**Protocol for image stack capture, 2D merging, and symbiont cell counting**

Written by Tyler Coleman and edited by Trevor Tivey

1. Image-stack capture and export
   1. Obtain two stacks of images of a single tentacle by taking a video while scrolling through the focus on the epifluorescent microscope first on bright field then using the laser immediately after.
      1. Note: approximately 30 tentacles can be imaged per hour in this way.
   2. Export the video into an image-stack using the image export in Zen, make sure Create a new folder is selected.
      1. Note 1: If you are exporting many videos you can use batch export
      2. Note 2: Batch exporting 300 videos as images takes between 30 minutes-50 minutes though it is fully automated and can be done from any computer that has the free Zen lite application.
2. Image-stack curation
   1. Make sure all of the image stacks consist of 50 or less images and are all in one folder then update “Automated\_Merging\_Sure.jsx” with all relevant paths
      1. Note: Delete images that aren’t in focus from the top and bottom of the stack, especially if there are more than 50 images.
   2. Separate brightfield (BF) and fluorescence (FL) image-stacks into two different folders.
      1. Note: If you want to restrict observations to focal tentacle, you will need to use both brightfield and fluorescence image-stacks and make sure that the folder directories are organized in the same order such that the nth image in the BF folder and the nth image in the FL folder are of the same tentacle.
3. Merge image-stack using Adobe Photoshop
   1. Open “Auto\_Blend.ana” in Adobe Photoshop then run “Automated\_Merging\_Sure.jsx” by selecting file>scripts>browse at which point you can locate “Automated\_Merging\_Sure.jsx” in the folder you have it placed in
      1. Note 1: This process is completely automated though takes a while, 288 stacks took ~5 hours.
      2. Note 2: Occasionally the process will stop due to taking up too much processing power. If this happens the process can be restarted after you remove folders that contain stacks that were already merged.
4. Fiji/ImageJ Analysis
   1. **Protocol A: Using brightfield images to restrict observations to focal tentacle**
      1. Once all of the stacks have been merged into 2D images separate brightfield (BF) and fluorescence (FL) images into two different folders.
      2. Make sure that the merged images in each folder are in the same order such that the nth image in the BF folder and the nth image in the FL folder are of the same tentacle

* + 1. Open ImageJ and make sure the following macro and plugins are installed:
       1. Color\_Pixel\_Counter.class
       2. CoustomAnalyzer.class
       3. ICTN\_.jar
       4. Particle\_Remover.class
       5. tc\_SURE\_master\_macro.ijm
    2. Run the “TC\_SURE\_macro.ijm” macro (for best results use a large and high resolution monitor for better thresholding)
       1. This is not fully automated and took me ~3 hours for 144 BF-FL image pairs
    3. Place “Combine\_Cell\_Locations.sh” and “Combine\_Cluster\_Size.sh” in the results folder from the “TC\_SURE\_macro.ijm” macro then run each of them using the bash command line
    4. Open “SURE\_Macros.xlsm” and make sure that macros are enabled in excel
    5. In Excel run the “Cell\_Location.ProcessFiles” macro and the “Cluster\_Size.ProcessFiles” macro.
       1. These each take ~2-3 minutes for 144 files
    6. Finally place one copy of “Combine\_csv.sh” in a folder containing all Cell\_Location files, and another copy in a folder containing all of the Cluster\_Size files.
    7. Run “Combine\_csv.sh” in each folder creating a master csv file for each.
  1. **Protocol B: Measuring clusters abundance and size by fluorescence images only using pixel location and size**
     1. Open FIJI/ImageJ2 and edit macro “cluster-object-processing.ijm”
     2. Set input folder and output folder directories.
     3. Run cluster-object-processing.ijm
        1. Opens image
        2. Turns it into 8-bit
        3. Runs Gaussian blur with a sigma of 1
        4. Subtract Background with a rolling ball of 50
        5. Performs Autothreshold using MaxEntropy
           1. Note 1: After running, check if threshold is not working for certain images. For these files can either manually perform thresholding, or further curate image-stack,
           2. Note 2: If a problem with a significant amount of files, may be best to go all the way back into Zen to make sure proper brightness and contrast was applied to images prior to image export.
        6. Converts to a Mask, and inverts
        7. Saves JPEG as data input
        8. Runs 3D Object Counter on Binary image providing statistics, summary, centres of mass, and objects
        9. Saves results output as a txt file
        10. Saves centres of mass as a JPEG
        11. Saves objects as a JPEG
        12. Saves log as a recurring file for history and object count summary purposes
     4. Check output file images (Mask JPEG, Object counting JPEGS) to ensure correct processing.
     5. Create folder for Data analysis / R input containing only Object count txt files: ./all-3dobj-counts/
     6. Proceed to object size conversion to symbiont count in R using verification with a subset of handcounted symbiont clusters.