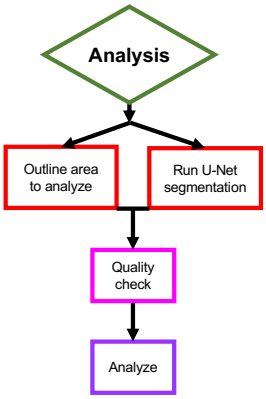
Analysis

Revision: 12/10/2022 Madi McElliott and Jeff Beamish



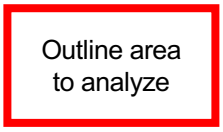
The following protocol creates cortex only masks of cross-sectional kidney sections and uses U-Net assisted analysis to capture positive area detection for higher order and nuclear stains. This allows for downstream analysis of total positive area detection, area analysis, and area fraction which can be modified for specific data needs. It is assumed that the "Pre-Processing" protocol has been completed.

BEFORE STARTING:
Establish macro directory in QuPath. First download the QuPath scripts (all end *.groovy). Note the location. Then go to QuPath→ Automate→"Set Script Directory"→select the folder where all the scripts are saved

You must have the "U-Net Segmentation" plugin installed in Fiji. If it is not installed, go to Help→"Update…" then select "Manage update sites" and select "U-Net Segmentation" which will add this package to Fiji. You will have to close and restart Fiji after adding.

Unless specified, all macros can either be installed and run OR opened in Fiji and run using the Macro Editor (which is our preference as it makes changing parameters easier, if needed).

***You must have directories of entire section images for all of the files you want to analyze. See the "**Preprocessing**" protocol for detailed instructions. As with all other aspects of these instructions, it is critical that images and associated files have the form: part1_part2_part3_part4_part5_part6...other info; for example: the "WT_GFP_M_0001_FA2_Ki67.tif" This protocol is directly built following the instructions of the "Preprocessing" protocol to split multichannel sections into individual, single channel .tiff files.

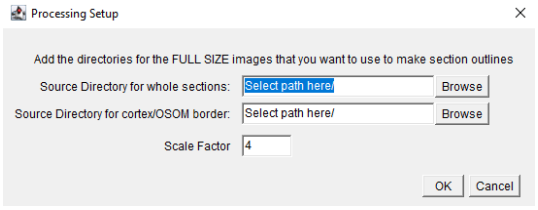


PART I: Generate Masks of Area to Analyze

This step makes an outline of the area of the section to analyze. Generally, we have used the cortex and outer stripe of the outer medulla (OSOM), but any area could be selected. We have found it easiest to first outline the entire section carefully (excluding defects, large vessels, collecting system); then in a second step, define the border of the OSOM. The protocol below describes this two step process.

STEP ONE: Make low resolution images more suitable for outlining sec tions (Fiji).

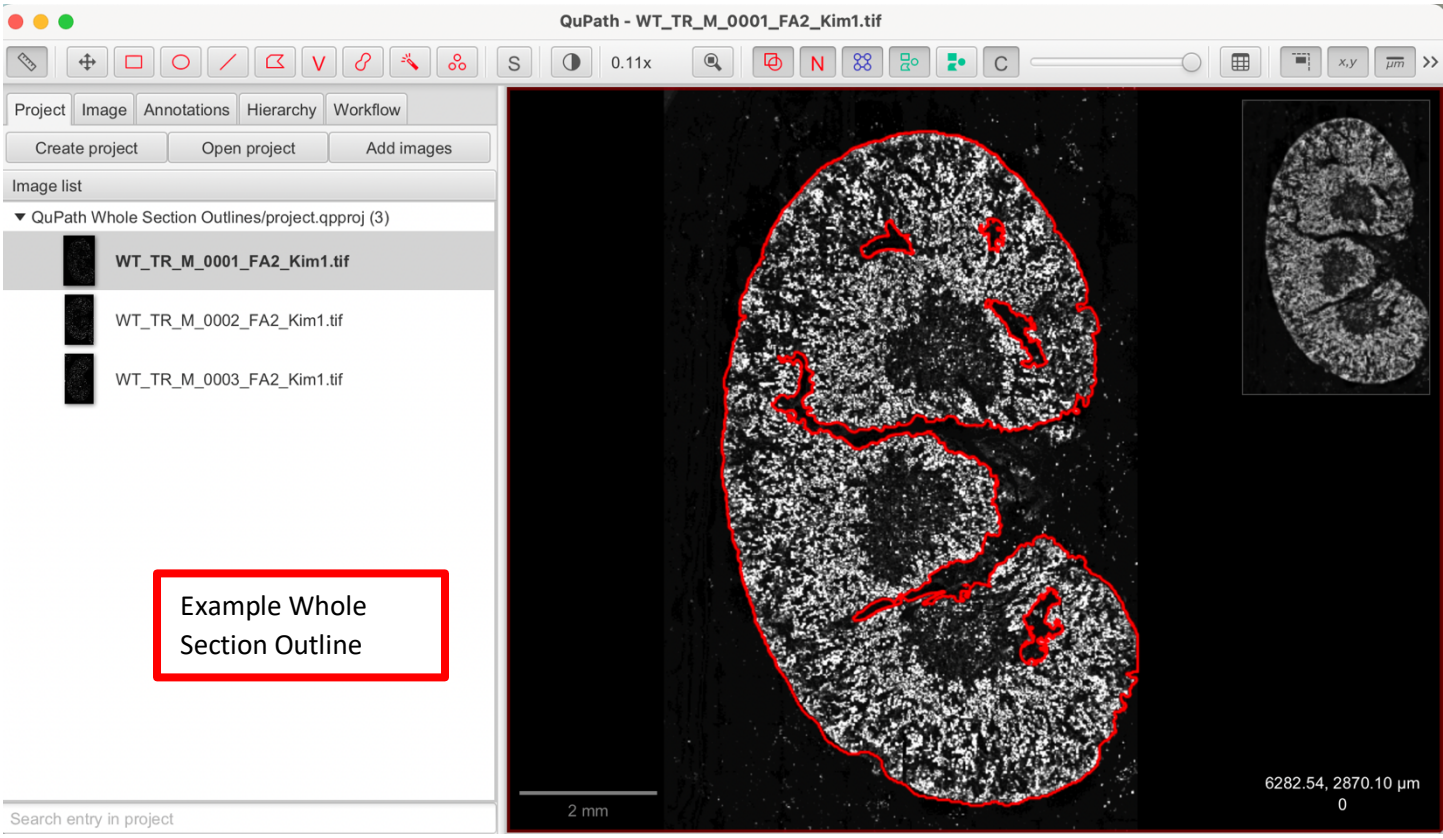
1) To speed up the QuPath processing, we first generate files of lower resolution
2) Run the macro "Analysis_STEP_ONE_Make Low Resolution Images.ijm".
    a. Select the directory of the files you want to use to make the whole section outlines. Any set of images will usually be adequate, but we often use the autofluorescence channel generated by our slide scanner.
    b. Select the directory of the files you want to use to define the cortex/OSOM border. Many of our animals have GFP expressed in the S3 segment so we use this channel if available, but any channel can work based on morphological parameters.
    c. Set the Scale Factor. A 4-fold reduction seems to work well to give files around 10 MB that QuPath can handle easily.
    d. Click OK to process the files. The macro will save the files in two separate folders with a name that ends in "(LOW RES)" for example "Autofluorescence(LOW RES)"



STEP TWO: Generate ROI masks that define the area of the section you want to analyze and excludes defects and collecting system (QuPath)

1) Create a new folder named "QuPath Whole Section Outlines" in the "Extracted_Decoded_Split_Analyzed" directory (generated in the Preprocessing protocol) and generate a new QuPath Project and import the "[channel] (LOW RES)" images for the whole section outlines (for example "Autofluorescence (LOW RES)")
2) *Optional: If image files are not at an optimal contrast level. You can run the QuPath script "Adjust BC.groovy". You will need to adjust the parameters in the code before running.
3) Use a combination of the Wand (W) and Brush (B) tools to define the whole area of the section. Include all areas of cortex and medulla that are directly contiguous (or would be if there were not artifacts from sectioning). Useful keyboard short cuts:
    - SPACEBAR-move the image without moving the annotation;
    - W-switch to the wand tool (used most of the time)
    - B-switch to the brush tool (useful when the brush tool is producing irregular results)
    - ALT-hold down while using other tools to REMOVE areas of the ROI
    - SHIFT-hold down while using other tools to ADD areas to the ROI that are not directly contiguous

    Save these annotations before moving onto the next step.



4) Run the "Export Section Outlines.groovy" script in QuPath. This will generate Fiji-compatible *.roi files with from the annotations in QuPath.
5) Create a subfolder in "Extracted_Decoded_Split_Analyzed" named "Whole Section Outlines **(LOW RES)**" and move all extracted *.roi files here. (After running the script, the *.roi files will be in the "QuPath Whole Section Outlines" folder)

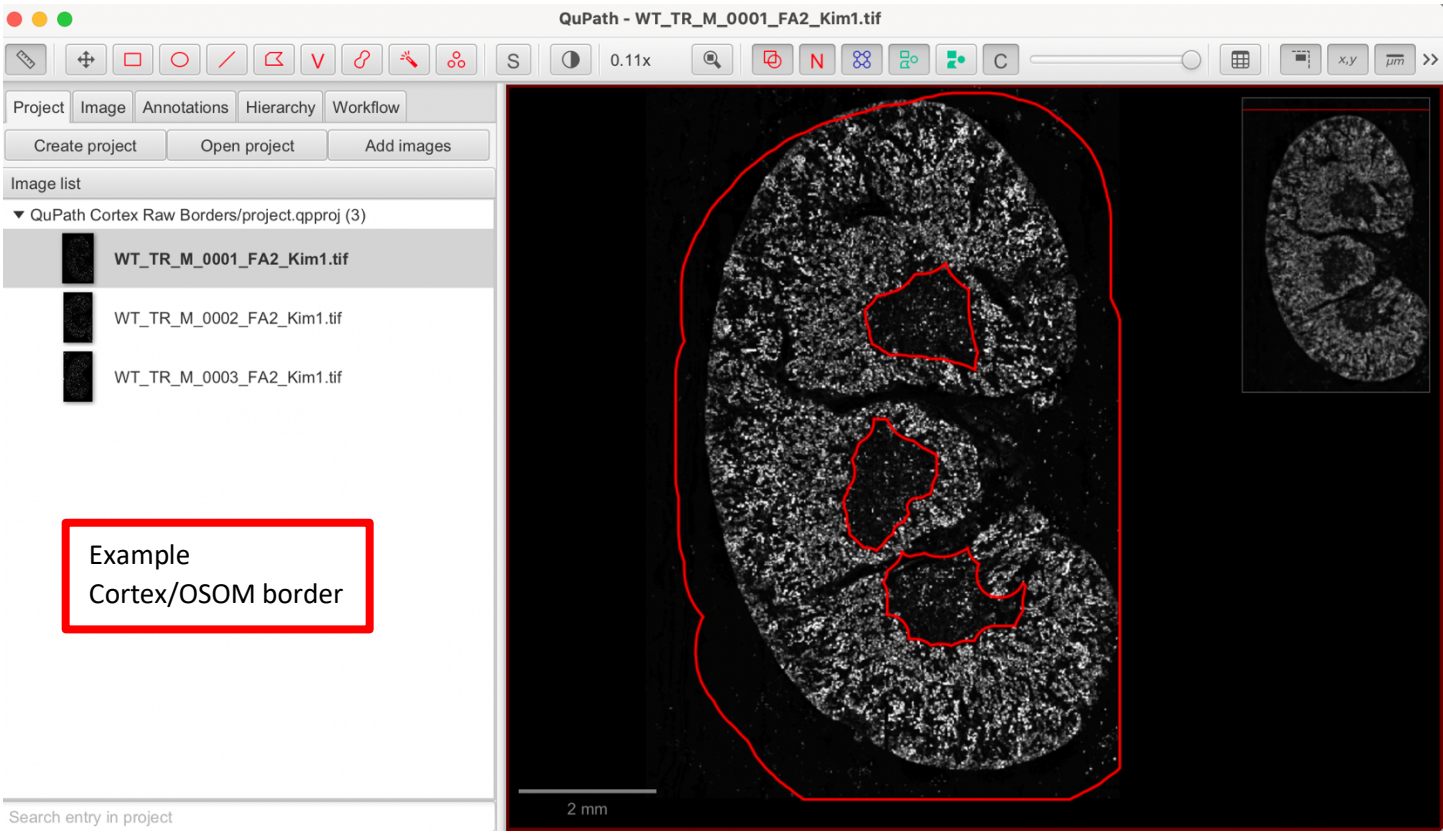
STEP THREE: Generate ROI masks that define the cortex (QuPath)

6) Generate a new QuPath Project in the "Extracted_Decoded_Split_Analyzed" named "QuPath Cortex Raw Borders" and import all of the low resolution images to be used for defining the cortex/OSOM border (**from STEP ONE – 2b**).

7) Use a combination of the Wand (W) and Brush (B) tools to define the area of the section.

   NOTE: Because the cortex will be generated at a later step by the intersection with the whole section ROI, only the cortico-medullary border is critical. The rest of the ROI can (and should) extend beyond the section itself and does NOT need to account for defects.

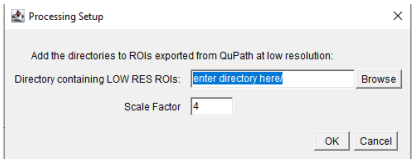
   NOTE: the brush tool is the most useful in this step (in contrast to 3b where the wand tool was best). However, the default sizes are too small. To change the default brush tool size in QuPath go to: "Edit|Preferences…" then under "Drawing tools" change "Brush diameter" to 80, which will work well for the low res images



8) Run the "Export Section Outlines.groovy" script in QuPath. This will generate Fiji-compatible *.roi files with from the annotations in QuPath.
9) Create a folder the "Extracted_Decoded_Split_Analyzed" named "Cortex Raw Borders **(LOW RES)**" and move all extracted *.roi files here. (After running the script, the *.roi files will be in the "QuPath Whole Section Outlines" folder.)

STEP FOUR: Enlarge the ROIs back to full size so they will work for downstream analysis (Fiji)

1) The ROIs generated were on lower resolution images so will be too small and won't align with the full-size images. Therefore, they need to be re-expanded.
2) Run the macro "Analysis_STEP_FOUR_Enlarge_Sections.ijm"
   a. In text pathway "Directory containing LOW RES ROIs:" enter the path to the "Whole Section Outlines (LOW RES)" created in **STEP TWO -- 5**
   b. Enter the same scale factor used in **STEP ONE – 2c**, usually 4.

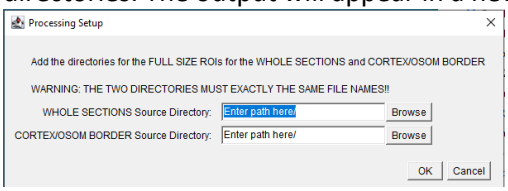


c. Once done: change the output file name from “Whole Section Outlines (LOW RES) (FULL SIZE)” to “Whole Section Outlines (FULL SIZE)” to clarify the contents.

3) Repeat step two for the “Cortex Raw Borders (LOW RES)” in the “Directory containing LOW RES ROIs”
4) Recommended: after completing steps 2 and 3, place all of the “LOW RES” folders/files into a new folder called “LOW RES”

STEP FIVE: Make the cortex only masks (Fiji)

a. Run the “Analysis_STEP_FIVE_Cortex_Only_Masks.ijm” macro, follow the prompt using the (FULL SIZE) directories. The output will appear in a new folder called “CORTEX ONLY OUTLINES”



Run U-Net segmentation

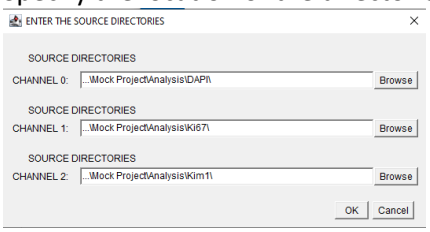
PART II: Segment the images using U-Net (Fiji) (can be done in parallel with PART I)

This part sets up a batch segmentation that can be run in the background. This process has two parts. First, we will construct a “Run File” that will contain all the required data for each segmentation (location of the image to segment, location of the model to use, etc). This will allow the batch to resume if there are failures (for example your spot EC2 instance is closed). Second, we will run the batch segmentation from the “Run File” which keeps track of progress and allows you to resume the batch if needed. Each batch can take several hours to run depending on the size and number of the images to analyze. Please see “**Setting up a remote AWS instance for U-Net Segmentation”** to configure the “…caffeemodel.h5” if starting from scratch.

STEP SIX: Batch segment images (Fiji)

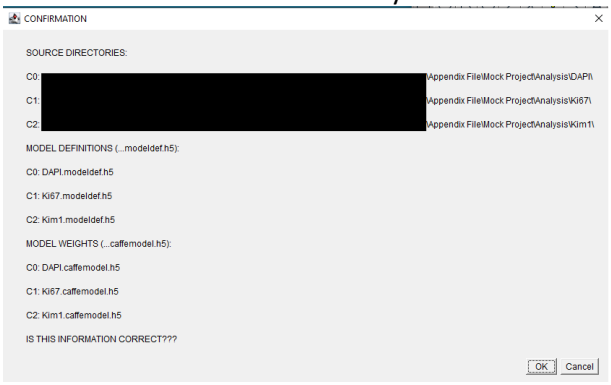
1) Start and configure an Amazon Web Services (AWS) EC2 instance (see **“Setting up a remote AWS instance for U-Net Segmentation**” protocol). You will need the “Public IPv4 address” and the “*.pem” version of the Key pair file you used when starting your instance.
2) Upload ALL weight files (…caffemodel.h5) for the segmentations to be performed, this will often included 3-4 models. (see **“Setting up a remote AWS instance for U-Net Segmentation**” protocol, **“STEP THREE: Upload model data”)**
3) Run the macro “Analysis_STEP_SIX_Make_Run_File.ijm” in Fiji. This macro constructs a .cvs table of all the data needed to segment each file.
4) Enter the number of directories that you plan to process and the location where you want to save your run file.

5) Specify the location of the directories that contain the .tif files that you want to segment



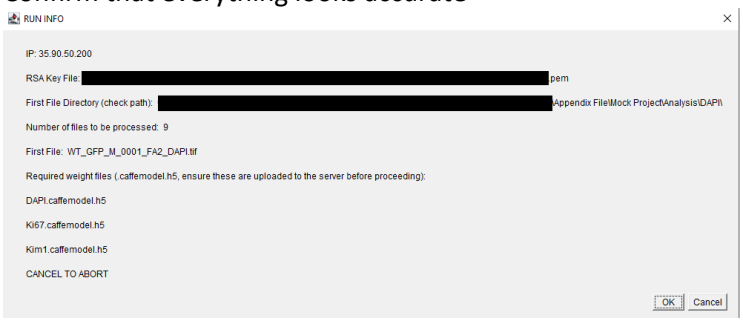
6) Specify the U-Net model information (both the .modeldef.H5 model definition file and the .caffemodel.h5 weight file.

7) Confirm that all the information you entered was correct.



8) Click OK to generate the run file (will take about 1 s).
9) Run the macro “Analysis_STEP_SIX_Run_Batch_from_Run_File.ijm” This will use the Run file to actually run the segmentations using the file created above.
10) Enter the location of the Run File, the *.pem Key file for your EC2 instance, and the “The “Public IPv4 address” for your EC2 instance.

11) Confirm that everything looks accurate



12) Click OK to start the segmentations. This will take several hours depending on the size and number of files. It is recommended that you monitor progress until the first file is complete. Practically, we usually run segmentations overnight or on a computer that will not be used for anything else. Follow progress in the Fiji log window.
13) The resulting segmentations will be stored in an output folder that is created in the same directory as the original images of the form (Channel_[timestamp](model), for example “Kim1_12112022_1730(Kim1)”) for each channel. In this folder, the macro will generate a full size *.zip file that contains the binary segmentation image (stored in the “Segmentation” subdirectory of the output folder) and a 2-fold reduced image *.zip file that shows the original image segmented and an overlay of the segmentation in semi-transparent yellow (stored in the “Overlays” subdirectory of the output folder). The later file is useful for checking the segmentation accuracy if there are issues with QC below.

PART III: Complete analysis

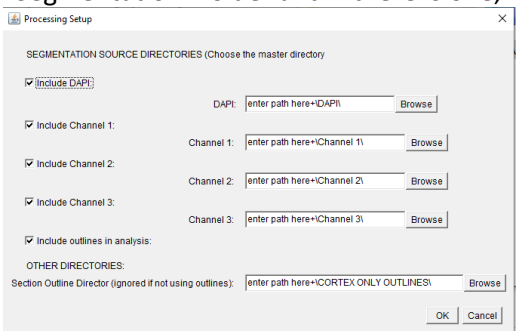
This part puts together the outputs of **PART I** and **PART II** to generate the data shown in the figures of the manuscript.

STEP SEVEN: Perform segmentation quality control (Fiji)

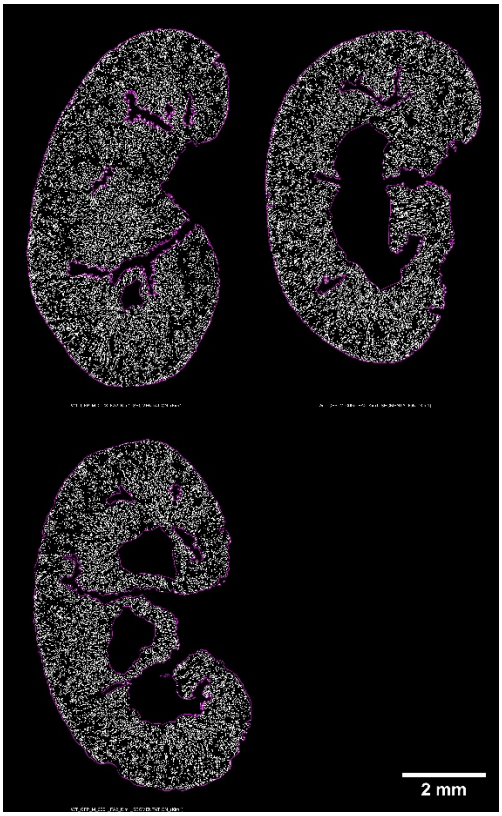
Quality check

*This step takes the section outlines (**PART I**) and the segmentation output (**PART II**) and generates montages of all the results to check for obvious artifacts or segmentation problems.*

1) Run the macro: “Preprocessing_and_Analysis_Make_QC_Montages”. This macro will generate montage images of the segmentation results.
    a. Along the left side of the user interface, select the channels that you want include in the processing (minimum of one channel). You can select DAPI and up to 3 channels additional channels.
    b. Select if you would like the montages to include the section outlines. If you select this, the outline will be shown on the montage and all of the segmentation results outside of this outline will be hidden so you can interpret only the segmentation results that will be used in subsequent analysis. If unchecked, the entire image will be shown.
    c. For each selected channel enter the path to the segmentation directories. For segmentations, you can select the root directory (for example “../Ki67_12112022_1730(Ki67”) instead of …/Ki67_12112022_1730(Ki67)/Segmentation). The macro will look inside each folder to see if there is a “Segmentation” folder and if there is one, it will pull the images from this folder).



    d. Click OK to run
2) The macro will generate montages for each channel and put them in a folder called “QC_montages([timestamp]). For each channel, by default this macro will sort and group the images by the first and fifth parts of the name the file name (for example for a file name of structure genotype_reporter_sex_tag_condition_channel..other info.zip it will sort by “genotype” and by “condition”, that is the image "WT_GFP_M_0001_FA2_Ki67_SEGMENTATION_(Ki67).zip” will be grouped in the same montage as all other images with “WT” in the first position and “FA2” in the second position. You can adjust this by editing the macro code on line 139 and 140.
3) Carefully review the output for any problems. These are large JPEG files but should have enough resolution to identify issues. The file name is printed below each image. (example below, WT animals at FA2 for stain Kim1)



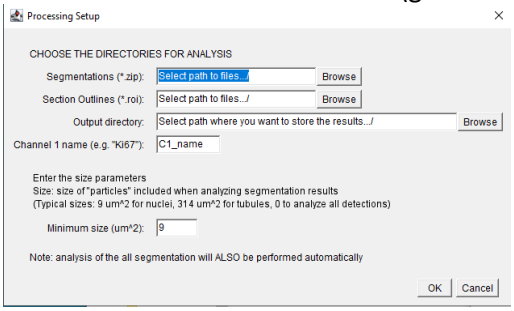
4) The QC_montage folder can be renamed to make it clear what it contains (for example, “QC_montages Post Segmentation”)
5) If there are any concerns for a particular image, you can go back to the associate “Overlays” folder (see **STEP SIX – 14**) and review the segmentation performance in detail relative to the input image.

STEP EIGHT: Analyze the segmentations (Fiji)

Analyze

*This step takes the section outlines (**PART I**) and the segmentation output (**PART II**) and generates a summary table that includes the total area analyzed, the area of the segmentation, and area fraction. In addition to these measurements, code also processes the segmentation with a minimum size filter that also generated area fraction and counts (for example if counting positive nuclei).*

1) In the “Extracted_Decoded_Split_Analyzed” folder make a subfolder to contain the analysis results (“Analysis”).
2) Run the macro “Analysis_Step3_Analyze_Segmentations.ijm”
3) Input the directory with the segmentation images (as *.zip files) in “Segmentations (*.zip):” (generated in **STEP SIX**)
4) Input the directory with the section outlines in “Section Outlines (*.roi):” (generated in **PART I**).
5) Input the directory where you want to put the results in “Output directory:” (Step 1 above)
6) Enter a name for the channel analyzed in “Channel 1 name (e.g. “Ki67”):
7) Enter the minimum size to count (guidelines in the macro).



8) Press “OK” to run (will take a few minutes)

9) The macro will generate a *.csv table with the results. This location of the table is in the directory you set up in #4 above.