3D imaging of cellular features in kidney glomeruli using expansion microscopy and fluorescent covalent stains

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Abstract. Objective: To develop a 3D imaging method for observing the structural features and identifying three types of cells in the glomerulus based on multiple optical sections of the specimen. Methodology: Following expansion microscopy procedures, the 50 μm thick sections of rat kidney tissues were expanded and cleared. The nuclear and cytoplasmic components of the glomerulus were then stained with dyes with optimized chemical and fluorescent properties and finally visualized by confocal microscopy. To further make the dataset friendly to pathologists, the images were pseudo-colored to resemble H&E. Results: Using the method presented here we were able to collect multiple sections of data. The different types of cells within the glomerulus can be well identified based on their anatomical features and gradual appearance changes along the thickness direction. Conclusion: Compared to two-dimensional data, our 3D multilayered data allows different glomerular cells to be more clearly identified, which lays the experimental foundation for more in-depth study of pathologic changes in the glomerulus.

1. Introduction
As the filtration barrier of kidney, the glomerulus is an important subject of study in pathological analysis of kidneys. There are three types of cells in the glomerulus—podocytes, endothelial cells and mesangial cells. Many renal diseases exhibit changes in the morphology of the glomeruli and the number of cells within it, so the identification of the cellular features in the glomeruli has important implications. The traditional diagnosis of glomerulonephritis is to examine the two-dimensional sections of the biopsy tissue by light microscopy, immunofluorescence microscopy and electron microscopy. However, these two-dimensional methods can only observe the pathological changes of a certain cross section of the glomerulus, which leads to limited information in the data when a three-dimensional tissue is only imaged in a two-dimensional section. In addition, the lesions of some diseases are local, and a single cross-section of imaging data may miss important information and affect the accurate diagnosis and treatment of the disease. Therefore, it is necessary to develop methods that allow imaging multiple cross-sections of the glomerulus.
To image glomerular cell morphology beyond two dimensions, previous researchers used serial section method to observe the glomerulus along the thickness direction [1], which is, however, quite tedious and costly for sample preparation. The other method is confocal microscopy of cleared tissues [2]. Such method could successfully identify all the podocytes in the glomerulus with the help of antibody labeling. However, the endothelial cells and mesangial cells could not be identified in the meantime, which may be due to issues with multi-color labeling, poor penetration of antibodies through thick tissues, or insufficient resolution.

Here, we use expansion microscopy (ExM) to examine the kidney glomerulus. ExM utilizes acrylamide- and acrylate-based hydrogel material to form a three-dimensional network that expands significantly in water but not dissolve. The biological tissues were immersed in hydrogel solution to form tissue-hydrogel hybrid, with the biomolecules chemically cross-linked with the hydrogel. The tissue-hydrogel hybrid can be physically expanded to allow sample magnification and thus achieve improved imaging resolution [3]. During expansion, the tissue was also cleared by lipid removal, which makes volumetric imaging of the tissue possible. In order to illuminate the morphology of the glomerulus, we use the recently reported fluorescent covalent staining method [4], which can reveal the general physiology of the biological sample, and meanwhile ensure sufficient staining throughout the thick tissue, since such labeling is based on small molecules instead of antibodies. Specifically, we use fluorescent N-hydroxysuccinimide (NHS) ester to react with the amine groups in the expanded tissue to provide rich structural details, which, together with nuclear staining, facilitates cell identification in the glomerulus.

2. Materials and methods

2.1. Experimental animals
Sprague-Dawley rats were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The experimental protocol was approved by the ethics committee of Nantong University with a project license No. 20200603-002.

2.2. Tissue sectioning and gelation
The renal cortex was harvested from one 8-week-old rat and fixed in a solution containing 4% paraformaldehyde and 20% acrylamide (pH 9.0). The tissue was embedded in tissue freezing media at -30 °C in a cryostat and then sectioned at 50 μm of thickness. The tissue sections were collected onto poly-L-lysine coated coverglass. The sample was then immersed in the ZOOM gel solution [5] containing 30% acrylamide, 0.01% N, N'-methylenebisacrylamide, 4% paraformaldehyde, and 0.1% VA-044 at 4 °C for 13 hours, followed by gelation at 50 °C for 10 hours.

2.3. Tissue expansion and fluorescent staining
Next, following previous protocols [6][7], the gelled samples were incubated in a solution containing 50 mM Tris-HCl, pH 9.0, 200 mM sodium chloride, 200 mM sodium dodecyl sulfate, and 200 mM dithiothreitol at 82 °C for 24 hours, for denaturation of proteins and removal of lipids in the tissues. To stain the cytoplasm and extracellular matrix, the expanded tissue was incubated with 0.2 mM CF568 NHS ester (Sigma, SCJ4600027) in phosphate buffered saline (PBS) for 10 hours at room temperature. After washing in PBS, it was further incubated in 0.01 mM Sytox Green (ThermoFisher, S7020) in PBS for 2 hours at room temperature to stain the nuclei. Finally, the tissue was placed in deionized water to achieve complete expansion.

2.4. Confocal imaging
The expanded and stained tissue was then placed in a glass-bottom dish for confocal imaging. The images were taken under a Zeiss LSM 800 confocal microscope using a 40× objective lens. The CF568 and Sytox Green stains were excited by 561 nm and 488 nm lasers, respectively. The z-stack function was used to scan the glomerulus along the thickness direction with z step of 0.8 μm.
2.5. **Digital pseudo-coloring of fluorescent images**

The two grayscale images, one representing the nuclear signal, and the other the cytoplasmic signal, were synthesized into a single pseudo-colored RGB image simulating the hues of hematoxylin and eosin (H&E) using the approach described previously [8][9]. The image processing was performed in MATLAB.

3. **Results**

3.1. **Measurement of expansion factor**

We first measured the degree of expansion of the hydrogel sample. By immersing the kidney sections with the ZOOM expansion solution developed by Park et al [5], the tissue got embedded into the hydrogel network after gelation at 50 °C. As shown in Fig. 1(A), the 50 μm thick tissue in the hydrogel before expansion appeared partially opaque. The specimen then underwent heat denaturation treatment to allow for subsequent expansion in a low-osmolarity solution. Fig. 1(B) shows the size of the gel after expansion. By comparing the sizes of the hydrogel before and after expansion, we obtained the linear expansion factor to be 2.5.

3.2. **Visualization of the expanded glomerulus**

We then stained the amine residues in the specimen with CF568 NHS ester, and DNA (which is mostly in the nucleus) with Sytox Green dye, which were then visualized by confocal microscopy using 488 nm and 561 nm excitation lasers respectively, with a total of 40-50 successive layers of image taken for each glomerulus. A typical layer was shown in Fig. 2, where the nuclei and cytoplasmic signals can be either separately displayed or merged together.

![Figure 1](image1.png) **Figure 1.** The size of the hydrogel sample before expansion (A) and after expansion (B). The gel sample was placed onto 0.6-cm grid paper.

![Figure 2](image2.png) **Figure 2.** One slice of confocal image of an expanded and fluorescently stained glomerulus. (A) Image with two channels merged where the nucleus and cytoplasm signals are shown in green and red respectively. (B)(C) Single channel images for each of the two channels. The image represents a tissue area of 106.5 μm × 106.5 μm after taking into account the expansion factor.
3.3. *Pseudo*-H&E display of the data
In order to facilitate clinical interpretation of our data by pathologists, we then adjusted the colors of our two-channel fluorescent images to resemble the classical H&E histology images, with the cell nucleus in purple and cytoplasm in pink (Fig. 3). We found that the pseudo-colored image clearly exhibits various structural details of the glomerulus, such as the glomerular basement membranes and the cellular chromatin, etc.

3.4. Cell identification
With the improvement in resolution and imaging volume, we were able to observe the prominent features for the three different cell types of the glomerulus: podocytes are attached to the outside of glomerular basement membranes with relatively large nuclei and mainly uncondensed chromatin; endothelial cells are located on the endothelial side of capillaries with smaller nuclei; mesangial cells are surrounded by mesangial matrix with small irregularly shaped nuclei and mostly condensed chromatin. Previously, identification of these cellular features usually requires the use of fluorescent antibodies [2][7]. Here, we were able to further make use of our 3D multilayered data to examine the cellular features in adjacent cross-sections of the glomeruli, which allowed clear identification of the different types of glomerular cells (Fig. 4).

4. Conclusion
In summary, we developed a method to image successive layers of glomerular specimen using ExM and fluorescent covalent labeling. By pseudo-coloring the images to simulate H&E, we have presented the
data in a pathologist-friendly manner, which allow clear identification of the podocytes, endothelial cells, and mesangial cells in the glomerulus. Compared to traditional two-dimensional methods, our method increases the sample size and information content and thus has the potential for more accurate analysis of pathological changes of the glomerulus.

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Figure 4. Identification of different types of cells in the glomerulus. (A) The cell labeled in asterisk (*) is identified as podocyte based on its anatomical features on two adjacent z-slices (0.64 μm apart). Similarly, the * cell in (B) can be identified as endothelial cell, and the * cell in (C) can be identified as mesangial cell. The images represent an area of 26 μm × 26 μm.

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