New technical approaches for 3D morphological imaging and quantification of measurements

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Abstract
3D imaging is becoming more and more popular, as it allows us to identify interactions between structures in organs. Furthermore, it gives the possibility to quantify and size these structures. To allow 3D imaging, the tissue sample has to be transparent. This is usually achieved by using optical tissue clearing protocols. Although using optical tissue clearing often results in perfect 3D images, these protocols have some pitfalls, like long duration of sample preparation (up to several weeks), use of toxic substances, damage to antibody staining, fluorescent proteins or dyes, high refractive indices, and high costs of sample processing. Recently we described [Huang et al., Scientific Reports 9(1): 521 (2019)] a fast, safe, and inexpensive ethyl cinnamate (ECi) based optical tissue clearing protocol. Here, we present extensions of our protocol with respect to the deparaffinization of old paraffin-embedded samples allowing 3D imaging of the blocks. In addition, we learned to remove ECi from the samples allowing the use of routine immunolabeling protocols. Furthermore, we demonstrate new pictures of lungs after expansion microscopy and adaptation of already existing protocols. The aim of our work is, in summary, to describe the advances in these methodologies, focusing on the morphological imaging of kidneys and lungs.

KEYWORDS
deparaffinization, ECi-OTC, ExM, immunolabeling, 3D imaging

1 | INTRODUCTION

The need for volumetric 3D imaging is related to the deeper understanding of morphogenetic and developmental processes in organs and whole organisms (Richardson & Lichtman, 2015). The worst drawbacks in 3D imaging are the lack of transparency of tissue and the light scattering phenomenon. Both of them are consequences of the specific tissue texture (Richardson & Lichtman, 2015). In fact,
the presence of molecules, membranes, organelles, and cells, with their own refractive indexes (RIs), does not allow light to travel in straight lines, as it should, but it is often reflected, causing a deterioration of the final 3D imaging (Richardson & Lichtman, 2015). In particular, the overall tissue RI is generally around 1.4–1.5 (Bolin, Preuss, Taylor, & Ference, 1989; Tuchin, 2015). Additionally, pigments such as hemoglobin, myoglobin, and melanin provide certain tissue coloration. These molecules are responsible for the major part of light absorption (Richardson & Lichtman, 2015). In addition, the lipids of the cellular membranes bilayers are also contributing to opacity, due to their high RI value and their tendency to create aggregates (Tuchin, 2015). The principle of optical tissue clearing (OTC) is to remove/reduce the lipid content of the sample and homogenize the RI of the tissue (Richardson & Lichtman, 2015) leading to optical transparency and/or translucency, which is essential in the 3D imaging.

Since the beginning of the 20th century (Spalteholz, 1911), many OTC protocols have been established, which may be grouped into solvent-based and aqueous-based clearing techniques. The first group is characterized by high RI values and a good potential for de-lipidation (Becker et al., 2014). The most important disadvantages of these clearing solutions are their toxicity and corrosive nature that might damage the microscope, as well as the fluorescent signal. Moreover, the dehydration causes a high degree of tissue shrinkage (up to 50%) (Becker et al., 2014). In order to overcome these limitations, new solvent-based OTC protocols have been described, like 3DISCO (Ertürk et al., 2011; Ertürk & Bradke, 2013), iDISCO (Liebmann et al., 2016; Renier et al., 2014), BABB (Dobosz, Ntziachristos, Scheuer, & Strobel, 2014; Renier et al., 2016).

Aqueous-based OTC protocols, however, minimize the tissue shrinkage as well as the fluorescent signal decay, as many fluorescent proteins require an aqueous environment to fluoresce (Richardson & Lichtman, 2015); although their worst disadvantage is that they are highly time consuming (days to several weeks). This is especially true for the protocols containing sucrose, fructose, or glycerol (Ke, Fujimoto, & Imai, 2013; Meglinski, Churmakov, Bashkatov, Genina, & Tuchin, 2003; Staudt, Lang, Medda, Engelhardt, & Hell, 2007; Tsai et al., 2009). Another aspect of some aqueous-based OTC protocols is the combination of the removal of lipids (by using detergents) with hydration (e.g., urea) of the sample, to reduce the RI of the tissue components, as described in CUBIC and Scale methods (Hama et al., 2011; Hama et al., 2015; Nojima et al., 2017). Other protocols are based on the use of hydrogels to prevent protein loss followed by the use of detergents (e.g., SDS) for lipid removal (performed passively or via electrophoresis) and incubation in RI matching solutions, as reported for CLARITY and PACT (Chung et al., 2013; Neckel, Mattheus, Hirt, Just, & Mack, 2016; Tomer, Ye, Hsueh, & Deisseroth, 2014; Yang et al., 2014). Although PACT was established to overcome some limitations of CLARITY (expensive equipment, difficulty to implement mounting solutions), one of the worst drawbacks remain the long sample preparation duration. These limitations had already been overcome by our group, where we have described an OTC protocol, based on the use of ethyl-3-phenylprop-2-enoate (ethyl cinnamate – ECi). ECi-based OTC is an optimization of the procedure published by Klingberg et al. (2017). ECi is the main component of the cinnamic acid and it is considered nontoxic, according to the European directive 67/548/EEG. It is an FDA approved food flavor and additive for cosmetic products (Wang, Zhang, Zhang, Chen, & Zhi, 2016). We described a new ECi-based OTC (ECi-OTC) protocol by using an automatic tissue processor and performing the whole sample processing at room temperature which optimizes the diffusion coefficient (Huang et al., 2019). Thereby, the time interval between organ harvesting and microscopic analysis (confocal or light-sheet microscopy) was reduced from 18 to 5 hr. Moreover, a cationic near-infrared fluorescent dye was developed for the staining of blood vessels, for example, in kidney, heart, liver, and lung (Huang et al., 2019).

Subsequently, we combined our ECi-OTC protocol with deparaffinization and 3D immunohistochemistry. Here, we present a new mode to deparaffinize and optically clear old paraffin-embedded samples using autofluorescence for 3D imaging. After ECi removal in decreasing ethanol concentrations we could perform immunolabeling of samples, previously optically cleared with ECi. As a consequence, the samples were also rehydrated, and this modified RI from high (ECi RI: 1.558 at 25°C) to aqueous RI (e.g., glycerol RI: 1.478 at 25°C). In addition, we improved the resolution of 3D imaging by using expansion microscopy (ExM). This technique was established to enable the super-resolution imaging of the glomerular filtration barrier, enhancing imaging resolution fivefold (Unnersjo-Jess et al., 2018). The protocol involves the synthesis of a swellable polymer network and the embedding of the specimen in this densely cross-linked polymer gel. Due to the swelling properties of the gel-specimen, it can be physically expanded resulting in physical magnification. In this work, we focused also on lung ExM, although, given the consistency of pulmonary tissue, the standard ExM procedure could have damaged the microstructures within it. Therefore, based on the original protocol developed by Unnersjo-Jess’s group in 2018, we decided to stop the expansion procedure directly after
the denaturation step, in order to obtain an expansion factor of ≈2. First, we tested this modified ExM procedure on fresh pulmonary tissue and afterward on deparaffinized tissue, combining in both cases an immunolabeling protocol. This was done in order to obtain 3D imaging of the pulmonary tissue with very high resolution, without running the risk to damage the finest lung structures.

In summary, we want to describe different optimized methodologies aiming to improve the 3D imaging with a particular focus on renal and lung tissue morphology as well as on vascular structures of the kidney and lungs.

2 | MATERIAL AND METHODS

As the aim of this work is to illustrate different methodologies, performed both on fresh and deparaffinized tissue, this paragraph has been divided according to the different protocols. First, sample imaging, data processing, and statistical analysis are described; second, the handling of fresh tissue is explained, and lastly, methodologies focusing on clearing and expansion of deparaffinized tissues are elucidated. For each topic, an animal used, the protocol of clearing/expansion, immunostaining, and details regarding the imaging have been clarified. Supplementary Tables (Table a, Table b, Table c) have been also provided to give a more detailed description of the material used for any protocol illustrated.

2.1 | 3D image acquisition

The samples were imaged using a confocal (Leica TCS SP8, from Leica Microsystems, Wetzlar, Germany) or a light-sheet microscope (Leica DLS, from Leica Microsystems).

In order to balance the acquisition time and the quality of the pictures, the resolution (1,024 × 1,024 for confocal microscopy and 2 × 2 binning for the light-sheet microscope) and speed of imaging were kept always constant. Parameters including laser intensity, the aperture of the pinhole, and depth (z-axis) assigned were fixed according to the characteristics of every single specimen. Depending on the type of specimen, its thickness and consistency, as well as the type of microscope, two different ways of sample mounting, were performed:

1 Confocal microscopy: on the border of a 60 mm tissue culture dish (Orange Scientific, Belgium) some adhesive paste (HAMA) was attached, in order to fix a G27 1/2 needle, which worked to anchor the specimen, that is, particular kidneys. After positioning the sample, the dish was filled by either immersion oil (Type F Immersion oil, from Leica. RI: 1.518 at 23°C) or some other hydrophilic mounting media, as 88% glycerol (RI: 1.478 at 23°C) or 1× PBS (RI: 1.34 at 25°C), which completely covered the specimen. Alternatively, for tissues with a softer surface (i.e., lung), which could be easily damaged by the insertion of the needle into the sample, we glued the sample to the center of the dish, by using a big drop of super glue (UHU);

2 Light-sheet microscopy: as the objective of this microscope is surrounded by a mirror cap with an orientation of 45°, the specimen should fit into this space. In particular, to image the mouse kidney, the most suitable method to anchor the samples was the use of a sticky piece of parafilm of 1 mm in thickness in the center of the glass bottom of the Petri dish (35 mm high, 60 μm in diameter, IBIDI, Martinsried, Munich, Germany). On that, a piece of the specimen, maximum 4 mm in width and 5 mm in height was pasted, by using the super glue. Finally, the dish was completely filled with ECI (RI: 1.558 at 25°C).

2.2 | Data processing, storage, and statistical analysis

Image reconstruction and analysis were performed with Las X software (Leica Microsystems). Las X software was also used to evaluate the glomerular size change.

All data acquired from both microscopes were stored in the Scientific Data Storage of the Computing Center of the University of Heidelberg, Germany.

Statistical analysis was performed using JMP genomic 7 software. Calculations of mean, SDs, analysis of variance (ANOVA), and paired t-students were performed with the same software. p values <.05 were considered statistically significant.

2.3 | Animals and perfusion

All the experiments were conducted in accordance with the German Animal Protection Law and approved by the local authority (Regierungspräsidium Nordbaden, Karlsruhe Germany in agreement with EU guideline 2010/63/EU). Eight weeks old female wild type C57/BL6 mice were anesthetized by intraperitoneal injection of Ketamine and Xylazine (16 mg/kg BW Xylazin and 120 mg/kg BW Ketamine) and, then, perfused with saline, fluorescent dye, saline to flush the unlinked dye and then 4% PFA for fixation, according to Huang et al. (2019). This perfusion method accomplished the removal of erythrocytes and allowed the immediate staining of all organs of the animal. Fresh kidneys and lungs were collected. After the organs were harvested, they were fixed and stored in 4% PFA, until further processing.
2.4 | ECI-based OTC (ECi-OTC)

Fresh renal tissues from C57/BL6 mice were rapidly cleared as reported in Huang et al. (2019). During the perfusion, the specimens were stained with the cationic MHH148-PEI and MHH148-PEI-opt (from now on named “Cy7-PEI” and “Cy7-PEIopt,” respectively) synthetized to stain anionic structures. These are near-infrared dyes, designed and produced at the Zentrum für Medizinische Forschung, Mannheim, Germany (Huang et al., 2019). Both dyes combine heptamethine cyanine dye (MHH148) and branched polyethylenimine (PEI), in order to visualize in 3D blood vessels and renal glomeruli. The main difference between them is in the molecular weight. For all structural and technical details see Huang et al. (2019).

2.5 | ECI removal and 3D immunohistochemistry

In order to perform the 3D immunohistochemistry, after ECI clearing, prior to the clearing procedure, sections of 1 mm of kidney were cut, by using either a Vibratome (VT1200/S, from Leica Biosystems, Nußloch, Germany) or a rat heart slicer matrix (65-2100, provided by AgnTho’s AB, Lidingö, Sweden).

ECi was removed by incubating the sample in an alcohol bath at different concentrations, pH 9.0 for all the concentrations: 99% 60 min, 99% 45 min, 80% 60 min, and 50% for 60 min per step. Due to the reduction in the alcohol concentrations, the sample was rehydrated and the RI adjusted to hydrophilic buffers, as, for example, glycerol. Room temperature was kept for all the steps of ECI removal. Afterward, the samples were stored in 1× PBS overnight at room temperature, on a roller.

Then antigen retrieval was performed, by incubating the sample in citrate buffer pH 6.0 at 37°C overnight. Afterward, a quenching step was performed to reduce the autofluorescence and the background noise of the samples. For that, the samples were incubated in 500 mM Glycine (Sigma-Aldrich, Germany, GE17-1323-01) for 1 day at room temperature, by alternating the use of the roller for 2 hr and the sonicator for 15 min. Lastly, the samples were incubated in the same buffer overnight at 37°C.

The permeabilization step was performed, by using Triton X-100 (Sigma-Aldrich, Darmstadt, Germany) in 1× PBS, totally for 1 hr at 37°C: 1% for the first 24 hr and 2% for the second 24 hr. The blocking in 5% Chicken Serum (Normal Unconjugated Chicken Serum, Jackson ImmunoResearch Europe Ltd., UK) occurred for 48 hr, at 37°C, by changing the buffer every 24 hr.

The incubation in the primary antibody (Polyclonal Goat Anti-Nephrin primary antibody (AF3159), R&D Systems, Germany; stock concentration 0.2 mg/ml, diluted 1:100, in a final volume of 5 ml) lasted for 48 hr, at 37°C, changing the buffer every 24 hr. The incubation in the secondary antibody (Chicken Anti Goat secondary antibody Alexa Fluor® 647 conjugated (A21469), Thermo Fisher Scientific, Germany; stock concentration 2 mg/ml, diluted 1:200, in a final volume of 5 ml) took 72 hr, at 37°C, changing the solution every 36 hr. Finally, a 48 hr washing step in 1× PBS was performed.

Lastly, a nuclear staining (SYTOX™ Green Nucleic Acid Stain (S7020), ThermoFisher, Germany Scientific; stock concentration 5 mM, diluted 1:20.000, in a final volume of 5 ml) was performed incubating the nuclear dye in 1× PBS with TritonX-100 at 37°C overnight. After washing in 1× PBS, the samples were stored in 88% glycerol (OmniPur® Glycerol, Merck, Darmstadt, Germany; RI: 1.475 at 20°C), until the imaging.

The samples were imaged using a confocal microscope. Depending on the purpose, we mainly used: a 20× (HC PL 20×/0.75 IMM CORR CS2—Leica Microsystems) in 88% glycerol; a 63× (HC PL APO 63×/1.40 Oil CS2—Leica Microsystems) in 88% glycerol, required for the imaging of cellular nuclei; a 16× (PL FLUOTAR 16×/0.50 IMM) in 88% glycerol.

The 1 mm kidney slices were mounted in a 60 mm tissue culture dish, glued by superglue (UHU) and completely filled by 88% glycerol. The emission channels used were 638 nm for Alexa Fluor detection and 488 nm for Sytox Green detection.

2.6 | ExM for lung and 3D immunohistochemistry

As the lung is a difficult tissue to clear, lung ExM was tested both on fresh tissue and deparaffinized samples (see below). Fresh lungs were, initially, incubated at 4°C for 2 days in Amplifying Hydrogel Solution (20% acrylamide, 0.05% bis acrylamide, 4% PFA, 10% sodium acrylate, 0.1% VA-044, 1× PBS). Afterward, the pulmonary tissue was incubated at 37°C for 2 hr in a humidified chamber and, then, cut into 500 μm slices using a vibratome (VT1200/S, Leica Biosystems). The sections were then incubated in a denaturation solution (10% SDS, 5 M NaCl, 1 M Tris–HCl, pH 9.0) at 70°C for 24 hr and at 95°C for 12 hr.

3D immunohistochemistry was performed by blocking [blocking buffer: 1× PBS, 2% BSA (A9647, Sigma-Aldrich), 1% TritonX-100 (Sigma-Aldrich)] at 37°C for 24 hr; thereafter, the sample was incubated in the primary antibody (primary antibodies used: Polyclonal Goat Anti-CD31 primary antibody (AF3628), R&D Systems; stock concentration 0.2 mg/ml, diluted 1:100; Monoclonal Rabbit Anti-E-Cadherin primary antibody (#3195),
Cell Signaling Technology, Germany; stock concentration 52 μg/ml, diluted 1:100), combined with the blocking solution, at 37°C for 48 hr. After washing the sample in the blocking buffer (37°C, 60 min ×3), the incubation in the secondary antibody (Donkey Anti Goat secondary antibody Alexa Fluor 647 conjugated (A21447), Thermo Fisher Scientific; stock concentration 2 mg/ml, diluted 1:200), combined with the blocking solution, followed. Finally, the sample was washed and stored in 1× PBS, until the imaging. The sample analysis was performed using a confocal microscope. Depending on the purpose, the most frequently used objectives were: a 20× (HC PL 20×/0.75 IMM CORR CS2—Leica Microsystems) in 1× PBS; a 40× (HC PL APO 40×/1.25 Oil CS2—Leica Microsystems) in 1× PBS; a 63× (HC PL APO 63×/1.40 Oil CS2—Leica Microsystems) in 1× PBS. The emission channels used were 638 nm for Alexa Fluor detection and 488 nm for autofluorescence detection.

2.7 | ECi clearing and deparaffinization

Twenty years old paraffin-embedded kidneys from PCK rats were deparaffinized and cleared by using a tissue processor, the Leica EM TP (Leica Biosystems). The paraffin removal was performed by incubating the block in an incubator at 60°C for ~20 min and, then, cutting the left-over paraffin with a disposable scalpel. Thereafter, the sample was processed in the tissue processor, according to the following set up: (a) xylol (60°C, 90 min ×2), (b) 100% ethanol (35°C, 90 min ×2; 35°C, 45 min ×1), and (c) ECi (35°C, 60 min ×2; 35°C and overnight ×1). The tissue processor was programmed in this way in order to wash the leftover paraffin away (a), to wash the xylol way (b), and to perform the ECi clearing (c). After the procedure, the specimens were stored in ECI at room temperature until imaging. No staining was performed, as only autofluorescence was tested during the imaging. The samples were imaged using a confocal microscope, where a 20× (HC PL 20×/0.75 IMM CORR CS2—Leica Microsystems), in immersion oil, was used for this purpose. The 488 channel was used to detect autofluorescence.

2.8 | ExM and deparaffinization

Twenty years old paraffin-embedded blocks of lungs from Sprague Dawley rats were used for these experiments. After the paraffin melting into an incubator at 60°C for ~20 min and prior to ExM, the deparaffinized lung samples were rehydrated in decreasing concentrations of ethanol (100, 90, 80, 70 at room temperature and 90 min for each step and stored in 1× PBS). Thereafter, the specimens were treated for the ExM, immunostained and imaged in the same way as already described for the fresh pulmonary tissue (refers to: ExM for Lung and 3D Immunohistochemistry section, above).

3 | RESULTS

3.1 | Optimization of ECi-based OTC

All samples were stained with Cy7-PEI (3 mg/ml perfusion solution) and cleared using ECi-OTC (Huang et al., 2019) unless stated otherwise.

Figure 1 shows a section of a mouse kidney, imaged using a confocal microscope, where the 3D image clearly shows renal glomeruli and a network of blood vessels, connecting the glomeruli to each other. Moreover, the depth code underlines the presence of the signal in all the points of the image.

Figure 2 depicts a mouse pole kidney, imaged using a light-sheet microscope. In contrast to Figure 1, the concentration of the dye in the perfusion solution was 1.5 mg/ml, in order to understand whether it could have been possible to reduce the initial concentration during the perfusion. The pole is arch-shaped. Although the blood vessels are not distinguishable, the glomeruli are. Despite the objective magnification (5×; HC PL FLUOTAR 5×/0.15 IMM DLS,
water/gly-Leica Microsystems), the renal glomeruli were imaged in a way that they could be counted and sized. Moreover, the background noise level is very low.

Figure 3 depicts in high-resolution part of a renal artery, despite a further reduction of the dye concentration (perfusion solution) to 1 mg/ml. Besides that, the main vessel is highly distinguishable as well as its branches and the glomeruli (indicated by yellow arrowheads) linked to that.

3.2 | ECi removal, RI adjustment, antibodies (3D immunohistochemistry)

After ECi-OTC, the RI of the sample is 1.558. Therefore standard immunohistochemistry protocols cannot be used. On the other hand, performing immunohistochemistry before the clearing may affect the signal. Here, we present results obtained with a new and innovative 3D immunohistochemistry protocol, performed after the ECi clearing. The key points of this procedure are the ECi removal, the RI adjustment with a subsequent immunohistochemistry.

Figure 4 illustrates the results of a mouse kidney stained with the aforementioned procedure. In (a) the nuclear staining (Sytox Green) is presented; in (b) the red channel (anti-nephrin Alexa Fluor 647 conjugated) staining only glomeruli, and in (c) the merged image is reported. All the renal structures are properly stained. Moreover, the very low background noise level confirms that the ECi removal and subsequent rehydration does not affect the efficacy of the clearing, but allows further staining.

Figure 5 shows images acquired in order to evaluate the goodness of the secondary antibody. To do so, first, a complete 3D immunohistochemistry experiment was performed (Figure 5a) and, afterward, only the secondary antibody was tested (Figure 5b), by staining a 1 mm mouse kidney with only chicken anti-goat secondary Alexa Fluor 647 conjugated. In both cases, a 16× objective in 88% glycerol was used. Both 638 (for the Alexa Fluor 647) and 488 (for autofluorescence) channels were used. In Figure 5a, the detection of both signals is recognizable: the glomeruli are detected in red, as expected; on the other hand, autofluorescence identifies only renal tubuli and the background noise level is barely perceptible. Indeed, the results shown in Figure 5b demonstrate that in absence of the specific primary antibody, the secondary does not link to any structure, as only renal tubuli are detected from the autofluorescence signal (green color) and no red signal was visualized.

In addition to the 3D imaging, we wanted also to ascertain the size of the nuclei of the epithelial cells. The nuclear diameter of nine cell nuclei was measured, by using a manual calculation (Table 1): the value of the scale bar was converted into magnification and, then, using this value the actual size of the object was calculated. The value range

found was between $\approx 6$ and $\approx 8 \mu m$, which is in accordance with the knowledge about the nuclear sizing. The goodness of the staining, which is located in the right place, and the measurement of the diameters confirm that they are nuclei from epithelial cells.

In order to assess whether the regain of water after ECi clearing could have affected the quality of the sample, we visually checked and documented the appearance of the kidney by photographs immediately after the perfusion (Figure 6a), ECi-OTC (Figure 6b), and ECi removal and rehydration (Figure 6c). Additionally, the diameter of 13 glomeruli was measured (Figure 7) immediately after the perfusion, ECi-OTC and ECi removal and rehydration using the Las X software. Afterward, a one way ANOVA test was performed.

A modification of the glomerular size was noticed, as follows: (a) between the glomeruli cleared and rehydrated versus glomeruli only perfused: $+6.35 \mu m$; (b) between the glomeruli cleared and rehydrated versus glomeruli cleared and not rehydrated: $+41.14 \mu m$; (c) between the glomeruli cleared and not rehydrated versus glomeruli only perfused: $-34.79 \mu m$. 

![Figure 4](image4.png) 3D Mouse kidney section (1 mm). After the ECi clearing, the ECi was removed by alcohol, rehydration and a following immunohistochemistry occurred with a primary antibody against nephrin, chicken anti-goat secondary antibody Alexa Fluor 647 conjugated and counterstained with Sytox Green for cellular nuclei detection, imaged by confocal microscopy, using a 63x objective in 88% glycerol and 488 and 632 channels, respectively, for Sytox Green and Alexa Fluor 647 detection. (a) Green channel detecting Sytox Green (epithelial cell nuclei). (b) Red channel detecting Alexa Fluor 647 (glomeruli). (c) Merging. Scale bars: 100 \mu m

![Figure 5](image5.png) 3D Mouse kidney section (1 mm). After the ECi clearing, the ECi was removed by alcohol, rehydration and a following immunohistochemistry occurred. The imaging occurred by confocal microscopy, using a 16x objective in 88% glycerol. (a) Complete 3D immunohistochemistry, showing glomeruli (in red) and renal tubuli (in green, from autofluorescence). Scale bar: 500 \mu m. (b) Control, obtained with only chicken anti-goat secondary antibody Alexa Fluor 647 conjugated imaged by confocal microscopy, using a 16x objective in 88% glycerol and 638 channels Alexa Fluor 647 detection. Scale bar: 500 \mu m
3.3 | Dye persistence

Here, we demonstrate that the dye injected during the perfusion “survived” for a long time, making the imaging of the same sample possible in an interval of 2 and 3 years (Figure 8). The specimen was stained by Cy7-PEI-opt 4F (3 mg/ml perfusion solution; 4F: four times filtered), cleared with ECi and imaged by a confocal microscope. The first imaging (Figure 8a) occurred immediately after the clearing procedure. Signals from both the dye and autofluorescence were detected. The same sample was reimaged after 2 years (Figure 8b) and surprisingly it was still possible to detect the fluorescent signal, coming from Cy7-PEI-opt 4F (no autofluorescence detection). Moreover, compared to the first results, the second imaging allowed (a) a deeper penetration depth and (b) the acquisition of a bigger region of interest, thanks to the update of the Las X software and the Stage Overview feature. Despite a slight decrease in the fluorescent signal and a little loss of structures, due to the time, it was possible to achieve a good quality in the 3D image. Renal glomeruli and blood vessels were still recognizable and they still appeared quite well-shaped and structured. After 3 years, the imaging was again repeated (Figure 8c): although the total depth and the signal detection appeared decreased, the results still show renal structures, such as renal glomeruli.

3.4 | Deparaffinization followed by ECi clearing and 3D imaging

Figure 9 was acquired after deparaffinization and clearing of a 20 years old paraffin-embedded PCK rat section. The inner structures survived the whole procedure. No staining was used for this imaging. Only autofluorescence

| Object number | Scale bar value (μm) | Conversion in magnification (×) | Diameter of Nuclei (μm) |
|---------------|----------------------|-------------------------------|------------------------|
| 1             | 2.11                 | 1,900                         | 6.84                   |
| 2             | 3.30                 | 1,818                         | 7.70                   |
| 3             | 3.35                 | 1,800                         | 6.7                    |
| 4             | 3.60                 | 1,700                         | 5.88                   |
| 5             | 3.70                 | 1,621                         | 6.79                   |
| 6             | 3.94                 | 1,777                         | 5.62                   |
| 7             | 5                    | 1,800                         | 6.7                    |
| 8             | 6.05                 | 1,818                         | 5.50                   |
| 9             | 7.71                 | 1,815                         | 6.61                   |

Note: The diameter of nine epithelial cell nuclei was scaled and afterward the value of the scale bar converted into the actual size of the object. Conversions from scale bars to cell diameters performed manually. Sample size: n = 9.

![Figures 6 and 7](image_url)
was used, which displays the presence of glomeruli and renal tubuli. The background noise level is low. Moreover, the depth reached for this imaging (here not shown) was 250 \( \mu \)m.

### 3.5 Expansion microscopy

The purpose of this section is to address the results obtained by ExM. The method was used both on fresh tissue as well as deparaffinized samples. In Figure 10, a perfused mouse pulmonary section (fresh tissue) is depicted. The sample was expanded, stained with antibody against E-cadherin Alexa Fluor 647 conjugated and the image was acquired by confocal microscopy. The figure reveals a bronchus in the central part (red signal, on the middle side), surrounded by the bronchial wall and pulmonary vessel, underneath the bronchus (green signal, on the left side). The overlay shown on the right side displays the perfect matching of both the signals, highlighting all the pulmonary inner structures. Although not shown, the final depth reached was 180 \( \mu \)m.

Figure 11 depicts a 20 years old paraffin-embedded Sprague Dawley rat lung section. After deparaffinization and expansion, this sample was stained with an antibody against CD31 Alexa Fluor 647 conjugated and imaged by using a confocal microscope. Despite the age of the sample, CD31 staining was still possible. In fact, the edges of the two pulmonary blood vessels, located at the left bottom and right upper part of the image, display a uniform staining. The green autofluorescence signal is detectable in the inner part of the vessel. This is due to a partial presence of erythrocytes, which exhibit autofluorescence.
4 | DISCUSSION

The following paragraph aims to discuss the results, in order to allow a better understanding of the procedures described. In this study, we aimed to extend the methodologies of ECi-based tissue clearing, for 3D imaging. After optimizing/accelerating the ECI-based clearing procedure, we aimed at performing immunostaining after the clearing as well as at clearing and imaging of very old paraffin-embedded specimens. In this context, it was

**FIGURE 10** 3D pulmonary section from a perfused C57/BL6 mouse, expanded and stained with an antibody against E-cadherin Alexa Fluor 647 conjugated and imaged by confocal microscopy, using a 20× objective in 1× PBS, with 488 and 638 channels for autofluorescence and Alexa Fluor 647 detection, respectively. (a) Pulmonary blood vessel and bronchial wall detected by autofluorescence only. (b) Bronchus detected by 638 channel for Alexa Fluor 647 detection. (c) Merged image. Scale bars: 100 μm

**FIGURE 11** 3D Lung section from 20 years old paraffin block of a Sprague Dawley rat, deparaffinized, expanded, stained with an antibody against CD31 Alexa Fluor 647 conjugated and imaged by confocal microscopy, using a 20× objective in 1× PBS. (a) Section of lung imaged by using 488 channel for autofluorescence detection and 638 channel for Alexa Fluor 647 detection. Scale bar: 500 μm. (b) Depth coding view. The depth scale on the right upper part of the figure denotes the depth reached during the imaging: this goes from the surface (0–80 μm, in blue–light blue), the middle area (80–200 μm, in green–yellow), up to the deepest part (200–240 μm, in orange–red). Scale bar: 200 μm
important to study the stability of our new dye and to provide a very long stability of the dye in the time, as the rapid decay of the fluorescent signal is still a remarkable drawback in many OTC protocols, which may reflect in a 3D imaging failure. Moreover, we want also to describe the ExM for lungs combined with immunolabeling, as another way to clear the sample, which makes the tissue transparent with high effectiveness. The main advantage of our ExM in the lung was in reducing the factor of expansion from 4 to $\approx 2$. This allowed us to expand finer structures, such as the alveolar structures, without running the risk to break them or lose epitopes, which might be a drawback for the final immunostaining. We discuss these results together with those obtained after extending our ECi-OTC for renal tissue.

4.1 | ECi-OTC, dye stability, and 3D imaging

We optimized an already published ECi-based procedure, by Klingberg’s group (2017). We saw the potential of this protocol, although, on the other hand, this was a highly cumbersome and still lengthy procedure. The first improvement performed was the acceleration of the sample preparation using a tissue processor (Leica TP1020). This reduced the preparation from 18 up to 5 hr, although the samples may stay longer in the ECi (Huang et al., 2019; Klingberg et al., 2017). The use of the TP1020 led to a standardization of the processing (Huang et al., 2019): (a) all the settings were easily programmed; (b) many samples can be processed in parallel; (c) the stronger and faster dehydration phase, as well as the use of room temperature instead of the cool temperature ($4^\circ$C), improved the diffusion coefficient of the solutions leading to a better penetration into the specimens; and (d) the tissue processor works with negative pressure preventing air bubble formation in the samples.

Comparing ECi-OTC with other OTC protocols is worthwhile. Most of the other protocols are very time consuming. Furthermore, the chemical solutions used are often harmful to the operator. Examples are Scale, BABB, CUBIC, but also active clarity technique or X-CLARITY, in which sample incubation is substituted by electrophoretic approaches (Azaripour et al., 2016; Hama et al., 2011; Lee et al., 2016; Nojima et al., 2017; Tomer et al., 2014). In addition, ECi-OTC does not require expensive equipment. Considering all of these aspects as well as the price of ECi and the possibility to reuse the solution several times allows to state that ECi-OTC protocol is a fast, safe, and economic method.

As shown in Figure 8, a long storage of the tissues in ECi affects only partially the preservation of the samples or the fluorescent signal. That is in accordance with Klingberg et al. (2017). Other OTC protocols have problems with the stability of fluorescence signals, as for instance in 3DISCO, where a rapid imaging immediately after the staining is required, in order not to lose the signal (Ertürk et al., 2012).

We also proved that the autofluorescence is a valid alternative to the staining, in order to acquire structural information in 3D. The reduced use of chemical staining might be a valid alternative in terms of time-saving and economy.

ECI-OTC was found to be useful in clearing many different samples/organs (Huang et al., 2019), demonstrating and validating the reliability of this protocol. This led us to combine our protocol with deparaffinization of old paraffin-embedded blocks and performing 3D immunohistochemistry (see below).

4.2 | ECi removal and 3D immunohistochemistry

Immunohistochemistry was only possible after ECi removal by using a decreasing ethanol concentration gradient, which also allowed the rehydration of the cleared samples. The importance of this step lies in the following facts: (a) the ECi hydrophobicity would have been in contrast with the hydrophilic environment, typically used in the standard immunohistochemistry; (b) the subsequent rehydration after ECi removal allowed a RI adjustment in the aqueous range, so that it became possible to use hydrophilic mounting media (e.g., glycerol) for 3D imaging.

Moreover, the specificity of the staining of renal glomeruli as well as of the epithelial cell nuclei with almost no background noise demonstrated that the regain of water is not a limiting factor, in terms of optical translucency. The immunohistochemistry in combination with ECi-OTC was primarily described by Klingberg et al. (2017). Compared to that, our protocol has several advantages:

1 First, in the original protocol, only antibodies injection was performed, before the OTC procedure. Indeed, in our protocol, the immunolabeling occurs after the clearing step, demonstrating the persistence of the epitopes even after the clearing procedure. Moreover, the immuno-incubation method could be used also on human samples, becoming a starting point for further studies also in the diagnostic field;

2 Second, generic antibodies were tested in the original protocol as the ones against CD11, CD31 (Klingberg et al., 2017), whereas our method, allowing the diffusion of more specific molecules, led to the detection of finer structures, including also nuclei from epithelial cells. In fact, in order to validate the goodness of
the nuclear staining, we also sized nine epithelial cell nuclei, where the value range was found between $\approx 6$ and $\approx 8 \, \mu m$, which is the standard value range for epithelial cell nuclei (Alvin, Virginia, & Laura, 2008);

3 In addition, the adjustment of the RI in the hydrophilic environment and further use of 88% glycerol as a mounting solution gives the opportunity to use a versatile mounting solution, which is more compatible with more objectives;

4 The only two downsides of this procedure could be identified in the large amount of buffers used and the number of days required to complete the protocol. Actually, both of these elements are essential for the better diffusion of the buffers into the tissue. Moreover, the amount of the different buffers was determined in order to stain a slice of 1 mm in thickness;

The structures of interest appeared not to be affected by the ECI removal and rehydration. In fact, the slight increase of the glomerular diameter, combined with the use of the 63x objective helped in defining microstructures as, for instance, the cellular nuclei.

4.3 | ECI-OTC and deparaffinization

We used old paraffin-embedded blocks from our archives to establish a method for deparaffinization and 3D imaging. Recently, the role of the liquid paraffin has been highlighted as a carrier for other hydrophilic solutions, for optically clearing deparaffinized samples (Wang et al., 2011). For instance, a CUBIC based method has been recently described to obtain a very high efficacy of clearing from normal and pathological human lungs and lymph nodes (Nojima et al., 2017).

Our group established a method to “revitalize” and clear samples from old paraffin-embedded blocks. Also, this protocol was automated to assure a high rate of efficacy and standardization in a short processing time. In order to understand whether it would have been possible to clear and analyze also pathological specimens, kidneys from PCK rats were tested, by using autofluorescence only. Our data confirmed that (a) old and pathological tissues survived our ECI clearing procedure. In addition, compared to the aforementioned data (Nojima et al., 2017) the short time of processing makes our procedure more attractive, especially in terms of routine procedures pathology, where speed and high performance are required; (b) Another important advantage is the use of autofluorescence in label-free samples: this could easily help in differentiating between physiological and pathological samples, without the need of specific staining (Wilson, Degan, Warren, & Fischer, 2012). In fact, autofluorescence is known also as primary fluorescence (Fred, 2017) and this is the natural emission of cells, given the presence of natural biological fluorescent components (Roshchina, 2012). Although autofluorescence is often considered as a nuisance, which may mask the real fluorescent signal, this is used as a guideline to distinguish the tissue morphology (Fred, 2017). Recently, it has been found that autofluorescence may be beneficial for the microscopic organism analysis (Roshchina, 2012). Our data confirmed that: from old paraffin-embedded blocks, we were able to clear, track, and image morphological structures, by using autofluorescence only. The preclearing treatment, in order to remove the paraffin, helped us to strongly reduce the nonspecific signals, which might increase the background noise level and contribute to the blurriness and opaqueness of the final image. By doing that, we were also able to discriminate between the nonspecific and the real signals, obtaining clear 3D images, depicting structures as glomeruli and renal tubuli.

4.4 | ExM for lung and 3D immunohistochemistry

ExM techniques have been recently developed (Chen, Tillberg, & Boyden, 2015). Since then, many protocols and variants have been established. The principal aim of this technique is to achieve 3D nanoscale resolution imaging of the specimens, by embedding the tissue in isotropic embedding solutions, followed by expansion in deionized water (Gao, Asano, & Boyden, 2017), which also can provide optical transparency when used in large amounts (Choziński et al., 2016; Gao et al., 2017). Usually, the expansion factor reached is about 4 (Choziński et al., 2016, Gao et al., 2017).

Kim et al. (2018) described the pulmonary tissue as a sponge. Due to that and to avoid the pulmonary tissue damage (e.g., tissue swelling) after stringent OTCs, our group, based on the protocol established by Unnersjo-Jess et al. (2018)), and contrary to the already existing studies, worked on normal lung expansion (both fresh and deparaffinized tissue) in order to reach a factor of expansion equal to $\approx 2$, by tissue embedding in an isotropic gel, followed by denaturation. In fact, due to the highly soft consistency of the normal lung, we found that expanding pulmonary tissue for four times could damage the finer structures, as alveoli. However, this variant offers the same advantages as the standard ExM (Choziński et al., 2016, Gao et al., 2017): (a) it provides optically transparent tissue, achieving both the expansion and the optical transparency at the same time; (b) no special or expensive equipment is needed; (c) a very easy to handle and fast procedure. The use of this protocol also on deparaffinized tissues makes it a promising tool for routine
diagnostics. In fact, some preliminary studies on metastases in the lung of rats are ongoing. In contrast to normal tissue, the lung tumor has a more solid consistency, allowing higher expansion factors without distorting the inner structures. Moreover, further immunostaining was possible. This protocol is very similar compared to the regular 2D immunolabeling. This is a very remarkable advantage of ExM: in fact, after ExM, the lipids are removed and hybrid porosity increases and this phenomenon accelerates the diffusion of molecular probes into the tissue. Thanks to that, it is possible to use conventional commercial antibodies for 3D imaging, detecting, in this way, the structures of interest. Consequently, the immunolabeling in thick tissues becomes possible, with less background noise than in the regular immunostaining protocol.

5 | CONCLUSIONS

One of the aims of our activities was to improve the ECi-OTC (Huang et al., 2019) and to make it more suitable also for medical diagnostics. Routine diagnostics is characterized by the use of 2D light microscopy analyzing only a few micrometer of tissue. OTCs in combination with the newest and most innovative microscopic technologies (such as confocal and/or light-sheet microscopies) could improve routine diagnostics by allowing 3D tissue visualization and analysis. The “reuse” of old paraffin-embedded blocks could allow analysis over time courses, if several samples of a patient are available.

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AUTHOR CONTRIBUTIONS

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