Journal of Visualized Experiments

Supervised machine learning for semi-quantification of extracellular DNA in glomerulonephritis

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61180R1
Full Title:	Supervised machine learning for semi-quantification of extracellular DNA in glomerulonephritis
Section/Category:	JoVE Immunology and Infection
Keywords:	Glomerulonephritis, Extracellular DNA, Kidney, Cell death, Supervised Machine Learning
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Melbourne, Victoria, Australia

JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

TITLE:

Supervised Machine Learning for Semi-Quantification of Extracellular DNA in Glomerulonephritis

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KEYWORDS:

Glomerulonephritis, Extracellular DNA, Kidney, Cell death, Supervised Machine Learning

SUMMARY:

Extracellular DNA (ecDNA) released during cell death is proinflammatory and contributes to inflammation. Measurement of ecDNA at the site of injury can determine the efficacy of therapeutic treatment in the target organ. This protocol describes the use of a machine learning tool to automate measurement of ecDNA in kidney tissue.

ABSTRACT:

Glomerular cell death is a pathological feature of myeloperoxidase anti neutrophil cytoplasmic antibody associated vasculitis (MPO-AAV). Extracellular deoxyribonucleic acid (ecDNA) is released during different forms of cell death including apoptosis, necrosis, necroptosis, neutrophil extracellular traps (NETs) and pyroptosis. Measurement of this cell death is time consuming with several different biomarkers required to identify the different biochemical forms of cell death. Measurement of ecDNA is generally conducted in serum and urine as a surrogate for renal damage, not in the actual target organ where the pathological injury occurs. The current difficulty in investigating ecDNA in the kidney is the lack of methods for formalin fixed paraffin embedded tissue (FFPE) both experimentally and in archived human kidney biopsies. This protocol provides a summary of the steps required to stain for ecDNA in FFPE tissue (both human and murine), quench autofluorescence and measure the ecDNA in the resulting images using a

machine learning tool from the publicly available open source ImageJ plugin trainable Weka segmentation. Trainable Weka segmentation is applied to ecDNA within the glomeruli where the program learns to classify ecDNA. This classifier is applied to subsequent acquired kidney images, reducing the need for manual annotations of each individual image. The adaptability of the trainable Weka segmentation is demonstrated further in kidney tissue from experimental murine anti-MPO glomerulonephritis (GN), to identify NETs and ecMPO, common pathological contributors to anti-MPO GN. This method provides objective analysis of ecDNA in kidney tissue that demonstrates clearly the efficacy in which the trainable Weka segmentation program can distinguish ecDNA between healthy normal kidney tissue and diseased kidney tissue. This protocol can easily be adapted to identify ecDNA, NETs and ecMPO in other organs.

INTRODUCTION:

Myeloperoxidase anti neutrophil cytoplasmic antibody associated vasculitis (MPO-AAV) is an autoimmune disease that results in renal failure from pathological glomerular injury with considerable cell death and release of deoxyribonucleic acid (DNA)[1,2]. DNA can activate the immune system by acting as a danger signal. Under normal healthy conditions, the nuclear location of DNA offers protection from exposure to the immune system. Self-DNA that is released extracellularly during either pathogenic processes or autoimmunity is seen by the immune system as a potent proinflammatory damage associated molecular self-protein (DAMP)[3]. Extra cellular DNA (ecDNA) is released from dying cells through several distinct mechanisms that are governed by distinct biochemical pathways, such as apoptosis, necroptosis neutrophil extracellular trap formation (NETs), necrosis or pyroptosis[4-8].

We describe herein methods to stain and measure ecDNA released from dying cells in sections of formalin fixed paraffin embedded (FFPE) kidneys from experimental anti-MPO GN and kidney biopsies from patients with MPO-AAV[9,10]. Multiple methods exist for the detection of circulating double stranded DNA (dsDNA) and DNA complexes from both serum and urine and from in vitro assays[11,12]. These methods, although accurate in determining the amount of ecDNA, do not determine where the ecDNA is released anatomically. There are methods that describe specific measurement of ecDNA such as tunel for apoptosis and measurement of cell debris[13,14]. There is no method that describes measuring ecDNA culminated from all forms of cell death in FFPE kidneys where the pathological damage occurs. This is important to determine if experimental therapeutic treatments are clearing the ecDNA from the sites of pathological injury in the actual target organ.

The acquisition of multiple images from kidney samples creates a high volume of data that is analyzed commonly by one single user. This is labor intensive, time consuming and can be subject to unreliable reproducibility by other users, due to user bias. Trainable Weka segmentation is an open-source software plugin for ImageJ that uses cutting edge bioinformatic tools to classify pixels using machine learning algorithms[15,16]. This method is “trainable” whereby it learns from the user’s classification of segments of pixels and applies the new learnt classification to other images. This method relies on common analysis tools within the ImageJ program that are used to “classify” each pixel in a segment as belonging to a specific “class”. Once the program learns the “classifiers”, they can be used to identify other similar classified segments within the same

image. This model is then saved and applied to other sets of images within the same experiment.

Current obstacles to determining ecDNA in situ in kidney sections is the endogenous autofluorescence from fixation in formalin and the labor-intensive analysis of the images. We describe here how to quench this autofluorescence, detect ecDNA, and use supervised machine learning for high throughput measurement of ecDNA. We have previously published the measurement of NETs and extracellular MPO (ecMPO) using a macro in ImageJ, we now demonstrate semi automation of these methods using supervised machine learning[1]. We demonstrate the adaptability of the machine learning tool, to classify an alternative stain for NETs and ecMPO within the same image. These staining methods described here for detecting ecDNA, NETs and ecMPO can be translated to other solid organs and diseases where ecDNA, NETS and ecMPO plays a role in perpetuating disease such as rheumatoid arthritis and lupus[17,18].

PROTOCOL:

This method enables detection of pan ecDNA from all forms of cell death. The same method and antibodies are used for human kidney biopsy tissue (from step 4) . All animal and human subjects had Ethics approval from Monash University, and Monash Health, Clayton, Victoria, Australia.

1. Staining for ecDNA with DAPI and β-Actin

1.1. Induce a 20 day model of anti-myeloperoxidase glomerulonephritis (anti-MPO-GN) in 8-10 week old C57/Bl6 mice, with controls[9].

1.1.1. Euthanize mice humanely using a CO_2 chamber.

1.1.2. Remove kidneys by making an anterior midline incision through the skin with scissors, and then carefully cut the peritoneum and pin back on a dissection board. Using forceps push aside the anterior renal fascia and parietal peritoneum and cut the kidney free by severing the ureter and blood vessels (renal artery and vein) at the renal pelvis. Remove with forceps and cut kidneys in half (sagittal plane) with a size 22 scalpel.

1.1.3. Place kidney in fixative (4% buffered formalin) for 16 hours at room temperature (RT). Remove and soak fixed kidney in 30% ethanol for 24 hours prior to processing.

1.2. Process kidneys using a standard 6-hour cycle:
70% ethanol for 15 min
90% ethanol for 15 min
100% ethanol for 15 min
100% ethanol for 20 min
100% ethanol for 25 min
100% ethanol for 30 min
Xylene for 20 min
Xylene for 30 min

Xylene for 40 min
Wax for 30 min
Wax for 35 min
Wax for 40 min

NOTE: This is important as kidneys should not be subjected to prolonged exposure to heat in the wax, as it can destroy antigens of interest.

1.3. Cut 3 µm sections on a microtome and mount on commercially available glass microscope slides coated with a positive charge. Allow to dry overnight.

1.4. Put glass slides into a slide rack into a 60 °C oven for 60 min.

NOTE: Steps 1.3 and 1.4 are crucial to ensure sections do not float off during the antigen retrieval step.

1.5. Immerse slides in two changes of solvent (xylene or substitute) for 40 min each, in a fume hood.

1.6. Rehydrate slides in 100% ethanol, 100% ethanol, 70% ethanol for 5 min each. Wash for 5 min in tap water.

1.7. Place a hot plate in a fume hood and preboil antigen retrieval solution (10 mM Tris, 1 mM EDTA pH 9.0) in a pressure cooker. As soon as the antigen retrieval solution starts to boil, place the slides in the pot horizontally using a pair of tongs and lock the lid. Boil on high (equivalent to 15 psi or 103 kPa) for 10 min.

NOTE: This step is crucial as it retrieves the antigen of interest by unmasking the antigen epitope (cross linked via formalin fixation) to allow epitope antibody specific binding the subsequent staining will not work without it.

1.8. Remove pressure cooker from heat and remove lid immediately by running cold tap on top of the lid in a sink. Leave slides to equilibrate for 20 min in the antigen retrieval solution.

1.9. Wash slides twice for 5 min in 0.01 M phosphate buffered saline (PBS) (pH 7.4) on an orbital shaker.

1.10. Use a hydrophobic pen to draw circles around the kidney tissue being careful not to let any tissue dry out in this time.

1.11. Block tissue in 10% chicken sera in 5% bovine serum albumin (BSA)/PBS (pH 7.4, 0.01 M) for 30 min, 60 µL per section. Do not wash after this step but carefully remove the blocking solution using a 60 µL pipette, do not let the sections dry out.

1.12. Apply primary antibody cocktail for β-actin diluted 1/1000 in 1% BSA/PBS (pH 7.4, 0.01 M), 60 µL per section and incubate overnight in a humidity chamber at 4 °C.

1.13. Wash slides twice for 5 min in PBS (pH 7.4, 0.01 M) on an orbital shaker.

1.14. Apply secondary antibody, chicken anti rabbit 488 (1/200), 60 µL per section diluted in 1%BSA/PBS (pH 7.4, 0.01 M) for 40 min at RT.

1.15. Wash the slides twice for 5 min in PBS (pH 7.4, 0.01 M) on an orbital shaker.

1.16. Immerse the slides in 0.3% Sudan Black in 70% ethanol for 30 min in a Coplin jar.

NOTE: This step is important as it quenches the autofluorescence caused by formalin fixation.

1.17. Wash the slides in tap water to remove precipitate and then immerse in PBS (pH 7.4, 0.01 M) for 10 min to prevent further Sudan Black precipitate from forming.

1.18. Mount slides onto confocal glass coverslips using three 60 µL drops of mounting solution with DAPI and seal the coverslips with nail polish by applying around the perimeter of the coverslip.

1.19. Allow slides to cure for 24 hours at RT (to allow DAPI to penetrate) and then store at 4 °C protected from light.

NOTE: Important to keep in the dark to prevent fading of fluorescent probes.

1.20. Image slides using a confocal laser scanning head attached to a microscope. Excite slides with the 405 laser for DAPI and the 488 Laser for Beta Actin. Capture single plane 1024 x1024 pixel images by using line-sequential capturing and 4-line averaging, which is essential for detecting ecDNA. 40x 1.0 NA oil objective is used.

1.20.1. Image a minimum of 20 glomeruli per sample. Save images as ND2 files.

2. DAPI and β-Actin analysis

2.1. Install ImageJ: https://imagej.net/Fiji/Downloads.

2.2. Check that the trainable Weka segmentation is available under **Plugins | Segmentation | Trainable Weka Segmentation**.

2.3. Drop the image onto the ImageJ tool bar. Click **Image | Color | Split Channels**. One image with DAPI in blue staining all nuclei will appear and 1 image with β-actin in green will appear, which delineates the glomeruli.

2.3.1. Create a merged file of the single images to draw a region of interest (ROI) for the glomerulus by clicking on **Color | Merge Channels**. A pop-up box will ask to assign a color to each channel. After assigning each channel a color, tick the **Create Composite** box and the **Keep Source Images** box and click **Ok**. A merged composite image will appear.

2.3.2. Using the β-actin staining as a guide, draw a ROI around the glomerular tuft. Keep this image aside to use the ROI at the end of this protocol.

2.4. Perform a Gaussian blur on the single blue DAPI image. Click **Process | Filters | Gaussian Blur**, put in 1-2.00. The image will now look a little blurred, but nuclei are now smooth and background softened.

NOTE: This step is preformed to remove noise to clean up the image from artifacts that may prevent relevant detection of image attributes to be analyzed.

2.5. Click on **Plugins | Segmentation | Trainable Weka Segmentation**.

2.6. Using the line tool on the tool bar in ImageJ, first trace around intact nuclei using the free hand tools (there are line, circular, and square tool options available to choose from) and then add to **Add Class 1** box.

2.7. Using the line tool on the tool bar in ImageJ delineate areas of background to **Class 2** box.

2.8. Click on the **Create New Class** button label on the Weka segmentation window and label "ecDNA." Use the line tool (from ImageJ tool bar) to delineate extranuclear DNA stained by DAPI.

2.9. Click on the **Train Classifier** button in the training menu in the trainable Weka segmentation window.

NOTE: The **STOP** button will appear in place of the **Train Classifier** button and remain until the training has finished. Do not to click on this button during training as the process will be interrupted.

2.10. Click on **Create Result** button to create an image with all the classified components, consisting of intact nuclei, the background and identified ecDNA.

2.11. Click on the **Get Probability** button. Toggle the mouse to give a black and white image of all the classes with the object of selection highlighted in white.

2.12. Duplicate this image by clicking on **Image | Duplicate**.

2.13. Toggle to the screen using the mouse button that contains the ecDNA. Apply a threshold to get only what has been identified as ecDNA. Click **Apply** when the threshold is sufficient.

2.14. If after thresholding, there are more areas of DNA that are not ecDNA, return to the original trained image, add more classifiers, and repeat steps 2.1-2.13.

2.15. Click **Image | Image Adjust | Make Binary**. Copy the glomerular ROI made in step 2.3 and click **Ctrl+Shift+E**. Activate the Weka window by **Edit | Selections | Restore**, which restores the selection. Click **Analyze Particles**, which will measure only the ecDNA particles within the glomerulus.

NOTE: A result sheet is computed with the count of particles, the area, averages pixels and the percentage of the glomerulus containing ecDNA. These results can be exported into a spreadsheet and saved, for later statistical analysis.

2.16. Save the classifier as ecDNA.classifier by clicking **Save Classifier** from the options menu into the ImageJ app on the desktop. Then click **Save Data** from the options menu and save as ecDNA.arff into the ImageJ folder. The ROI will have to be manually added each time as glomerulus size and shape will change, however the program has learnt what ecDNA is.

2.17. To reapply the model to subsequent images, repeat steps 2.1-2.5 with a new image. Click **Load Classifier** from the option menu and then **ecDNAmodel.classifier** in the ImageJ folder from step 2.16. The log menu will pop up and run the model.

2.18. Once the model has run, click on **Load Data** in the options menu and select the ecDNA.arff file saved in the ImageJ folder from step 2.16. Click on **Create Result**. If the resulting image has failed to pick up all the nuclei, background or ecDNA click on re-train classifier and add more classifiers and save the new model. Complete the analysis process by repeating steps 2.9-2.16.

NOTE: Several images can be used to generate the final model used to detect ecDNA. This is achieved by applying the model to subsequent images, adding more classifiers and saving the new model and data into the ImageJ folder. This can be particularly important when different samples have higher background. The Weka Segmentation program is also compatible with ImageJ macro language which enables many of the commands to be macro recordable to automate some of the steps.

3. Measurement of neutrophil extracellular traps and ecMPO

NOTE: This method identifies NETs by colocalization of extracellular DNA, Citrullinated Histones peptidyl arginase 4 (PAD4) and MPO.

3.1. Induce a 20 day model of anti-myeloperoxidase glomerulonephritis (anti-MPO-GN) in 8-10 week old C57/Bl6 mice, with controls[9].

3.1.1. Euthanize mice humanely using a CO_2 chamber.

3.1.2. Remove kidneys by making an anterior midline incision through the skin with scissors, and

then carefully cut the peritoneum and pin back on a dissection board. Using forceps push aside the anterior renal fascia and parietal peritoneum and cut the kidney free by severing the ureter and blood vessels (renal artery and vein) at the renal pelvis. Remove with forceps and cut kidneys in half (sagittal plane) with a size 22 scalpel.

3.1.3. Place kidney in fixative (4% buffered formalin) for 16 hours at room temperature (RT). Remove and soak fixed kidney in 30% ethanol for 24 hours prior to processing.

3.2. Process kidneys using a standard 6-hour cycle:
70% ethanol for 15 min
90% ethanol for 15 min
100% ethanol for 15 min
100% ethanol for 20 min
100% ethanol for 25 min
100% ethanol for 30 min
Xylene for 20 min
Xylene for 30 min
Xylene for 40 min
Wax for 30 min
Wax for 35 min
Wax for 40 min

NOTE: Kidneys should not be subjected to prolonged exposure to heat in the wax, as it can destroy antigens of interest.

3.3. Cut 3 µm sections on a microtome and mount on commercially available glass microscope slides coated with a positive charge. Allow to dry overnight

3.4. Put glass slides into a slide rack into a 60 °C oven for 60 min.

NOTE: Steps 3.3-3.4 are crucial to ensure sections do not float off during the antigen retrieval step.

3.5. Immerse slides in two changes of solvent (xylene or substitute) for 40 min each, in a fume hood.

3.6. Rehydrate slides in 100% ethanol, 100% ethanol, 70% ethanol for 5 min each.

3.7. Wash for 5 min in tap water.

3.8. Place a hot plate in a fume hood and preboil antigen retrieval solution (10 mM Tris-1 mM EDTA pH 9.0) in a pressure cooker. As soon as the antigen retrieval solution starts to boil place the slides in the pot horizontally using a pair of tongs and lock the lid. Boil on high (equivalent to 15 psi or 103 kPa) for 10 min.

NOTE: This step retrieves the antigen of interest by unmasking the antigen epitope (cross linked via formalin fixation) to allow epitope antibody specific binding the subsequent staining will not work without this step.

3.9. Remove the pressure cooker from heat and remove the lid immediately by running cold tap on top of the lid in a sink. Leave slides to equilibrate for 20 min in the antigen retrieval solution.

3.10. Wash slides twice for 5 min in phosphate buffered saline (PBS) (pH 7.4, 0.01 M) on an orbital shaker.

3.11. Use a hydrophobic pen to draw circles around the kidney tissue being careful not to let any tissue dry out in this time.

3.12. Block tissue in 10% chicken sera in 5% bovine serum albumin (BSA)/PBS (pH 7.4, 0.01 M) for 30 min, 60 µL per section. Do not wash after this step but carefully remove the blocking solution using a 60 µL pipette. Do not let the sections dry out.

3.13. Make a cocktail of the primary antibodies diluted in 1%BSA/PBS (pH 7.4, 0.01 M) in 1 tube. Apply 60 µL per kidney sections. The concentration of the primary antibodies are as follows:
Rabbit anti human/mouse H3Cit 1/100
Mouse anti human/mouse PAD4 1/50
Goat anti human/mouse MPO 1/200

3.14. Incubate overnight at 4 °C in a humidity chamber.

3.15. Wash slides twice for 5 min in PBS (pH 7.4, 0.01 M) on an orbital shaker.

3.16. Make a cocktail of the secondary antibodies in 1 tube. Secondary antibodies are applied in 1% BSA/PBS, 60 µL per section as follows:
Chicken anti rabbit 488 1/200.
Chicken anti mouse 647 1/200.
Chicken anti goat 594 1/200.

3.17. Incubate at RT for 40 min.

3.18. Wash slides twice for 5 min in PBS (pH 7.4, 0.01 M) on an orbital shaker.

3.19. Apply secondary antibody 1:200 in 1%BSA/PBS (pH 7.4, 0.01 M) for 40 min at RT 60 µL per section.

3.20. Wash slides twice for 5 min in PBS (pH 7.4, 0.01 M) on an orbital shaker.

3.21. Immerse slides in 0.3% Sudan Black in 70% ethanol for 30 min.

3.22. Wash slides in tap water to remove precipitate and then immerse in PBS (pH 7.4, 0.01 M) for 10 min to prevent further Sudan Black precipitate from forming.

3.23. Mount slides onto confocal glass coverslips using three 60 µL drops of mounting solution with DAPI. Seal the coverslips with nail polish by applying around the perimeter of the coverslip.

3.24. Allow slides to cure for 24 hours at RT (to allow DAPI to penetrate) and then store at 4 °C protected from light. Keep in the dark to prevent fading of fluorescent probes.

3.25. Image slides using a confocal laser scanning head attached to a microscope. Excite slides with the 405 laser for DAPI and the 488 Laser for H3Cit, 561 for MPO, and 647 for PAD4. Capture single plane 1024x1024 pixel images by using line-sequential capturing and 4-line averaging which is essential for detecting ecDNA. Use a 40x 1.0 NA oil objective. Image a minimum of 20 glomeruli per sample. Save images as ND2 files.

4. Neutrophil extracellular traps and ecMPO Analysis

4.1. Install ImageJ: https://imagej.net/Fiji/Downloads.

4.2. Check that the trainable Weka segmentation is available under **Plugins | Segmentation | Trainable Weka Segmentation**.

4.3. Drop the image into ImageJ. Click **Image | Color | Split Channels**. One image with DAPI in blue staining all nuclei will appear, one image with H3Cit in green, one image with MPO in red, and one image with PAD4 in white will appear.

4.3.1. Create a merged file of the single images to draw a ROI for the glomerulus by clicking on **Color | Merge Channels**. A pop-up box will ask to assign a color to each channel. After assigning each channel a color, tick the **Create Composite** box and the **Keep Source Images** box and click **Ok**. A merged image appears. Unlike the protocol for ecDNA previously we will use the composite image to perform the rest of the steps.

4.4. Perform a Gaussian blur on the composite image. This allows smoothing out of the nuclei. Click **Process | Filters | Gaussian Blur**, put in 1.00-2.00. The image will now look a little blurred but nuclei are now smooth and background softened.

NOTE: This step is preformed to remove noise to clean up the image from artifacts that may prevent relevant detection of image attributes to be analyzed.

4.5. Click on **Plugins | Segmentation | Trainable Weka Segmentation**. A whole new window will appear with the image to be analyzed and Weka segmentation specific menu tools.

4.6. Using the line tool on the tool bar (in ImageJ), first trace around intact nuclei not positive for all 4 NET components (MPO, PAD4, DAPI and H3Cit) using the free hand tool and then add to the **Add Class 1** box.

4.7. Using the line tool on the ImageJ tool bar delineate areas of background to the **Class 2** box.

4.8. Click on the **Create New Class** button label in the label menu and label "NETs". Use the line tool to delineate NETS identified as co-localization DAPI (blue), MPO (red), PAD4 (white) and citrullinated histones (green).

4.9. Click on the **Create New Class** button label in the label menu and label "ecMPO". Use the line tool to delineate MPO (red) that is cell free.

4.10. Click on the **Train Classifier** button in the training menu in the Trainable Weka Segmentation window.

NOTE: The **STOP** button will appear in place of the **Train Classifier** button and remain until the training has finished. Do not to click on this button during training and the process will be interrupted.

4.11. Click on **Create Result** button in the training menu and an image is created with all the classified components, consisting of intact nuclei, the background and what was identified as ecMPO.

4.12. Click on the **Get Probability** button in the training menu. Toggle the mouse to give a black and white image of all the classes with the object of selection highlighted in white.

4.13. Duplicate both the NET probability and the ecMPO probability image by clicking on **Image | Duplicate**.

4.14. Toggle to the screen using the mouse button that contains NETs. Apply a threshold to ensure only highlighting of identified NETs. Click **Image** on the ImageJ menu tool bar **| Adjust | Threshold**. Move the sliding toggle bars until on the NET components are highlighted. Click **Apply** when satisfied threshold. Repeat the same steps for ecMPO.

4.15. If after thresholding there are still detecting areas of ecMPO or NETs, go back to the original trained image and add more classifiers and reapply steps 4.1-4.14.

4.16. Click **Image | Image Adjust | Make Binary**. Copy the glomerular ROI made in step 4.3, click **Ctrl+Shift+E**. Activate Weka window by **Edit | Selections | Restore**, which restores the selection. Click **Analyze Particles** to measure only the NET particles within the glomerulus.

4.17. Toggle to the screen using the mouse button that contains ecMPO. Apply a threshold to

ensure that only identified ecMPO are obtained. Click **Apply** when satisfied with the threshold. Repeat step 4.16 above.

NOTE: A result sheet is computed with the Count of particles, the area, averages pixels and the percentage of the glomerulus containing both NETs and ecMPO. These results can then be put into a spreadsheet and saved, for later statistical analysis.

4.18. Save the classifier as NETs.classifier by clicking **Save Classifier** from the options menu and save file into the ImageJ app on the desktop (ImageJ folder). Then click **Save Data** from the options menu and save as NETs.arff file. The glomerular ROI will have to be manually added each time as glomerulus size and shape will change, but the program has learnt what both NETs and ecMPO are.

4.19. To reapply the model to subsequent images, repeat steps 4.1-4.5 with a new image. Click **Load Classifier** from the option menu. Click on **Nets.classifier** in the ImageJ folder. The log menu will pop up and run model, once the model has run click on **Load Data** in the options menu and select the NETs.arff file.

4.19.1. Click on **Create Result**. If the resulting image has failed to pick up all the nuclei, background or ecDNA click on re-train classifier and add more classifiers and save the new model. Complete the analysis process by repeating steps 4.11-4.19.

NOTE: Several images can be used to generate the final model used to detect ecDNA, ecMPO and NETs. This is achieved by applying the model to subsequent images, adding more classifiers and saving the new model and data into the ImageJ folder. This can be particularly important when different samples have higher background. The Weka Segmentation program is compatible with ImageJ macro language which enables many of the commands to be macro recordable to automate some of the steps.

REPRESENTATIVE RESULTS:

These images represent the multiple steps required to successfully use trainable Weka segmentation to minimize the labor-intensive manual measurement of ecDNA in fluorescently stained FFPE kidney tissue from a mouse with induced anti-MPO GN. These steps are summarized in **Figure 1** and **Figure 2** with images taken directly from the Weka segmentation program, outlining every step in the analysis process. Measurements from this analysis is then shown in **Figure 3** demonstrating the ability of the program to determine the different amounts of ecDNA deposited in the glomerulus, in control tissue, without induced anti MPO GN. **Figure 4** demonstrates that the model for ecDNA can be adapted to identify ecDNA in kidney biopsy specimens from a control patient (Minimal Change Disease patients have minimal glomerular damage evident at a histological level) and compared to that of a kidney biopsy from a patient with MPO-AAV. **Figure 5** demonstrates the translational capacity of this program to other stains within kidney tissue. We have used a representative sample from a mouse kidney with induced experimental anti-MPO GN to stain for NETs and ecMPO. The trainable Weka segmentation program is then used to identify both NETS and ecMPO within the same image. **Figure 6**

demonstrates there is no significant difference in the outcome of results in the amount of ecDNA quantification on the same data set analyzed by two independent users creating 2 different models designed to semi-quantitate ecDNA.

FIGURE AND TABLE LEGENDS:

Figure 1. Images illustrating classification of nuclei, background and extracellular DNA within mouse kidney glomeruli from experimental MPO-ANCA GN using trainable Weka segmentation. (**A**) Demonstrates single channel images of DAPI to stain DNA (blue), β actin (green) to delineate glomerular area, and the composite file with a region of interest (ROI) indicating the glomerular area to be measured. (**B**) Classification of intact nuclei to develop the model (pink) and unclassified nuclei (blue). (**C**) Classification of what is considered to be background (green). (**D**) Classification of what is considered to be ecDNA (purple). (**E**) The model generated by trainable Weka segmentation showing nuclei in red, background in green and ecDNA area in purple.

Figure 2. Images demonstrating the supervised component of the model to reduce the inaccuracy. The Weka model generates the probability of recognizing each classifier in unclassified components. (**A**) Model generated classification of what intact nuclei is. (**B**) Model generated classification of what is considered background. (**C**) Model generation of what ecDNA is considered. (**D**) Illustrates the image of classified ecDNA unthresholded. (**E**) Shows the adjustment of the threshold to rule out any errors in what has been identified as ecDNA, identified ecDNA shown in red. (**F**) Threshold is applied to image and made into a binary image for particle analysis. (**G**) The glomerular ROI is superimposed on the image so only glomerular ecDNA is analyzed. (**H**) Shows the summary of results generated from the analysis.

Figure 3. Images illustrating classification of nuclei, background and extracellular DNA within mouse kidney glomeruli from a control mouse without induced experimental MPO-ANCA GN using trainable Weka segmentation. (**A**) Shows the original merged image with the glomerular region to be analyzed, the training and the trained model result (background green, nuclei red and ecDNA identified in purple. (**B**) Shows the model probabilities of identifying, nuclei, background and ecDNA. (**C**) Results of what the model classified and identified as ecDNA, displayed in arbitrary units.

Figure 4. Image illustrating trainable Weka segmentation is adaptable for the analysis of ecDNA in human kidney biopsies from a patient with minimal change disease and a patient with MPO-ANCA vasculitis. (**A**) Illustrates that minimal ecDNA is detected using trainable Weka segmentation intact nuclei (red), background (green) and ecDNA (purple). (**B**) Demonstrates considerable quantities of ecDNA in a patient kidney biopsy from a patient with MPO-ANCA vasculitis intact nuclei (red), background (green) and ecDNA purple. Results demonstrate that 8 particles of ecDNA were found within the glomerular region of a patient with MCD compared to a patient with active MPO ANCA vasculitis (180 particles).

Figure 5. Trainable Weka segmentation can used to identify NETS and ecMPO within the same image and model analysis, in mouse kidney tissue from experimental MPO-ANCA GN.

(**A**) Demonstrates a glomerulus with NETs [co-localization of green (Citrullinate histone 3), red (MPO) DAPI (nuclei) and PAD4 (white)]. ecMPO is considered to be cell free. (**B**) Training for identification of the classifiers, Red (Intact nuclei), Green (background), Purple (NETs) and yellow (ecDNA)]. (**C**) The model trainable Weka segmentation uses to classify NETs and ecMPO, Red (Intact nuclei), Green (background), Purple (NETs) and yellow (ecDNA). (**D**) Particle analysis of what the model determined to be NETs. (**E**) Particle analysis of what the model determined to be ecMPO. (**F**) Results sheet from the particle analysis for both NETs and ecDNA.

Figure 6. Comparison of 2 independent users in designing a model for the detection of ecDNA. (**A**) Original image showing the DAPI, Beta Actin and Merged images to be analyzed. (**B**) Comparison of training model, classifiers, thresholding and glomerular ROI between 2 independent users. The yellow arrow indicates that User 2 had to retrain the model to remove background**.** (**C**) Results generated showing the comparison of ecDNA count, total area, % area and perimeter, between 2 users. (**D**) Graph of results showing no significant difference between the number of ecDNA detected within glomeruli and % area from two independent investigators. Statistical analysis performed using Mann-Whitney U test with significance set at <0.05. Sample size is n=6.

DISCUSSION:

Multiple protocols exist that measure proinflammatory markers in the serum and urine of both patients and mouse models of glomerulonephritis. This described protocol allows analysis of the products of cell death (ecDNA, NETs and ecMPO) within the glomerulus directly. The most crucial steps in this protocol is the tissue preparation and imaging. The major restricting element of using a fluorescent staining method for analysis is the tissue autofluorescence. Formalin fixed paraffin tissue is subject to autofluorescence that can obscure specific fluorescent staining. The final step in the staining method where slides are immersed in Sudan black, attenuates autofluorescence of the tissue, and allows the illumination of the antibody specific staining through reduction in signal to noise ratio[19]. The imaging of the tissue must be performed with at least 40x oil magnification to be able to detect smaller fragments of ecDNA and MPO. When imaging is acquired, it is crucial that it is done in a line sequential manner to ensure no bleed through of fluorescence of one marker to another.

An advantage of the protocol for analysis is that it is available in open access through ImageJ for anyone to access[16]. We have demonstrated herein that the method can be easily adapted to measure different fluorescent markers within kidney tissue. Once the model in trainable Weka segmentation has been determined it can be applied to subsequent images with no bias and in the exact same manner each image has been analyzed. The supervised nature of the analysis allows any error in segmentation to be adjusted through the additional steps of thresholding the "trained" images, or retraining the program and adding more classifiers. The advantage of this program is that two different users will get similar result using the same model, provided they accurately delineate the glomerular tuft. The biggest inaccuracy in reproducibility by two different end users is created by the different manner in which people trace around the glomerular tuft. For example, if one person draws a rough circle around the glomerular tuft and another user carefully draws around the outermost capillary loops the area being examined will

differ (as demonstrated in the results). Therefore, it is essential that both users are trained to identify the glomerular tuft in an identical manner. The practical application of this program would be for two users to design the model together on multiple images to build a robust model to be applied to further data sets. The more images that are used to train the classifiers the more accurate the model will be.

Limitations of this protocol would be measurement of fragments of ecDNA smaller than what the confocal microscope can detect. This could be overcome with the use of capturing images using super resolution microscopy methods and applying the trainable Weka segmentation to those images. The supervised component of the machine learning adds extra steps and reduces the ability to batch process large sets of images. However, as we demonstrated within the results unsupervised models have reduced accuracy and introducing the supervised component reduced inaccuracy significantly.

We have previously published that in addition to neutrophils producing extracellular traps, monocytes/macrophages were also observed to produce extracellular traps (termed METs) in human ANCA vasculitis, but in smaller proportions[1]. The current methods described herein do not distinguish, between extracellular traps produced by neutrophils or monocytes/macrophages. This is difficult to achieve as most confocal microscopes are limited by the number of lasers. Identification of NETs requires 4 different lasers therefore limiting the number of cell markers able to be processed via standard confocal imaging. If the MPO positive cell of origin is required a second serial section can be stained with either a neutrophil or macrophage/monocyte marker, to identify the cell type producing the extracellular trap.

Pathological features of MPO-ANCA vasculitis include the deposition of ecDNA, NETS and ecMPO within the glomeruli of the kidney[20]. Therapeutically targeting DNA within NETs and ecMPO as well as measuring them in human biopsies as markers of disease has been the subject of recent studies[1,20-22]. The significance of these methods in this field is accurately determining the relative proportion of ecDNA, NETs and ecMPO within the target organ, in a reproducible, less time-consuming manner. In conclusion we have demonstrated a supervised machine learning tool trainable Weka segmentation to semi automate the analysis of large data sets of acquired images for ecDNA, NETs and ecMPO. The use of this tool will reduce image analysis time considerably and the techniques can be easily adapted to other stains in other organs.

ACKNOWLEDGMENTS:
We acknowledge Monash Micro Imaging for the use of Nikon C1 upright confocal laser scanning microscope and the Monash Histology Platform for the processing of kidney tissue.

DISCLOSURES:
Nothing to disclose.

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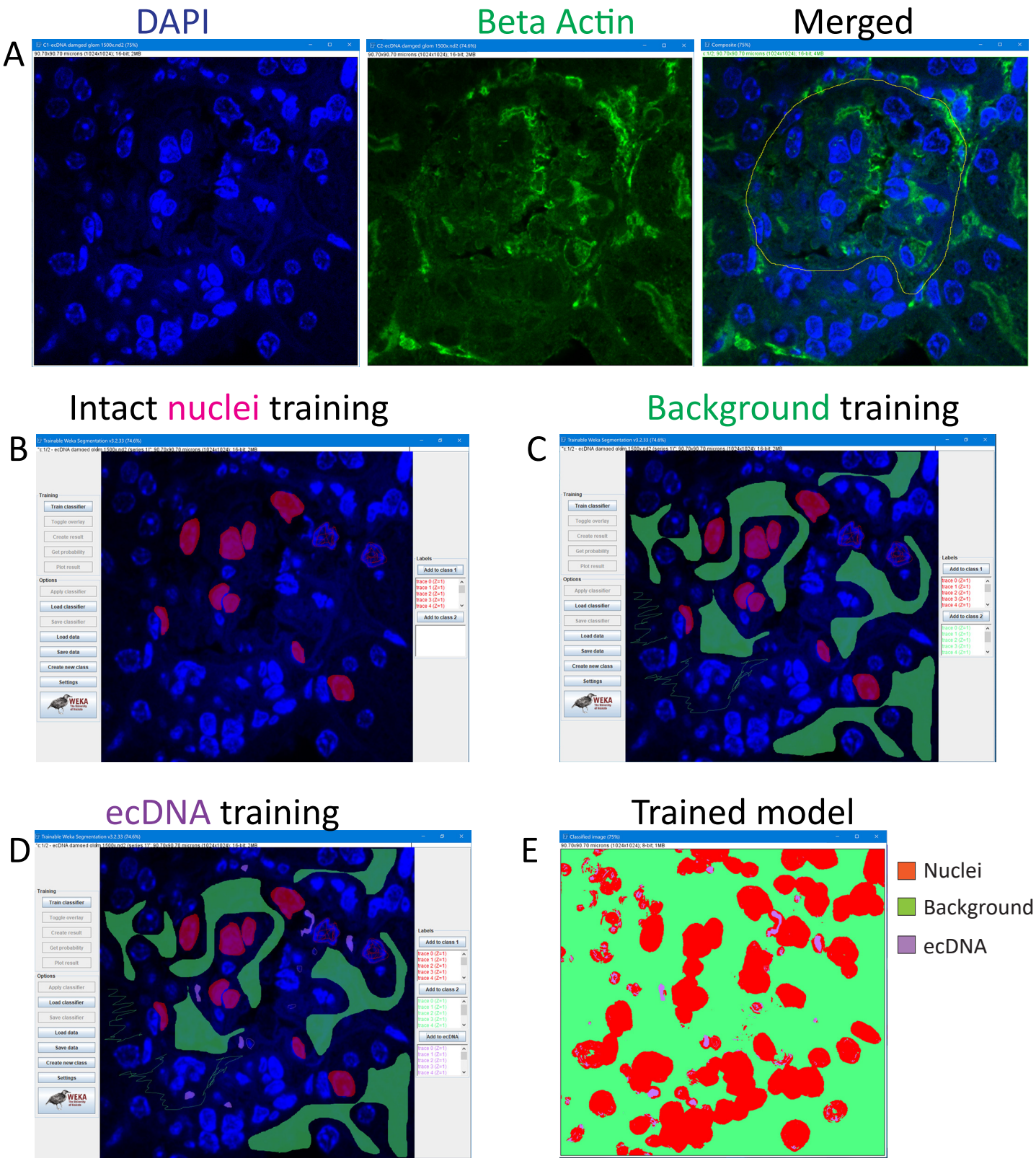
Figure

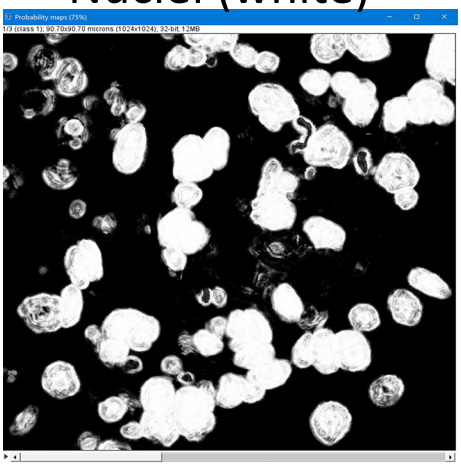
Click here to access/download;Figure;figure 1.ai



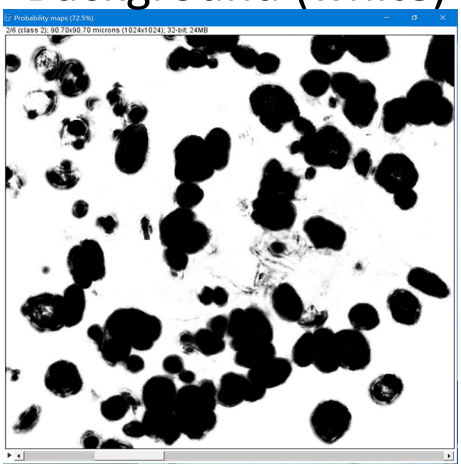
Figure

Click here to access/download;Figure;Figure 2.ai

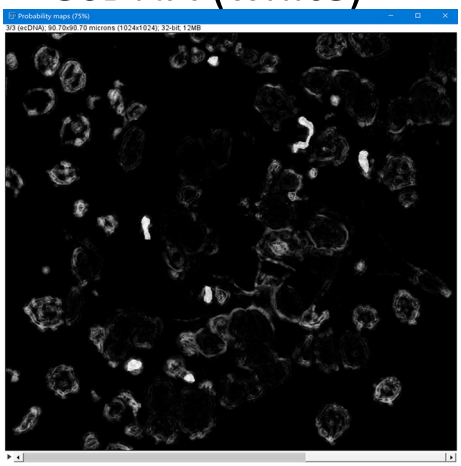
A Nuclei (white)



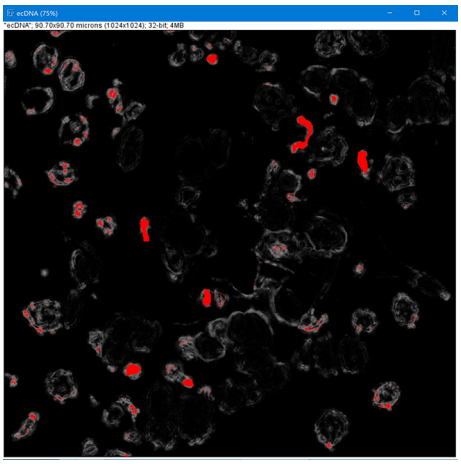
B Background (white)



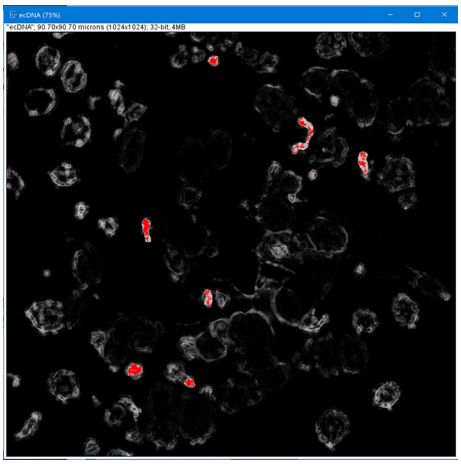
C ecDNA (white)



D Unadjusted threshold

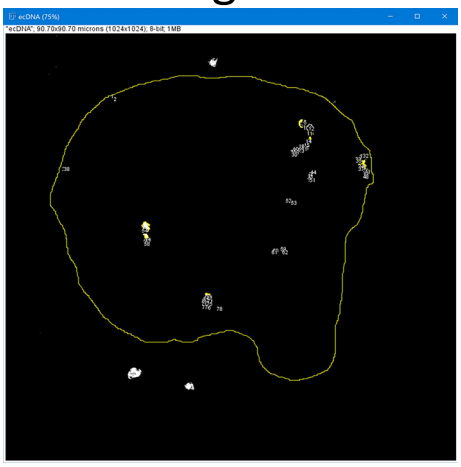


E Adjusted threshold



F Make binary

Measure glomerular ecDNA



Summary of results

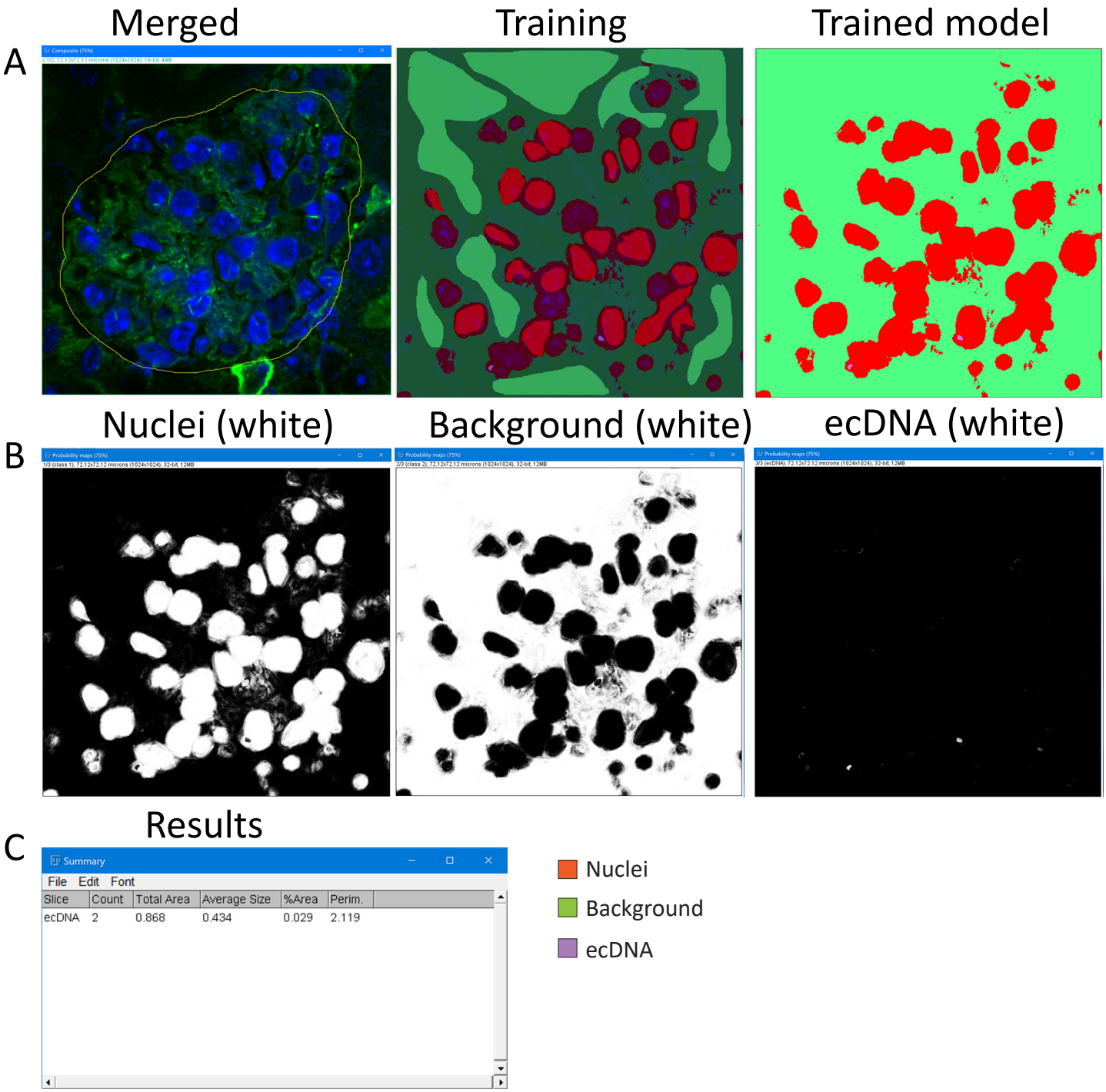
Summary

File Edit Font

Slice	Count	Total Area	Average Size	%Area	Perim.
ecDNA	78	11.212	0.144	0.361	1.297

Figure

Click here to access/download;Figure;Figure 3.ai



Figure

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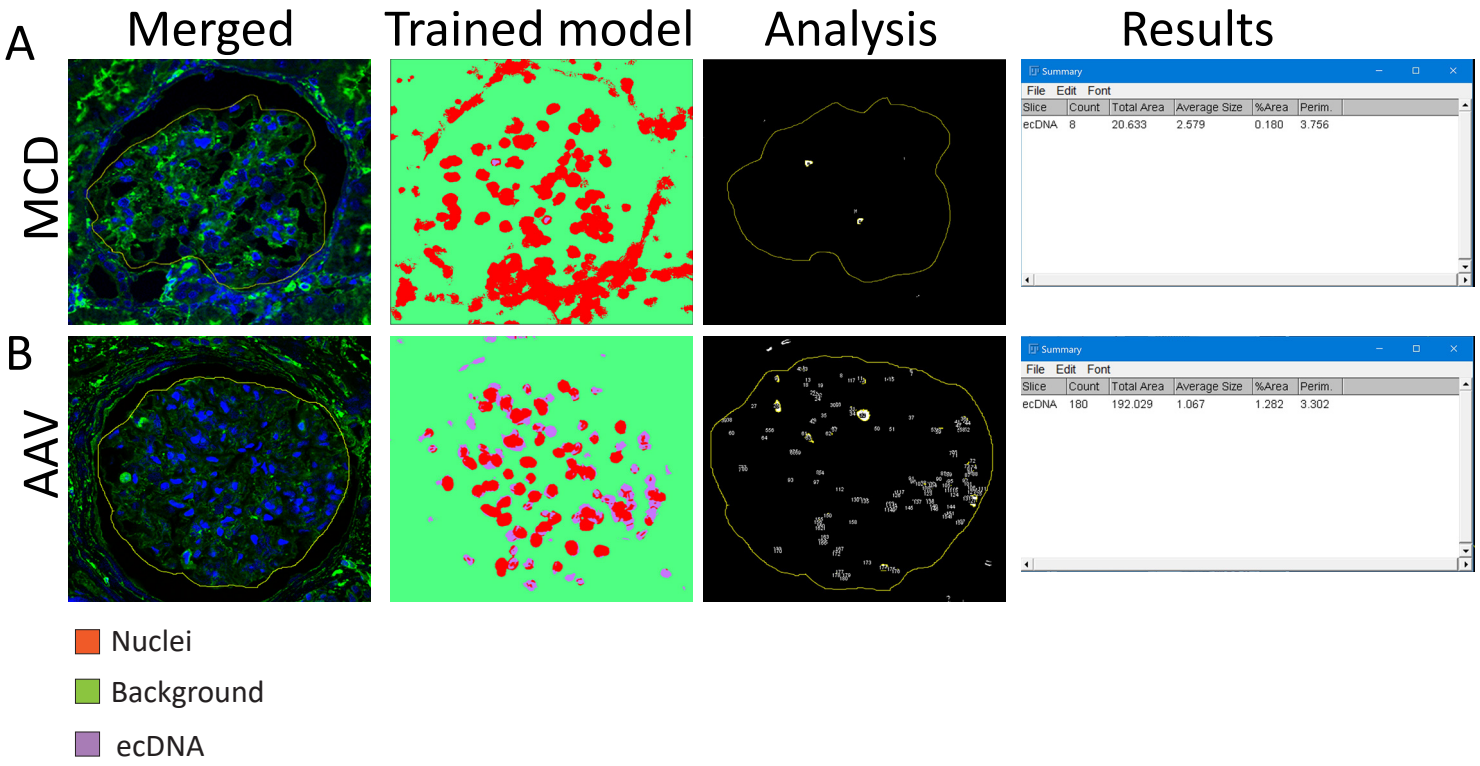
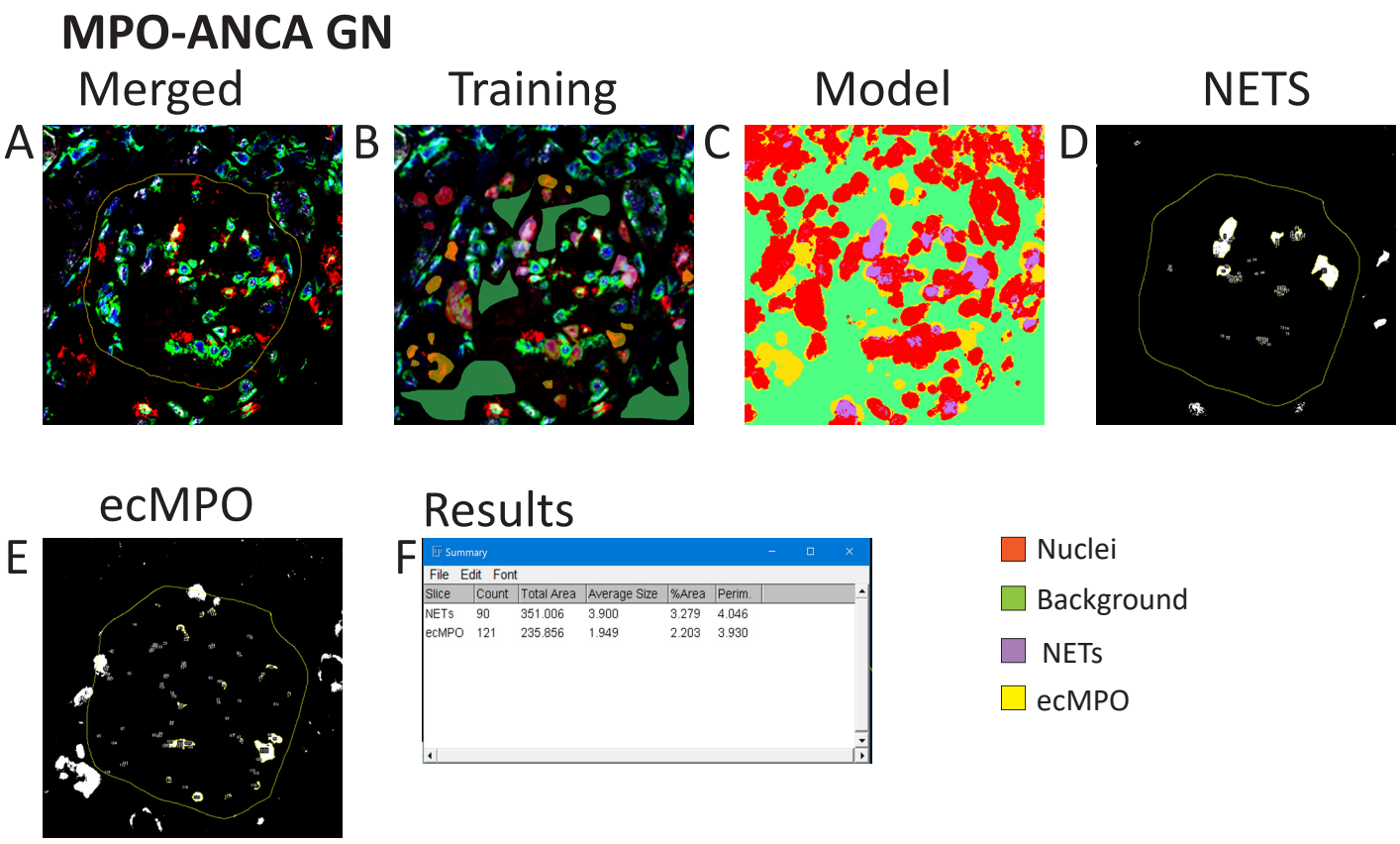
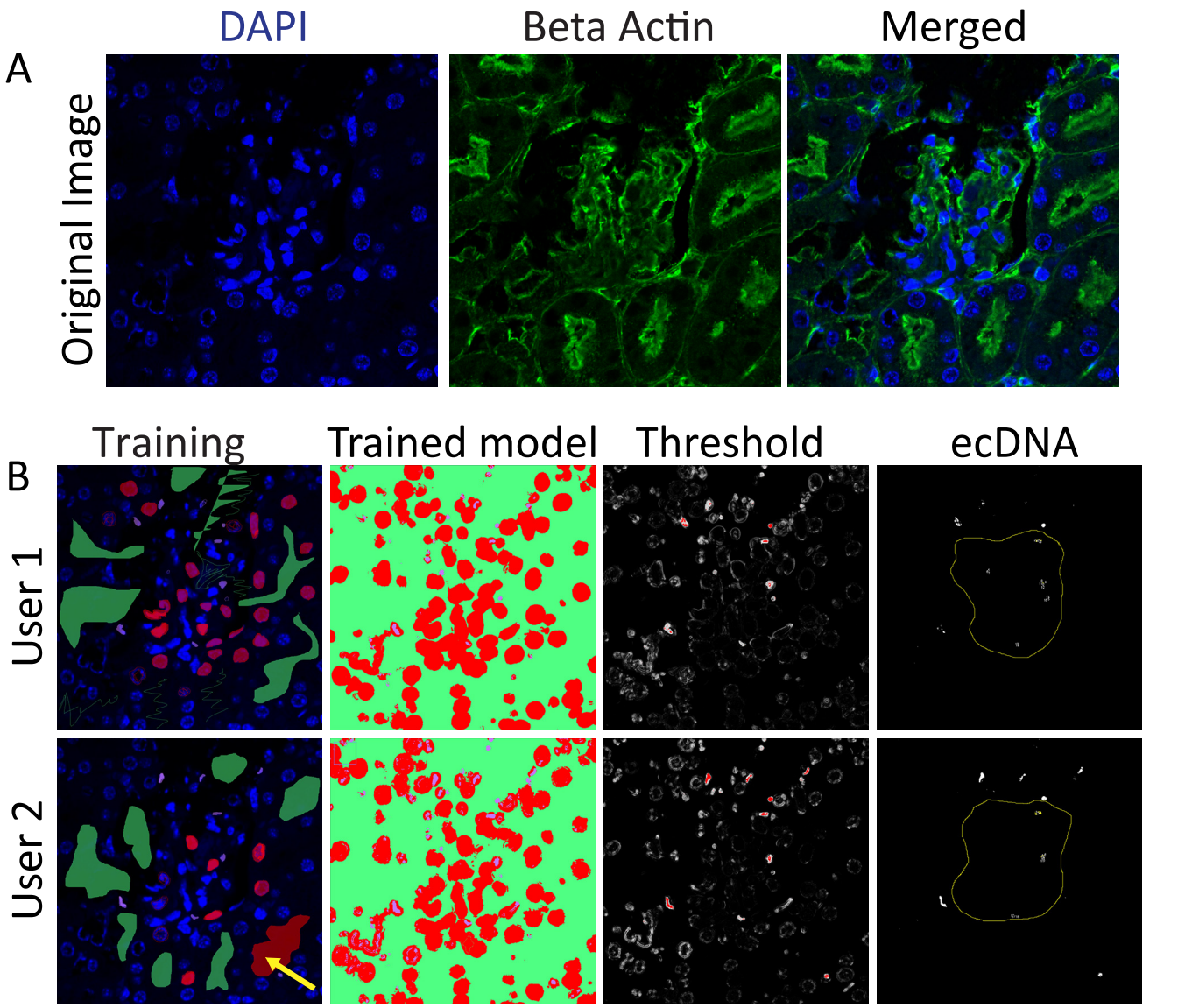
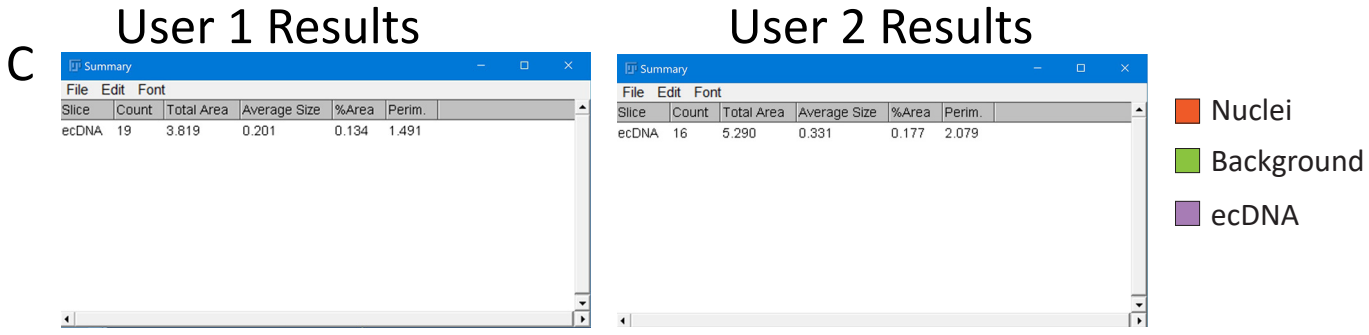


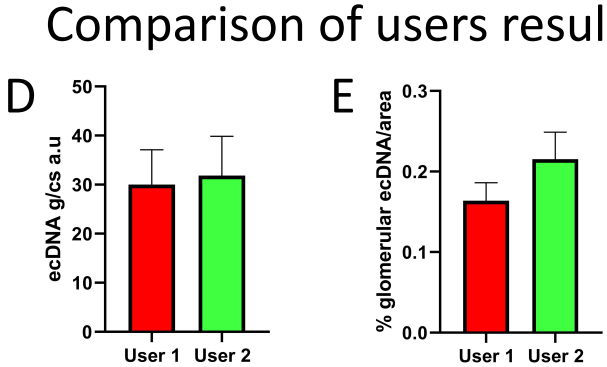
Figure

Click here to access/download;Figure;Figure 5.ai









Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Bovine Serum Albumin	SIGMA	A2153	5% and 1% solutions are made up in PBS, can be made in bulk and frozen- discard once thawed.
Chicken anti Goat IgG (H+L) cross absorbed antibody Alexa Fluor 594	ThermoFisher Scientific	A-21468	Spin in mini centrifuge for 1 minute prior to use to avoid any free conjugate in your antibody cocktail
Chicken anti mouse IgG (H+L) cross absorbed antibody, Alex Fluor 647	ThermoFisher Scientific	A-121468	Spin in mini centrifuge for 1 minute prior to use to avoid any free conjugate in your antibody cocktail
Chicken anti rabbit IgG (H+L) Cross absorbed antibody Alexa Fluor 488	ThermoFisher Scientific	A-21441	Spin in mini centrifuge for 1 minute prior to use to avoid any free conjugate in your antibody cocktail
Chicken sera	SIGMA	C5405	Made up in 1%BSA/PBS
Coverslips 24 x60 mm	Azerscientific	ES0107222	#1.5 This is not standard thickness- designed for use in confocal microscopy
EDTA 10mM	SIGMA	E6758	Add TRIS and EDTA together in distilled water and pH to 9, for antigen retrieval, can be made up in a 10x Solution
Ethanol 30%, 70% and 100%	Chem Supply	UN1170	Supplied as 100% undenatured ethanol- dilute to 30% and 70% using distilled water
Formaldehyde, 4% (10% Neutral buffered Formalin)	TRAJAN	NBF-500	Kidney is put into a 5ml tube containing 3ml of formalin for 16 hours at RT, formalin should be used in a fume hood
Glass histology slides- Ultra Super Frost, Menzel Glaze, 25x75 x1.0mm	TRAJAN	J3800AM4	Using positive charged coated slides is essential. We do not recommend using poly-L-lysine for coating slides as tissue dislodges from slides during the antigen retrieval step
Goat anti human/mouse MPO antibody	R&D	AF3667	Aliquot and freeze at minus 80 degrees upon arrival

Histosol	Clini Pure	CPL HISTOSOL 08	Used neat, in 200ml staining rack containers, use in a fume hood
Hydrophobic pen	VECTOR Labs	H-400	Use to draw circle around kidney tissue
Name of Material/ Equipment	**Company**	**Catalog Number**	**Comments/Description**
Mouse anti human/mouse Peptidyl arginase 4 (PAD4)	ABCAM	ab128086	Aliquot and freeze at minus 80 degrees upon arrival
Nikon C1 confocal scanning laser head attached to Nikon Ti-E inverted Microscope	Coherent Scientific		Aliquot and freeze at minus 80 degrees upon arrival
Phosphate Buffered Saline	SIGMA	P38135	0.01M PB/0.09% NaCl Make up 5L at a time
Pressure Cooker 6L Tefal secure 5 Neo stainless	Tefal	GSA-P2530738	Purchased at local homeware store
Prolong Gold DAPI	Life Technologies	P36962	Apply drops directly to coverslip
Rabbit anti human/mouse Beta Actin antibody	ABCAM	ab8227	Aliquot and freeze at minus 80 degrees upon arrival
Rabbit anti human/mouse H3Cit antibody	ABCAM	ab5103	Aliquot and freeze at minus 80 degrees upon arrival
Staining rack 24 slides	ProScitech	H4465	Staining rack chosen has to be able to withstand boiling under pressure and incubation in 60 degree oven
Sudan Black B	SIGMA	199664	0.3 % Add 3g to a 1L bottle in 70% Ethanol, filter and protect from the light- stable for 6 months at room temperature
Tris 10mM	SIGMA	T4661	Add TRIS and EDTA together in distilled water and pH to 9, for antigen retrieval, can be made up in a 10x solution
Xylene	Trajan	XL005/20	Must be use used in a fume hood

MONASH University

05/02/2020

Dr. Nam Nguyen
Manager of Review
JoVE

Dear Dr. Nam Nguyen,

Please consider our revised manuscript entitled "**Supervised machine learning for semi-quantification of extracellular DNA in glomerulonephritis"** for the Journal of Visualized Experiments as an invited methods article. This material has not been previously published and is not under consideration for publication elsewhere. The authors have read the manuscript and concur with submission.

We thank both the editors and reviewers for their comments, and have responded below:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version

Have carefully proofread the manuscript and am confident it is free from spelling and grammatical errors.

2. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

The table has now been revised.

3. Please revise the title to be more concise and direct.

The title has been revised to "Supervised Machine Learning for Semi-quantification of extracellular DNA from cell death in glomerulonephritis".

4**. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Super Frost Ultra, Histosol, etc.**

All trademark symbol and registered symbols, company names and commercial language have been removed. The commercial products have been referenced in the Table of Materials and Reagents.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Safety procedures and the removal of any text not written in the imperative tense has been amended.

6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Any discussion elements have been moved to the discussion

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Bullets and dashes have been removed and the Jove numbering utilised.

8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

The how part of the protocol has been carefully revised to allow the reader to reproduce every step.

9. How are the mice euthanized? Please specify the mouse gender/age.

This has been included in lines 126-127 (track change version)

10. How are the kidneys removed?

This has been added in line 128-132 (track change version)

11. What is used to cut the kidneys in half?

This has been added to line 132 (track change version)

12. What is the standard protocol to process the kidneys?

This has been added to manuscript on line 138-150 (track change version).

13. How are the glass slides coated? What are they coated with?

The slides are commercially obtained and come pre coated with a positive charge to ensure electrostatic attraction of tissue samples. The company do not reveal what it is coated with. In the note section of the materials we have added a note to suggest these slides should not be substituted- we have tried other brands, and coated slides ourselves with poly-L-Lysine, and tissue has still floated off during antigen retrieval.

14. 60 degrees C presumably?

Yes this has been added to the manuscript

15. Please provide units and more parameters on the pressure cooker usage for standardization.

The units in both psi and kPa have been added.

16. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

This has been highlighted.

17. Please do not abbreviate journal titles.

The endnote style provided by JoVE has been used.

Reviewers' comments:

Reviewer #1:

Major Concerns:

I have a few questions:

Delineating the areas of ecDNA and NETs is initially done by an (one) investigator. All subsequent semi-automated quantification steps rely on that initial data set. Did the authors perform analyses

where 2 or 3 investigators independently started the delineation/classification processes? It would be important to see if the resulting quantification after machine learning gave similar results. I am saying this because I find it difficult to unequivocally delineate ecDNA for example in Figure 1D. Some of the DAPI spots that were not marked purple could still be ecDNA. Thus, what is the gold standard and could it be improved by using independent investigators? Additional data and a critical discussion should be added to the manuscript.

Thank you for these comments. We have added data from a second investigator and new figures showing the model for detecting ecDNA classified by two different investigators. The results are then compared in new figure 6. We show that there is a non-significant variation between 2 investigators using the same images to train a model over several images. We have added to the protocol a description on how to add more classifiers if your first model is inaccurate, and need to retrain on the same image. Although we present here in the results the same images analysed by 2 independent users who create two independent models, ideally the practical application of this machine learning would be for two or more investigators to train and use the **one** model. The more classifiers and more images used to build the model will improve accuracy. The resulting model would then be used on multiple data sets, opposed to 2 investigators using two different models on the same data set. This is discussed in lines:575-585 (track change version) of the discussion, and in the results section lines:492-495, and figure 6.

It sounds to me that the initial investigator-dependent delineation/classification process is only done once and on one image and that it does not have to be repeated with the next section (e.g. another mouse or patient)? Please, clarify this issue in the manuscript.

Thank you for pointing this out. This has not been made clear in the manuscript and has now been added to the protocol to demonstrate that it is done over several images, and a description of how to add classifiers in subsequent images is outlined. Lines:262-275, and 456-468 (track change version).

NETs refer to neutrophils as a source. Is it possible that monocytes are also contributing to the ecDNA (MPO/PAD4/histone structures? This point should be discussed in the manuscript.

Yes, monocytes are likely to contribute to the ecDNA(MPO/PAD4/histone structures). This has already been published by our group in Kidney International in 2015 in human ANCA vasculitis. We have added this to the manuscript with advice on how to determine if the extracellular traps are of neutrophil or monocyte origin. Lines 595-603 (track change version).

Minor Concerns:

There are several issues with typos and lacking specifications. Please, go over the text carefully. Here are only some examples:

Line 48: is NETs a form of cell death or is it then NETosis?

NETs is a form of cell death, the term NETosis is used to describe the active events that lead to cell death, and its use is widely accepted in publications. However we have addressed your concerns and streamlined the expression to the term NETs.

Line 129: please, specify 60 degree (tilted or °C)

This has been amended to indicate °C

Line 181: should be 1.00-2.00 (as written later)?

Thank you, this was an oversight and has been amended.

Line 287: One " Click on" too much-

Thank you, this has been removed.

Why was some of the text marked yellow?

MONASH University

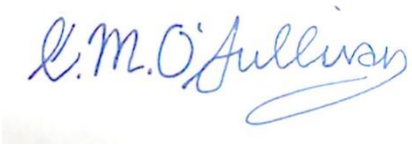
The text highlighted in yellow is the request from JoVE to indicate which part of the protocol will be filmed by the production crew.

Table of Material: Glass hisology needs an t.

The table of materials has been amended and checked thoroughly for spelling errors

Thank you for considering this manuscript for publication.

Yours sincerely,



Dr. Kim Maree O'Sullivan