

## **Parietal epithelial cell and podocyte quantification in human nephrectomies**

### **Analysis**

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**Overview:** This protocol will use full resolution SVS files of human nephrectomy biopsy samples to quantify parietal epithelial cell and podocyte densities on per glomeruli and per patient level. SVS files will be tiled apart and restitched together at a decreased resolution. U-Net segmentation will be performed to identify all glomeruli. Further segmentation will be performed on identified glomeruli to create a rim around the Bowman's capsule and to identify all WT1+ nuclei.

#### **Before Starting:**

The version of Fiji used in this analysis was ImageJ 1.54p with Java version 1.8.0\_322. You must have the "U-Net Segmentation" plugin and "SlideJ" plugin installed in Fiji. If not installed, go to Help→ "Update..." then select "Manage update sites" and select "U-Net Segmentation" and "SlideJ" 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).

#### **Step One: Split full resolution SVS files into full resolution tiled images**

The full resolution SVS are too large to be opened in Fiji, but tile sized images can be created and downsized in subsequent steps.

- 1) Open Fiji and select "Plugins" then "SlideJ"
- 2) In the "input" section select your directory containing full resolution SVS files
- 3) In the "output" section select the directory you want to store the split tiles
  - a. You will need to separate the tiles into separate folders per sample afterward
- 4) In the "macro" section select "**STEP ONE Split Images SlideJ.ijm**" macro
- 5) Select a Tile size of 13000
- 6) Leave Overlap as 0
- 7) Cancel temporary tiles select "No"

### **Step Two: Downscale Tiles by 4-fold and 16-fold**

The full resolution SVS are too large to be analyzed in Fiji, so the purpose of the rescaling is to allow for analysis in Fiji while maintaining relatively high-quality images.

- 1) Open the "**STEP\_TWO\_Rescale.ijm**" macro in Fiji
- 2) Input the number of directories of samples you will be working on
- 3) In the "Tiled Images" section select the directory of the tiled images from Step One
- 4) In the "Output Folder" section select the new directory you want to store the rescaled images in.
- 5) Input 4 or 16 in the "Scaling Factor" section. You will need to rescale the tiles by 4-fold and 16-fold and save them in separate directories, meaning the x and y lengths are reduced by dividing by the scaling factor.

### **Step Three: Downscale Tiles by 4-fold and 16-fold**

The purpose of this step is to allow for analysis of entire samples at a time by restitching the tiled images back together in a single image.

- 1) Open "**STEP\_THREE\_Restitch\_Tiles.ijm**" macro in Fiji
- 2) You will restitch each sample for the 4-fold and 16-fold tiled images separately
- 3) Input the number of directories of samples you will be working on
- 4) Input the scaling factor (4 or 16) of the folders you will be restitching
- 5) Input the tile size (13000)
- 6) This macro should output a file with the original sample name + "Rescale 4 (or 16)"

### **Step Four & Five: Perform Glomeruli Detection U-Net Segmentation**

This part sets up a batch segmentation. 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 to identify all the glomeruli from the 4-fold restitched images from the "Run File" which keeps track of progress and allows you to resume the batch if needed.

- 1) Start and configure an Amazon Web Services (AWS) EC2 instance. You will need the "Public IPv4 address" and the "\*.pem" version of the Key pair file you used when starting the instance
- 2) Upload all weight files (...caffemodel.h5) for the segmentations to be performed.
- 3) Model used in this analysis can be found via this DOI: <https://doi.org/10.7302/3np6-ze40>
- 4) Open "**STEP\_FOUR\_Make\_Run\_File.ijm**" macro in Fiji
- 5) Input the number of directories of samples you will be working on
- 6) Select the place you would like to store the CVS run file

- 7) In each “Channel” section select the path to the directory with the 16-fold restitched images created in Step Three.
- 8) \*For the “Model Definition (...modeldef.h5) file” section select the path to “GloSixteenFold.modeldef.h5” model
- 9) For the “Model Weight (...caffemodel.h5) file” section select the path to “GloSixteenFold.caffemodel.h5” model
- 10) If everything looks correct, select “Ok” run the macro.
- 11) Open “**STEP FIVE Run Batch from Run File.ijm**” macro in Fiji
- 12) For the “RUN FILE (.csv)” section select the path to the CSV file created in Step Four
- 13) For the “RSA Key File” select the path to the \*.pem Key file for your EC2 instance and enter the “The “Public IPv4 address” for your EC2 instance in the “Unet Host IP Address” section.
- 14) Click OK to start the segmentations.
- 15) This macro should create a folder labeled “Segmentation” with the U-Net segmentations.

### **Step Six, Seven, & Eight: Extract and quality check 4-fold extracted glomeruli**

The purpose of these steps is to use the 16-fold resolution identified glomeruli from steps four and five and to create ROIs (Regions of Interest) for each glomerulus. This macro will identify all glomeruli that have a greater than 300  $\mu\text{m}^2$  and with a circularity between 0.25-1.00 from the segmented 16-fold image from Step Five. The ROIs will be scaled up by 4-fold and used to extract higher 4-fold resolution glomeruli from the 4-fold restitched image for further analysis. There original glomeruli ROIs are enlarged by 50  $\mu\text{m}$  and bonding box is created around the glomerulus as a buffer.

- 1) Open “**STEP SIX Process Segmentations for Glomerulus ROIS.ijm**” macro in Fiji
- 2) Input the number of directories of samples you will be working on
- 3) Select the “Segmentation” folder created in Step Five
- 4) This will create a folder labeled “pp1” with the 16-fold glomeruli ROIs.
- 5) Open “**STEP SEVEN Scale Up Glomerulus ROIS.ijm**” macro in Fiji
- 6) Input the number of directories of samples you will be working on
- 7) In each “Channel” section select the path to the directory with the 4-fold restitched images created in Step Three.
- 8) In each “Channel # ROI Output from PP1” section select the path to the directory “pp1” output folder created in Step Six.
- 9) This will create a folder labeled “pp2” with 4-fold glomeruli ROIs.
- 10) Open “**STEP EIGHT Crop Glomeruli.ijm**” macro in Fiji
- 11) Input the number of directories of samples you will be working on

- 12) In each “Channel # Full Resolution Restitch Image” section select the path to the directory with the 4-fold restitched images created in Step Three.
- 13) In each “Channel # ROI Output from PP2” section select the path to the directory “pp2” output folder created in Step Seven.
- 14) This will create a folder labeled “cropped” with 4-fold resolution images of each identified and processed glomerulus.

### **Step Nine: Create DAB Images**

The purpose of this step is to create DAB images of each glomerulus from Step Eight for clearer identification of WT1 nuclei. Using Color Deconvolution with RGB vectors of (0.651, 0.701, 0.29), (0.269, 0.568, 0.778), and (0.633, -0.713, 0.302). These images will be used to segment all WT1+ nuclei for PEC and podocyte quantification.

- 1) Input the number of directories of samples you will be working on
- 2) In each “Original Cropped Images” section select the path to the directory “cropped” output folder created in Step Eight.
- 3) This will create a folder labeled “DAB” with DAB converted glomeruli images

### **Step Ten: Quality Check Segmentations and Remove Abnormal Glomeruli**

The purpose of this step is to quality check the segmentations done by U-Net. This step allows for the remove of non-glomeruli segmentation. Additionally, this step can be used to remove abnormal glomeruli, such as globally sclerotic glomeruli or segmental glomerulosclerosis glomeruli.

- 1) Open “**STEP Ten Quality Check Glomeruli.ijm**” macro in Fiji
- 2) In the “Cropped Glomeruli” section select the path to the directory “cropped” output folder created in Step Eight.
- 3) In the “DAB Glomeruli” section select the path to the directory “DAB” output folder created in Step Nine.
- 4) Select the folder you want the saved DAB glomeruli to be stored in.

### **Repeat Step Four & Five: Segment WT1+ Nuclei and Precise Outlines of Bowman’s Capsule**

The purpose for these steps is to U-Net segment the WT1+ nuclei from the DAB images for PEC and podocyte detection. These steps will also allow for segmentation of precise outlines of Bowman’s capsule for each glomerulus for future creation of a rim around Bowman’s capsule for WT1+ nuclei differentiation for PEC and podocyte quantification. These segmentation processes can be done in parallel.

- 1) Repeat steps Four and Five from above

- 2) For Precise outlines of Bowman's capsule segmentation in Step Four, in each "Channel" section select the path to the directory with the cropped glomeruli from Step Eight, \*for the "Model Definition (...modeldef.h5) file" section select the path to "SingularGlo07302024.modeldef.h5" model, and for the "Model Weight (...caffemodel.h5) file" section select the path to "SingularGlo07302024.caffemodel.h5" model
- 3) For WT1+ nuclei segmentation in Step Four, in each "Channel" section select the path to the directory with the quality-check DAB images from Step Ten, \*for the "Model Definition (...modeldef.h5) file" section select the path to "WT1DAB07302024.modeldef.h5" model, and for the "Model Weight (...caffemodel.h5) file" section select the path to "WT1DAB07302024.caffemodel.h5" model
- 4) Follow Step Five as previously explained.

### **Step Eleven & Twelve: Precise Outlines of Bowman's Capsule Extraction and Rim Creation**

The purpose of this step is to extract the outlines of Bowman's capsule from the segmentation as ROIs. These ROIs will be used to create a rim around Bowman's space. The inner rim is reduced to 0.82 the original size and the outer rim is expanded to 1.05 the original size. Since PECs reside along Bowman's Capsule and podocytes are in the glomerular tuft, the rim will be used to spatially identify as each WT1+ segmented nuclei as either a PEC or podocyte.

- 1) Open "**STEP ELEVEN Extract Glomeruli Outlines**" macro in Fiji
- 2) Input the number of directories of samples you will be working on
- 3) In each "Channel # Segmented Images" section select the path to the directory with the segmentation of the precise outlines of Bowman's capsule created in the repetition of Steps Four and Five.
- 4) This will create a folder labeled "Outlines" with the outlines for each glomerulus
- 5) Open "**STEP TWELVE Create Rim for Glomeruli**" macro in Fiji
- 6) In each "Channel # Segmented Glomeruli" section select the path to the directory of cropped glomeruli images from Step Nine.
- 7) In each "Channel # Outline ROI" section select the path to the directory of outlines from Step Eleven.
- 8) This will create a folder labeled "Rims" with the outlines for each glomerulus

### Step Thirteen: PEC and Podocyte Differentiated Images

The purpose of this step is to use the rims created in the previous step and the WT1+ nuclei segmentations to differentiate each WT1+ nuclei as either a PEC or podocyte depending on its spatial position. If either 70% of the area is inside the rim while crossing the inner edge of the rim or 30% of the area is inside the rim while crossing the outer edge of the rim, the nuclei is considered a PEC. If 70% of the nuclei area is inside the inner edge of the rim, then the nuclei is considered a podocyte. The result of these macros will be images of PECs and images of podocytes that will be use for analysis.

- 1) Open “STEP\_THIRTEEN\_Make\_PEC\_Images” macro in Fiji
- 2) Input the number of directories of samples you will be working on
- 3) In each “Channel # Segmented Images” section select the path to the directory of WT1+ segmented nuclei images created in the repetition of Steps Four and Five.
- 4) In each “Channel # Outline ROI” section select the path to the directory of rims from Step Twelve.
- 5) Select the folder you want the saved PEC nuclei images to be stored in.
- 6) Open “STEP\_THIRTEEN\_Make\_Podocyte\_Images” macro in Fiji
- 7) Input the number of directories of samples you will be working on
- 8) In each “Channel # Segmented Images” section select the path to the directory of WT1+ segmented nuclei images created in the repetition of Steps Four and Five.
- 9) In each “Channel # Outline ROI” section select the path to the directory of rims from Step Twelve.
- 10) Select the folder you want the saved podocyte nuclei images to be stored in.

### Step Fourteen & Fifteen: PEC and Podocyte Density Quantification and Data Extraction

The purpose of these steps is to quantify PEC and podocyte density on both a per glomerulus (Step Fourteen) and per sample (Step Fifteen) level. Some additional data, such as glomerular area, glomerular perimeter, nuclei count, etc. are also extracted in these steps.

- 1) Open “**STEP FOURTEEN Per Glomeruli Data Extraction**” macro in Fiji
- 2) Input the number of directories of samples you will be working on
- 3) In each “Channel # Segmented PEC Images” section select the path to the directory of PEC images created in Step Thirteen.
- 4) In each “Channel # Segmented Podocyte Images” section select the path to the directory of PEC images created in Step Thirteen.
- 5) In each “Channel # Segmented PEC Images” section select the path to the directory of outlines created in Step Eleven.
- 6) Select the folder you want the CSV per glomerulus data file to be stored in.
- 7) Open “**STEP FIFTEEN Per Patient Data Extraction**” macro in Fiji

- 8) Input the number of directories of samples you will be working on
- 9) In each “Channel # Segmented PEC Images” section select the path to the directory of PEC images created in Step Thirteen.
- 10) In each “Channel # Segmented Podocyte Images” section select the path to the directory of PEC images created in Step Thirteen.
- 11) In each “Channel # Segmented PEC Images” section select the path to the directory of outlines created in Step Eleven.
- 12) Select the folder you want the CSV per sample data file to be stored in.

\*All models were trained with a x and y tile shapes (px) of 492. The learning rate was 1E-5 and 3,000 iterations