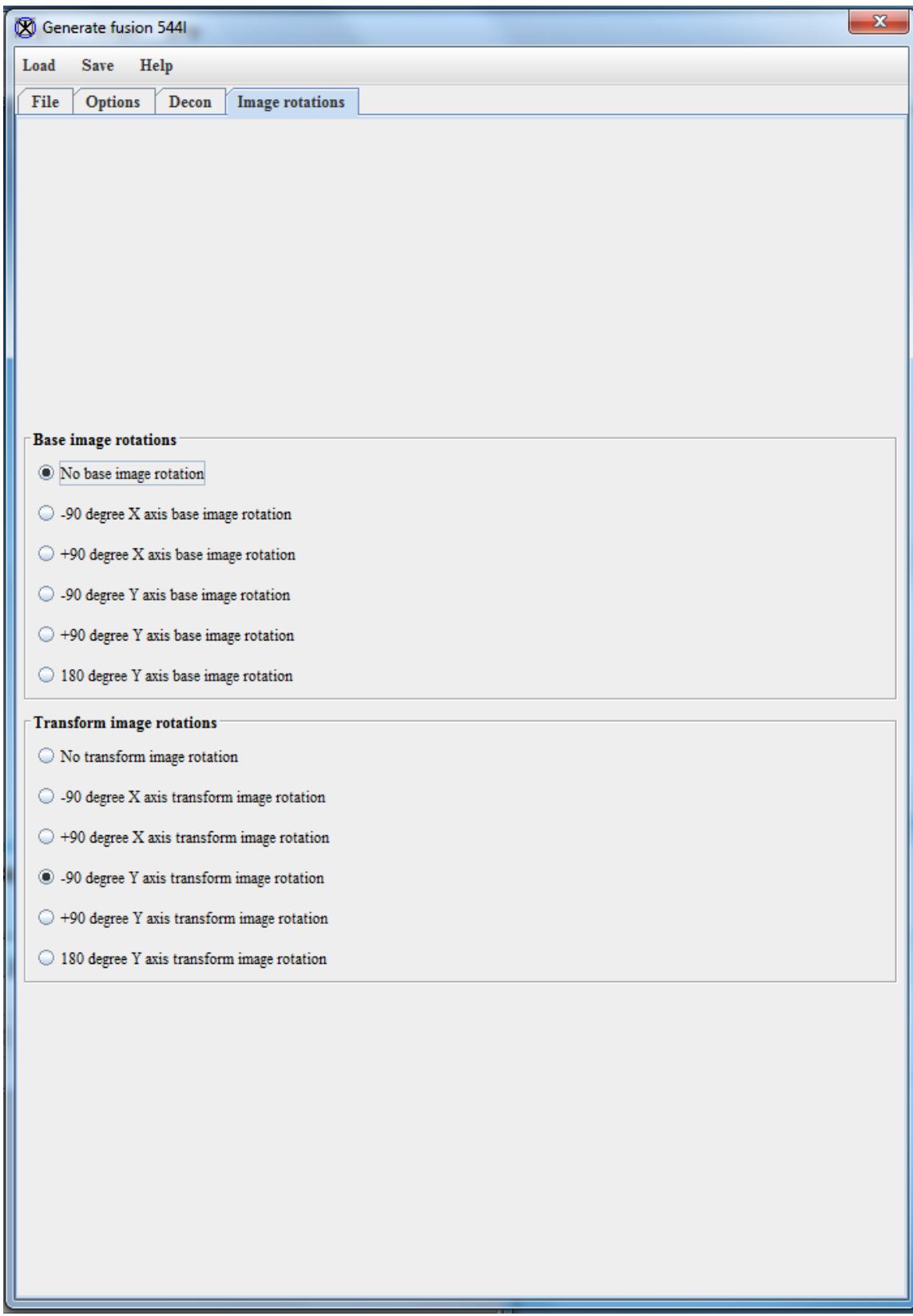




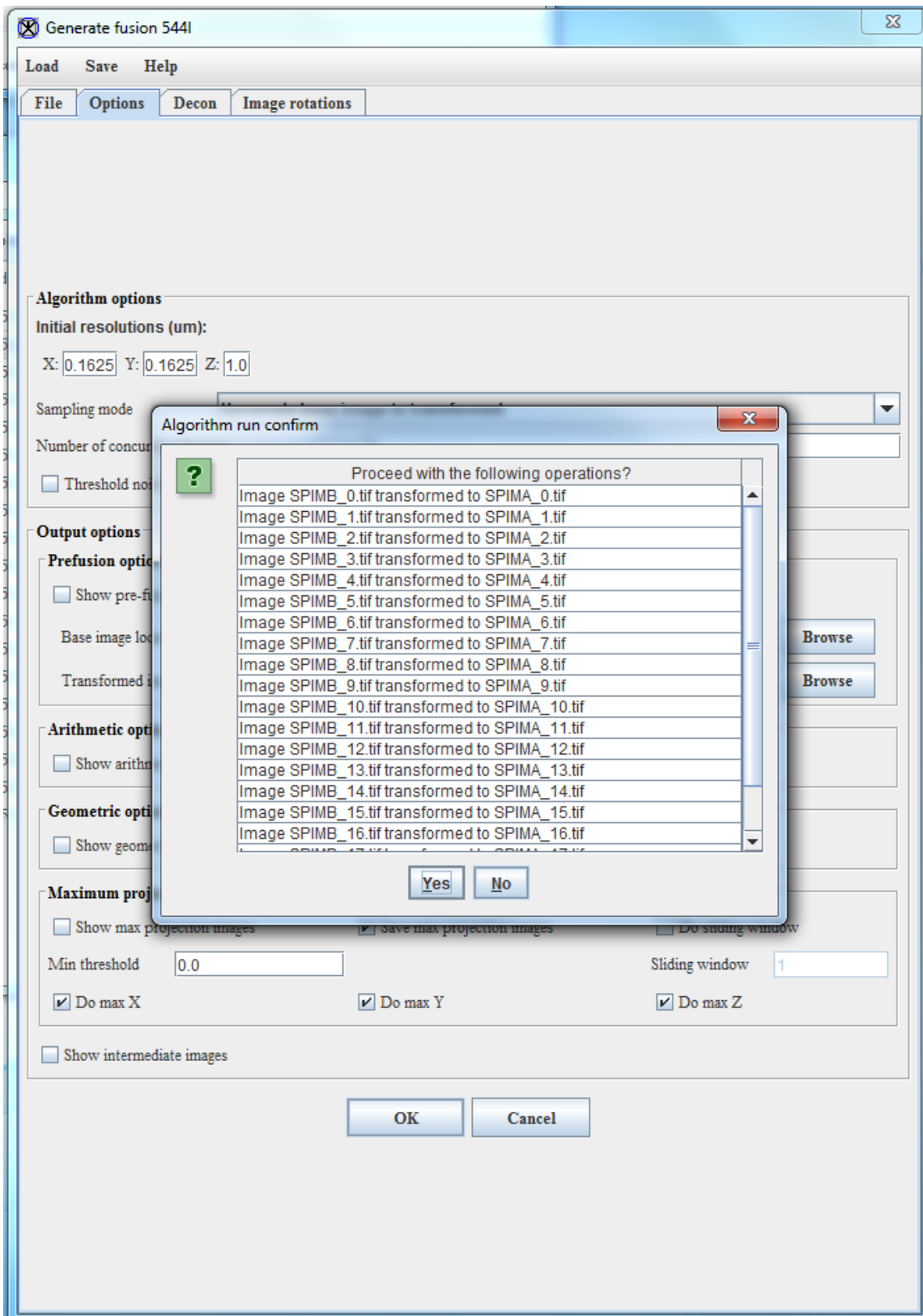
5. The Decon and Image rotations windows should be set to defaults and not

need changing. They will have the following settings:





6. Click on OK at the bottom of the Options Window. Verify that the correct number of images will be processed, and then click Yes on the Algorithm run confirm window.



7. MIPAV will now begin fusion and deconvolution.

8. MIPAV will create several new folders: PrefusionBase, which holds the base images, PrefusionTransform, which holds the transformed images, and Deconvolution, which holds the images created after transformation, fusion, and deconvolution. The deconvolved image of the embryo will look like the following:

