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3D Visualization of the Glomerulus within Kidney Tissue Made Transparent through Passive Optical Clearing

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3D Visualization of the Glomerulus within Kidney Tissue Made Transparent through Passive Optical Clearing

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

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THE UNIVERSITY OF WESTERN ONTARIO

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List of Abbreviations

2D – two-dimensional

3D – three-dimensional

MicroMRI – microscopic magnetic resonance imaging

OCT – optical coherence tomography

FFOCT – full-field optical coherence tomography

SeeDB – see deep brain

3DISCO – three-dimensional imaging of solvent cleared organs

GFP – green fluorescent protein

YFP – yellow fluorescent protein

GBM – glomerular basement membrane

PBS – phosphate buffered saline

PBST – phosphate buffered saline with triton

PFA – paraformaldehyde

sRIMS – sorbitol refractive index matching solution

Abstract

The understanding of structure/function relationships in complex cellular systems is enhanced by 3D visualization of their organization at microscopic resolution. Primarily for e-learning purposes, we recently developed a digital 3D model of the renal corpuscle derived from serial histologically stained semi-thin sections using Amira 5.1. Since this technique is labour-intensive and time-consuming, in this study we applied the optical clearing method CLARITY to render kidney tissue optically transparent for 3D visualization of specific structures within the renal corpuscle by immunocytochemistry and confocal microscopy. Mouse kidneys were infused with a crosslinked hydrogel network. Cortical kidney tissue was then cut into 1mm thick sections and passively cleared for 40 days in clearing solution. The resultant optically transparent tissue was labelled for 4 days with primary antibodies against podocyte specific antigens such as nephrin, a transmembrane protein of the slit diaphragm, followed by incubation for 4 days with fluorescent secondary antibodies. 3D visualization by confocal microscopy provides detailed morphological information of the filtration barrier in the kidney glomerulus. High-resolution 3D imaging of complex cellular structures using passive optical clearing methods thus may prove useful for histology education and in histopathological inquiries.

Key Words: 3D visualization, optical clearing, glomerulus, immunohistochemistry

Chapter I: Introduction and Literature Review

1.1 Three-Dimensional Anatomy and Histology Education

Histology is the study of anatomical and cellular structures at the microscopic level. It is an important tool in our understanding of structure-function interrelationships in often complex multicellular tissues and organs. Tissues are generally visualized histologically in two-dimensional (2D) microscopic images. However, when performing studies in the life sciences, it is necessary to examine the multiple aspects and regions of the samples in order to grasp a greater understanding of the spatial and structural organization of biological systems.[1] Computer-based three-dimensional (3D) reconstruction of tomographic data allows the use of multiple tools that can be used to navigate and interpret 3D systems at a level of understanding unachievable through the analysis of a series of 2D serial sections.[2]

Recently, developments in e-learning technologies have enabled the use of computer-based 3D simulated models in anatomy and histology education. This digital approach facilitates the interactive visualization of anatomical and histological structures and their relationships.[3,4] Although earlier work has failed to show that virtual 3D models provide a significant advantage over more traditional learning tools,[5,6] more recent studies suggest that computer-based 3D models are effective educational tools that increase understanding and allow students to cognitively transpose 2D images onto a 3D anatomical structure.[7,8,9] In a study performed by Nicholson et al., participants with

access to a virtual 3D model of the middle ear performed significantly better on an anatomical quiz than the control group lacking the digital 3D model.[10]

Despite these results suggesting that 3D virtual models are effective learning tools, a general consensus on the efficacy of 3D learning tools has not been formed. However, compared to traditional learning methods, virtual 3D models may hold significant advantages to student learning. Digital 3D models allow for the visualization, movement and manipulation of 3D anatomical and histological structures. It also allows for the combination and visualization of the key spatial information one would see in a prosection or museum model and the detailed information demonstrated in a textbook.[3] In this respect, 3D reconstruction, imaging, and analysis of tissues at microscopic levels may improve our understanding of histology and provide important insight in biological functions and pathological conditions.[11]

Certain 3D imaging techniques are providing high resolution images of tissue structures and can display cellular structures at resolutions down to the micrometer level. Modern tomographic technologies, such as X-ray microtomography, can be applied to image soft tissues at a scale of a few micrometers.[12] However, X-ray absorbance of soft tissues is quite low. Microscopic magnetic resonance imaging (microMRI) allows the visualization of biological systems at relatively high resolutions, but is limited to producing images at a resolution of 10 μm or more.[2] Optical coherence tomography (OCT) and full-field optical coherence tomography (FFOCT) have proven to be more effective tools for performing fast histology on both fresh and formalin-fixed tissues. OCT and FFOCT are cross-sectional, three-dimensional imaging methods that produce images at relatively high resolution by measuring the echo delay of light.[13] This

method allows for the use of fluorescent stains to image specific cellular structures and gene expressions.[14] A major limitation of OCT is the lack of cellular details in the images produced.[14] In fact, few 3D techniques can achieve the resolution of 2D histological images gathered via light microscopy.[2] Light microscopy techniques in conjunction with histochemical and immunohistochemical molecular labeling methods have shown to be useful tools for visualizing important structural and functional aspects of cellular construction.[15] However, light microscopy does not allow for imaging through larger volumes of intact tissue due to the opacity of the tissue leading to light scattering. In addition, important information regarding the 3D spatial organization of these structures may be missed or misinterpreted when inspecting 2D images of complex cellular structures.[16] To circumvent these limitations, studies have applied thin serial sectioning techniques followed by imaging and 3D reconstruction to visualize micro-anatomical structures of tissues.[1] The most common methods for reconstructing sections of histological data into a 3D representation involve iso-surface rendering and volume rendering with an image processing software such as Amira. An iso-surface is a 3D representation of different points of a constant value within a volume of space, while volume rendering involves the development of a 3D image through simulation of absorption and emission values of each volumetric pixel (voxel) in a tomographic data set.[2] Simulating the transmission of light through a voxel allows the visualization of a data set from any orientation without the construction of an iso-surface. Volume rendering can also be combined with iso-surface rendering. Iso-surface rendering results in the construction of polygonal surface models. These 3D rendering techniques can provide comprehensive 3D representations of 2D biological data sets.

A digital 3D model of the kidney renal corpuscle was recently developed through semi-thin sectioning of cortical kidney tissue and 3D reconstruction with Amira 5.1 software (Fig. 1.1).[17,18] This was performed with the goal of generating a virtual 3D model of a complex cellular structure to help bridge the cognitive gap between 2D histological visualization and 3D comprehension of the structural organization of the renal corpuscle. Several studies suggest that virtual 3D models have exciting potential to improve histology education and understanding.[7,8,9] Nonetheless, serial sectioning and 3D reconstruction can be time-consuming, labour intensive, and difficult to incorporate into panoramic 3D images

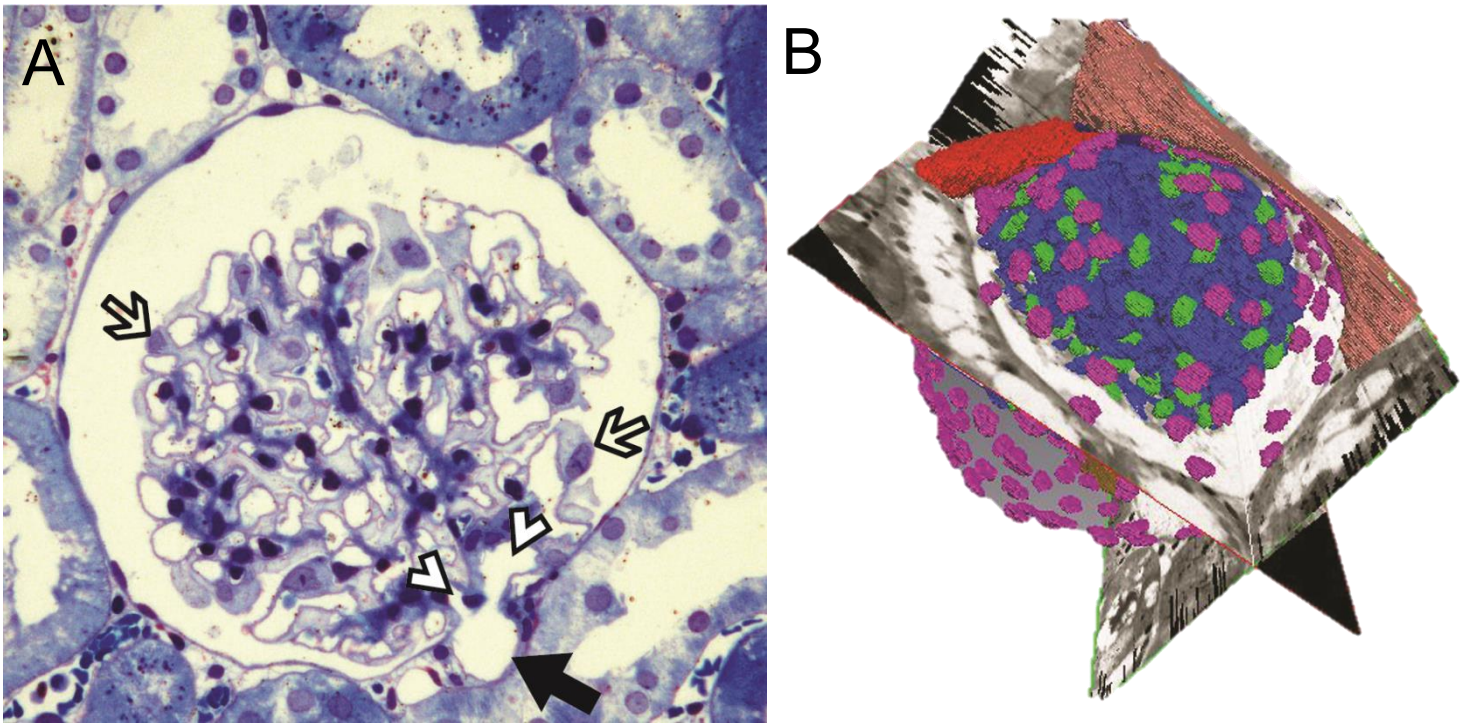

Figure 1.1 | 3D reconstruction from serial sections of the renal corpuscle. A) An image from the original z-stack of serial sections of the kidney renal corpuscle. B) Virtual 3D model of the renal corpuscle created using Amira.[17,18]

1.2 Optical Clearing

To bypass the labour intensive process of serially sectioning and manual segmentation in order to form a 3D reconstruction, in this study, we apply relatively new passive optical clearing methods in conjunction with immunohistochemistry and confocal microscopy to visualize kidney tissues in 3D. These optical clearing methods, such as SeeDB and SCALE, have been developed that render tissues transparent by

reducing the amount of light scattering that occurs within the tissue (Fig. 1.2).[19,20] Although effective for certain applications, these techniques are not compatible with immunohistochemical approaches. This is due to the transparent tissue remaining mostly impenetrable to macromolecules such as antibodies.[19] 3DISCO, a new optical clearing technique, has been used to render different tissues optically transparent and macromolecule-permeable. This is achieved by reducing lipid barriers to antibody labels through the application of hydrophobic clearing solutions to the tissue (Fig. 10.3).[21] However, as with many other clearing protocols, 3DISCO's clearing reagents (tetrahydrofuran and DBE) cause quenching of fluorescent labels in tissue samples.[22] These limitations inspired the recent development of CLARITY, a relatively new optical clearing method that involves polymerization of a hydrogel network within tissue samples and the extraction of lipids via incubation in a hydrophilic clearing solution (Fig. 1.4). This method renders tissues optically transparent and permeable to macromolecular probes and labels, while maintaining the ultrastructural integrity of the sample and the fluorescence of molecular labels (Fig. 1.5).[23] The original CLARITY protocol involved active lipid extraction through electrophoretic tissue clearing. This process is financially more costly and has been shown to cause damage to fine structural details within the tissue, epitope loss, and browning of the tissue due to heating.[15] Consequently, updated CLARITY protocols have been developed that apply passive lipid extraction to render tissues optically transparent while maintaining the cellular integrity of the tissue. Although the optical clearing of tissues has long proven useful for anatomical and biomedical studies, earlier clearing methods have only been optimized and presented for clearing brain tissue, spinal cord, or whole embryos.[24]

These tissues contain relatively large amounts of lipid and little dense connective tissue. A recent study has addressed this issue by slightly modifying the passive optical clearing seen in CLARITY and applying it across different tissues.[24] The result was successful clearing, immunostaining, and low resolution imaging across different organs such as the kidney, liver, gut, and heart (Fig. 1.6). This same study introduced a whole-body clearing method that uses the newly developed CLARITY method as a base. This protocol results in whole-body clearing of mice by delivering a clearing solution containing sodium dodecyl sulfate throughout the circulatory system of the subject.[24] Although this method resulted in optically transparent non-neural organs and the labelling of markers within these organs, imaging of fine structural details at high resolution in optically cleared tissues other than the brain is still lacking. For example, Figure 1.6 demonstrates passively cleared kidney tissue at low resolution. Although the glomeruli are visible in this figure (arrow heads), the glomeruli are not visualized at a scale suitable for the high resolution imaging necessary for the construction of a virtual 3D model. In this thesis, we demonstrate that passive optical clearing is suitable for producing high resolution images of complex microscopic structures in non-neural or embryological tissue such as the kidney glomerulus. From these high resolution images, we develop a virtual 3D model of the glomerulus.

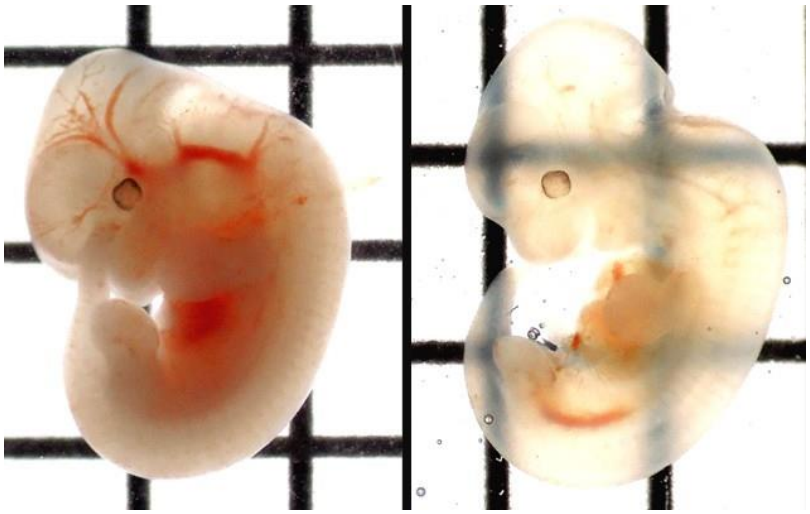

Figure 1.2 | Whole embros rendered optically transparent using SeeDB protocol.[19]

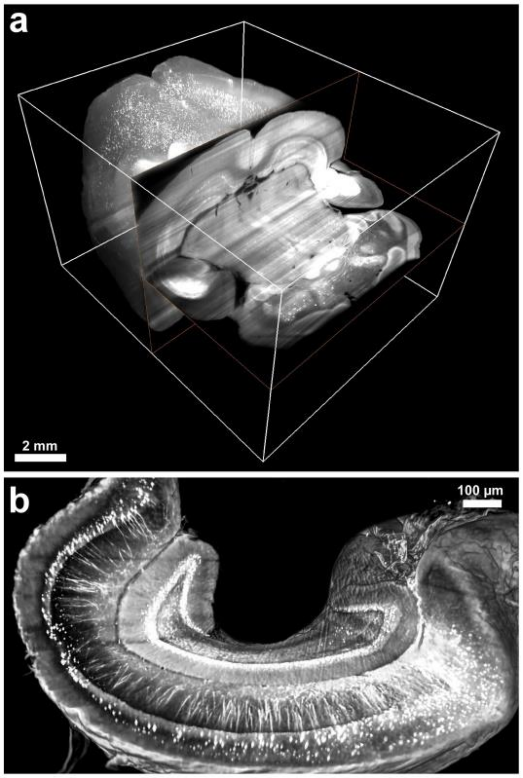

Figure 1.3 | Imaging of brain and hippocampus cleared with 3DISCO. These images demonstrate the neuronal networks of GFP–labeled mice.[22]

Figure 1.4 | Mouse brain tissue at different stages of the CLARITY protocol. A) Optically opaque mouse brain. B) Optically transparent mouse brain following CLARITY. C) Immunolabelled optically transparent mouse brain following CLARITY.[23]

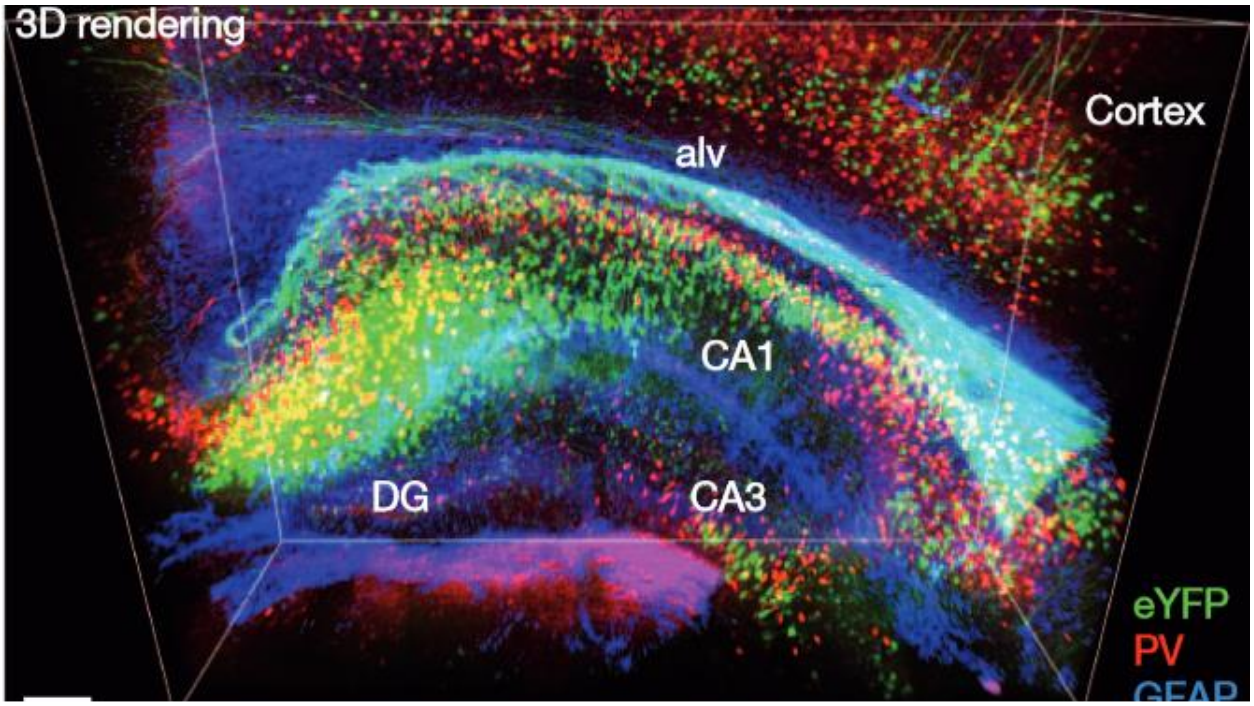

Figure 1.5 | Three-dimensional rendering of mouse hippocampus optically cleared using CLARITY. Green represents eYFP-expressing neurons, red demonstrates parvalbumin-positive neurons, and blue represents GFAP expressing neurons.[23]

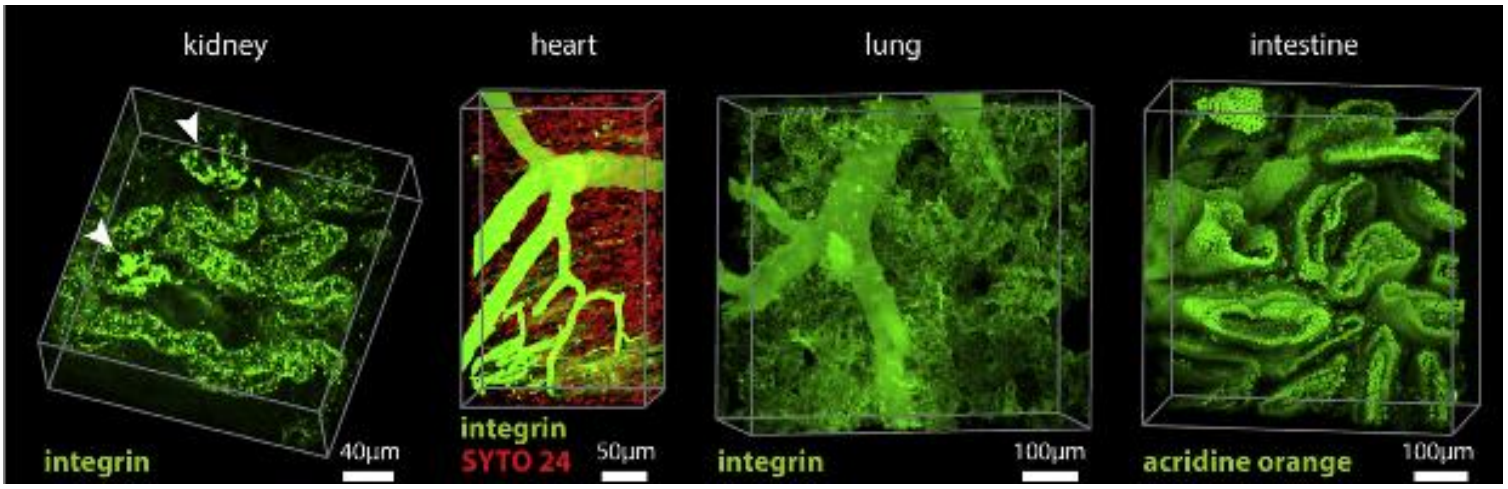

Figure 1.6 | 1 mm thick section of mouse kidney, heart, lung, and 400um section of mouse intestine stained with anti-integrin antibodies, SYTO24 (a green, fluorescent nucleic acid), and acridine orange. These tissues were optically cleared using a protocol similar to a passive CLARITY method. Notice the low resolution images of the kidney glomeruli (arrows).[24]

1.3 The Renal Corpuscle

The renal corpuscle and the glomerulus are complex cellular structures with various cell types. Due to the complex spatial and structural organization of these structures, they are ideal for the development of a 3D model. In addition, by visualizing the renal corpuscle of optically cleared tissue, we demonstrate that passive optical clearing is suitable for producing high resolution images of complex microscopic structures in non-neural or embryological tissue. The glomerulus is a unique network of capillaries found in the renal corpuscle that functions mainly in the filtration of blood. The glomerular capillaries are formed by a fenestrated endothelium as part of the urinary filtration barrier. The glomerular basement membrane, containing type IV collagen, laminins, fibronectin and proteoglycans, is produced by both endothelial cells and podocytes.[25] During glomerulogenesis, the basement membrane of developing endothelial cells fuses with the basement membrane of the developing podocytes. This fusion of two basement membranes results in the glomerular basement membrane (GBM).[26] The composition of the differentiating GBM changes during development. Early in development, the GBM consists of laminin α1β1γ1, whereas the fully developed GBM is composed of laminin α5β2γ1.[27] It has been suggested that laminin α5 may be necessary for proper development and maintenance of fully differentiated glomeruli. In fact, mice that lack laminin α5 fail to form fully developed glomeruli and die before birth.[28] Collagen IV, one of the major structural components of the basement membranes, consists of six known isoform α-chains. These isoforms make three different triple helical heterotrimers that are localized in different parts of the developed glomerulus (Fig. 1.7).[29] Type IV collagen α1α2α1 is located within the mesangial matrix

of the renal corpuscle and type IV collagen α3α4α5 is localized to the GBM. The basement membrane of the parietal epithelial layer of Bowman's capsule of the renal corpuscle is comprised of type IV collagen α1α2α1 and type IV collagen α5α6α5.[26] Immunostaining of collagen type IV and laminin in the adult kidney allows for visualization of the GBM and Bowman's capsule.

Podocytes are epithelial cells that constitute the visceral layer of the Bowman's capsule in the renal corpuscle. These cells form interdigitating processes that are connected by a filtration slit diaphragm.[30] Although the molecular composition of the podocyte cell adhesion complexes has mainly been determined, the composition of the slit diaphragm had remained relatively unclear.[31] Recently, however, several novel proteins of the slit diaphragm have been identified.[32] Nephrin, a glycoprotein, has been identified in the intercellular junctions of podocyte secondary foot processes (Fig. 1.8). Nephrin is a cell adhesion molecule of the immunoglobulin superfamily and participates in cellular interactions within the slit diaphragm.[33] A mutation in the gene coding for this protein has been found to be the underlying cause of congenital and acquired nephrotic syndrome. This disease manifests when glomerular podocytes fail to form normal foot processes or slit diaphragms.[31] The localization of nephrin within the filtration slit and the fact that the protein is mutated in congenital nephrotic syndrome indicates an important role for the slit diaphragm in the production, preservation and permeability of the GBM.[31] Immunolocalization of nephrin has also proven a useful tool for whole mount immunostaining when imaging the glomerulus.[32] Further investigation of this protein may also provide new avenues for examination of various histochemical inquiries.

Immunostaining of these antigens specific to the podocytes and GBM of the renal corpuscle, followed by confocal microscopy and 3D rendering may provide important physiological and anatomical insight into the true spatial and functional organization of the glomerular filtration barrier by allowing visualization of specific structures of the renal corpuscle such as the GBM, Bowman's capsule, and the surrounding renal tubules (Fig. 1.9). Visualization of these structures through immunolabelling will enable the development of 3D virtual models of the renal corpuscle for e-learning purposes.

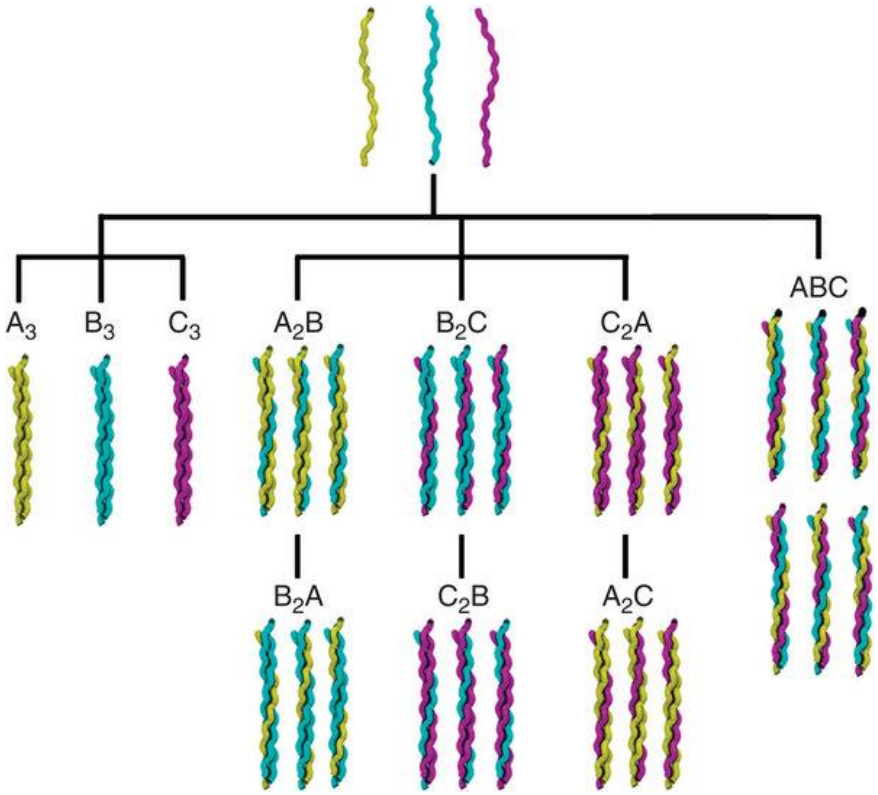

Figure 1.7 | Schematic representation of the possible collagen IV heterotrimer isoforms that can form.[34]

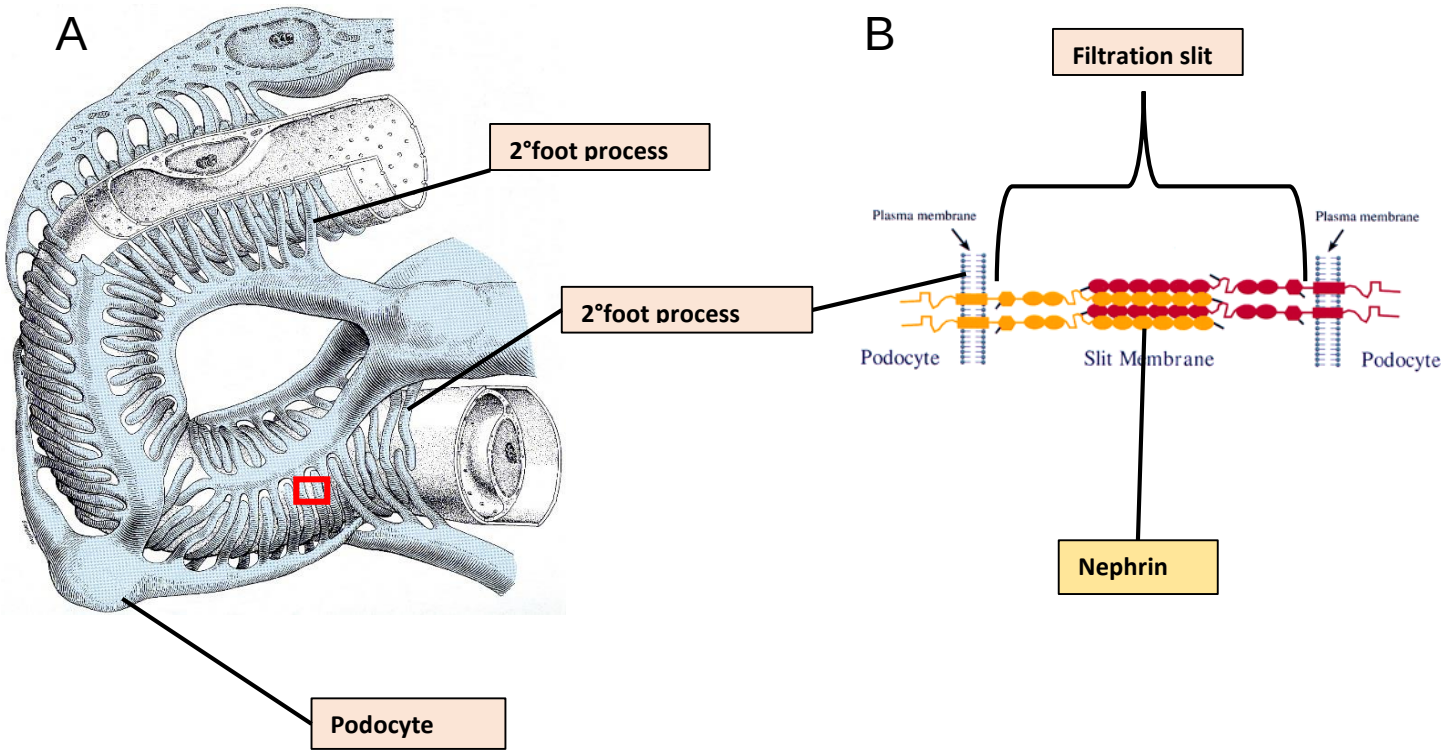

Figure 1. 8 | A) Representation of the podocytes forming an interdigitating filtration barrier around the glomerular capillary via secondary foot processes. B) Representation of an individual filtration slit. Notice the presence of nephrin within the filtration slit. (Copyright © by the National Academy of Sciences)[35,36]

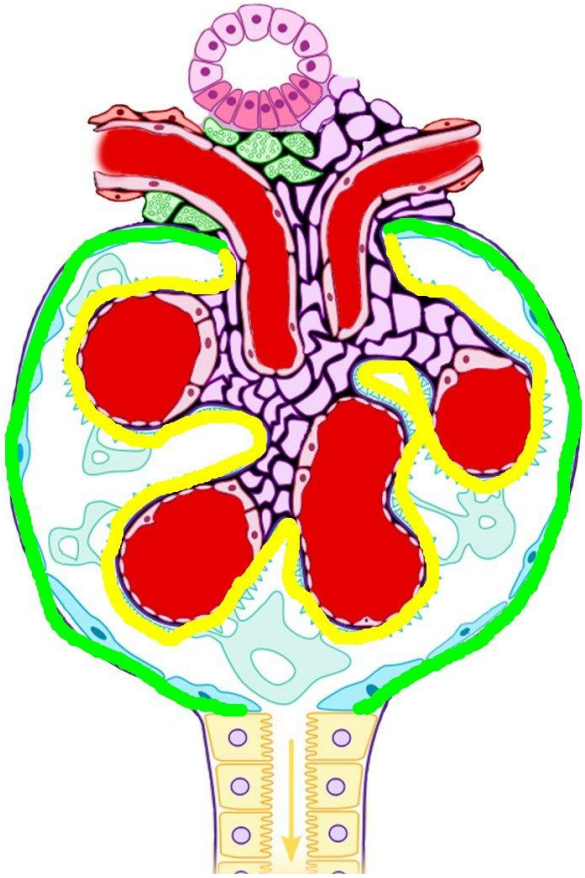

Figure 1.9 | With the goal of creating a comprehensive 3D model of the kidney renal corpuscle, cortical kidney tissue was immunolabelled for nephrin or a combination of nephrin and collagen IV/Laminin α5. Staining of nephrin will localized to filtration slit barrier of the glomerulus (red). Collagen IV and laminin α5 would be expected to localize to the filtration barrier and Bowman ’s capsule of the renal corpuscle. **(**Michal Komorniczak, Poland)

1.4 Purpose and Objectives

The purpose of this study was to test the hypothesis that optical clearing in conjunction with immunohistochemical staining of select cellular components allows 3D visualization of the renal corpuscle at high resolution superior to 3D reconstruction from serial histological sections for research and education. To achieve these goals we set the following objectives:

1. Optically clear kidney tissue using CLARITY passive optical clearing.
2. Visualize podocyte specific antigens in the glomerulus and renal corpuscle by immunohistochemistry and confocal microscopy
3. Visualize antigens specific to the Bowman's capsule of the renal corpuscle, the surrounding tubules, and the glomerular basement membrane by immunohistochemistry and confocal microscopy.
4. Create a 3D reconstruction of the kidney glomerulus for future e-learning purposes.

Chapter II: Materials and Methods

2.1 Reagents

The samples to be imaged were extracted from wild type mice. The chemicals used in preparation of the hydrogel monomer solution are listed in **table 2.1**. The chemicals used in preparation of the clearing solution are listed in **table 2.2**.

Table 2.1. Recipe: Hydrogel Solution (400mL)

Ingredient	***Add:***	***Final Concentration***	***Supplier***
Acrylamide (40%)	*40 mL*	*4%*	(40% (wt/vol); Bio-Rad, cat. no. 161-0140
Bis (2%)	*10 mL*	*0.05%*	2% (wt/vol); Bio-Rad, cat. no. 1610142
VA-044 Initiator	*1 g*	*0.25%*	Wako, cat. no. VA-044
10X PBS	*40 mL*	*1X*	-
16% PFA	*100 mL*	*4%*	-
dH2O	*210 mL*	*-*	-

Table 2. Recipe: Clearing Solution (10L)

Ingredient	***Add:***	***Final Concentration***	***Supplier***
Boric Acid	*123.66g*	*200mM*	Sigma-Aldrich, cat. no. B7901
Sodium Dodecyl Sulfate	*400g*	*4%*	Sigma-Aldrich, cat. no. L337
dH2O	*Fill to 10L*	-	-
NaOH	*To pH 8.5*	-	EMD, cat. no. SX0590-3

The following primary and secondary antibodies were purchased for immunostaining (**Table 3**). All antibodies were diluted with PBST (1X PBS with Triton-X-100 (0.1% wt/vol)).

Table 2.3. Antibodies for staining 1-mm-thick cortical kidney tissue block

Concentration/Dilution	***Antibody***	***Supplier***
0.2 mg/ml / 1:20	*Goat Anti-Nephrin*	*R&D Systems, cat. no. AF3159*
0.9 mg/ml / 1:20	*Rabbit Anti-Podocalyxin*	*Novus Biological, cat. no. NB110-41503*
1:20	*Rabbit Anti-Collagen IV*	*AbD Serotec, cat. no 2150-1470*
	Rabbit Anti-Laminin α	*Santa Cruz Biotech, cat. no. sc-20145*
1:200	*Donkey Anti- Goat NL 557*	*R&D Systems, cat. no. NL001*
1:100	*Donkey Anti-Rabbit NL493*	*R&D Systems, cat. no. NL006*

The following chemicals and equipment were used for sample mounting and imaging:

- 70% sorbitol (w/v) (Sigma-Aldrich, cat. no. S1876)
- Glass-bottom-dishes, MatTek Corporation, cat. no. P35G-1.5-14-C
- Coverslips
- Zeiss Multiphoton Confocal Microscope (Carl Zeiss LSM 510 META)

2.2 Tissue Preparation

Mice were transcardially perfused with 20 mL of cold 1X PBS followed by 20 mL of thawed hydrogel solution containing acrylamide and paraformaldehyde. Hydrogel solution is stored at -20℃. The fixed kidneys were then excised and immediately placed in 20 mL of cold hydrogel solution and incubated at 4℃ in a 50 mL conical tube for 3 days to allow for diffusion of the hydrogel solution into the tissue.

2.3. Hydrogel Tissue Embedding

After the kidneys have been prepared via perfusion and hydrogel solution incubation, oxygen must be removed from the system to allow for proper hydrogel polymerization. The system was degassed by transferring the kidney sample and hydrogel solution to a smaller conical tube such that the volume of the solution completely filled the container and there was little to no excess air inside. There are several alternatives to the degassing procedure that can also be used to remove oxygen inhibition during hydrogel polymerization. After degassing, the hydrogel solution containing the sample was polymerized by incubating at 37℃ in a water bath rotating at 37 r.p.m for 3 hours (Fig. 2.1). In a fume hood, the embedded kidney samples were then removed from the crosslinked hydrogel and freed from externally adhering gel. The sample was then washed with 50 mL of clearing solution for 24 hours at 37℃ to dialyze out remaining hydrogel monomers and PFA. This step was repeated two more times to ensure removal of residual PFA and monomers, thereby preventing further polymerization and crosslinking inside the tissue.

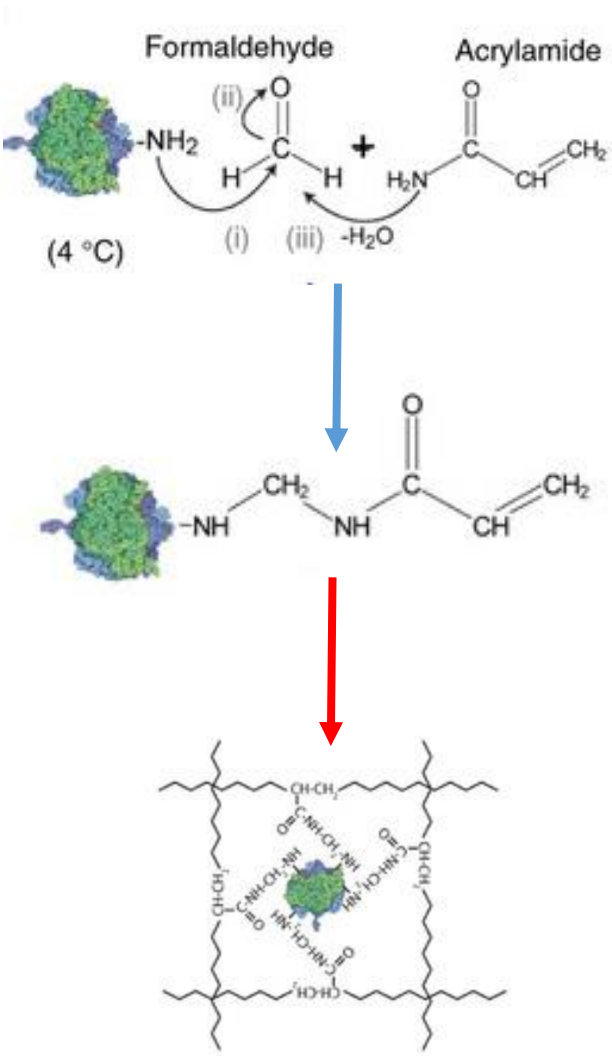

Figure 2.1 | Hydrogel embedding overview. The kidney sample is perfused with cold HM solution that contains a mix of acrylamide, bis-acrylamide, paraformaldehyde and thermal initiator. Formaldehyde mediates cross-linking of biomolecules to acrylamide monomers via protein amine groups. Incubation of the sample at 37℃ initiates hydrogel polymerization resulting in a crosslinked meshwork within the tissue.[15]

2.4 Passive Optical Clearing

Sectioning of the tissue before passive optical clearing is recommended if only studying smaller subsections of the tissue sample. Since passive optical clearing and immunostaining occurs more quickly in smaller tissue sections rather than in whole organs, the medulla of the kidneys was removed and cortical tissue was cut into 1mm thick slices. At this point in the process, the tissue biomolecules (mainly proteins and amino acids) other than lipids are linked to the polymerized hydrogel matrix. The clearing solution is composed of an ionic detergent that removes all lipids from the tissue. This leads to an optically transparent tissue. Kidney tissue was incubated in clearing solution in a water bath of 37℃ rotating at 37 r.p.m.. This process was continued until the tissue was visibly transparent (approximately 40 days) (Fig. 2.2).

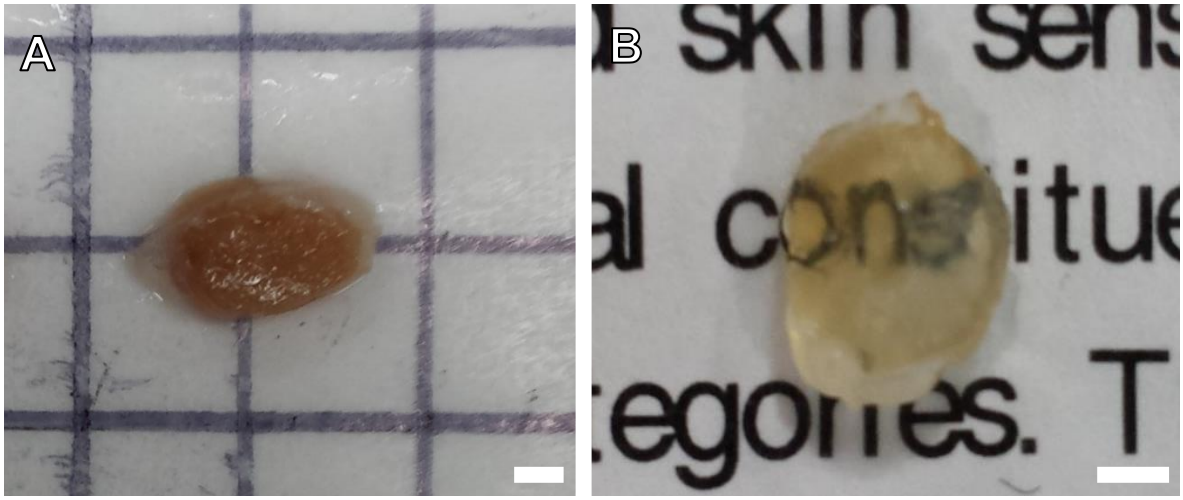

Figure 2.2 | Passive clearing overview. Lipid membranes are extracted by passive optical clearing at 37℃ in clearing solution. The resulting tissue is optically transparent and permeable to macromolecular labels. The cleared tissue can undergo multiple rounds of immunohistochemistry and microscopy. A) Optically opaque kidney before optical clearing. B) Optically transparent kidney section following optical clearing. Bar = 2 mm

2.5 Immunostaining

Following optical clearing, kidney tissues were washed in PBST buffer for 2-5 days to remove residual clearing solution. The samples were then incubated with goat anti-nephrin, rabbit anti-podocalyxin, rabbit anti-laminin alpha 5 or rabbit anti-collagen IV primary antibodies in PBST (1X PBS with Triton-X-100, pH to 7.4) at 37℃ for 4-7 days. Some samples underwent double labelling of nephrin and collagen IV, or nephrin and laminin alpha 5. These samples were incubated in a solution mix of goat anti-nephrin and rabbit anti-collagen IV (1:10-1:20), or goat anti-nephrin and rabbit anti-laminin alpha 5 (1:10-1:20), respectively. Samples were then washed with PBST for 48 hours then incubated with donkey anti-goat IgG Northern Lights 557 and donkey anti-rabbit IgG Northern Lights 493 secondary antibodies (1:200) in PBST at 37℃ for 4-5 days. Samples were then washed with PBST over the course of 2 days.

2.6 Sample Imaging and Processing

Following a 2 day PBST buffer wash, the kidney sections were incubated in sorbitol refractive index matching solution (sRIMS - 70% sorbitol (w/v) (Sigma S1876) in 0.02 M phosphate buffer with 0.01% sodium azide, pH to 7.5 with NaOH) for 1-4 days to re-establish transparency. When ready for imaging, the samples were mounted in sRIMS using 3.5 mm glass bottom culture dishes. Images were captured on a confocal microscope (Carl Zeiss) using a 40X water-immersion objective lens and saved in uncompressed TIFF format at 8-bit RGB color depth. Subsequently, the images were edited using Zen (Zeiss Imaging Software). Images were cropped and smoothened

using the Zen Software to reduce background noise. Images were then edited using Image J. First, dust and artifact were removed using the ‘Despeckle’ tool. Images were adjusted for colour using the ‘Levels’ tool. Finally, overall image brightness and contrast were adjusted. All images were stored in both 8-bit RGB and 8-bit grayscale uncompressed TIFF format. The grayscale images were used for constructing a virtual 3D model of the glomerulus.

2.7 3D Renal Corpuscle Reconstruction

Grasycaled image z-stacks were loaded into the 3D program, Amira 4.1 (Mercury Computer System Inc., Chelmsford, MA) using the import function. The imported z-stacks are arranged in the XY axis. The stack can then be visualized from the XY axis, XZ axis and YZ axis using the *orthogonal slices* tool. Amira allows the visualization of multiple orthogonal slices in different orientations. Orthogonal slices in each axis can be manipulated to display different slices across each axis.

In order to develop an accurate isosurface rendering of the renal corpuscle image stack, modification of the black and white threshold values must occur. This separates background pixels from the histological structures of interest. Once the proper threshold values were determined, the data set was resampled with a coarser resolution. This step is not necessary, but results in increased rendering performance. Following resampling of the image stack, an isosurface of the data was created using the *Isosurface* module. This results in a 3D representation that encompasses all parts of the volume of the glomerulus that are brighter than the threshold value set earlier.

Volume rendering is another useful tool for developing a virtual 3D model. This technique produces a 3D representation of the data set based on the emission and absorption spectra of light at each voxel of the structure of interest. A volume rendering of the renal corpuscle was produced using the *Voltex* module in Amira.

Chapter III: Results

3.1 Optical Clearing

After hydrogel embedding of the tissue, 1 mm thick sections of the cortex were optically cleared by incubation in optical clearing solution at 37℃ (Fig. 3.1). Tissues were placed on printed text or on paper with drawn grids to evaluate optical transparency. Tissues became transparent over a period of 40 days. Clearing was rather homogeneous throughout the tissue and printed text was clearly visible underneath the tissue (Figure 3.1).

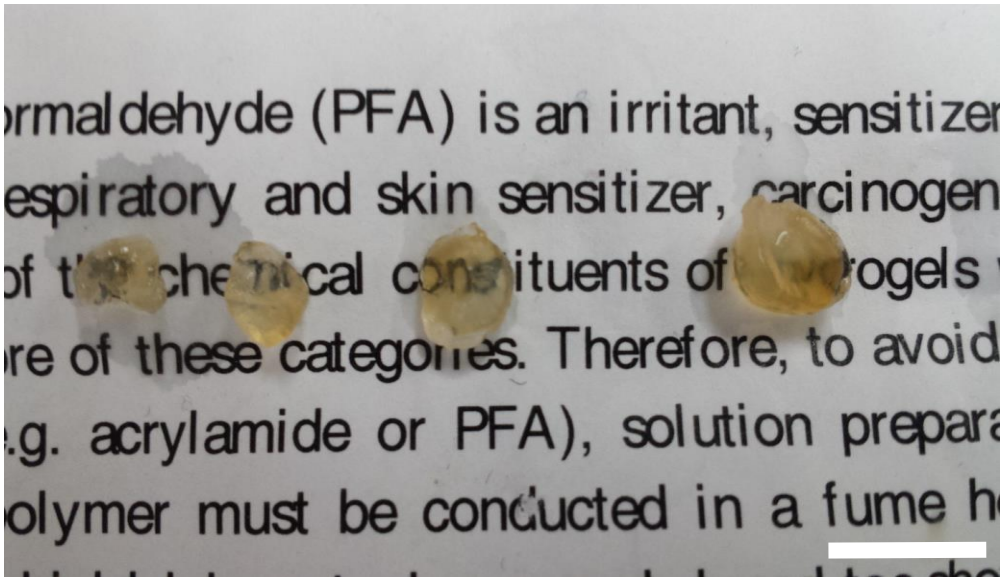

Figure 3.1 | Mice kidney sections following approximately 40 days of passive optical clearing. Homogenous transparency is seen throughout the tissue. These sections were sufficiently transparent for imaging without further optical clearing. Bar = 10 mm

3.2 Immunohistochemistry and Imaging

After rendering kidney sections optically transparent, the tissues were labeled with anti-nephrin, anti-collagen, and anti-laminin antibodies to confirm that the optically cleared tissues were sufficiently macromolecule permeable, and to allow for labeling of specific proteins of the glomerulus and renal corpuscle. As shown in Figure 3.2, we attained effective antibody penetration through 1 mm thick cortical kidney tissue. After attaining immunostained, optically transparent kidney tissue, the next challenge is to visualize to suitable depths within the tissue. Through laser scanning confocal microscopy, we were able to image 1 mm sections of optically cleared cortical kidney tissue. Figure 3.3 demonstrates a glomerulus of a 1 mm section of kidney tissue following antibody labeling of nephrin and confocal microscopy. We were able to scan the entirety of a glomerulus (diameter = 80 μm) without further need for sectioning. A z-stack of 100 sections at increments of 0.8 μm was produced. Using Zeiss 3D reconstruction software, we were able to produce a 3D representation of the kidney glomerulus (Figure 3.3 D).

Immunostaining of antigens specific to the podocytes and GBM of the renal corpuscle, followed by confocal microscopy and 3D rendering may provide important morphological insight into the structural organization of renal corpuscle. Labelling of nephrin (localized to the filtration slit) and collagen IV or laminin α5 (expected to localize to the GBM and Bowman's capsule) will enable the development of a comprehensive 3D virtual model of the renal corpuscle for e-learning purposes.

Figure 3.4 demonstrates a composition of one slice from a 1mm thick image stack of cortical kidney tissue double-labelled for both nephrin (red) and type IV collagen (green) or laminin α5 (green). As we expected, nephrin staining was localized to the filtration slit diaphragm and appeared to tightly cover the glomerular capillary network. This filtration barrier covers the fused basement membrane of the endothelial cell and podocytes. As we anticipated, collagen IV was localized to the Bowman's capsule and surrounding tubules of the kidney. However, to our surprise, there was no signal attained in the GBM through labelling of collagen IV. Instead, we observed substantial staining of the mesangial matrix. This suggests that the antibody may recognize a collagen IV isotype that is enriched in the mesangial extracellular matrix, but lacking in the GBM. Laminin α5 was localized to the Bowman's capsule of the renal corpuscle as well as to the glomerular basement membrane. Co-localization of nephrin and laminin α5 (indicated by superimposition of both nephrin and laminin α5 signals) demonstrate areas of the endothelial surface within the glomerulus that is in contact with the filtration barrier (Figure 3.4).

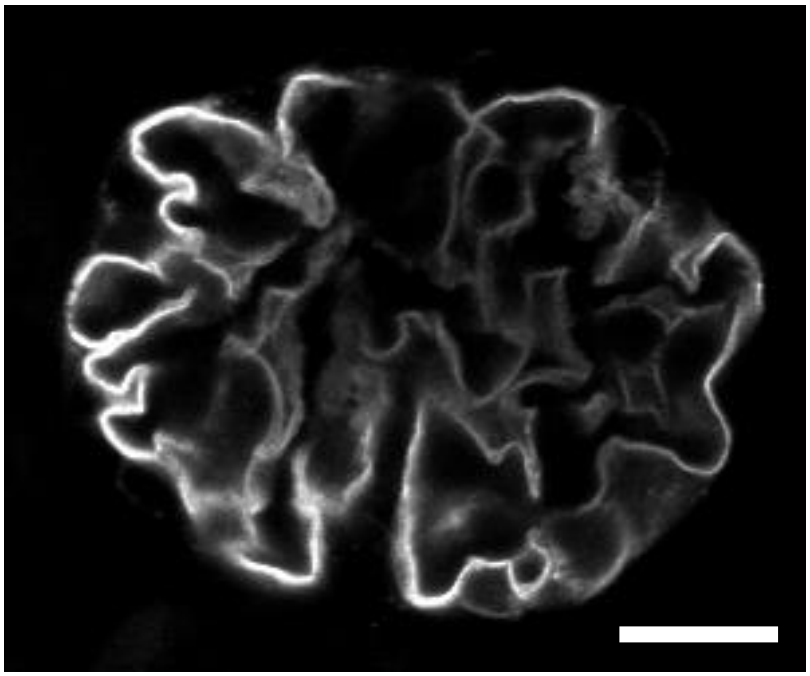

Figure 3.2 | Glomerulus from 1 mm section of cortical kidney tissue stained with anti-nephrin antibodies. As observed in this sample, successful primary and secondary antibody penetration was attained in 1 mm thick cortical kidney sections. Nephrin is localized to the filtration slit of the filtration barrier of the glomerular capillaries and podocyte secondary foot processes. Bar = 20 µm

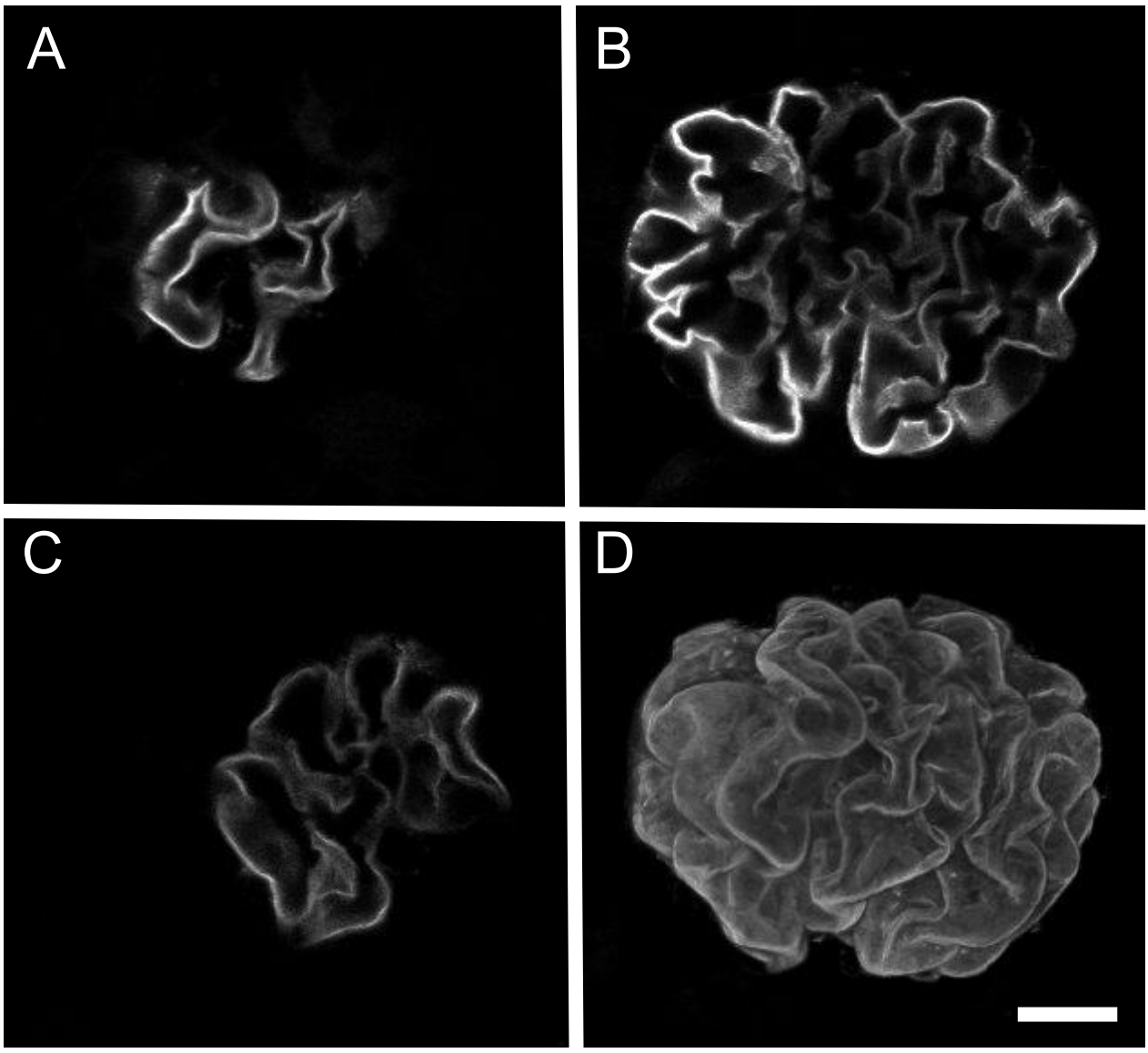

Figure 3.3 | Confocal images of individual optical sections (A-C) in a Z-stack through a renal corpuscle show that nephrin is localized to the filtration barrier of the glomerular capillaries and podocyte secondary foot processes. The software-generated 3D reconstruction of the entire Z-stack (100 optical sections) visualizes the surface of the glomerular capillaries that participate in urinary filtration. Bar = 20 µm

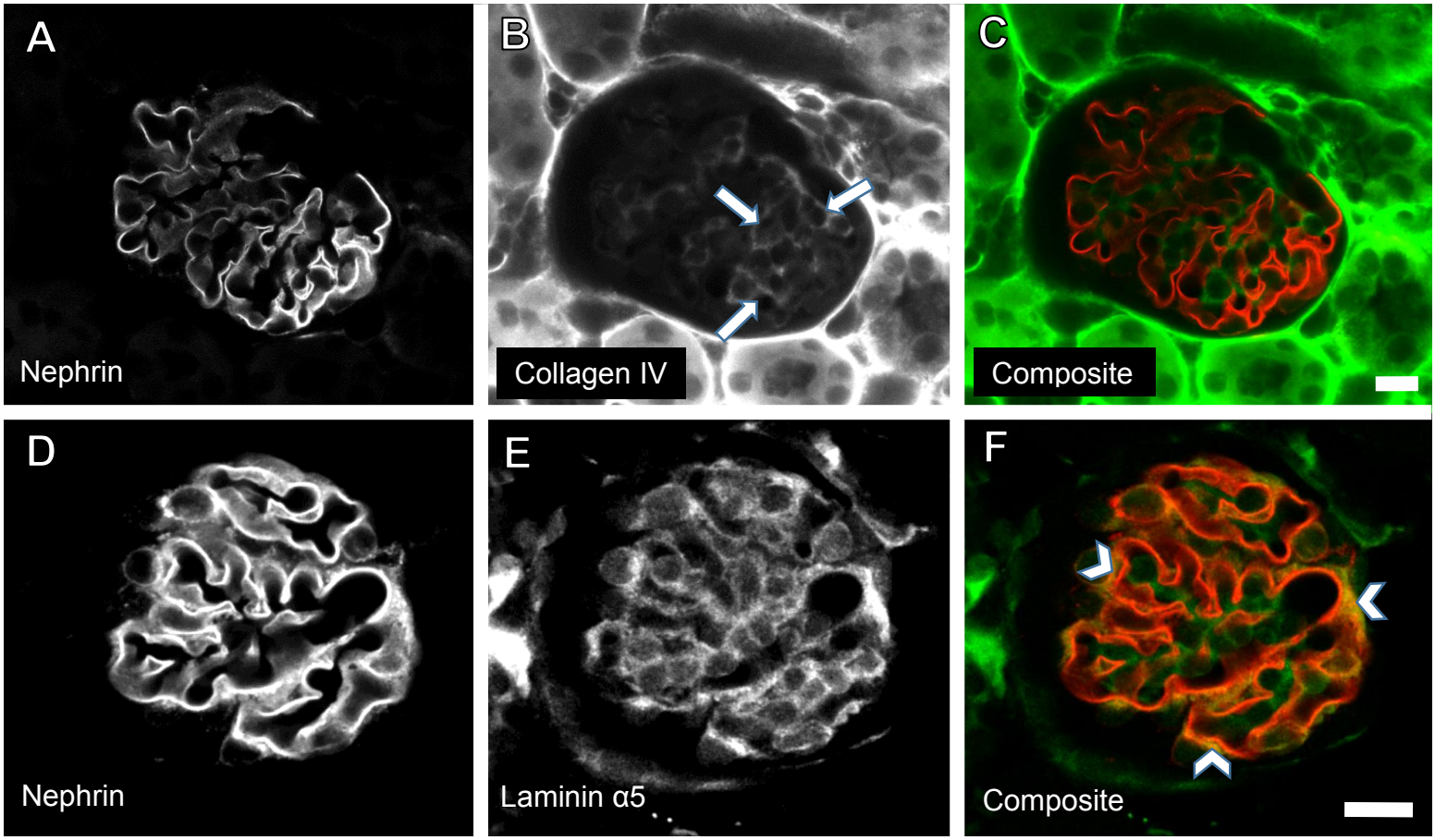

Figure 3.4 | Confocal images of individual optical sections through a renal corpuscle stained for nephrin & Collagen IV (A-C); and for nephrin & Laminin α5 (D-F). Nephrin staining is localized to the filtration barrier of the glomerular capillaries and podocyte secondary foot processes (A & D). Collagen IV is localized to the basement membrane of the renal tubules, the Bowman's capsule, and the mesangial matrix (B). Laminin α5 is localized to the Bowman's capsule and glomerular basement membrane (E). The composite images reveal that only Laminin α5 co-localizes with nephrin to basement membrane regions in the glomerulus (F – arrow heads). Collagen IV appears to be enriched in the mesangial ECM and does not co-localize with nephrin (B –arrows & C). Bar = 20 µm

3.3 Reconstruction and Virtual 3D Model

Amira is an ideal software for 3D modelling of complex cellular structures as it allows visualization of data sets as image volumes or geometrical iso-surfaces. 3D structures can easily be analyzed, quantified, and manipulated for greater understanding of the structures of interest.

A 3D reconstruction of a kidney glomerulus immunolabelled for nephrin-antigens was developed through volume rendering (Figure 3.5 A-C) and surface rendering (Figure 3.5 D). Certain modules within Amira, such as the orthogonal slices tool, allow the user to scroll through an image stack in any axis and visualize the corresponding 2D slice of that section of the 3D structure in any orientation. Amira also provides the user with the ability to view the 2D sections of the 3D model in any plane through the generation of images of virtual sections that can be visualized in any orientation. The software therefore serves as a “virtual microtome”, allowing visualization of digital sections of tissue from reconstructed 3D model.

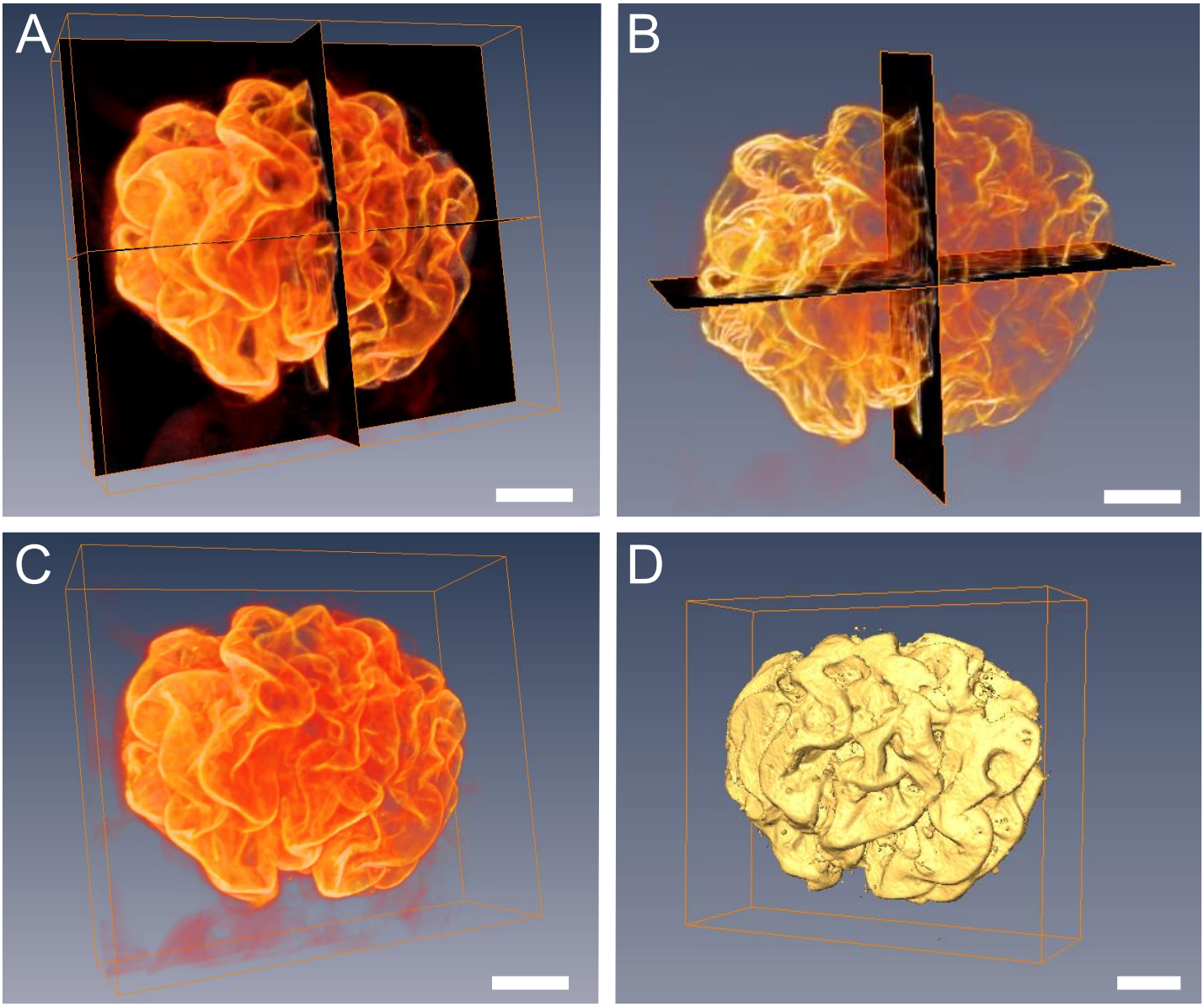

Figure 3.5 | Confocal image stacks of a 1mm section of cortical kidney were imported into Amira software. 3D reconstruction of a renal corpuscle labelled for nephrin through either volume rendering (A-C) or isosurface rendering (D) provides a virtual model of the glomerulus. The virtual model can be viewed with orthogonal slices in all three (XY, XZ, YZ) axes (A). These slices can be manipulated through the model to display different slices. Bar = 20 µm

Chapter IV: Discussion

In this thesis, we outline the steps necessary for developing a morphologically accurate virtual 3D representation of parts of the renal corpuscle using optically cleared kidney tissue for use as a virtual tool in E-learning settings. In particular, we imaged by confocal microscopy the distribution of the podocyte marker nephrin that identifies the filtration slit diaphragm overlying glomerular capillaries. We then used Amira software to reconstruct the filtration barrier in 3D. When studying histological structures, students generally learn by viewing 2D images. They must then infer the 3D organization of the biological structures from the 2D images in order to develop an improved understanding of the often complex spatial relations and morphology of the 3D histological structures. It is hypothesized that by viewing 2D images of a histological structure in conjunction with a virtual 3D model of the same structure will help students bridge the cognitive gap between 2D histological visualization and 3D comprehension of the organization and morphology of the structure. However, there is a scarcity of research on the subject. Nonetheless, there has been an exponential growth in the use of virtual 3D images and models in anatomy education.[7,9,37,38] The visualization of 2D neuroanatomical images is subject to similar cognitive constraints as the visualization of 2D histological images. In both fields, it is necessary to infer complex 3D information from 2D images. Ruisoto et al. analyzed whether 3D visualization of the anatomy of the brain aids students to discover and identify subcortical structures more accurately than viewing typical cross-sectional 2D images used in neuroanatomy education.[37] Participants were either provided with a 3D volumetric model of brain or 2D-cross sectional images and were tasked with identifying sub-cortical brain structures. The percentage of correct

identification of subcortical structures and level of confidence in responses were significantly higher in the 3D group. Additionally, the response time was significantly lower in the 3D group compared to the 2D group. Furthermore, 3D visualization facilitated the localization of difficult subcortical structures more than the 2D visualization.[37] This study confirms the benefits of 3D visualization as opposed to 2D visualization of complex structures. It is suggested that this mode of learning is promising for anatomical education as it allows learners to develop a more precise understanding of 3D structures and their spatial relationships.[37] The virtual 3D reconstruction of the kidney glomerulus developed in this report allows for the visualization and navigation of the 3D structure, while incorporating the ability to view the 2D immunohistochemical images at different orientations through the structure. Since this approach incorporates the ability to visualize and manipulate 2D images in the context of an interactive 3D virtual tool, students should be able to analyze the 2D histological sections with greater understanding and confidence.[37] Further research is required to determine the efficacy of 3D histological models in histology education and how these digital tools may help students interpret important biological structures and improve understanding of complex histological concepts. The development of virtual 3D models of histological structures through the methods outlined in this thesis, therefore, may prove useful in histology education.

Traditionally, histological 3D models are developed through reconstruction of histologically-stained semi-thin sections. In a study performed by Roth et al., a virtual 3D model of a renal corpuscle was developed from ribbons of serial histological sections for incorporation into digital 3D e-learning tools.[17] The kidney tissues were first

fixed by perfusion and embedded. The embedded tissue was then trimmed with a stereo microscope. The slides used to mount the tissues then needed to be properly treated prior to sectioning. The kidney tissues were then sectioned into ribbons of semi-thin sections (25 sections/ribbon). Ribbons were then guided onto the treated slide, dehydrated, and stained. The slides were then imaged, processed, and imported into Amira for development of a 3D model of the renal corpuscle. This process requires the manual segmentation of a region of interest from each image. Once all the structures of interest were segmented, polygonal mesh surfaces were separately created for each individual structure.[17] Compared to other modes of 3D imaging such as microCT, microMRI and optical coherence tomography, the major drawback of 3D rendering of physical sections is the time and labour demanded by the technical procedure of producing multiple semi-thin serial sections. The process of using serial section image stacks to develop high resolution 3D rendering is time-consuming and prone to many potential error sources.[39] The production of histological sections may result in tissue damage or loss and distortion during histological processing.[1] The processing of serial physical sections may also be prone to misalignment, geometric distortion and variation in staining.[39] Furthermore, trimming and sectioning is difficult to master and requires many hours of practice to learn. In addition to the time required for manual sectioning of the tissue, the manual segmentation of each individual section required in this procedure is very time consuming and it is not always possible to segment individual cells of histologically stained tissues due to potential lack of resolution and necessary magnifications.[17] Further studies would have to be performed to determine the implications of these limitations in histology education. Although the total amount of

man-hours required of this procedure depends greatly on the number of structures that have to be individually segmented and the number of sections needed, this procedure is substantially more time-consuming and labour intensive than the procedure of using optically cleared, immunostained tissues for the creation of a 3D reconstruction. Therefore, while the development of an accurate virtual 3D model of histologically stained tissues is achievable, the amount of labour and man-hours required of this process may prove detrimental and debilitating in the creation of digital 3D learning tools. In this respect, the use of passive optical clearing techniques that circumvent the need to physically section the tissues and manually segment individual structures may prove beneficial in the production of virtual 3D tools for use in histology education.

Herein, we utilize a passive optical clearing method that renders kidney tissue optically transparent in order to avoid the need for physical sectioning. This allows imaging of thick (>1 mm) sections of tissues by confocal microscopy while maintaining the structural integrity of the tissue and preserving fluorescent signals. Passive optical clearing techniques such as CLARITY allows for the visualization of biological systems at unprecedented depths and resolution. The hydrogel-tissue hybridization preserves important biomolecules such as proteins and nucleic acids while the removal of lipid membranes allows for molecular labelling of the retained biomolecules through passive diffusion of macromolecular probes such as antibodies.[15] The preservation of the structural integrity of the tissue through the hydrogel-tissue hybridization and the porous nature of the optically cleared tissue allows for multiple rounds of antibody staining, destaining and restaining that would not normally be possible with tissues fixed with conventional methods.[40] Confocal microscopy is appropriate with passive optical

clearing because the excitation and emission wavelengths of light can penetrate relatively deep into the optically transparent tissue.[40] The transparency of the optically cleared tissues allows for high resolution 3D visualization of complex cellular structures such as the glomerulus without the necessity of labour-intensive and time-consuming serial sectioning. In addition, this process circumvents the need for manual segmentation of each individual structure of interest as channels depicting different structures can be split, incorporated into Amira, and merged. This may prove useful in histological education, as we are able to visualize specific cellular structures in 3D to grasp a greater understanding of the true spatial organization of biological systems.

Whether through immunolabelling of optically cleared tissue or through the physical sectioning of histologically stained tissue, the use of biologically accurate 2D images to construct a virtual 3D model holds several advantages. By developing a virtual 3D model based on morphologically accurate 2D images rather than on diagrammatic representations, the model will be a true depiction of the spatial organization and relationships of the structure of interest.[17] Therefore, by using true image stacks to form the 3D reconstruction, the model is accurate and unlikely to represent false morphological and structural information.[17] In addition, the original 2D images can be incorporated into the completed 3D model, thereby allowing the user to visualize the 2D slices in conjunction with the 3D reconstruction. This allows the incorporation of both 2D and 3D modes of learning into a single model.

4.1 Limitations

CLARITY is a relatively new technique that will require optimization as certain limitations currently exist. Firstly, the procedure of passively clearing 1mm sections of kidney tissue took approximately 40 days. Although there is very little labour involved in this process, this is still a substantial amount of time. In addition, when labelling thick optical sections, antibody penetration of the entire section can be difficult to attain. Fortunately, by altering the concentration of hydrogel monomers with the hydrogel solution, optical clearing time can be reduced and macromolecular penetration can be increased. Yang et al. hypothesized that a decrease in hydrogel monomer concentration would result in faster lipid extraction and increased macromolecular penetration into thick tissue during immunohistochemistry.[24] They found that the efficiency of tissue clearing and the depth of antibody penetration increased significantly when lower concentrations of formaldehyde and acrylamide were used in the hydrogel solution. It was also found that the lower concentration hydrogel monomer solution was sufficient for the preservation of tissue morphology and structure, with very little protein leaking found from the crosslinked tissue.[24] In this thesis, we used high incubation temperature and time to ensure sufficient antibody penetration of 1mm thick cortical kidney sections. However, when staining whole organs, reduction of hydrogel monomer concentration may be necessary to ensure complete macromolecular penetration. We detected very little background fluorescence and little to no photobleaching occurred 7-10 days post-staining when imaging 1 mm sections of kidney tissue. In addition, tissues remained suitable for imaging up to two months after initial staining with little to no signal loss. However, confocal microscopy on optically cleared tissues has been shown

to induce substantial photobleaching of fluorescent labels when applying slow, high resolution, whole-organ imaging.[23] Two-photon microscopy addresses this issue, at the cost of increased imaging times. Certain microscopy techniques such as selective plane illumination microscopy may be more suitable to optical clearing techniques when imaging large samples at high resolution and high image acquisition rates.[40] Although imaging of optically cleared, antibody-labeled tissue with conventional confocal microscopy techniques provides high resolution visualization of detailed cellular structures, the level of resolution doesn't quite match the resolution seen with electron microscopy.[23] Fortunately, optically cleared tissues may also be compatible with electron microscopy. However, due to the lack of lipid membranes within the cleared tissues, conventional electron microscopy staining does not currently provide enough contrast to be useful in the visualization of all relevant ultrastructural features.[41] Therefore, further optimization of this technique may be necessary for compatibility with optically cleared tissues.

Although the procedure of developing a 3D reconstruction from optically transparent tissues is far less labour-intensive and time consuming than the procedure of physically sectioning and reconstructing multiple semi-thin slices, this procedure may hold certain disadvantages to physical sectioning. When creating a 3D reconstruction from optical sections of transparent tissue, the number of potential structures that can be individually segmented in the 3D model is limited to the number of distinct structures that can be labelled through immunohistochemical techniques. In addition, the success of this procedure is highly reliant on the use of good antibodies that demonstrate high affinity for their specific epitope. Due to the thickness of the samples being stained, high

quality antibodies are needed to exhibit specific staining and penetration of the entire section being labelled.

The success of this technique is also highly reliant on the 3D software used for the development of the 3D reconstruction. Amira may not be the ideal software for developing a learning tool. It has an expensive license, high graphical processing requirements, and the interface of the program is too complex to serve as an interactive virtual model to be used easily by students. Nonetheless, due to this software's highly effective 3D rendering capabilities, a 3D reconstruction developed in Amira can serve as a framework for a future learning tool.

4.2 Future Directions

Without being incorporated into a learning tool, the glomerulus model created is not yet accessible or useful for education in histology courses. Therefore, further work in the development of a usable, interactive learning tool of the kidney renal corpuscle is needed. This tool should incorporate the ability to view the original 2D biological sections in the context of the reconstructed 3D representation. Upon the development of an interactive 3D model of the renal corpuscle, further studies investigating the efficacy of virtual 3D models of complex cellular structures in 3D histology education should be performed. In addition, studies investigating the efficacy and usefulness of the developed tool should be performed to determine if the tool is easy to use or too complex to be effective as learning tool. The result of these studies would provide

insight with regards to the potential benefits of using virtual 3D tools in histology education and the ways in which these tools could be improved for future use.

An interesting feature of the 3D model developed is the ability to visualize 2D orthogonal slices in conjunction with the 3D reconstruction. This allows the incorporation of both 2D and 3D modes of learning into a single model. The efficacy of this combination of both 2D visualization and 3D comprehension warrants further investigation.

Passive optical clearing in conjunction with antibody labeling within the renal corpuscle may be of value when evaluating specific cellular features such as the area of the glomerular capillary network that is covered by podocyte secondary foot processes compared to the area that is exposed to filtration slit. This would provide a greater understanding of the composition and organization of the glomerular filtration barrier. In addition, when analyzing renal corpuscle-specific proteins such as nephrin, this technique may also prove beneficial in histopathological studies relating to congenital and acute nephrotic syndrome, as well as many other renal pathologies. Other useful markers for 3D visualization of the glomerulus exist. For example, podocalyxin, the major sialoprotein of the glomerular epithelial cell, has been detected in the glycocalyx of glomerular podocytes. Podocalyxin is believed to play an important role in the maintenance of the characteristic and unique foot processes and slit organization of the glomerular podocytes. Diminished expression of podocalyxin has been associated with minimal change nephrosis – a disease of the kidney which can lead to nephrotic syndrome.[42] Continued analysis of this protein may prove useful for future histopathological inquiries regarding kidney disease.

4.3 Conclusion

CLARITY optically clears kidney tissue sufficiently for analysis by immunohistochemistry. Immunohistochemical labelling of nephrin in optically transparent kidney tissue allows for microscopic, high resolution visualization of the glomerular capillaries that participate in urinary filtration. 3D visualization of basement membrane structures in the renal corpuscle largely depends on good antibodies to specific isoforms of extracellular matrix proteins such as Collagen IV and Laminin α5. 3D reconstruction of 2D optical sections obtained from fluorescently labelled, optically transparent kidney tissue produces a biologically accurate model of the kidney glomerulus. In summary, the development of an interactive 3D model of the kidney glomerulus through passive optical clearing in conjunction with immunohistochemical techniques allows for an accurate representation of the kidney glomerulus while avoiding the disadvantages of 3D reconstruction from physical sectioning.

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APPENDIX C – Curriculum Vitae

Tristan Conciatori

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1-519-566-1555, tconciat@uwo.ca

Education

Master of Clinical Anatomy **Anticipated Completion: April 2015**

The University of Western Ontario, London, Ontario, Canada

Bachelor of Science *(Honours in Biological Sciences)* **April 2013**

The University of Windsor, Windsor, Ontario, Canada

- President's Honour Roll (2009-2013)
- Dean's List (2009-2013)
- Degree Conferred with Great Distinction

Teaching Experience

Head Teaching Assistant - Systemic Human Anatomy **2013-2014**

Department of Anatomy and Cell Biology, Western, London, ON

- Organized tutorial discussion for approximately 100 students to ensure efficient and timely tutorial seminars.
- Organized classroom stations in preparation of weekly demonstrations.
- Recorded student attendance to weekly tutorial sessions to ensure active participation of weekly seminars.

Teaching Assistant - Systemic Human Anatomy **2013-2014**

Department of Anatomy and Cell Biology, Western, London, ON

- Presented six 30-minute tutorial sessions on a weekly basis to approximately 10-15 students.
- Facilitated student learning with an interactive approach by using models, cadaveric material, videos and diagrams to describe anatomical structures and relationships in three dimensions.
- Attended to students' learning needs during and after tutorials, which helped them improve their anatomical knowledge, critical thinking, and preparation for exams.

Teaching Assistant - Functional Human Anatomy **2013-2014**

Department of Anatomy and Cell Biology, Western, London, ON

- Facilitated two-hour-long lab sessions on a weekly basis.
- Supervised student dissection of cadaveric specimens and demonstrated proper dissection technique.
- Guided students through outlined lab material and related cadaveric anatomy to clinical cases.
- Provided verbal assessments using cadaveric specimens to test student knowledge of functional and gross anatomy.

Teaching Assistant - Mammalian Histology **2014-2015**

Department of Anatomy and Cell Biology, Western, London, ON

- Presented pre-lab tutorial sessions to approximately 90 students.
- Attended to students' inquiries regarding histological information presented in class and tutorial sessions.
- Demonstrated proper light microscopy techniques.
- Marked weekly assignments and quizzes to record student learning and progress with the material.

Teaching Assistant - Gross Anatomy for 1st and 2nd year medical students *Schulich School of Medicine & Dentistry, Western, London, ON* **2014-2015**

- Facilitated small group lab tutorials for two cadaver stations.
- Supervised student dissection of cadaveric specimens and demonstrated proper dissection technique.
- Guided students through outlined lab material and related cadaveric anatomy to clinical cases.

Guest Lecture – Functional Human Anatomy **2014**

Department of Anatomy and Cell Biology, Western, London, ON

- Presented a 50 minute lecture on the anatomy of the anterior forearm to approximately 120 physiotherapy and kinesiology students.

Peer Tutor – Summer School **July 2008 - August 2009**

Greater Essex County District School Board, Windsor, Ontario, Canada

- Identified the learning style of each student and helped them thoroughly understand concepts of mathematics, biology, chemistry, physics, and English.
- Facilitated student learning and provided tips and methods for students to refine their study habits and exam preparation.

Lab Experience

Cadaver Lab - Prepare and maintain anatomical prosections for use in teaching and research.

Department of Anatomy and Cell Biology, Western, London, ON

Volunteer Experience

Hospital Volunteer in Physiotherapy Department **2011 - 2013**

Windsor Regional Hospital, Windsor, Ontario, Canada

- Aided physiotherapists with patient care.
- Clearly explained and demonstrated exercise techniques to ensure proper rehabilitation for patients.
- Recognized patients' individual needs and patiently interacted with them to help ease their emotional and physical stress.

Hospital Volunteer at Cardiac Wellness Center **2011 - 2013**

Windsor Regional Hospital, Windsor, Ontario, Canada

- Educated patients on proper use and benefits of exercise equipment.
- Recorded patient heart rates, blood pressure and tested blood glucose levels.
- Acknowledged patients' concerns and ensured that proper care was administered to relieve their anxieties.
- Interacted with patients over the phone and in person to confirm appointment times and address any complaints or concerns.

Soccer Coach **2010 - 2013**

Tecumseh Soccer Club, Tecumseh, Ontario, Canada

- Taught young soccer players proper soccer techniques and strategies in an interactive and fun environment.
- Organized practices and team events.
- Attended to players' needs and helped them with any difficulties.
- Cooperated with parents to create a fun, safe, and educational experience for their children.

Member of Students Orientating Students **2009 - 2013**

University of Windsor, Windsor, Ontario, Canada

- Assisted with orientations to help new students adjust successfully to university life.
- Led groups of new or aspiring university students through campus to help introduce them to the university experience.
- Aided new students with their course selection to ensure they fulfill all program requirements.

Scholarships and Academic Honours

Renewable Entrance Scholarship valued at $10,000 **2008 - 2013**

University of Windsor, Windsor, Ontario, Canada

Member of the Golden Key National Honours Society **2009 - present**

- Recognizes outstanding scholastic achievement among university students.
- Membership to the society is administered only to the top 15% of university students.

Languages

- **English** – native level of competence
- **French** – advanced level of competence

Certification and Training

Biosafety **2013**

Comprehensive WHMIS Certification **2013**