Protocol for ImageJ Analysis

**Protocol for AF013 to AF030 and AF071 to AF078:**

1. Open MATLAB and open drive O:\
2. Add to path in “DataAnalysis”: “Segmentation”, “PAT-US\_matimg”, and “ARCrecon\_Ivan\_Mar04\_2016”
3. Open and run “O:\DataAnalysis\ARCRecon\_Ivan\_Mar04\_2016\ArcReconPAT\_July7-2015\GUIs\TheGUIMaster.m”
4. Close ArcReconGUI and Visualization GUI, keep CoRegisterGUI open
5. Check “Show image” boxes located adjacent to “US image ID:” and “PAT image ID:”
6. From AF074 on, change the “depth” parameter in the CoRegisterGUI from the default of 70 to 85.
7. Select desired image folders under “US image load path:” and “PAT image folder path:” from “O:\DataAnalysis\PAT-US\_matimg\AF###-AF###\Recon” – match non-flip US with non-flip PAT and match flip US with flip PAT. Use only 930nm wavelength PAT images.
8. Run using big button “>>”
9. Montage images of PAT and US will appear once co-registration is complete. Save each montage in “O:\DataAnalysis\PAT-US\_matimg\AF###-AF###\CoReg\” in the appropriate folder, which will be entitled AF###matimgUS or AF###matimg930. The montages should be saved using the following formats: “AF### US Montage”, “AF### 930 Montage”, “AF### US\_flip Montage”, or “AF### 930\_flip Montage”,
10. Open (Fiji is just) ImageJ
11. In ImageJ, open “O:\DataAnalysis\Segmentation\SegmentingPAT” by using the “File>Import>Image Sequence”, open “slice01.png” and select “OK”.
12. Repeat step ten for “O:\DataAnalysis\Segmentation\SegmentingUS”
13. Use montages as described in step 8 to identify the useable range of each stack. The number of slices should be the same in each stack. Use “Image>Stacks>Tools>Stack Sorter” to “Delete n” slices. Note the range of slices in the excel document entitled, “Data Analysis Progress”.
14. Save each stack in in the appropriate folder under “O:\DataAnalysis\Analysis\_ImageJ\AF???\US” or “O:\DataAnalysis\Analysis\_ImageJ\AF###\PAT 930”. For US stack, save as a tiff using the following format: “### US initial stack”. For PAT 930 stack, save as a tiff using the following format: ““### PAT 930 initial stack”.
15. “Analyze>Tools>ROI Manager”
16. Select “Freehand selections” button – the icon is a heart shape.
17. Trace the outermost edges of the sample, making sure to not include any shadows, sutures, or illumination of the plastic bag.
18. Once an enclosed trace has been completed, use the “Add[t]” button on ROI Manager to add it to the list of ROI.
19. Repeat step 16 and 17 for each slice of the US stack. Select all ROI in the ROI manager and use “More>Save and save the ROI in “O:\DataAnalysis\Analysis\_ImageJ\AF###” using the format “034 ROI (your initials in caps)”
20. Click on the US stack window and then to the first ROI in the ROI Manager. Then use “Edit>Clear Outside” and select “No” in the pop-up window. Click the next ROI and repeat “Edit>Clear Outside” and select “No” in the pop-up window. Repeat this for each ROI in the manager.
21. Repeat step 19 with the PAT 930 stack.
22. Save each stack as a tiff in the appropriate folder using the following formats: “### US final stack (your initials in caps)”; “### PAT 930 final stack (your initials in caps)”, “### US final combined stack (you initials in caps)”; “### PAT 930 final combined stack (your initials in caps)”.

**Protocol for AF078 to AF098:**

Follow the steps as outlined for AF013 to AF030 and AF071 to AF078; however, use “flipped stacks” as they are the only images available for these samples. Also, the PAT stacks must be reversed in order because the US probe is now imaging from the bottom. The PAT stacks must also be translated in the x and y directions to match up with the US images. Additionally, during co-registration, make sure the depth is set to 85 mm from AF074 on.

**Protocol for AF031 to AF070:**

The sample started being imaged from both sides (flipped) starting at AF021; starting at AF031 illumination changed from the top and bottom to only the bottom of the sample, rendering only half of each PAT and US scan useable. As a result, the stacks created and then traced are a combination of the flipped and the non-flipped scans.

1. Follow the same protocol as described above for AF013 to AF030 and AF071 on. However, steps six to thirteen must be completed twice – once with flipped scans of US and PAT 930, and once with non-flipped scans. Note – once one group of scans have been co-registered, ensure the stacks have been created out of the “Segmentation” folder as running the CoRegisterGUI again will replace the slices in those folders.
2. When reducing number of slices in the stacks, first use non-flip US to identify the range of the stack. Then use the number of slices in this stack to work backwards with on other stacks as other stacks will not have a defined start point (they should have a defined ending point). Be sure to note which slices are the ones that are actually used in the excel document entitled “Data Analysis Progress”.
3. At this point, there should be four stacks created and saved as tiff’s: “### US initial stack”, “### US initial flipped stack”, “### PAT 930 initial stack”, and “### PAT 930 initial flipped stack”.
4. For AF031 to AF035: delete the beginning halves of each stack, then “Image>Stacks>Tools>Reverse” each flipped stack, use various transformations under “Image>Transform” to co-ordinate the orientation and location of the flipped and non-flipped stacks. Do the transformations only on the flipped stacks. Co-ordinate and transform the images using the last slice of the reversed flipped stack and the first slice of the non-flip stack (these are consecutive slices in the eventual combined stack). A typical transformed flipped stack will likely require a horizontal or vertical flip, rotation of n degrees, and translations in the x and y directions. Save the flipped stacks after they have been transformed (overwrite the previous version). Finally, “Image>Stacks>Tools>Concatenate” the flipped and non-flipped stacks of US and PAT 930, respectively. The flipped stack goes first in concatenation. Save the concatenated stack as a tiff in the appropriate folder using the following formats: “### US initial combined stack”; “### PAT 930 initial combined stack”. Follow steps 14 – 21 above to complete the tracing using the combined stacks. Note – sometimes the combined stacks of the US and PAT 930 do not line up properly when applying the US traces to the PAT 930. When this happens, use “Image>Tools>Transform>Translate” to co-ordinate the traces and the images.
5. For AF036 to AF071: follow step four; however, delete the second half of both US stacks rather than the first half. When concatenating, the non-flip US goes first in the combined stack. This occurs because the US probe was switched from imaging from the top to imaging at the bottom. Follow the rest of the steps as described above in step four.

**How to make final stack into 3D image:**

1. Open ImageJ (not Fiji ImageJ).
2. Open the desired stack.
3. “Plugins>3D>3DViewer”.
4. Click OK on the pop-up menu.