Quantification and Characterization of Glomeruli Across Diverse Stains Using HALO AI™

INTRODUCTION

Glomeruli are the filtering functional unit of the kidney and their enumeration and characterization by volume are commonly measured in renal physiology, as glomerular hypertrophy can be an early marker of kidney disease. While many diseases can impact glomerular function, the leading cause of glomerular disease is diabetic nephropathy. Damage to glomeruli can be seen in inflammation or swelling of glomeruli, called glomerulonephritis, or in scarring or hardening of the glomeruli, called glomerulosclerosis. Glomerulosclerosis and other chronic renal diseases are also characterized by fibrosis, which is deposition of excessive extracellular matrix proteins that impair kidney function.[1-6]

In this Application Note, we train the DenseNet algorithm in **HALO AI™** to detect and segment glomeruli within stained rat kidney tissue cross sections. We demonstrate that a robust tissue classifier can be built that accurately segments glomeruli from surrounding tubules in the kidney cortex across a range of stains including H&E, trichrome, periodic acid methenamine silver (PAMS), and DAB with hematoxylin. Using the same dataset, we further show how the HALO AI tissue classifier can be embedded within **HALO® modules** to quantify additional features inside and outside of the segmented glomeruli, including 1) cell quantification (**Multiplex IHC module**), 2) analysis of cell-based IHC stains e.g. inflammatory or apoptotic biomarkers (Multiplex IHC module), 3) pixel-based quantification of fibrosis (**Area Quantification BF module**), and 4) RNAscope to elucidate gene expression (**ISH module**).

This study highlights the potential for **HALO®** and HALO AI to quantify biomarkers, gene expression, and morphological changes in kidney disease, during development, and in toxicological studies.

METHODS AND RESULTS

To train a HALO AI DenseNet algorithm to recognize glomeruli across stain types with high sensitivity and specificity, 15 images of kidney cross sections were selected, where two were stained with PAMS, four were stained with Trichrome, seven were stained with H&E, and two were stained with DAB and hematoxylin. **Figure 1** shows representative images from the dataset.

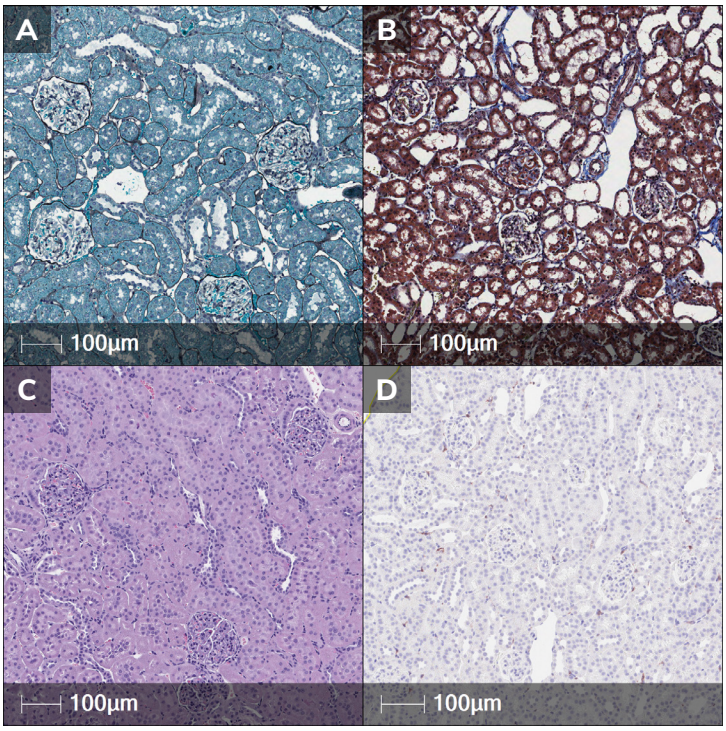

Figure 1. Representative images from kidney dataset. A. PAMS stain. **B.** Trichrome stain. **C.** H&E stain. **D.** DAB and hematoxylin stain.

In order to create a classifier robust enough to reliably detect glomeruli across a large cohort, as much variability as possible was captured in the training data. The classifier included two classes that were 'Glomeruli' and 'Background', where 'Background' included all other kidney tissue and glass area on the slide. The DenseNet network of HALO AI was chosen as it is capable of handling multiple stain types in a single classifier.

This network requires many training examples and more training time than the MiniNet network, which cannot handle multiple stain types in a single classifier. Training of the DenseNet algorithm was performed with the Resolution of 2 µm/px and the default Minimum Object Size of 1000 μm^2. The algorithm was allowed to train overnight for a total of 88,575 Iterations and was then evaluated across the four sample types. At this timepoint, the classifier was performing with near perfect sensitivity and specificity across the PAMS, Trichrome, and H&E

samples, but the DAB and hematoxylin sample had a few false negative glomerular events **(Figures 2-3)**. The fact that several-fold fewer training data was available for this sample type likely explains this performance result.

To determine if further training would improve the classifier performance, the classifier was allowed to train for a total of 191,595 Iterations and the performance was again evaluated. At this time point, there was some improvement in classifier performance as judged by fewer false negatives, but more training data is needed to further improve performance of this classifier **(Figure 3)**.

When a HALO AI Classifier is run, the output data include the Region Perimeter and Region Area for each object identified by the classifier, as well as the x and y coordinates **(Figure 4)**. This data is easily exportable as a .csv file for further analysis. With this type of analysis, one can enumerate glomeruli, determine number of glomeruli per area classified, and answer questions as to whether glomerulonephritis has occurred by examining control vs. experimental Region Area in the Glomeruli class.

To characterize the number of cells or phenotype of cells in each glomerulus or in the surrounding tubules, a HALO AI Classifier needs to be run in the context of a HALO module. **Figure 5** demonstrates a HALO AI Classifier that is embedded within the Multiplex IHC module of HALO. In this analysis, HALO AI first identifies the Glomeruli and Background classes and then for each object identified as a glomerulus, a Multiplex IHC analysis is performed. In the PAMS analysis shown in **Figure 5**, the number of cells in each glomerulus can be enumerated. If a phenotypic analysis is desired, a multiplex IHC assay with up to 5 biomarkers or stains can be quantified.

Figure 6 shows an example of Multiplex IHC analysis where a HALO AI Classifier was embedded, and the

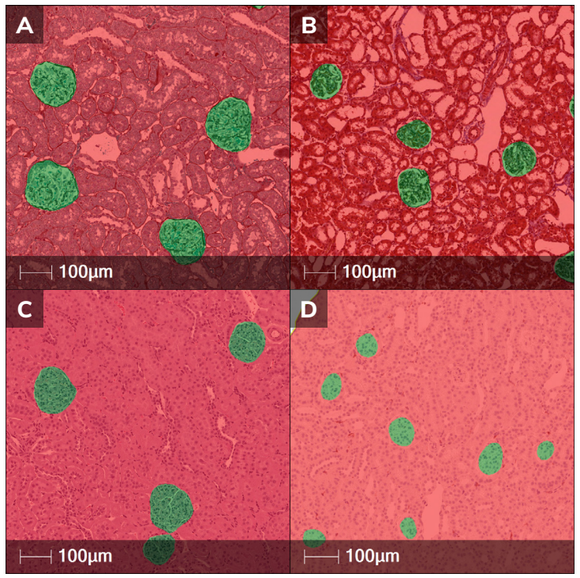

Figure 2. HALO AI Classifier performance across test set images after 88,575 Iterations. Green indicates the Glomeruli class and red indicates the Background class. **A.** PAMS stain **B.** Trichrome stain **C.** H&E stain. **D.** DAB and hematoxylin stain. Images represent same regions shown in **Figure 1**.

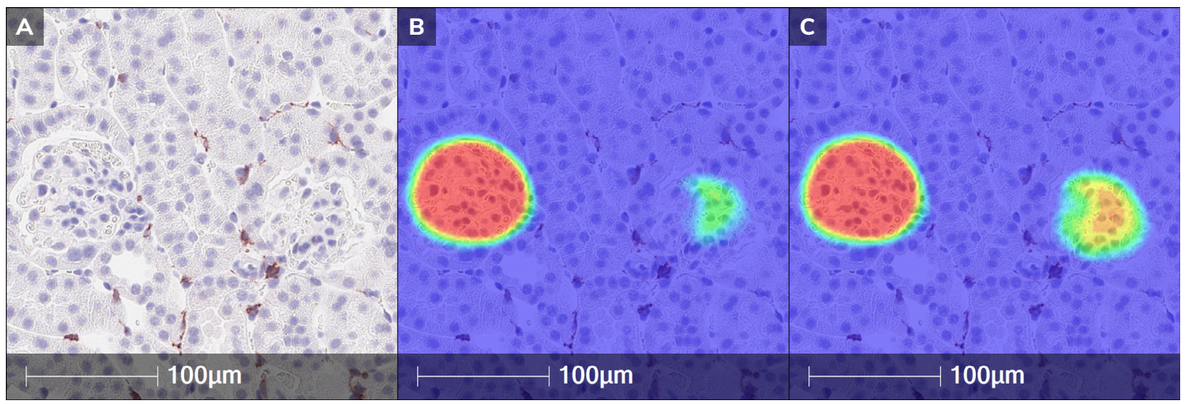

Figure 3. HALO AI Classifier performance on DAB and hematoxylin sample with Probability Map markup image. Blue color indicates the lowest probability of being a glomerulus, while green, yellow, and orange indicate intermediate probability, and red indicates highest probability. **A.** DAB and hematoxylin stain. **B.** HALO AI Classifier at 88,575 Iterations. The classifier is detecting only a portion of the glomerulus on the right, and with low probability. **C.** HALO AI Classifier performance at 191,595 Iterations. The classifier is detecting a larger portion of the glomerulus on the right and the red and yellow markup image indicates higher probability.

Object Data

Object Actions

1 Object(s) Selected

Classifier Label	Region Perimeter (µm)	Region Area (µm²)	Id	XMin	XMax	YMin	YMax
Background	120332	26363500	0	2728	24187	2291	30412
Glomeruli	280	3884	1	10595	10880	7652	7921
Glomeruli	268	3376	2	15868	16122	4123	4400
Glomeruli	276	3576	3	15464	15702	4583	4892
Glomeruli	320	4872	4	16249	16550	5090	5423
Glomeruli	212	2152	5	17962	18160	5360	5582
Glomeruli	328	5100	6	17288	17565	5384	5757
Glomeruli	224	2268	7	16376	16574	6248	6494
Glomeruli	300	4216	8	19080	19366	6272	6581
Glomeruli	240	2776	9	15258	15519	6383	6597
Glomeruli	272	3516	10	19715	20000	7113	7366

Figure 4. HALO AI Classifier Object data. The Region Perimeter and Region Area for each object identified by the classifier are output in the Object Data in addition to the XMin, XMax, YMin, and YMax coordinates.

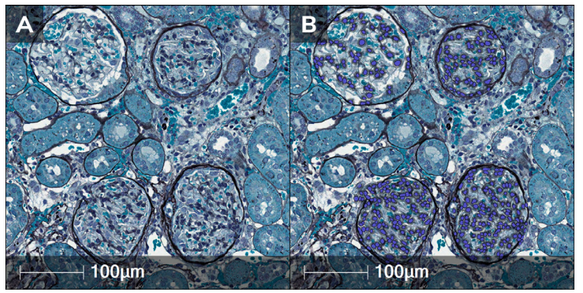

Figure 5. Performing Multiplex IHC analysis with the embedded glomerular HALO AI Classifier. HALO AI was also used for nuclear segmentation. **A.** PAMS image **B.** PAMS image with Multiplex IHC markup.

Background class was analyzed to examine DAB expression in the surrounding tubules. With this type of analysis, HALO provides the number of DAB negative, weak, moderate, and strong expressing cells in the class(es) selected for analysis. If a biomarker of interest localized to the glomeruli and not the surrounding tubules, the analysis could easily be updated to examine DAB positivity within the glomeruli.

To analyze fibrosis in the tissue surrounding the glomeruli, the Area Quantification BF module can be used with an embedded HALO AI classifier. The Area Quantification BF module quantifies pixels and is ideal for quantification of extracellular localization. **Figure 7** demonstrates quantification of fibrosis in the Background class identified by HALO AI.

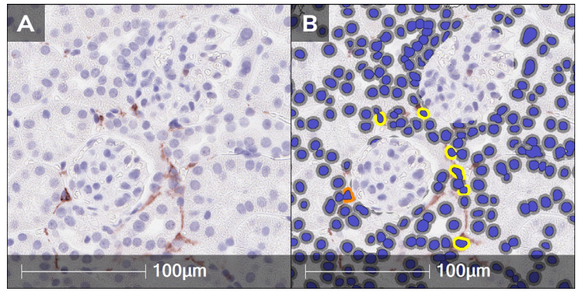

Figure 6. Multiplex IHC analysis of Background layer identified by HALO AI. A. DAB and hematoxylin image **B.** HALO markup image where yellow indicates low DAB expression, orange indicates moderate expression, and red indicates high expression.

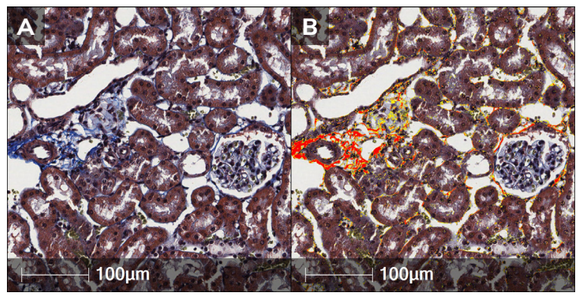

Figure 7. Analysis of fibrosis in a Trichome stained image with the Area Quantification BF module and an embedded HALO AI Classifier. A. Trichrome image. **B.** Blue collagen is quantified in the Background class where yellow indicates low intensity, orange indicates medium intensity, and red indicates high intensity.

To analyze an in-situ hybridization image, provided by ACD, a Bio-Techne brand, the HALO AI classifier that was developed and used in **Figure 2-7**, was tested on this new stain type. Even though the classifier was not trained on an RNAscope image, the hematoxylin staining present in both the RNAscope image, and a training image (DAB and hematoxylin) was sufficient to enable the classifier to identify with high sensitivity and specificity. **Figure 8** demonstrates HALO analysis using the ISH module where the analysis is run on the Glomeruli class.

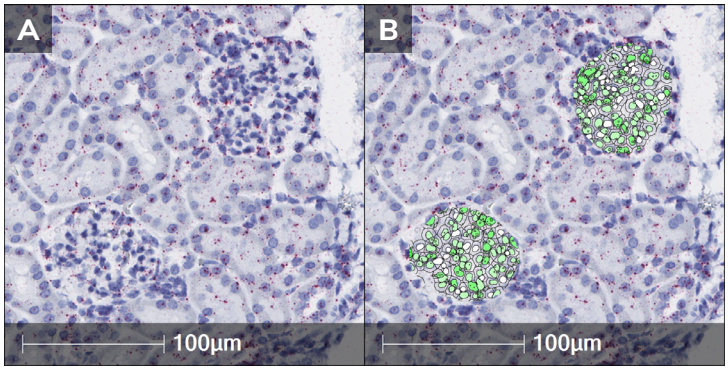

Figure 8. RNAscope analysis with embedded HALO AI glomeruli classifier. Polr2a is in magenta and a hematoxylin counterstain is present. A. RNAscope image **B.** HALO analysis markup with the ISH module and embedded classifier. Green indicates cells with probe positivity in the nucleus and probes are represented by green foci.

CONCLUSIONS

Identifying glomeruli with high sensitivity and specificity is critical for digital image analysis of kidney sections for evaluation of kidney diseases including glomerulosclerosis and glomerulonephritis. Across stains including H&E, trichrome, PAMS, and DAB with hematoxylin, glomeruli appear dramatically different in color, texture, and morphology. Despite this high variability, a HALO AI algorithm is able to be trained to reliably recognize glomeruli across all four staining methods. Once glomeruli are detected, a variety of HALO image analysis algorithms can be applied to examine glomerular area, perimeter, fibrosis, biomarker positivity, and gene expression. Additional AI-based kidney applications include creating a classifier to analyze tubule atrophy, to differentiate normal glomeruli from diseased ones, or an AI-based phenotyper could be trained to identify different cell types within and around glomeruli.

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RESOURCES

HALO customers can sign-up for **Indica Labs Learning Portal** (https://learn.indicalab.com) to access additional resources, including module user guides, video tutorials, and masterclass webinars.

For specific information about the image analysis methods used in this application note, please email our Applications Scientists at info@indicalab.com.