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Multiplex immunofluorescence staining of coverslip-mounted paraffin-embedded tissue sections

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Animal and human tissues are used extensively in physiological and pathophysiological research. Due to both ethical considerations and low availability, it is essential to maximize the use of these tissues. Therefore, the aim was to develop a new method allowing for multiplex immunofluorescence (IF) staining of kidney sections in order to reuse the same tissue section multiple times. The paraffin-embedded kidney sections were placed onto coated coverslips and multiplex IF staining was performed. Five rounds of staining were performed where each round consisted of indirect antibody labelling, imaging on a widefield epifluorescence microscope, removal of the antibodies using a stripping buffer, and then re-staining. In the final round, the tissue was stained with hematoxylin/eosin. Using this method, tubular segments in the nephron, blood vessels, and interstitial cells were labeled. Furthermore, by placing the tissue on coverslips, confocal-like resolution was obtained using a conventional widefield epifluorescence microscope and a 60x oil objective. Thus, using standard reagents and equipment, paraffin-embedded tissue was used for multiplex IF staining with increased Z-resolution. In summary, this method offers time-saving multiplex IF staining and allows for the retrieval of both quantitative and spatial expressional information of multiple proteins and subsequently for an assessment of the tissue morphology. Due to the simplicity and integrated effectivity of this multiplex IF protocol, it holds the potential to supplement standard IF staining protocols and maximize use of tissue.

Key words: Histopathology; immunostaining; microscopy; multiplex; pathology; renal/kidney; image resolution; tissue; coverslips.

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Research in physiology and pathophysiology relies on the use of experimental model systems like laboratory animals and human tissue samples (*i.e.*, biopsies). Generally, the access to human biopsies is limited and the use of experimental animals should be restricted to a minimum. Typically, researchers are interested in investigating the expression levels and subcellular location of specific proteins within tissues. Here, immunofluorescence (IF) microscopy of paraffin-embedded tissue sections provides both quantitative and spatial information of protein expression and allows for staining of multiple fluorophores on the same tissue section [1, 2].

In standard protocols, staining is followed by mounting of a thin coverslip with mounting medium to the microscope slide before imaging. To optimize the use of valuable tissue, new strategies have recently been developed allowing for the removal of the coverslip and previously bound antibodies for subsequent re-staining with new antibodies, so-called multiplex IF [1–6]. Not only does this maximize the use of each tissue section but it also allows for staining with multiple antibodies on the same section. In these protocols, strong chemicals like β-mercaptoethanol [7], $KMnO_4$ [8], or $NaBH_4$ [1] are often used to remove bound antibodies. Other protocols use fluorophore bleaching, with low pH and light, to inactivate the fluorophore instead of removing the antibodies [2, 4, 9]. However, these methods require that the primary antibodies are directly labeled which is more expensive and less sensitive than using indirect labelling

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(*i.e.*, a set of primary with secondary, fluorescently labeled antibodies) [6]. Furthermore, these protocols require removal of mounting media and coverslips between stainings, which can damage the sections and is time consuming.

The overall aim of this study was to establish a new and easy protocol for multiplex immunofluorescence staining of paraffin-embedded tissue sections. Thus, the specific objectives were as follows: (1) to place paraffin-embedded tissue sections on coated coverslips to increase Z-resolution, avoid mounting media, and reduce handling time compared to using conventional microscope slides; (2) to remove previously bound antibodies using conventional and cheap reagents; and (3) to perform multiplex immunofluorescence staining on specific targets combined with conventional staining using hematoxylin and eosin for a general assessment of tissue morphology.

MATERIALS AND METHODS

Precoating of coverslips

Glass coverslips (25 mm Ø and 0.16 mm thick) were washed in deionized water with hand soap, then sprayed with 70% ethanol on both sides and dried. Next, the coverslips were incubated five times for 5 s in a preheated (45 °C), filter sterilized (0.45 µm filter unit, Sartorius, Goettingen, Germany) gelatin-coating solution (0.5% gelatin, 1 mM chromium potassium sulfate dodecahydrate (Sigma-Aldrich, Co., St. Louis, MO, USA) and deionized water), containing 1 µm FluoSpheres (F13081, 1:4000; ThermoFisher, Invitrogen, Eugene, OR, USA). Finally, the coverslips were dried at room temperature for 48 h in the dark. The gelatin-coating solution protocol was adapted from R&D Systems [10].

Multiplex immunofluorescence

Formalin fixed and paraffin-embedded kidney tissue from humans and mice were used. The use of human kidney tissue was approved by the Central Denmark Region Committees on Biomedical Research Ethics (Journal number 1-10-72-211-17) and the Danish Data Protection Agency. The tissue samples were obtained from functional (*i.e.*, eGFR >60 mL/min/1.73 m^2) and macroscopically healthy renal cortical tissue from patients following nephrectomies, as described previously [11,12]. All participants gave written informed consent. Mouse kidney tissue was obtained from healthy 8–9-week-old C57BL/6 male mice (Janvier Labs, Le Genest-Saint-Isle, France) and the animal protocols were approved by the Department of Clinical Medicine, Aarhus University, according to the licenses for the use of experimental animals issued by the Danish Veterinary and Food Administration (Approval no. 2020-15-0201-00617). Kidney sections (3 µm) were placed onto the coated coverslips, heated on a hotplate at 60 °C for 10 min, and subsequently placed in xylene overnight, or a minimum of 2 h at room temperature. The sections were then rehydrated in descending concentrations of ethanol starting from 99% (3 × 5 min), 96% (2 × 5 min), 70% (5 min), and lastly washed three times in deionized water. Next, the sections were placed in slide holders containing 200 mL retrieval buffer (0.5 mM EGTA [Merck] in 10 mM Tris-base buffer pH 9) and boiled in a microwave oven for 10 min at 375 Watt to maintain constant mild boiling. Afterwards, the sections were placed on ice and cooled to room temperature. Then, the sections were placed in 50 mM NH_4Cl buffer for 30 min and subsequently washed three times in blocking buffer for 10 min (1% bovine serum albumin [BSA], 0.2% gelatin and 0.05% saponin in PBS). This was performed by placing the coverslips on a drop of buffer, which was placed on parafilm. The coverslips were inverted so the tissue was facing down on the drop (drop incubation). Next, the sections were drop incubated with 70 µL primary antibodies (see Table 1 below) diluted in staining buffer (0.1% BSA and 0.3% Triton X-100 in PBS). The incubation was performed in a humidity chamber; first for 30 min at room temperature and then overnight at 4 °C. The following day, the sections were placed at room temperature for 1 h and washed three times in PBS and subsequently incubated with secondary antibodies (see Table 1 below) and the nuclear stain Hoechst (33 342; ThermoFisher, Invitrogen) for 50 min. Finally, the sections were washed three times in PBS. For imaging, the coverslips were placed in a Chamlide Magnetic Chamber (Live Cell Instrument Co, Republic of Korea) and filled with PBS as for live-cell imaging (albeit here cell culture media is used instead of PBS) [13].

Table 1. Antibodies used in this study

Target	Source	Specificity includes	Dilution	Manufacturer
Primary antibodies				
AQP1	Rabbit	Human, mouse	1:200	AB2219; Merck-Millipore
AQP2	Rabbit	Mouse	1:200	sc-515770; Santa Cruz Biotechnology
CD34	Rabbit	Human, mouse	1:200	ab81289; Abcam
PDGFRα	Goat	Mouse	1:20	AF1062; R&D Systems
Podocin	Rabbit	Human	1:500	ab181143; Abcam
Uromodulin	Sheep	Human, mouse	1:500	AB733; Merck-Millipore
Vimentin	Rabbit	Human	1:200	92 547; Abcam
Secondary antibodies				
Rabbit	Donkey		1:500	IR-dye 680; Invitrogen
Mouse	Donkey		1:500	Alexa Fluor 660; Invitrogen
Goat	Donkey		1:500	Alexa Fluor 680; Invitrogen
Sheep	Donkey		1:500	Alexa Fluor 594; Invitrogen

AQP, Aquaporin; CD, Complementary domain; PDGFRα, Platelet-derived growth factor receptor alpha.

Antibody stripping

To remove the antibodies and the Hoechst staining from the tissue, the sections were incubated with a reducing antibody stripping buffer (20% 0.35 M SDS, 20% 0.5 M Tris-base, pH 6.8, and 1% 1 M DTT in deionized water). First, the SDS and the Tris-base were mixed with deionized water and heated to 56 °C and just before use, the DTT was added to the mixture and the sections were incubated for 50 min at 56 °C. This was followed by five washes for a total of 30 min in TBS-Ts (0.01% Tween 20 and 30% sucrose in TBS). Antibody staining and stripping could be repeated a minimum of five times and after the last imaging, the sections were counterstained with hematoxylin and eosin (H&E) using a standard protocol, dehydrated, and mounted in hydrophilic mounting medium on a microscope slide.

For the comparison studies of the image resolution between tissue attached to coverslips or microscope slides, 3 µm human kidney sections were attached to Superfrost Plus Adhesion Microscope Slides (Epredia), treated as previously described, and mounted with Glycergel antifade mounting medium (DAKO; Agilent Technologies, USA).

Image acquisition and registration

Images of IF-stained sections were acquired on a Nikon T*i* Eclipse inverted fluorescence microscope equipped with a CoolLED pE-300 unit. Acquisition was performed with an Andor Zyla 5.5 Mpixel camera (Andor Technology Ltd., Belfast, UK) with a 12-bit setting. For visualization of the different channels, we used the following excitation (ex)/emission (em) filter cubes: 370/460 nm, 460/510 nm, and 550/670 nm. Mouse kidney sections were imaged with a 40× air objective (NA 0.65) and 17 × 17 images were stitched automatically to capture the whole kidneys. Human kidney sections were imaged with a 60× oil objective (NA 1.40) and 4 × 4 images were stitched. Stitching was performed during acquisition using NIS-elements AR software (version 4.51.01). All images were processed in Fiji (ImageJ) [14] by first combining all the three-stack images using "Concatenate" and then aligned with the plugin "HyperStackReg" (V5.6) [15] using the green (em 510 nm) and blue channel (em 460) as references. For visualization of the H&E-stained kidney sections, bright-field images were acquired on a ZEISS upright Axio Imager 2 equipped with an Axiocam 506 and a 20× air objective (NA 0.8) objective was used to produce 10 × 10 images that were automatically stitched together using ZEN 2 (blue edition) software.

RESULTS

A multiplex immunostaining protocol for paraffin-embedded tissue sections using coverslips instead of microscope slides

The aim was to establish a new protocol for IF staining of paraffin-embedded tissue sections on glass coverslips that enables multiple rounds of staining and imaging, which could be terminated with a hematoxylin–eosin stain to visualize tissue morphology. Such a protocol would optimize the use of valuable tissue, reduce costs, and improve image quality of widefield fluorescence images.

In multiplex IF, a section is normally placed on a microscope glass, stained, and a thin coverslip is mounted using mounting media. Following imaging, the coverslip and mounting media are removed before the section can be re-stained. To eliminate these steps, the tissues were placed on round coverslips, which fit in a chamber for live-imaging, and covered with PBS (Fig. 1). Imaging was performed on an inverted widefield epifluorescence microscope using the far-red channel (550/670 nm) to visualize the target protein, the green channel (460/510 nm) to visualize the fluorescent beads (505/515 nm) as well as the prominent autofluorescence observed from kidney tissue [16] and the blue channel (370/460 nm) to visualize Hoechst staining of DNA.

Antibody stripping enables multiple rounds of IF staining on the same tissue

To evaluate effectiveness of the antibody stripping method, sections were first stained for the renal water channel protein, aquaporin-1 (AQP1) (Fig. 2A) and imaged. Following imaging, the sections were stripped for antibodies and the same region was imaged. No specific signal from the antibodies nor the Hoechst staining was observed after stripping. It has previously been shown that the autofluorescence signal declines after repeated staining and stripping [1]. To investigate this, autofluorescence was quantified after each round of stripping which revealed that not only the intensity of the autofluorescence, but also the repeating Hoechst staining, decreased between 4% and 10% (Fig. 2B) per round. After four rounds of stripping, the accumulated Hoechst intensity decreased with 24% and the autofluorescence decreased with 19%, compared to the intensity after the first staining (where no antibody stripping was performed). Note that the decrease in autofluorescence was heterogeneous (Fig. 2C, red inserts). Moreover, the tissue architecture was slightly altered after four rounds of stripping (Fig. 2C). In particular, different tissue artifacts (*i.e.*, folding of the tissue and minor cracks and holes) (Fig. 2C, yellow inserts) were observed.

Placing the tissue section on thin coverslips increases image resolution

To test whether Z-resolution could be increased using coverslips instead of microscope slides, serial sections were analyzed. Two adjacent sections were placed onto either a coated coverslip or a Superfrost Plus Microscope slide and stained against

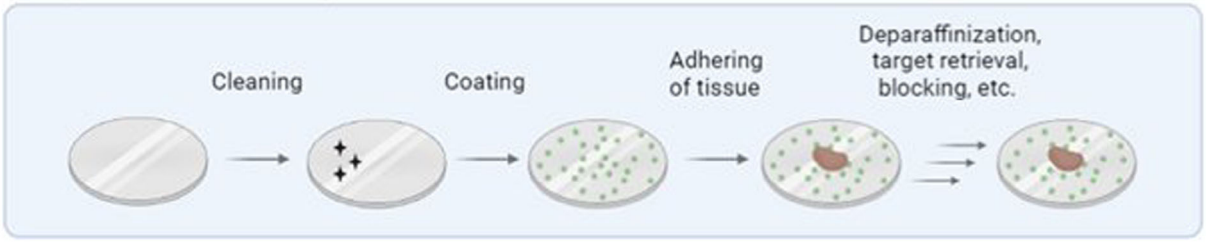

Fig. 1. Preparation of coverslips and attachment of tissue. Coverslips (25 mm Ø and 0.16 mm thick) were cleaned in soapy water and 70% ethanol and dipped in a gelatin-coating solution containing chromium and autofluorescent beads (FluoSpheres). Three micrometer human or mouse sections were adhered onto the coated coverslips. The coverslips were prepared according to standard protocols (see Multiplex immunofluorescence) and incubated with primary (overnight) and secondary antibodies (for 50 min). AB, antibody.

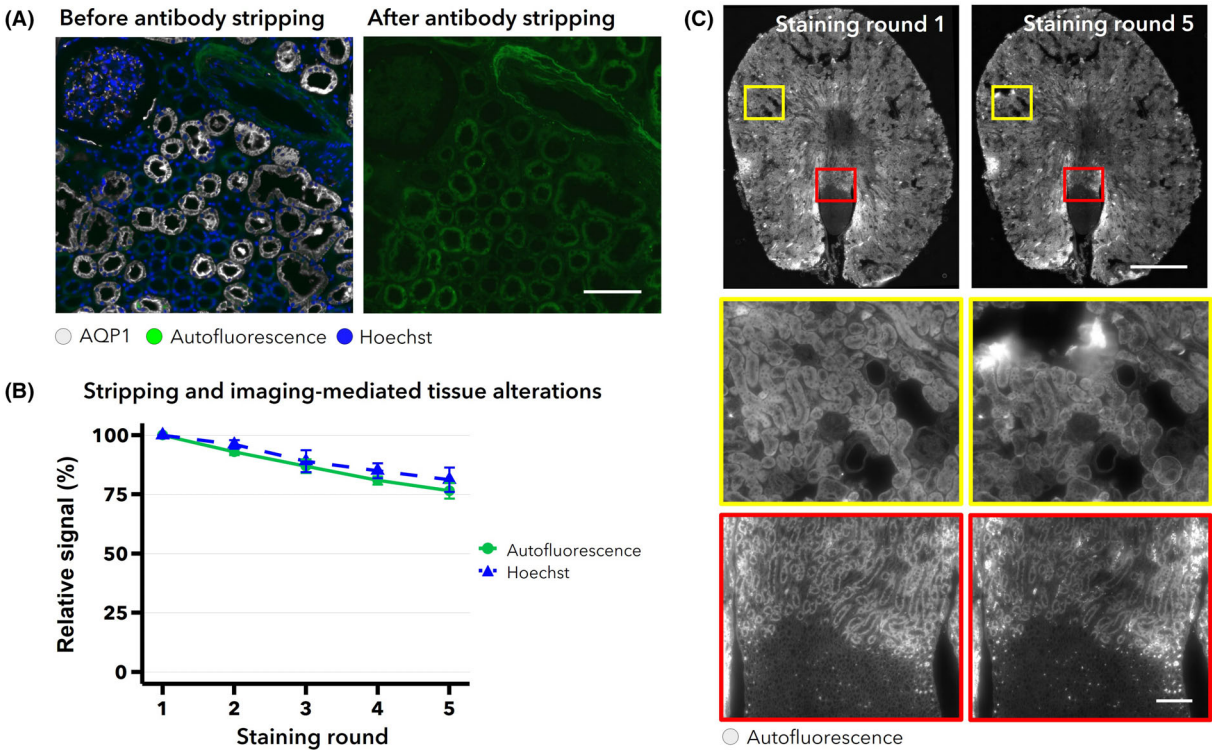

Fig. 2. The antibody stripping solution is effective but decreases both the autofluorescence and Hoechst staining signal for every round. (A) Representative images of kidney tissue sections stained against AQP1 and counterstained with Hoechst before (left image) and after (right image) incubation with the antibody stripping solution. The images were acquired under identical settings. Objective: 60× oil. Scale bar: 100 µm. (B) Cycle-dependent decrease of nuclei intensity strength (em 460 nm) (blue) and kidney autofluorescence (em 550 nm) from mouse kidney tissue (n = 4) expressed as % change relative to the first round (cycle round 1) from mouse kidney sections. The images were acquired with identical microscope settings and measured from images of equal size using the "Measure" function in Fiji. n = 4 per IF staining round. Data are represented as ± SD. (C) Difference in tissue architecture and autofluorescence intensity from first (left column) to fifth cycle (right column); the four lower contoured images are magnified views of the marked areas on the two top images. Objective: 40× air. Scale bar (upper images): 1000 µm. Scale bar (lower images): 100 µm. AF, autofluorescence; em, emission; nm, nanometers.

podocin (a marker of the podocytes forming the slit diaphragm of the glomerulus) to compare the difference in the image resolution between the two techniques (Fig. 3A). The contours of the membrane of podocytes appeared sharper in the images from the coverslip-attached tissue section vs the

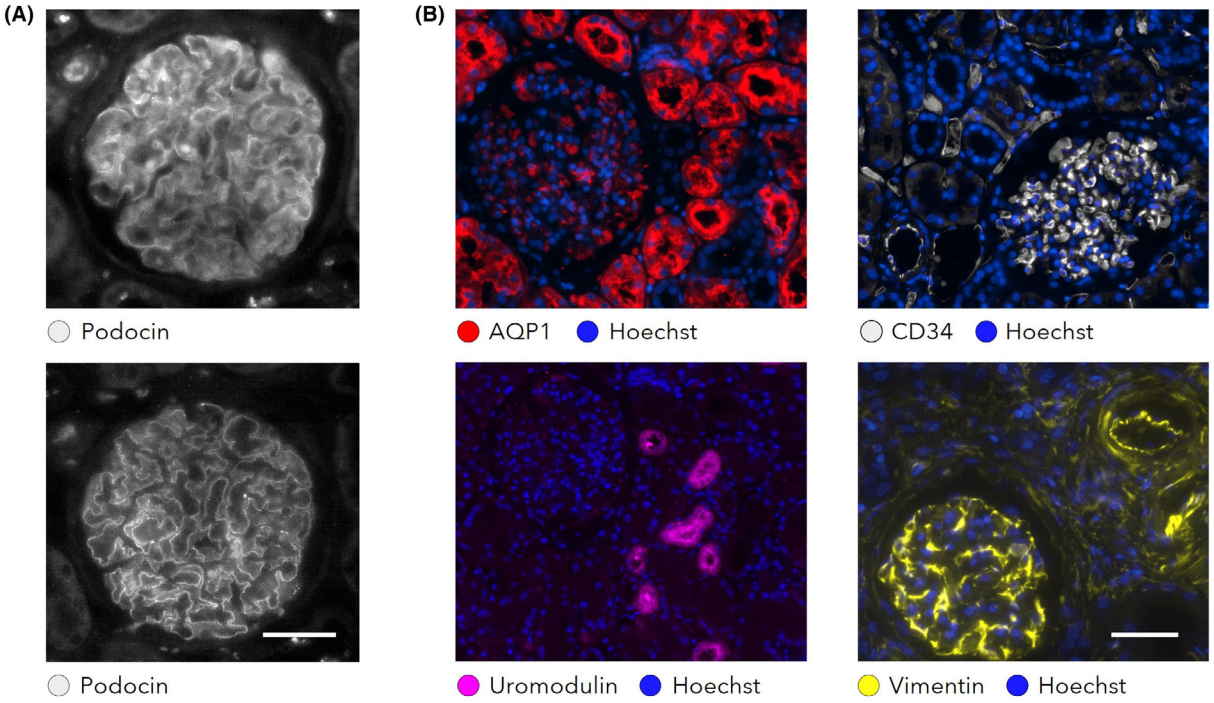

Fig. 3. Attaching tissue directly on a glass coverslip increases image resolution. (A) Human kidney blocks were serial sectioned and placed onto microscope slides (top) or coverslips (bottom) and stained against podocin. The sections were treated and imaged under identical conditions. Objective: 60× oil. Scale bar: 50 µm. (B) Human kidney sections on coverslips were stained with Hoechst (nuclear staining) and against AQP1 (stains proximal tubules, thin descending limb of Henle, and descending vasa recta), CD34 (stains endothelial cells), uromodulin (stains the thick ascending limb of Henle and the convoluted distal tubule), and vimentin (stains podocytes, the intertubular interstitium, and the tunica media of arterioles). Objective: 60× oil. Scale bar: 50 µm.

sections attached onto a microscope slide where imaging was performed through the mounting medium (Fig. 3A bottom vs top). Furthermore, the background noise was also decreased using the coverslips. To showcase the confocal-like resolution, a small collage of images acquired from tissue attached to coverslips are displayed in Fig. 3B.

Multiple rounds of IF staining on the same tissue can be followed by a H&E counterstain

Next, a sequence of five stainings of a mouse kidney section was performed to visualize the different tubular segments of the nephron, the blood vessels, and the interstitial cells of the mouse kidney (Fig. 4A). The multiplex IF stainings were acquired in the following order: AQP1, AQP2, Uromodulin, CD34, and PDGFRα. All antibodies but one (the PDGFRα antibody) were removable using our stripping buffer, thus, the PDGFRα antibody was used last in the sequence of stainings (Fig. 4A). To visualize kidney morphology, the kidney sections were stained with H&E following the multiplex IF staining. The H&E staining revealed tissue morphology, but the staining was less intense than normal, and revealed some artifacts originating from the repeated handling of the tissue (Fig. 4B).

DISCUSSION

IF microscopy is widely used in research laboratories as it provides information about the expression and subcellular location of specific proteins. However, standard protocols only allow for one staining round per sample. Therefore, the aim in this study was to develop a method suited for multiplex IF staining of coverslip-attached paraffin-embedded tissue. First, we showed that this approach was compatible with standard deparaffinization and microwave-mediated target retrieval. Next, we performed IF staining and acquired images with confocal-like resolution using a widefield epifluorescence microscope while showing that our antibody stripping protocol effectively removed the previously bound antibodies. We performed five rounds

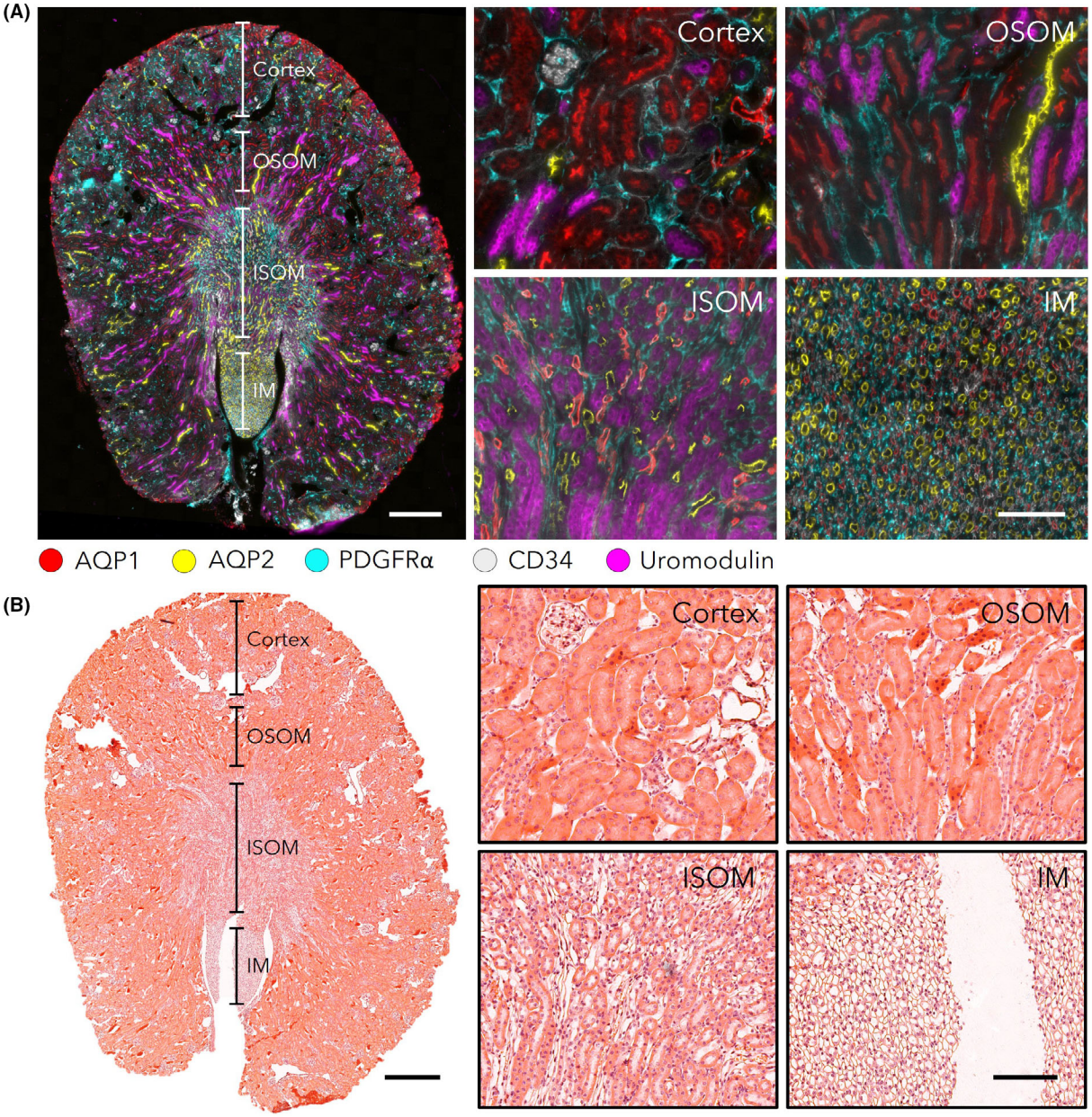

Fig. 4. Mouse kidney sections stained with multiplex immunofluorescence. (A) Left panel: Mouse kidney section placed on a coverslip and stained with markers for five distinct segments: AQP1 (red, proximal tubules, thin descending limb of Henle, and descending vasa recta), CD34 (gray, fenestrated capillaries of the glomeruli and in the remaining endothelial cells), and uromodulin (magenta, ascending thick limb of Henle and distal convoluted tubules). AQP2 (yellow, collecting duct principal cells) and PDGFRα (cyan, interstitial cells within the glomeruli and in the intratubular space). Objective: 40× air. Scale bar: 500 μm. Right panels: Representative areas of mouse kidney zone. Scale bar: 100 μm. (B) Left panel: Mouse kidney section stained with H&E after five rounds of IF staining. Objective: 20× air. Scale bar: 500 μm. Right panels: Same representative areas of each zone of the mouse kidney as in A. ISOM, Inner stripe of outer medulla; OSOM, Outer stripe of outer medulla; IM, Inner medulla.

of staining (Fig. 5) finalizing with an H&E stain for tissue morphology assessment.

Multiplex IF allows for several rounds of staining on the same tissue and depending on antibody species and the microscope setup multiple fluorophores can be imaged in each round. This allows for maximized use of each tissue section and for more advanced colocalization studies. Moreover,

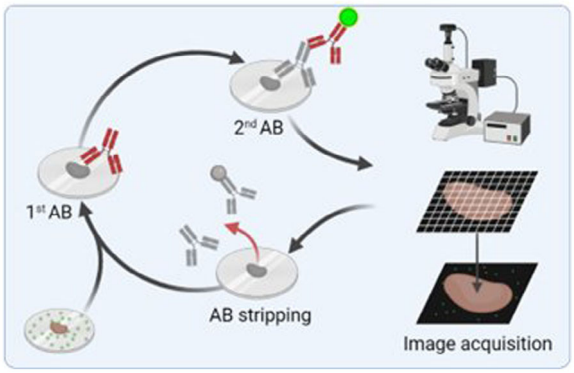

Fig. 5. Multiplex immunofluorescence staining. The kidney sections were imaged at either 40× or 60× magnification and stitched using NIS-elements software. After image acquisition, the sections were stripped, incubated with blocking buffer, then with primary and secondary antibodies, and imaged. These steps (orange arrows) could be repeated a minimum of five times . AB; antibody.

antibodies raised in the same species can be used on the same tissue section. All reagents and instruments used are commercially available, have low cost, and require no specialist expertise. This contrasts with other described methods employing directly labeled antibodies [2,5], spectral imaging [17], and confocal microscopes [4,18]. To assemble the separate images of the same tissue sections, tissue-specific structural features were used for alignment using ImageJ. In this study, fluorescent beads were added in the gelatin-coating solution and used as guidelines for the image-alignment software. However, the HyperStackReg-plugin, used for image alignment, was as efficient in alignment using the green autofluorescence from the kidney tissue. However, in less autofluorescent tissues, these beads may be essential for correct image alignment.

In recent years, a variety of different protocols for multiplex IF staining on tissue sections have been published [1,2,4,5,9,19]. These protocols showed ineffective stripping of high-affinity antibodies or highly stable fluorophores like the PDGFRα antibody used in this study. Therefore, it is recommended to test all antibodies for their ability to be removed by the stripping buffer. Alternatively, the buffer can be supplemented with β-mercaptoethanol to remove rigid antibodies [1]. The stripping buffer used here was designed to be relatively mild to protect the tissue. Still, the tissue architecture was slightly compromised after four rounds of antibody stripping and decreased intensity of both the kidney autofluorescence and nuclear staining was observed (Fig. 2C). These changes in the tissue could also explain the unusual color reaction observed in the H&E staining performed after four rounds of antibody stripping (Fig. 4B). In a related study, Bolognesi *et al.* [1] found that after four rounds of antibody stripping, the kidney autofluorescence signal was reduced by 20% similar to the observation using this protocol (Fig. 2B). Likewise, they showed a heterogeneous reduction in the autofluorescence intensity throughout the kidney, implying a selective inactivation of highly fluorescent tissue components. These changes may be explained by a combinatory effect of repetitive light exposure that bleaches the fluorophores and the direct chemical effect of the stripping buffer on the tissue. Consequently, the gradual decrease of tissue integrity must be considered when designing the experiments and especially if quantifications of the staining intensity of specific proteins are made on the final images. Therefore, it is recommended to place antibodies against less abundant targets, or targets that will be quantified, earliest in the sequence of stainings, while antibodies against high abundance targets or antibodies against structural proteins or markers of specific tissue segments can be placed last in the staining sequence. As an example, if the expression of two proteins within specific compartments of a given tissue are quantified, the two proteins should be placed in the two first rounds of staining, while markers against the different compartments should be placed in round three, four, and five. An additional consequence of the repetitive staining and antibody stripping was that the signal intensity was increased around the edges compared to the rest of the kidney because the antibodies bound on both sides of the tissue due to slight detachment of the tissue. For optimal post-processing of the images, the tissue sections should be placed in the center of the coverslip; this decreases the possibility of image drifting and fluctuations in the focus plan across the sample, which becomes a problem when acquiring large images. For this reason, it was not possible to acquire the images of the full mouse kidney sections with the 60× oil objective, as the objective came too close to the wall of the imaging chamber.

Using coverslips compared to microscope slides increased Z-resolution, which reflects that when imaging mounted tissue, the mounting medium increases the distance between the objective and sample exceeding the optimal working distance of the objective and that when light passes a liquid medium it scatters before reaching the detector and if there is a mismatch between the refractive indices of the mounting medium and the immersion oil, the scattering is increased [20]. Attaching tissue to coverslips increases the signal-to-noise ratio, thus creating confocal-like image resolution. An additional

benefit of using coverslips instead of microscope slides is that the evaporation during the overnight antibody incubation is minimized because the sections are placed on top of a drop of antibody solution. This reduces the total amount of antibody solution needed per staining round. Moreover, as no removal of the mounting medium-adhered coverslip was needed to expose the tissue between the staining rounds, the method is more time-efficient than using microscope slides.

Combined, this new method is compatible with all the different parts of IF staining and is suited for multiplex IF including a final H&E staining. We therefore believe that this new method holds the potential to supplement the conventional methods and save tissue when availability is scarce, like for human biopsies, as well as reduce the number of experimental animals.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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