Journal of Visualized Experiments

Optical clearing and imaging of immunolabeled kidney tissue

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60002R1
Full Title:	Optical clearing and imaging of immunolabeled kidney tissue
Keywords:	optical clearing, whole-mount immunolabeling, kidney, tubule, tissue clearing, three-dimensional analysis
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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.	Aachen, North Rhine-Westphalia, Germany

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To
Phillip Steindel, Ph.D.
and the Editors of *JoVE*

Aachen, May 2, 2019

REVISION OF MANUSCRIPT: JoVE60002

Dear Dr. Steindel,

On behalf of all co-authors I would like to thank the editorial team and the external reviewers for the thoughtful comments to our manuscript entitled:

Optical clearing and imaging of immunolabeled kidney tissue

We also thank the editorial team for offering us the opportunity to revise our manuscript and to resubmit a revised version to your journal.

The revised version is now entitled "–R1" as appendix to the manuscript number. In the rebuttal letter we included a detailed, point-by-point reply to the correspondence with the editorial team and the external reviewers. Changes in the revised manuscript called "R1" are highlighted within the text. Incorporating the reviewers` comments to our manuscript has substantially improved the clarity and increased the scientific quality of the manuscript.

We are looking forward to receiving the editorial comments to the revised version of our manuscript. All co-authors have seen and approved the revised version of the manuscript.

Sincerely,

On behalf of the authors
Dr. med. Turgay Saritas

TITLE:

Optical Clearing and Imaging of Immunolabeled Kidney Tissue

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KEYWORDS:

optical clearing, whole-mount immunolabeling, kidney, tubule, tissue clearing, three-dimensional analysis

SUMMARY:

The combination of antibody labeling, optical clearing, and advanced light microscopy allows three-dimensional analysis of complete structures or organs. Described here is a simple method to combine immunolabeling of thick kidney slices, optical clearing with ethyl cinnamate, and confocal imaging that enables visualization and quantification of three-dimensional kidney structures.

ABSTRACT:

Optical clearing techniques render tissue transparent by equilibrating the refractive index throughout a sample for subsequent three-dimensional (3-D) imaging. They have received great attention in all research areas for the potential to analyze microscopic multicellular structures that extend over macroscopic distances. Given that kidney tubules, vasculature, nerves, and glomeruli extend in many directions, which have been only partially captured by traditional

two-dimensional techniques so far, tissue clearing also opened up many new areas of kidney research. The list of optical clearing methods is rapidly growing, but it remains difficult for beginners in this field to choose the best method for a given research question. Provided here is a simple method that combines antibody labeling of thick mouse kidney slices; optical clearing with cheap, non-toxic and ready-to-use chemical ethyl cinnamate; and confocal imaging. This protocol describes how to perfuse kidneys and use an antigen-retrieval step to increase antibody- binding without requiring specialized equipment. Its application is presented in imaging different multicellular structures within the kidney, and how to troubleshoot poor antibody penetration into tissue is addressed. We also discuss the potential difficulties of imaging endogenous fluorophores and acquiring very large samples and how to overcome them. This simple protocol provides an easy-to-setup and comprehensive tool to study tissue in three dimensions.

INTRODUCTION:

The growing interest in studying entire organs or large multicellular structures have led to the development of optical clearing methods that involve imaging of transparent tissue in three dimensions. Until recently, the best methods to estimate cell number, length, or volume of whole structures have been stereology or exhaustive serial sectioning, which is based on the systemic sampling of tissue for subsequent analysis in two dimensions[1-3]. However, these methods are time-consuming and need a high level of training and expertise[4]. Optical clearing methods overcome these problems by equilibrating the refractive index throughout a sample to make tissue translucent for 3-D imaging[5-7].

Several optical clearing methods have been developed which fall into two main categories: solvent-based and aqueous-based methods. Aqueous-based methods can be further divided into simple immersion[8,9], hyperhydration[10,11], and hydrogel embedding[12,13]. Solvent-based methods dehydrate the tissue, remove lipids and normalize the refractive index to a value around 1.55. Limitations of most solvent-based methods are quenching of endogenous fluorescence of commonly used reporter proteins such as GFP, solvent toxicity, capacity to dissolve glues used in some imaging chambers or objective lenses, and shrinkage of tissue during dehydration[14-21]. However, solvent-based methods are simple, time-efficient, and can work in a number of different tissue types.

Aqueous-based methods rely on the immersion of the tissue in aqueous solutions that have refractive indices in the range of 1.38–1.52[8,11,12,22-24]. These methods were developed to preserve endogenous fluorescent reporter protein emission and prevent dehydration-induced shrinkage, but limitations of most aqueous-based clearing methods include a longer duration of the protocol, tissue expansion, and protein modification (i.e. partial denaturation of proteins by urea in hyperhydrating protocols such as Sca*le*A2)[7,11,23,25]. Sca*le*S addressed tissue expansion by combining urea with sorbitol, which counterbalances by dehydration the urea-induced tissue expansion, and preserved the tissue ultrastructure as evaluated by electron microscopy[10]. Tissue shrinkage or expansion affects the absolute sizes of structures, distances between

objects, or cell density per volume; thus, the measurement of size changes upon clearing of the tissue may help interpret the obtained results[7,26].

In general, a protocol for optical clearing consists of multiple steps, including pretreatment, permeabilization, immunolabeling (if required), refractive index matching, and imaging with advanced light microscopy (e.g., two-photon, confocal, or light-sheet fluorescence microscopy). Most of the clearing approaches have been developed to visualize neuronal tissue, and emerging studies have validated their application in other organs[5]. This comprehensive tool has been previously demonstrated to allow reliable and efficient analysis of kidney structures, including glomeruli[27,28], immune infiltrates[28], vasculature[28], and tubule segments[29], and it is an ideal approach to better the understanding of glomerular function and tubule remodeling in health and disease.

Summarized here is a solvent-based method that combines immunostaining of kidney tubules; optical clearing with cheap, non-toxic, and ready-to-use chemical ethyl cinnamate (ECi); and confocal microscopy imaging that allows complete tubule visualization and quantification. This method is simple, combines antigen-retrieval of kidney slices with staining of commercial antibodies, and does not require specialized equipment, which makes it accessible to most laboratories.

PROTOCOL:

NOTE: All experimental procedures described here were approved by the Institutional Animal Care and Use Committee (IACUC) of Oregon Health and Science University, Portland, Oregon, USA, and relevant local authorities in Aachen, Germany.

1. Retrograde abdominal aortic perfusion and fixation of mouse kidneys

1.1. Prepare solutions the same day or evening before and store in a fridge overnight. Warm solutions to room temperature (RT) before using.

1.2. Make a fresh batch of 3% paraformaldehyde (PFA) in 1x phosphate-buffered saline (PBS). About 50–100 mL PFA is needed per mouse.

1.2.1. To make 1 L of 3% PFA: Weigh 30 g of PFA and add 800 mL of distilled water in the fume hood. Stir and heat to 50–60 °C. Do not heat above 70 °C.

CAUTION: PFA is toxic. Prepare PFA in the fume hood.

1.2.2. Slowly add several drops of 2N NaOH. Wait a few minutes until PFA goes into the solution or add a few more drops. Some small chunks will not dissolve.

1.2.3. Remove the solution from heat. Add 50 mL of 20x PBS. Chill on ice to RT.

1.2.4. Adjust the pH to 7.3–7.4 with HCl. Add distilled water to 1 L.

1.2.5. Remove any undissolved particles by filtering 1x PBS and 3% PFA with 0.22 µm filter.

1.3. Add 1000 units of Heparin to 1 L of 1x PBS. Transfer 1x PBS containing heparin and 3% PFA in 1x PBS into separate 50 mL plastic syringes.

NOTE: If available, pressure-controlled pump set at 80–100 mmHg or hydrostatic pressure (drip method, the height of the perfusion solutions: 160–200 cm above animal) can be used for kidney perfusion.

1.4. Connect the PBS, PFA and a blunted 21 G butterfly needle to a three-way stopcock. Make sure there are no air bubbles in the whole system.

1.5. Label a 15 mL conical tube and dispense 10 mL of PFA into it.

1.6. Deeply anesthetize a male or female C57BL/6 mouse 12–24 weeks of age using 120 mg/kg bodyweight ketamine and 16 mg/kg bodyweight xylazine. The animal must be checked for complete absence of responsiveness by pinching the reflexes before proceeding to surgery (e.g., toe pinch reflex).

1.7. Once the animal has reached a surgical plane of anesthesia, place it on its back under the dissecting microscope. Surgically open the abdomen with a midline abdominal incision using an operating scissors and expose the abdominal aorta.

1.8. Clamp the abdominal aorta right above the branching to the iliac artery with a curved hemostat. Then clamp the abdominal aorta right below the renal arteries with a micro serrefine. Make a small incision (1 mm) on the abdominal aorta between the two clamps with vannas scissors. Insert the butterfly needle into the incision slowly and be careful not to rip the abdominal aorta open.

1.9. Ligate the right renal artery with an 5-0 silk suture and remove the right kidney for other analysis if only one kidney is needed for perfusion and fixation.

1.10. Remove the micro serrefine, transect the portal vein with vannas scissors, and immediately perfuse with 50 mL of PBS containing heparin, then switch and perfuse with 50 mL of 3% PFA.

NOTE: High perfusion pressure through abdominal aorta is required to open renal tubules for better antibody diffusion through tissue. Perfusion through heart may not open renal tubules.

1.11. Collect the perfused kidney carefully and avoid puncturing or squeezing the tissue.

1.12. Remove the capsule and cut the kidney into 1 mm thick coronal slices. Use a slicer matrix **(Table of Materials)** to standardize slice thickness.

NOTE: Alternatively, consider using a vibratome.

1.13. Immerse the kidney slices with the prepared PFA in the labeled 15 mL conical tube.

1.14. Dispense 10 mL of PFA to another labeled 15 mL conical tube. Flush the three-way stopcock and butterfly needle with PBS before moving to the next mouse.

1.15. Carry out post-fixation overnight at RT protected from light.

2. Tissue preparation and immunostaining

2.1. After post-fixation, wash the kidney slice twice with 1x wash buffer **(Table of Materials)** for 1 h on a horizontal rocker at RT.

2.2. Perform antigen retrieval. Heat up 300 mL of 1x antigen unmasking solution **(Table of Materials)** in a 500 mL beaker to 92–98 °C. Enclose the slice in embedding cassette permeable to the heated buffer with stirring for 1 h at 92–98 °C. Remove the beaker from heat and leave it to cool to RT.

NOTE: Some vendors test their antibodies for immunohistochemistry application and will include a suggested antigen retrieval method in the datasheet. Therefore, some epitopes may require a more basic buffer (e.g., pH 9).

2.3. Transfer the slice into 10 mL of 1x wash buffer with 0.1% Triton X-100 and rock overnight at RT. Wash the slices 2x with 10 mL of fresh 1x wash buffer for 1 h the next day.

2.4. Dilute the primary antibody in 500 µL of normal antibody diluent **(Table of Materials)**. Begin with a concentration of 1:50–1:100. Gently rock the kidney slice in diluted primary antibody for 4 d at 37 °C.

NOTE: Since each antibody has unique properties, temperature during antibody incubation and dilutions of antibody need to be optimized for individual probes. For secondary antibody-only controls, incubate kidney tissue in diluent without primary antibody. Instead of commercial antibody diluent, 1x PBS with 0.1% Triton X-100 and 0.01% sodium azide can be used.

2.5. Wash the kidney slice in 10 mL of 1x wash buffer overnight at RT with one change of wash buffer after 8 h.

2.6. Dilute the secondary antibodies (e.g., 1:100 for Alexa Fluor-conjugated secondary antibodies) in 500 µL of normal antibody diluent. Incubate the kidney slices in diluted secondary antibody for 4 d at 37 °C. From this step onwards, protect the kidney slices from light.

2.7. Wash the kidney slices in 10 mL of 1x wash buffer overnight at RT with one change of wash buffer after 8 h.

3. Tissue clearing

3.1. Transfer kidney slice to 5 mL of high grade 100% ethanol **(Table of Materials)** for 2 h at RT with gentle rocking (with one change to fresh ethanol after 1 h). This step is for tissue dehydration.

NOTE: High grade ethanol is required to achieve a high tissue translucency in the next step. Methanol or tetrahydrofuran are alternative dehydration reagents with high delipidation potential.

3.2. Immerse kidney slice in 2 mL of ECi **(Table of Materials)** with gentle rocking at RT (with one change to fresh ECi after 2 h) overnight.

NOTE: The freezing/melting point of ECi is 6–8 °C. Therefore, do not store samples in the fridge. Conduct immersion in a properly ventilated fume hood and avoid direct contact with clothes and skin (ECi is a non-toxic Food and Drug Administration-approved compound but has a strong odor). Use regular Eppendorf tubes or glass vessels (no polystyrene vessels).

3.3. Tissue translucency can be achieved after ECi immersion and when the kidney slices are ready for imaging.

4. Confocal imaging and image analysis

NOTE: For imaging, other microscopy techniques can be used as long as the refractive index matching solution is compatible with the objective lens. This protocol uses an inverted confocal microscope.

4.1. Add 600–1000 µL of ECi into the glass bottom dish **(Table of Materials)**.

NOTE: Avoid use of regular cell culture dishes, because ECi is an organic solvent that will dissolve the plastic dishes. Similarly, ECi might attack plastic parts/insulation rings on objective lenses. Refer to the appropriate reports for an overview of compatible imaging dishes[30] and self-made 3-D printed chambers[28].

4.2. Transfer the translucent kidney slice into the dish. Place a round coverslip on the kidney slice to apply light pressure towards the glass bottom. Seal the dish with paraffin film **(Table of Materials)** to avoid leakage of ECi.

NOTE: Whole organs or several millimeter-thick tissue slices may require a border (dental cement or silicone elastomer**;** see **Table of Materials)** around the tissue to make an ECi-pool for the sample.

4.3. Place the dish onto the microscope imaging platform.

4.4. Take several z-stacks and perform stitching. Start with a z-step size of 5 µm.

NOTE: Consider using long working distance (>5 mm) and high numerical aperture (>0.9) objectives for imaging of very thick tissue slices or organs. After imaging, transfer the tissue back to ethanol and store in wash buffer or PBS with 0.02% sodium azide.

4.5. Analyze the image using 3-D rendering with software **(Table of Materials).**

REPRESENTATIVE RESULTS:

Kidneys are complex organs comprised of 43 different cell types[31]. Most of these cells form large multicellular structures such as glomeruli and tubules, and their function is highly dependent on interactions with each other. Classical 2-D histological techniques partially capture these large structures and may miss focal changes within intact structures[31]. Thus, 3-D analysis using optical clearing techniques helps to understand how they function in health and disease.

Most solvent-based optical clearing techniques will at least partially quench the endogenous fluorescent reporter protein signal. Quenching of YFP-tagged Parvalbumin was observed upon dehydration and optical clearing with ECi (**Figure 1**). However, other strong fluorescent proteins may resist signal-quenching, and adjustment of pH to basic levels (pH 8–11) may stabilize endogenous fluorescent proteins[14,20,28,30]. In addition, aqueous-based methods should be considered if fluorescent protein emission preservation is desired. In this current report, it is demonstrated that this solvent-based clearing protocol is compatible with antibody labeling; although, it remains challenging to achieve deep immunolabeling of tissue, especially in a dense cell-rich organ such as the kidney.

Retrograde abdominal aortic perfusion of the kidneys was then performed to remove blood cells and open up tubules (**Figure 2A,B**). This approach decreases autofluorescence and improve antibody diffusion. Antibody concentration depends on the size of tissue and abundance of the antigen. Therefore, the superficial signal can relate to either poor antibody penetration or insufficient amount of antibody (**Figure 3A,B,** see also **Movie 1**). For large samples or extremely abundant markers, higher concentrations of antibody and replenishment of antibody after 1–2 days may be required. If the antigen of interest is expressed in kidney vasculature, intravascular delivery of the antibody should be considered (**Figure 3C**). However, antibodies targeting proteins in the apical membrane of tubule epithelial cells do not cross the glomerular filtration barrier due to molecular size, thus causing unspecific signals in blood vessels and glomeruli (**Figure 3-D**).

This protocol can be applied to kidney slices or whole kidneys (**Figure 4A,B**). To test antibody-specificity, only controls were subjected to secondary antibody (**Figure 4C**). The tissue can be labeled with antibodies to detect a specific cell population (e.g., proliferating cells, **Figure 4D**) or to visualize whole tubule segments using segment-specific antibodies (**Figure 4E**). Moreover, the combination of multiple antibodies provides the opportunity to colocalize different proteins in 3-D (e.g., to detect segment-specific tubule hyperplasia as it occurs in tubule remodeling upon different stimuli) (**Figure 4F,G**; also see **Movie 2**).

FIGURE AND TABLE LEGENDS:

Figure 1: Quenching of endogenous fluorescent reporter protein signal. (A) The endogenous fluorescent reporter protein derived from Parvalbumin^{YFP+} transgenic mouse is detectable in 5 µm thin PFA-fixed frozen kidney sections. The arrows mark some fluorescent-labeled parvalbumin^{+} cells located in the early part of the distal convoluted tubule. **(B)** No evident fluorescent signal after the optical clearing of kidney tissue is observed.

Figure 2: Evaluation of the kidney perfusion quality. (A) Periodic Acid Schiff (PAS)-stained paraffin embedded tissue (5 µm thin sections) shows few tubules with a slightly opened lumen (typically proximal tubules which possess brush border; see arrows). **(B)** All tubules are opened up after good kidney perfusion.

Figure 3: Troubleshooting whole-mount immunolabeling. (A,B) A thick kidney slice was stained with an antibody against sodium-chloride cotransporter-2 (NKCC2), which is expressed in the thick ascending limb of the loop of Henle. The signal is detectable at the surface of the tissue (arrows in (B)). However, the antibody did not penetrate into the tissue [see arrowhead in (B)]. **(C)** Intravascular antibody injection targeting proteins expressed in the vessels (e.g., CD31) allows fast and homogenous blood vessel staining. The round structures with a strong signal are glomeruli. **(D)** However, antibodies targeting proteins expressed in the apical membrane of tubule epithelia (e.g., phosphorylated sodium-chloride cotransporter (phospho-NCC) which is expressed in the distal convoluted tubule) will not cross the glomerular filtration barrier, thus causing unspecific signals in the vasculature (see arrowhead pointing at afferent arterioles and other vessels) and glomeruli (see arrows).

Figure 4: Representative results of immunolabeled optically cleared kidney tissue. (A,B) This protocol allows optical clearing of the whole kidney. **(C)** A representative z-stack to show absence of non-specific binding of secondary antibody as control without prior incubation. **(D)** 3-D visualization of a representative z-stack shows proliferating bromodeoxyuridine (BrdU)$^{+}$ cells in kidney medulla. **(E)** Medullary collecting ducts are visualized using an antibody against aquaporin-2 (AQP2). **(F,G)** Analysis and quantification of BrdU^{+} cells within AQP2^{+} medullary collecting ducts. For (F,G), see also **Movie 2**. This figure has been modified from Saritas et al.[29].

Movie 1 (Related to Figure 3): 3-D visualization of NKCC2^{+} thick ascending limb of the loop of Henle. The movie demonstrates poor antibody penetration in an optically cleared kidney slice.

Movie 2 (Related to Figure 4): 3-D visualization of BrdU$^+$ cells and AQP2$^+$ medullary collecting ducts in an optically cleared kidney slice. BrdU$^+$ cells (in red) and AQP2$^+$ medullary collecting ducts (in green) are shown. Imaris spot and surface algorithms were used to determine BrdU$^+$ cells outside (turquoise spots) or inside (pink spots) AQP2$^+$ tubules (green surfaces). This movie originally appeared in Saritas et al.[29].

DISCUSSION:

Optical clearing techniques have received wide attention for 3-D visualization and quantification of microanatomy in various organs. Here, solvent-based clearing method (ECi) was combined with immunolabeling for 3-D imaging of whole tubules in kidney slices. This method is simple, inexpensive, and quick. However, other research questions may be best answered with other clearing protocols[5]. It is also important to keep in mind that solvent-based methods cause tissue-shrinkage at variable degrees, mainly due to the dehydration step[14,18]. Most solvent-based methods (e.g., ECi[14]) also at least partially quench endogenous fluorescence of reporters such as GFP or tdTomato; thus, FDISCO[32], CLARITY[12], or CUBIC[11] may serve as alternative protocols. However, the quenching effect of ECi is variable and depends on individual constructs of each reporter mouse[28]. In addition, use of 1) a fluorescent antibody against GFP and other reporters or 2) modified solvent-based protocols that preserve endogenous fluorescence signals[30,32] can also be an alternative approach in this context. It is worth mentioning that several groups have tested the compatibility of aqueous-based protocols[24,33,34] to visualize RNA in 3-D, while solvent-based clearing methods have not yet been tested. Thus, aqueous-based clearing methods such as the modified version of CLARITY[33] should be preferred when RNA analysis is considered.

Cells or gene products of interest can be visualized using transgenic mice with endogenous fluorescent protein expression, but the generation of genetically engineered mouse lines is time-consuming and expensive. Therefore, antibody labeling is more practical and provides more flexibility, although the immunolabeling of large tissue is challenging. In contrast to the original ECi protocol[14], our approach combines a modified ECi optical clearing method and immunolabeling, which uses an antigen-retrieval step. Heat-induced antigen-retrieval will denature proteins, but helps to recover loss of antigenicity during PFA-fixation[35] and improves antibody-binding.

There are a few critical steps in this protocol. First, it is important to perform good perfusion of the kidney. Hemoglobin containing red blood cells limits the imaging depth[7] and expanded tubules with open lumen enhance antibody penetration. The opening of tubules also allows better distinguishing of proteins expressed in the epithelial apical membrane from other apically expressed proteins on the contralateral side. However, artificially expanded tubules may negatively influence tubule structure and mask specific pathophysiological response of the kidney (e.g. dilation of injured proximal tubules,) but not of healthy tubules[36]. Second, antibody incubation at 37°C rather than 4 °C or RT improves antibody penetration[27]. However, each antibody has unique properties; thus, temperature during antibody incubation needs to be

optimized for individual probes. Third, the use of Alexa Fluor-647 (far-red spectrum) secondary antibodies helps increase the signal-to-noise ratio, especially since kidneys emit a large amount of autofluorescence in the blue-green spectrum[37].

Retro-orbital injection[14] or perfusion of kidney[38] with fluorophore-conjugated antibodies against proteins expressed in blood vessels is an elegant and fast way to label kidney vasculature. However, proteins in the apical membrane of tubules remain inaccessible by intravascular injection since antibodies cannot cross the glomerular filtration barrier with its cut-off molecular weight for filtration of proteins in the range of 60–65 kDa. Therefore, the use of small antibody fragments and engineered variants such as Fab fragments (~55 kDa), diabodies (~50 kDa), tandem scFv (~28 kDa) or nucleic-acid aptamers (~6–30 kDa) with preserved molecular recognition properties of antibodies may provide an opportunity to access the apical membrane of tubules[38,39]. In addition, the combination of small antibody fragments with electric fields[40] or pressure[13] or the use of sodium dodecyl sulfate (SDS)-based clearing protocols to remove lipids[24,41,42] may enable fast and efficient antibody penetration of tissue.

Several groups demonstrated that perfusion with clearing reagent not only reduces the protocol time, but also increases tissue transparency[19,24,43,44]. Therefore, cannulation of the abdominal aorta or the renal artery with subsequent perfusion of the kidney with dehydrating and refractive index matching solutions should be considered to achieve better and faster tissue clearing. However, we did not perfuse the whole kidney with clearing reagents and used sliced kidneys for several reasons. First, different antigens can be visualized by antibody labeling of multiple kidney slices from one kidney. Second, less time and antibody are needed to perform immunolabeling of a kidney slice compared to a whole kidney. Third, 3-D imaging of large samples generates data sets up to several terabytes, which can be challenging to manage for most workstations. Therefore, data from tissue slices or subsets of bigger files are more convenient to perform complex operations such as automated counting or distance measurements.

In this protocol, confocal microscopy is used to perform imaging with single-cell resolution, which is particularly relevant in colocalization studies. However, confocal imaging requires laser scanning, since its imaging speed is only practical for small pieces of cleared tissue. To perform 3-D morphometric analysis of multicellular structures such as long tubule segments or even whole organs, faster microscope techniques such as light sheet fluorescent microscopes (LSFM) are necessary. LSFM allow fast imaging when the highest cellular resolution is not essential. For example, the lengths of distal convoluted tubules were recently assessed by combining whole-mount immunolabeling, optical clearing based on CLARITY[12], and LSFM[29]. Unfortunately, commercial LSFM is expensive and not always compatible with solvent-based clearing protocols. In fact, CLARITY was chosen for this study, since our particular LSFM with an objective customized for CLARITY was not compatible with ECi[29]. However, Klingberg et al. demonstrated that ECi is in principle compatible with LSFM[14].

In conclusion, a simple ECi-based optical clearing method is demonstrated, which can be applied to any research project using fixed tissue slices ranging from ~100 µm to several

millimeters in thickness. It also allows feasible analyses that previously required almost exhaustive efforts to complete, and eliminates the required assumptions and inferences associated with two-dimensional analysis of morphology. The combination of whole-mount immunolabeling, optical clearing, and advanced light microscopy will help advance the understanding of cellular function in health and disease.

ACKNOWLEDGMENTS:

T. S. is supported by grants from the DFG German Research Foundation (332853055), Else Kröner-Fresenius-Stiftung (2015_A197), and the Medical Faculty of the RWTH Aachen (RWTH Returner Program). V. G. P. is supported by research fellowships from Deutsche Gesellschaft fur Nephrologie, the Alexander von Humboldt Foundation, and the National Health and Medical Research Council of Australia. D. H. E is supported by Fondation LeDucq. R. K. is supported by grants from the DFG (KR-4073/3-1, SCHN1188/5-1, SFB/TRR57, SFB/TRR219), the State of Northrhinewestfalia (MIWF-NRW) and the Interdisciplinary Centre for Clinical Research at RWTH Aachen University (O3-11).

DISCLOSURES:

The authors have nothing to disclose.

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Figure 2

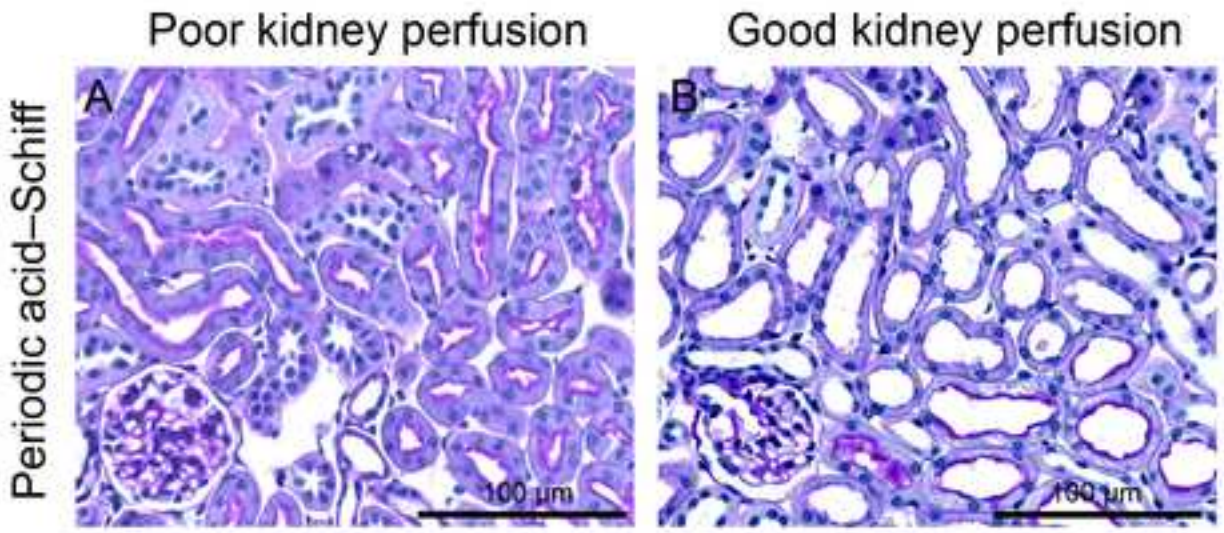


Figure 3

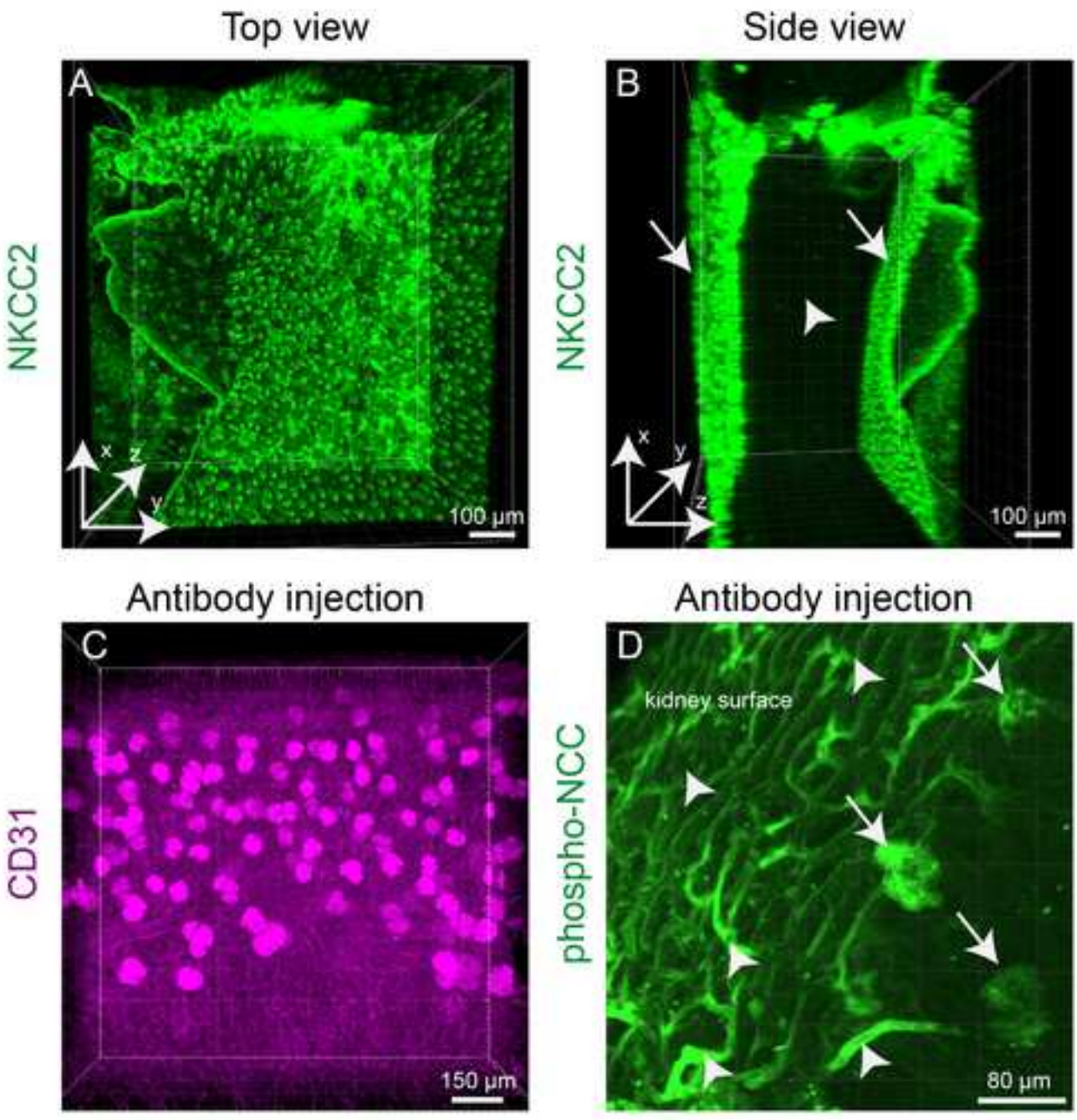


Figure 4

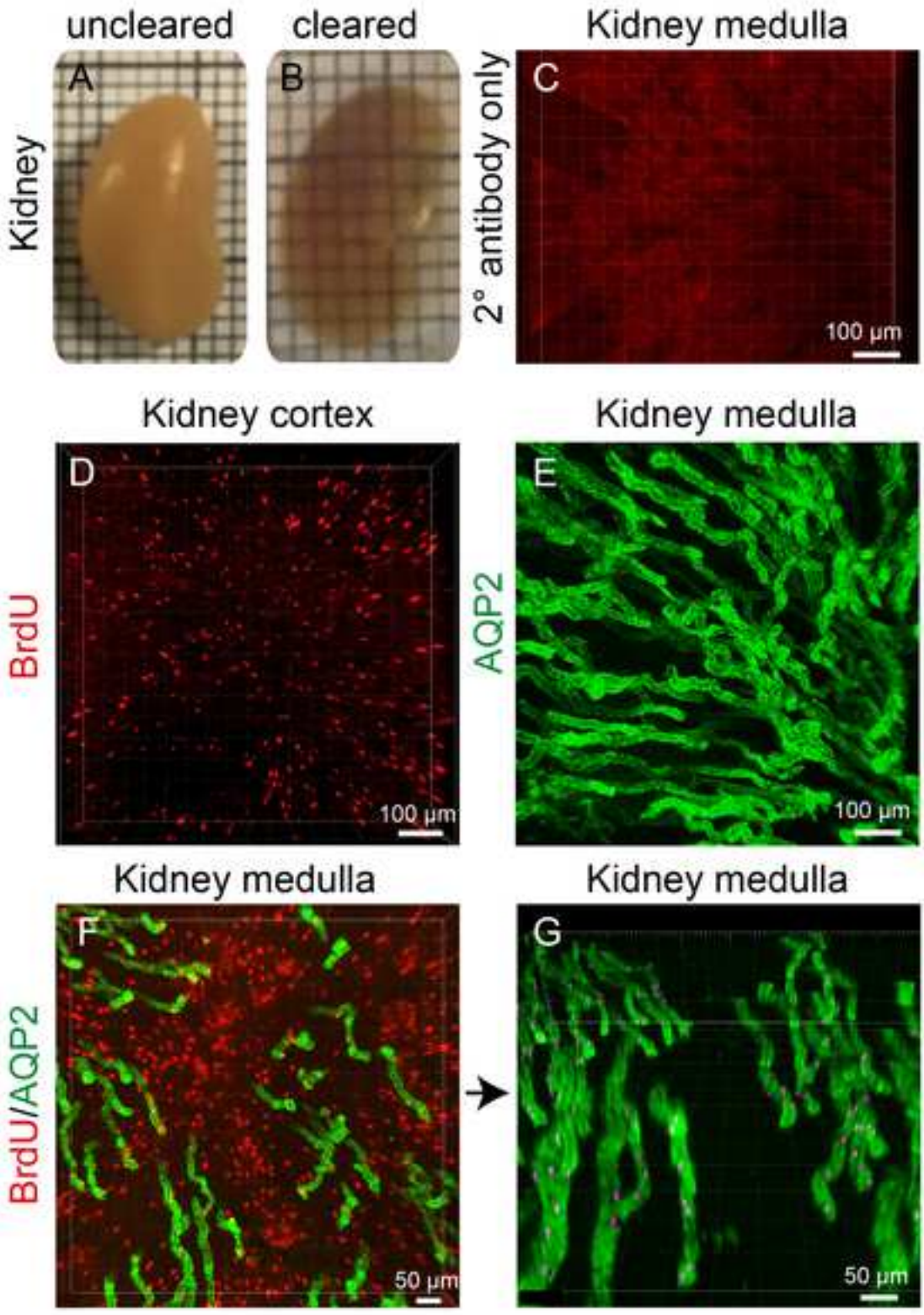
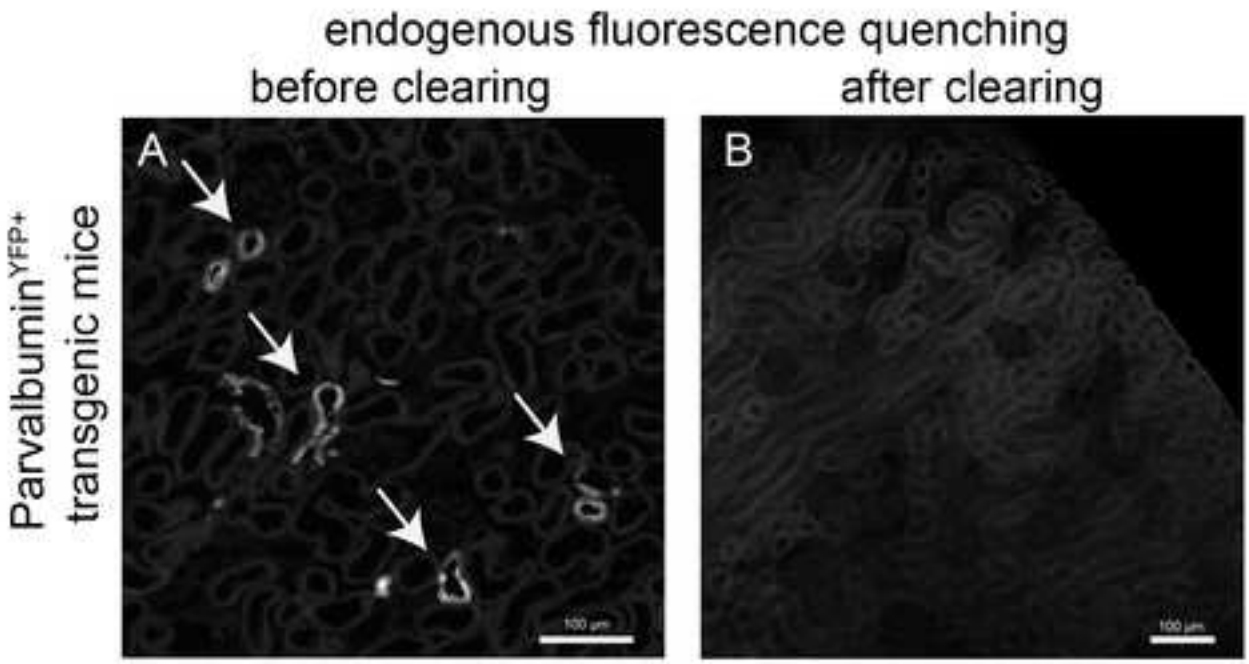


Figure 1



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0.22 µm filter	Fisher Scientific	09-761-112
15 mL conical tube	Fisher Scientific	339650
21 gauge butterfly needle	Braun	Venofix
3-way stopcock	Fisher Scientific	K420163-4503
3D analyis software	Bitplane AG	IMARIS
3D analyis software	Cellprofiler	
5-0 silk suture	Fine Science Tools	18020-50
50 ml plastic syringes	Fisher Scientific	14-817-57
Anti-BrdU monoclonal antibody	Roche	11296736001
Antibody diluent	Dako	S0809
CD31-647	BioLegend	102516
Citrate-based antigen retrieval solution	Vector Laboratories	H-3300
curved hemostat	Fisher Scientific	13-812-14
Dako Wash Buffer	Agilent	S3006
dissecting microscope	Motic	DSK-500
Embedding cassettes	Carl Roth	E478.1
Ethanol	Merck	100983
Ethyl cinnamate	Sigma-Aldrich	112372
Flexible film/Parafilm M	Sigma-Aldrich	P7793
Goat anti-AQP2	Santa Cruz Biotechnology	sc-9882
Guinea pig anti-NKCC2	N/A	N/A
HCl	Carl Roth	P074.1
Heparin	Sagent Pharmaceuticals	401-02
hemostat	Agnthos	312-471-140
horizontal rocker	Labnet	S2035-E
Imaging dish	Ibidi	81218
Ketamine	MWI Animal Health	501090
Micro serrefine	Fine Science Tools	18052-03
NaOH	Fisher Scientific	S318-500
Operating scissors	Merit	97-272
Paraformaldehyde	Thermo Fischer Scientific	O4042-500
Rabbit anti-phoshoThr53-NCC	PhosphoSolutions	p1311-53

Silicone elastomer	World Precision Instruments Kwik-Sil	KWIK-SIL
Sodium azide	Sigma-Aldrich	S2002
Tissue slicer	Zivic Instruments	HSRA001-1
Triton X-100	Acros Organics	AC215682500
Vannas scissors	Fine Science Tools	15000-00
Vibratome	Lancer	Series 1000
Xylazine	MWI Animal Health	AnaSed Inj SA (Xylazine)

Comments/Description

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DOI: 10.1681/ASN.2012040404

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Rebuttal Letter

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Response: As requested, several native speakers did proofreading of the manuscript.

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Response: We uploaded the copyright permission and corrected the figure legends.

3. Please revise lines 81-82, 340-342, and 376-381 to avoid previously published text.
Response: We thank for the comment and revised the text accordingly.

4. Authors and affiliations: Please provide an email address for each author.
Response: We added the email addresses of the co-authors on the title page.

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Response: As requested, we removed commercial language from our manuscripts.

6. 1.5: Please specify the age, gender and type of the animal used. Please also specify the dose of ketamine and xylazine.
Response: We added additional information in the protocol section (1.5.).

7. Please specify all surgical tools used throughout the protocol.
Response: As requested, all surgical tools were specified in the manuscript and table of materials.

8. Lines 143, 150: How large is the incision?
Response: We added additional information in the protocol section.

9. 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.

Response: 2.75 pages or less of the protocol was highlighted in yellow for the video.

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Response: We highlighted completed sentences and made sure that at least one action is written in imperative tense.

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: As requested, all relevant details that are required to perform the step were highlighted.

12. References: Please do not abbreviate journal titles.

Response: We corrected the references accordingly.

13. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Response: We sorted the items in alphabetical order.

Reviewer #1:

Manuscript Summary:

This protocol describes the optical clearing and fluorescent labelling of kidney slices using a solvent-based clearing method called ECi. The protocol is clearly stated and easy to follow for any scientist with some experience with handling animals. However, I have a few comments regarding the introduction, results and discussion.

Minor Concerns:

1. It is mentioned in the introduction that 2D analysis with thin slices is time-consuming. At the same time, the presented protocol is 14 days long (which is pretty long for clearing and staining 1 mm thick tissue slices, other protocols would perform faster, like FACT). This should be mentioned somewhere in the paper.

Response: As suggested by Reviewer #1, we highlighted other protocols in the discussion which might perform better antibody penetration of the tissue. See lines 427-429.

2. When grouping different clearing protocols in the introduction, solvent-based and aqueous-based clearing protocols are mentioned, and hydrogel-based (or SDS-based)

clearing techniques are omitted. Of course, one could argue that hydrogel-based clearing is an aqueous-based technique, but the SDS-delipidation is such a crucial step of these protocols in terms of clearing efficiency and antibody penetration that I think they deserve to be mentioned separately. Also, hyper-hydrating protocols like CUBIC and Scale should maybe be mentioned on their own.

Response: We thank Reviewer #1 for his comment. We mentioned hydrogel-based embedding and hyper-hydrating methods on their own in the introduction. See lines 73-74.

3. The authors mention mild tissue expansion as a problem of aqueous-based techniques. I think this should be more clearly explained to the general reader, why is this a problem? Citation?

Response: We added citations and explained in the introduction that tissue deformation through shrinkage or expansion will affect absolute morphometrics. See lines 85-90.

4. I think the authors have to elaborate (or cite a paper) on what they mean with "protein modification" as a problem with aqueous techniques. Most people would argue that the antigen retrieval step (at 92-98°C) of the protocol presented in this manuscript is also modifying (denaturing) proteins.

Response: We absolutely agree with Reviewer #2. We detailed “protein modification” in the introduction and provided citations (see lines 84-85). We further mentioned in the discussion section that our protocol will denature proteins (see lines 396-398).

5. Our lab has experience with most clearing techniques. We also tried the ECi-protocol, and in our hands it caused a quite high background in kidney tissue. This is of course fine for very abundant targets where the staining is strong, but we found it to be a problem for less abundant targets where other techniques (like hydrogel-based or SDS-based techniques, or other aqueous immersion-based techniques) performed better in our experience. I think this could be mentioned in the discussion (if the authors have the same experience).

Response: In our hands, we did not experience a problematic signal-to-noise ratio when we used the ECi protocol. In fact, we obtained similar results when we compared the ECi-protocol with other protocols (see Puelles et al, Kidney International, Article in Press, 2019). However, we found that good perfusion (removal of red blood cells) and high-grade 100% ethanol at the dehydration step aids to lower autofluorescence and to achieve high penetration depth. In addition, we used antibodies against abundant proteins and had access to strong fluorescent reporter mice, thus we do not have enough experience to state whether this protocol might not be well suited for less abundant targets.

6. The "opening" of the tubules is mentioned as a crucial positive thing in the protocol. It should also be mentioned as a possible artifact when studying tubular proteins or structures.

Response: As requested, we mentioned in the discussion section (see lines 402-406) that artificially opened tubules may have a negative effect when studying tubule

structure. However, we also stated that the opening of tubules would also allow to better distinguish proteins expressed in the apical membrane from other proteins located in the apical membrane on the contralateral side.

7. In our hands (and showed by other groups, like in the PACT publication by Yang et al. 2014) SDS-based clearing like the PACT or FACT protocols are the single best way to increase antibody penetration. This could be mentioned in the "troubleshooting" as a possible solution.

Response: We thank Reviewer #2 for his suggestion, and mentioned SDS-based clearing methods as alternative approaches to increase antibody penetration (see lines 428-429).

8. Finally, it might be of interest for the reader to know why the authors used CLARITY for the publication cited in the discussion. When is this protocol favourable (apart from when fluorescent proteins need to be preserved)?

Response: As requested, we explained why we used CLARITY in our recent publication (see lines 451-452). In addition, we highlighted that aqueous-based protocols including CLARITY is compatible with RNA analysis while no studies have tested solvent-based methods to visualize RNA yet (see lines 386-389).

Reviewer #2:

Manuscript Summary:

The authors describe a simple method to combine immunolabeling of thick kidney slices, optical clearing with ethyl cinnamate and confocal imaging that enables visualization and quantification of three-dimensional kidney structures. This method is very useful for the 3D image analysis of tissue structure.

Major Concerns:

1. Protocol 1.2., lines 123-124:

The sentence that "Transfer 1x PBS and 3% PFA in 1x PBS to 50 mL plastic syringes." should be changed to "Transfer 1x PBS containing Heparin and 3% PFA in 1x PBS to separate 50 mL plastic syringes."

Response: As requested, we reworded this for clarity in 1.2 (protocol section).

2. Protocol 1.9., line 151:

The sentence that "----- perfuse with 50 mL PBS, then switch and perfuse with 50 mL PFA." should be changed to "----- perfuse with 50 mL PBS containing Heparin, then switch and perfuse with 50 mL 3% PFA."

Response: We changed this sentence in 1.9 (protocol section).

3. Protocol 4.1. and 4.2., lines 228 and 236:

"ethyl cinnamate" should be changed to "ECi".

Response: We changed "ethyl cinnamate" to "ECi" in 4.1 and 4.2 (protocol section)

4. Representative Results, lines 261:
Please discuss on the merits of this method compared with the aqueous-based methods such as Scale S method in the Discussion.

Response: As suggested, we highlighted the advantage of aqueous-based methods including ScaleS in the manuscript (see lines 80-90, lines 301-302, lines 386-389).

5. Table 1. Table of materials and equipment.
The sentence "Citrate-based antigen retrieval soluVector Laboratories Cat#H-3300" should be corrected to "Citrate-based antigen retrieval solution Vector Laboratories Cat#H-3300 or H-3301".

Response: Thank you. We updated the table of materials.

6. Table 1. Table of materials and equipment.
Please add the Vibratome (Protocol 1.10. Note, line 158).

Response: As requested by Editor and Reviewer #2, the vibratome was listed in the table of materials.

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Estimated size of new article (number of pages)	14
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