Methods in Molecular Biology
DOI 10.1007/7651_2014_93

Advanced Fixation for Transmission Electron Microscopy Unveils Special Extracellular Matrix Within the Renal Stem/Progenitor Cell Niche

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Abstract

As well in light as in transmission electron microscopy can be seen that the renal stem/progenitor cell niche shows a special arrangement of two different kinds of stem/progenitor cells. Epithelial cells are found in the tip of an ureteric bud derived CD ampulla encircled by a special basal lamina. Mesenchymal cells are separated from them by a striking interstitial interface. Specimens fixed by conventional glutaraldehyde solution show that the interface looks bright and unremarkable. In contrast, fixation of specimens with glutaraldehyde in combination with cupromeronic blue, ruthenium red, or tannic acid illustrates that the interface contains a remarkable network of extracellular matrix spanning between epithelial and mesenchymal stem/progenitor cells. After unpacking this particular extracellular matrix for electron microscopy, elaboration of related functions such as structural composition of contained molecules, binding of morphogenetic factors, and influence on parenchyma development is under current experimental work.

Keywords: Kidney, Stem/progenitor cell niche, Interstitial interface, Transmission electron microscopy, Glutaraldehyde, Cupromeronic blue, Ruthenium red, Tannic acid, Extracellular matrix

1 Introduction

From the fetal and up to the neonatal phase development of renal parenchyma takes place exclusively in the presumptive outer cortex and always in close vicinity of the organ capsule (1). At this exposed site the renal stem/progenitor cell niche controls as well nephron as collecting duct formation (2). For performance of these complex tasks it harbors two different types of stem/progenitor cells. Epithelial stem/progenitor cells are contained within the ureteric bud derived tip of a collecting duct ampulla, while mesenchymal stem/progenitor cells are found in the covering cap condensate (3). Between both types of stem/progenitor cells numerous morphogenetic molecules such as FGF2/9/20, Wnt9b, LIF, c-Ret, GDNF, SOX8, Wnt4, PAX2, and BMP7 are exchanged to regulate survival, proliferation, induction, and differentiation during development of parenchyma (4, 5). As a result epithelial cells within an ampulla develop into Principal and Intercalated Cells of the

collecting duct, while surrounding mesenchymal cells perform a mesenchymal-to-epithelial transition (MET) to differentiate into various segments occurring in a nephron (6).

As compared to other organs the renal stem/progenitor cell niche exhibits special morphological features. For example, electron microscopy of specimens fixed in conventional glutaraldehyde solution shows that epithelial stem/progenitor cells are wrapped within the tip of a collecting duct ampulla by a striking basal lamina (7). Further mesenchymal cells do not stand in close contact with epithelial cells but are separated by a bright interstitial interface (8). Up to date it was assumed that the interface between both kinds of stem/progenitor cells is an artifact, which appears to be filled by interstitial fluid and may be caused by suboptimal fixation or crude handling of specimens during embedding for electron microscopy. However, recent immunohistochemical data raised by light microscopy exhibit that microfibers labeled by Soybean Agglutinin (SBA), anti-collagen I, II, III, and IV originate at the tip of a collecting duct ampulla, cross the interstitial interface to end between neighboring mesenchymal stem/progenitor cells (9). Further on, special contrasting of specimens by glutaraldehyde in combination with cupromeronic blue, ruthenium red, or tannic acid for transmission electron microscopy demonstrates that the interstitial interface is authentic. It can be recognized that the interface contains filigree and heterogeneously composed extracellular matrix. An unexpected finding was further that strands of extracellular matrix line in parallel to projections of mesenchymal cells, cross the interface, penetrate the basal lamina, and contact via tunneling nanotubes epithelial stem/progenitor cells at the tip of a collecting duct ampulla (1).

Up to date it is unknown, why in transmission electron microscopy advanced fixation by glutaraldehyde including cupromeronic blue, ruthenium red, or tannic acid does exhibit essential features at the interstitial interface of the renal stem/progenitor cell niche, while traditional fixation solely by glutaraldehyde solution does not show these characteristics. Thus, to shed more light on the matter and to elucidate related functional aspects, reliable protocols are presented here to unpack this special kind of extracellular matrix. It is most probable that these techniques are able to reveal new morphological details not only in the renal but also in other kinds of stem/progenitor cell niches.

2 Materials

2.1 Animals

To investigate morphological features in the interstitium of the renal stem/progenitor cell niche one-day-old male and female New Zealand rabbits (Seidl, Oberndorf, Germany) were used to isolate both kidneys to perform immersion fixation for transmission electron microscopy (Fig. 1).

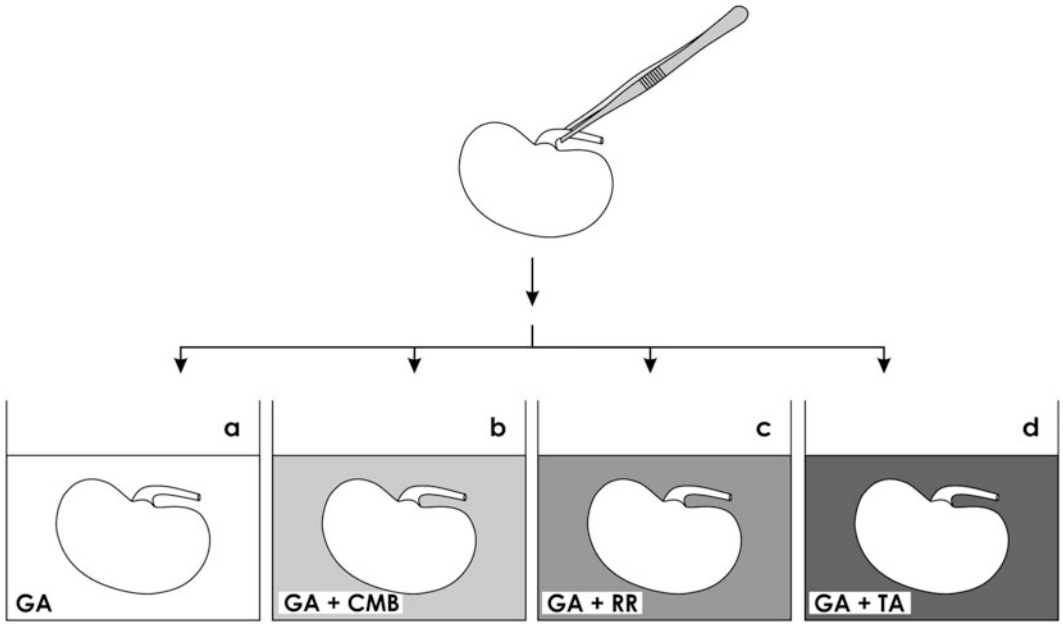

Fig. 1 Schematic illustration of immersion fixation. Entire kidneys with intact capsule were fixed in (**a**) glutaraldehyde (GA; *see* Section 2.2.1), (**b**) GA including cupromeronic blue (CMB; *see* Section 2.2.2), (**c**) GA including ruthenium red (RR; *see* Section 2.2.3), or (**d**) GA including tannic acid (TA; *see* Section 2.2.4)

2.2 Solutions for Fixation, Postfixation, and Block Contrasting

For the current protocol, fixation as well in conventional glutaraldehyde solution as fixation for advanced contrasting was adapted for investigations on embryonic renal parenchyma (*see* **Note 1**). The procedure is based on techniques as earlier introduced for the analysis of ECM in mouse tectorial membrane (10) and proteoglycans in cardiovascular structures (11).

2.2.1 Glutaraldehyde (GA) Solution

1. As stock solution prepare 0.15 M sodium cacodylate solution, pH 7.4.

   Weigh 3.21 g cacodylic acid sodium salt trihydrate (MW 214.03 g/Mol; Roth, Karlsruhe, Germany) in a measuring cylinder and fill to 95 mL with bidistilled water. Dissolve it and adjust the pH to 7.4 with an 1 N hydrochloric acid. Make up to 100 mL with bidistilled water (*see* **Note 2**).
2. For fixation use 5 % glutaraldehyde in 0.15 M sodium cacodylate solution, pH 7.4.

   Immediately before fixation of specimens prepare the GA solution. Pipet 8 mL of the 0.15 M sodium cacodylate (*see* Section 2.2.1, item 1) in a 20 mL glass vessel with a snap-on cap (Fisher Scientific, Schwerte, Germany) and add 2 mL glutaraldehyde solution (25 %, Serva, Heidelberg, Germany) for a final concentration of 5 % glutaraldehyde in 0.15 M sodium cacodylate.
3. For postfixation use 0.15 M sodium cacodylate solution, pH 7.4 including 1 % osmium tetroxide.

Pipet 3 mL osmium tetroxide (4 %, Science Services, München, Germany) in a 20 mL glass vessel with a snap-on cap and fill it up to 12 mL with 0.15 M sodium cacodylate (*see* Section 2.2.1, item 1)

2.2.2 Glutaraldehyde (GA) Solution in Combination with Cupromeronic Blue (CMB)

1. For fixation use 5 % GA buffered with 0.15 M sodium cacodylate solution, pH 7.4.

   Prepare 10 mL of GA solution (*see* Section 2.2.1, item 2) and pipet it into a 20 mL glass vessel with a snap-on cap.
2. For improved contrast use 0.1 % cupromeronic blue and 0.1 M magnesium chloride hexahydrate dissolved in 0.2 M sodium acetate buffer, pH 5.6.

   Weigh 0.14 g sodium acetate anhydrous (MW 82.03 g/Mol; Sigma, Taufkirchen, Germany) in a glass measuring cylinder and dissolve it in 9 mL bidistilled water. Then dilute in a glass beaker 1 mL acetic acid anhydrous solution (100 %; Merck, Darmstadt, Germany) by the addition of 9 mL bidistilled water to a 10 % solution. Use this solution to adjust the dissolved sodium acetate to pH 5.6. Then fill it up to 10 mL with bidistilled water. Finally add 0.203 g magnesium chloride hexahydrate (MW 203.30 g/mol; Sigma) and 0.01 g cupromeronic blue (Santa Cruz, Heidelberg, Germany) under permanent stirring.
3. For counterstaining use 0.5 % sodium tungstate dehydrate in bidistilled water.

   Weigh 0.05 g sodium tungstate dehydrate (Sigma) in a 20 mL glass vessel with a snap-on cap and dissolve it in 10 mL bidistilled water.

2.2.3 Glutaraldehyde (GA) Solution in Combination with Ruthenium Red (RR)

1. For fixation use 5 % GA buffered with 0.15 M sodium cacodylate solution, pH 7.4 including 0.5 % ruthenium red.

   Prepare 10 mL of GA solution (*see* Section 2.2.1, item 2) and pipet it into a 20 mL glass vessel with a snap-on cap. Add 0.05 g ruthenium red (Fluka, Taufkirchen, Germany) under permanent stirring.
2. For postfixation use 0.15 M sodium cacodylate solution, pH 7.4 including 0.5 % ruthenium red and 1 % osmium tetroxide.

   Weigh 0.045 g ruthenium red in a 20 mL glass vessel with a snap-on cap and dilute it in 9 mL 0.15 M sodium cacodylate (*see* Section 2.2.1, item 1). Finally add 3 mL osmium tetroxide (4 %; Science Services).

2.2.4 Glutaraldehyde (GA) Solution in Combination with Tannic Acid (TA)

1. For fixation use 5 % GA buffered with 0.15 M sodium cacodylate solution, pH 7.4 including 1 % tannic acid.

   Prepare 10 mL of GA solution (*see* Section 2.2.1, item 2) and pipet it into a 20 mL glass vessel with a snap-on cap. Add 0.1 g tannic acid (Sigma) under permanent stirring.

2. For postfixation use 0.15 M sodium cacodylate pH 7.4 including 1 % osmium tetroxide.

   Pipet 3 mL osmium tetroxide (4 %; Science Services) in a 20 mL glass vessel with a snap-on cap and fill it up to 12 mL with 0.15 M sodium cacodylate solution (*see* Section 2.2.1, item 1).

2.3 Solutions for Dehydration

Before embedding in Epon™ resin specimens have to be dehydrated in graded series of ethanols (50, 70, 80, 90, 99.8 %, *see* **Note 3**).

1. Dehydration step I: Ethanol 50 %.
   Dilute in a glass beaker ethanol (99.8 %; Roth) to 50 % by the addition of 50 mL bidistilled water and 50 mL ethanol 99.8 %.
2. Dehydration step II: Ethanol 70 %.
   Dilute in a glass beaker ethanol (99.8 %; Roth) to 70 % by the addition of 30 mL bidistilled water and 70 mL ethanol 99.8 %.
3. Dehydration step III: Ethanol 80 %.
   Dilute in a glass beaker ethanol (99.8 %; Roth) to 80 % by the addition of 20 mL bidistilled water and 80 mL ethanol 99.8 %.
4. Dehydration step IV: Ethanol 90 %.
   Dilute in a glass beaker ethanol (99.8 %; Roth) to 90 % by the addition of 10 mL bidistilled water and 90 mL ethanol 99.8 %.
5. Dehydration step V: Ethanol pure 99.8 %.
   Ethanol (99.8 %; Roth) is ready for use.

2.4 Solutions for Pre-embedding

Before embedding in Epon™ resin the specimens have to be incubated with fluid/resin intermedia.

1. Preparation of Epon™ resin.

   For 50 g Epon™ resin weigh 23 g epoxy embedding medium (synonym: Epon™ 812 substitute; Fluka), 14.25 g epoxy hardener DDSA (synonym: dodecenylsuccinic anhydride; Fluka), and 12.4 g epoxy hardener MNA (synonym: methyl nadic anhydride; Fluka) in a glass beaker. Mix the fluid under slow but permanent stirring for 30 min. Then add slowly 0.75 g epoxy accelerator DMP-30 (synonym: 2,4,6-tris (dimethylaminomethyl)phenol; Fluka) under permanent stirring. Then stop stirring and wait for 30 min until gas bubbles are disappeared (*see* **Note 4**).
2. Intermedium fluid I: Ethanol 99.8 % and Epon™ resin in a ratio 2:1.

   Pipet 10 mL ethanol (99.8 %; Roth) in a glass vessel with a snap-on cap and add 5 mL Epon™ resin (*see* Section 2.4, item 1) under permanent stirring.
3. Intermedium fluid II: Ethanol 99.8 % and Epon™ resin in a ratio 1:1.

Pipet 7.5 mL ethanol (99.8 %; Roth) in a glass vessel with a snap-on cap and add 7.5 mL Epon™ resin (*see* Section 2.4, item 1) under permanent stirring.

4. Intermedium fluid III: Ethanol 99.8 % and Epon™ resin in a ratio 1:2.
   Pipet 5 mL ethanol (99.8 %; Roth) in a glass vessel with a snap-on cap and add 10 mL Epon™ resin (*see* Section 2.4, item 1) under permanent stirring.

2.5 Coating of Slot Grids

The ultrathin sections have to be placed on grids for electron microscopy coated with Pioloform as it was earlier described (12).

1. Selection of grids.
   Use copper slot grids with a diameter of 3.05 mm and 2 mm × 1 mm slot; Nr. G2500C (Plano, Wetzlar, Germany).
2. Preparation of a 1.5 % Pioloform solution for coating.
   Weigh 1.5 g Pioloform (FN65, Plano) in a 100 mL dark glass bottle and dissolve it with 100 mL Chloroform (Merck) under permanent stirring (*see* **Note 5**).

2.6 Solutions for Contrasting Ultrathin Sections

The ultrathin sections have to be contrasted before use in the electron microscope.

1. Preparation of a 1 % uranyl acetate solution.
   Weigh 0.5 g uranyl acetate (Merck) in a sealable dark glass bottle and dissolve it with 50 mL absolute methanol (Merck) under gentle stirring (*see* **Note 6**).
2. Preparation of a 3 % lead citrate solution.
   Lead citrate solution (3 %, Ultrostain II; Leica, Wetzlar, Germany) is ready for use.

2.7 Richardson Solution for Staining of Semithin Sections

Semithin sections of embedded specimens have to be stained with Richardson solution before they can be analyzed under the light microscope.

1. Preparation of stock solution I.
   Weigh 0.5 g di-sodium tetraborate decahydrate (synonym: Borax; Merck) in a glass beaker and dissolve it with 50 mL bidistilled water for a final concentration of 1 % Borax solution. Then add to this solution 0.5 g methylene blue (Merck).
2. Preparation of stock solution II.
   Dissolve in a glass beaker 0.5 g Azur II powder (Fluka) in 50 mL bidistilled water.
3. Preparation of Richardson staining solution.
   Mix in a 100 mL glass bottle 50 mL stock solution I (*see* Section 2.7, item 1) and 50 mL stock solution II (*see* Section 2.7, item 2) (*see* **Note 7**). For application soak the solution up in a

plastic syringe. Then filtrate the staining solution immediately before use by a nitrocellulose filter, pore size 0.45 μm (Millipore, Billerica, USA).

3 Methods

3.1 Kidney Isolation

To investigate extracellular matrix at the interstitium of the renal stem/progenitor cell niche in transmission electron microscopy, one day old male and female New Zealand rabbits (Seidl) are anesthetized with ether (Merck) and killed by cervical dislocation. Both kidneys are immediately surgically removed (Fig. 1).

3.2 Fixation and Postfixation of the Entire Kidney

Each kidney is isolated with an intact capsule. Then it is separated from its fatty covering and transferred as quickly as possible by small anatomical forceps to a 20 mL glass vessel with a snap-on cap filled with 10 mL solution for fixation.

3.2.1 Fixation with Glutaraldehyde (GA)

1. Fixation of an entire kidney is performed in glutaraldehyde solution (GA; Fig. 1a; *see* Section 2.2.1, item 2) for 24 h at room temperature.
2. Then the specimens are washed in sodium cacodylate solution (*see* Section 2.2.1, item 1) three times each for 5 min.
3. In the next step postfixation is performed for 1 h in 12 mL sodium cacodylate solution including 1 % osmium tetroxide (*see* Section 2.2.1, item 3).
4. Finally the specimens are rinsed in sodium cacodylate solution (*see* Section 2.2.1, item 1) three times each for 5 min.

3.2.2 Fixation with Glutaraldehyde Containing Cupromeronic Blue (GA + CMB)

1. Fixation of an entire kidney is performed in glutaraldehyde containing cupromeronic blue (GA + CMB; Fig. 1b; *see* Section 2.2.2, item 1) for 24 h at room temperature.
2. Then the specimens are washed in sodium cacodylate solution (*see* Section 2.2.1, item 1) three times each for 5 min.
3. To improve contrast specimens are incubated with 10 mL 1 % cupromeronic blue and 0.1 M magnesium chloride hexahydrate dissolved in 0.2 M sodium acetate buffer (*see* Section 2.2.2, item 2) over night.
4. For the following counterstaining the specimens are kept for 5 h in 10 ml 0.5 % sodium tungstate dehydrate dissolved in bidistilled water (*see* Section 2.2.2, item 3).

3.2.3 Fixation with Glutaraldehyde Containing Ruthenium Red (GA + RR)

1. Fixation of an entire kidney is performed in glutaraldehyde containing ruthenium red (GA + RR; Fig. 1c; *see* Section 2.2.3, item 1) for 24 h at room temperature.
2. Then the specimens are washed in sodium cacodylate solution (*see* Section 2.2.1, item 1) three times each for 5 min.

3. In the next step postfixation is performed for 1 h in 12 mL sodium cacodylate solution including 0.5 % ruthenium red and 1 % osmium tetroxide (*see* Section 2.2.3, item 2).
4. Finally the specimens are rinsed in sodium cacodylate solution (*see* Section 2.2.1, item 1) three times each for 5 min.

3.2.4 Fixation with Glutaraldehyde Containing Tannic Acid (GA + TA)

1. Fixation of an entire kidney is performed in glutaraldehyde containing tannic acid (GA + TA; Fig. 1d; *see* Section 2.2.4, item 1) for 24 h at room temperature.
2. Then the specimens are washed in sodium cacodylate solution (*see* Section 2.2.1, item 1) three times each for 5 min.
3. In the next step postfixation is performed for 1 h in 12 mL sodium cacodylate solution including 1 % osmium tetroxide (*see* Section 2.2.4, item 2).
4. Finally the specimens are rinsed in sodium cacodylate solution (*see* Section 2.2.1, item 1) three times each for 5 min.

3.3 Orientation of Parenchyma Before Embedding

To obtain a comparable view to the renal stem/progenitor cell niche in different experimental series, it is essential to orientate exactly the parenchyma (Fig. 2) (*see* **Note 8**).

1. First each kidney is divided between both poles by a new knife of a scalpel (Fig. 2a; 1. cutting line). Following this strategy the cutting plane of parenchyma is yet orientated along the straight cortico-medullary course of collecting ducts (CD in Fig. 2b). Looking to the section plane renal stem/progenitor cell niches are found in the outer cortex and in close vicinity to the organ capsule (C). Each niche contains epithelial stem/progenitor cells in the tip of an ureteric bud derived CD ampulla (A). Mesenchymal stem/progenitor cells can be detected in the space between the tip of a CD ampulla (A) and the organ capsule (C).
2. In the next step the parenchyma is vertically cut to lining CDs on the border between inner cortex and medulla (Fig. 2b; 2. cutting line).

3.4 Processing of Parenchyma Before Embedding in Epon™ Resin

Before embedding of parenchyma it has to be dehydrated and incubated with intermedia fluids to support penetration of Epon™ resin (*see* **Note 8**).

1. The parenchyma is transferred from sodium cacodylate solution (*see* Section 2.2.1, item 1) to a 20 mL glass vessel with a snap-on cap filled with 10 mL 50 % ethanol (*see* Section 2.3, item 1)
2. Dehydration is performed with 10 mL 50 % ethanol (*see* Section 2.3, item 1) for 20 min.
3. Dehydration is performed with 10 mL 50 % ethanol (*see* Section 2.3, item 1) for 20 min.

4. Dehydration is performed with 10 mL 70 % ethanol (*see* Section 2.3, item 2) for 20 min.
5. Dehydration is performed with 10 mL 70 % ethanol (*see* Section 2.3, item 2) over night.
6. Dehydration is performed with 10 mL 80 % ethanol (*see* Section 2.3, item 3) for 20 min.
7. Dehydration is performed with 10 mL 80 % ethanol (*see* Section 2.3, item 3) for 20 min.
8. Dehydration is performed with 10 mL 90 % ethanol (*see* Section 2.3, item 4) for 20 min.
9. Dehydration is performed with 10 mL 90 % ethanol (*see* Section 2.3, item 4) for 20 min.
10. Dehydration is performed with 10 mL 99.8 % ethanol (*see* Section 2.3, item 5) for 20 min.
11. Dehydration is performed with 10 mL 99.8 % ethanol (*see* Section 2.3, item 5) for 20 min.
12. Incubation is performed with 15 mL intermedium fluid I (*see* Section 2.4, item 2) for 2 h.
13. Incubation is performed with 15 mL intermedium fluid II (*see* Section 2.4, item 3) for 2 h.
14. Incubation is performed with 15 mL intermedium fluid III (*see* Section 2.4, item 4) for 24 h in an open glass vessel so that ethanol can evaporate.

3.5 Embedding of Parenchyma in Epon™ Resin

1. For embedding an orientated block of parenchyma is transferred to a silicone mold (Plano; Fig. 2c) (*see* **Note 8**).
2. The tissue is placed so that the plane of the 1. cutting line (Fig. 2a) is in front and the plane of the 2. cutting line (Fig. 2b) faces the bottom of the mold. Following this kind of orientation the organ capsule covers the cortical parenchyma.
3. Then Epon™ resin is slowly added (*see* Section 2.4, item 1) by a 10 mL plastic syringe until the tissue block is totally covered.
4. To eliminate gas bubbles, the specimens are placed in an incubator for 6 h at 30 °C.
5. Finally polymerization is performed at 60 °C for 48 h.

3.6 Semithin sections

1. To produce semithin sections, the fully cured Epon™ resin block with embedded parenchyma is taken out of the mold and mounted in a tissue holder.
2. Then the block is trimmed by a diamond drill (Leica) and clamped in the holder of an ultramicrotome EM UC6 (Leica) for slicing (Fig. 2d). The block of parenchyma is correctly placed, when the plane of the 1. cutting line (Fig. 2a) faces

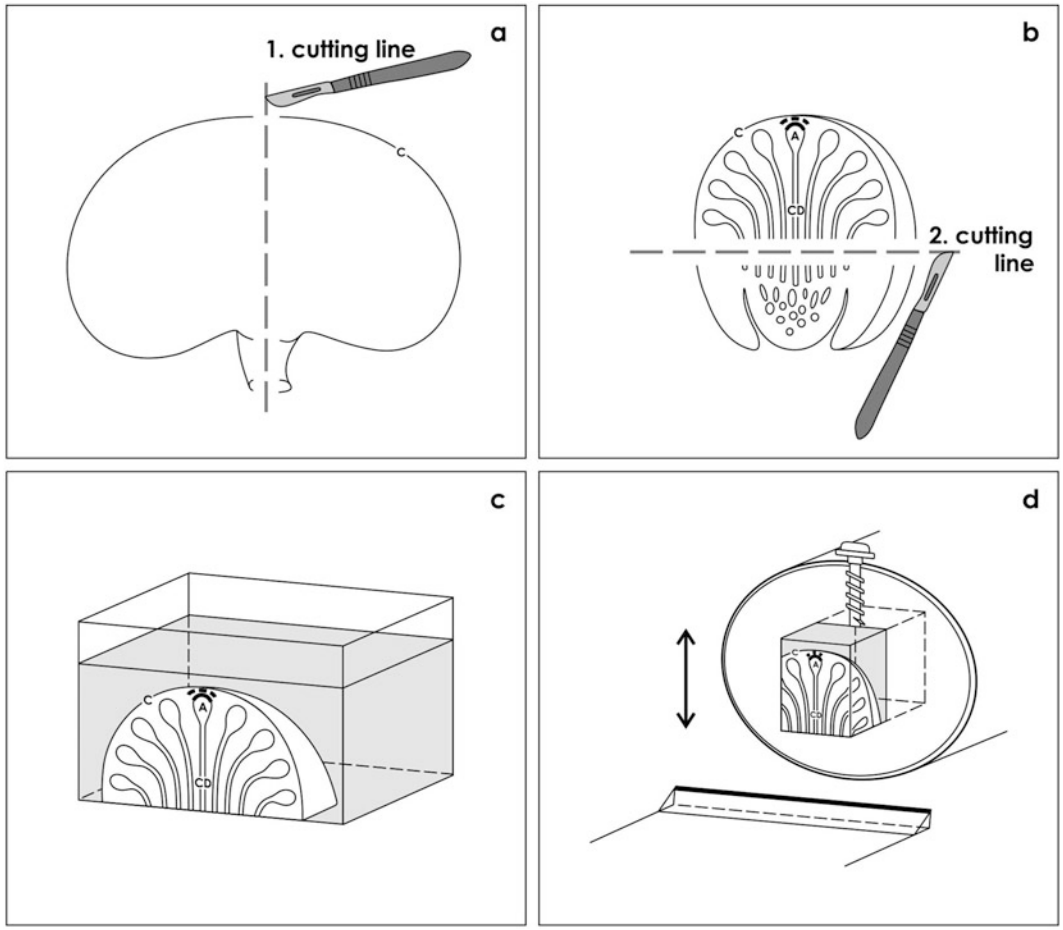

Fig. 2 Compelling morphological view to the renal stem/progenitor cell niche requires accurate orientation of parenchyma. (**a**) For embedding a fixed kidney is first cut between both poles (1. *cutting line*). (**b**) The section plane is yet in parallel to the lumen of lining collecting ducts (CD) and perpendicular to the organ capsule (C). Epithelial stem/progenitor cells are found in the outer cortex within the tip of a CD ampulla (A). Mesenchymal stem/progenitor cells surround the basal aspect of a CD ampulla. For embedding the tissue block is then cut vertically to lining CDs at the frontier between the cortex and medulla (2. *cutting line*). (**c**) Embedding of parenchyma is performed in a silicone mold. The parenchyma is yet orientated so that the 1. *cutting plane* is in *front*, while the 2. *cutting plane* faces the *bottom* of the mold. (**d**) To produce semithin and ultrathin sections, the embedded parenchyma is mounted in a tissue holder for slicing in a microtome. The 1. *cutting plane* of tissue has to face the blade of the diamond knife

the blade of the diamond knife (Diatome, Biel, Switzerland; Fig. 2d). According to this orientation sections can be made in parallel to lining collecting duct tubules (CD) and the ampullae (A) found beyond the organ capsule (C).

3. For light microscopy first a semithin section (2 μm thick) is made and placed together with a drop of bidistilled water on a glass slide (Roth).

4. Then the section is dried on a thermo plate (Medax-Nagel, Kiel, Germany) at 60 °C for 10 min.
5. For staining the section is incubated with a drop of Richardson solution (*see* Section 2.7, item 3) for 2 min at 60 °C on the thermo plate.
6. After staining the specimen is rinsed with bidistilled water and dried.
7. For control a semithin section is analyzed under the light microscope. Yet it can be seen that the renal parenchyma is exactly orientated along a cortico-medullary axis and in parallel to a lining collecting duct (CD) (Fig. 3a). The ureteric bud derived tip of a CD ampulla (A) harbors epithelial stem/progenitor cells. Between the covering organ capsule and the tip of a CD ampulla the cap condensate is seen containing 2–3 layers of mesenchymal stem/progenitor cells.

3.7 Coating of Slot Grids

Ultrathin sections of parenchyma have to be attached to slot grids coated by a Pioloform film (FN65) according established methods (12).

1. First a glass slide (76 mm × 26 mm; Roth) is thoroughly cleaned with bidistilled water and dried with a freshly washed cotton tissue.
2. Then a staining box (Roth) is filled with 100 mL 1.5 % Pioloform solution (*see* Section 2.5, item 2) and the glass slide is slowly sunk into the box until 2 cm of the glass border is left. After 4 s the slide is evenly pulled out of the Pioloform solution and dried under atmospheric air.
3. The adherent film is cut by a razor blade (Science Services) at the edges of the glass slide. This procedure separates in part the film from the glass.
4. Before removing the complete Pioloform film a petri dish (20 cm diameter and 5 cm deep; Roth) is totally filled with bidistilled water. Then the glass slide is sunk in a sharp angle into the petri dish until the film is floating off. On the swimming Pioloform film the slot grids (*see* Section 2.5, item 1) are yet placed side by side.
5. With a strip of Parafilm® M (3 cm × 7 cm; Novodirect, Kehl, Germany) the coated grids are separated by catching them from the water surface.
6. The grids are dried in a glass petri dish. Finally single coated grids are separated by cutting the Pioloform film with a sharp needle (*see* **Note 9**).

3.8 Ultrathin Sections

1. The Epon™ resin block with embedded parenchyma is mounted in a tissue holder and is trimmed to a maximum size of 1 mm × 2 mm by a diamond drill (Leica).

2. Then the holder is clamped in an ultramicrotome EM UC6 (Leica) to produce ultrathin sections (Fig. 2d). The parenchyma is correctly placed, when the plane of the 1. cutting line (Fig. 2a) faces the blade of the diamond knife (Diatome; Fig. 2d).
3. Before cutting the tank of the knife is filled with bidistilled water so that ultrathin sections (70 nm thick) can be fished with a Pioloform coated slot grid (*see* Section 3.7).
4. Finally the sections are dried under a simple lamp until the water is evaporated (*see* **Note 10**).

3.9 Contrasting of Ultrathin Sections

1. For contrasting a strip of Parafilm® M is cut to 3 cm × 15 cm. Onto the Parafilm® M a drop of bidistilled water is pipetted.
2. A grid with a mounted section is placed on top of a drop so that the section faces the fluid.
3. Then the grid is transferred to an isolated drop of 1 % uranyl acetate solution (*see* Section 2.6, item 1) and incubated for 30 min.
4. In the next step the grid is washed in 15 separated drops of bidistilled water.
5. Finally the grid is exposed to a drop of 3 % lead citrate solution (*see* Section 2.6, item 2) for 1 min (*see* **Note 11**).
6. The grid is washed again in 15 separated drops of bidistilled water and dried under a simple lamp.

3.10 Analysis of Specimens by Transmission Electron Microscopy

Analysis of ultrathin sections is performed at 80 kV using an EM 902 transmission electron microscope (Zeiss, Oberkochen, Germany).

Comparable areas of specimens fixed in glutaraldehyde (GA; *see* Section 2.2.1; Figs. 3b, c and 4a), glutaraldehyde including cupromeronic blue (GA + CMB; *see* Section 2.2.2. Fig. 4b), ruthenium red (GA + RR; *see* Section 2.2.3; Fig 4c), or tannic acid (GA + TA; *see* Section 2.2.4. Fig. 4d) are investigated under low and high magnification by the electron microscopy.

1. Specimens fixed by **glutaraldehyde** (GA) show under low (Fig. 3b) and higher (Fig. 3c) magnification the renal stem/progenitor cell niche. Epithelial stem/progenitor cells are found enclosed within the tip of a CD ampulla (A). Mesenchymal stem/progenitor cells can be detected in the space between the inner side of the organ capsule (C) and the basal aspect of a CD ampulla. In each of the cases both kinds of stem/progenitor cells are separated by a bright interstitial interface (asterisk). Under high magnification it can be further seen that the ureteric bud derived tip of a CD ampulla harboring epithelial stem/progenitor cells is covered by a consistently developed basal lamina

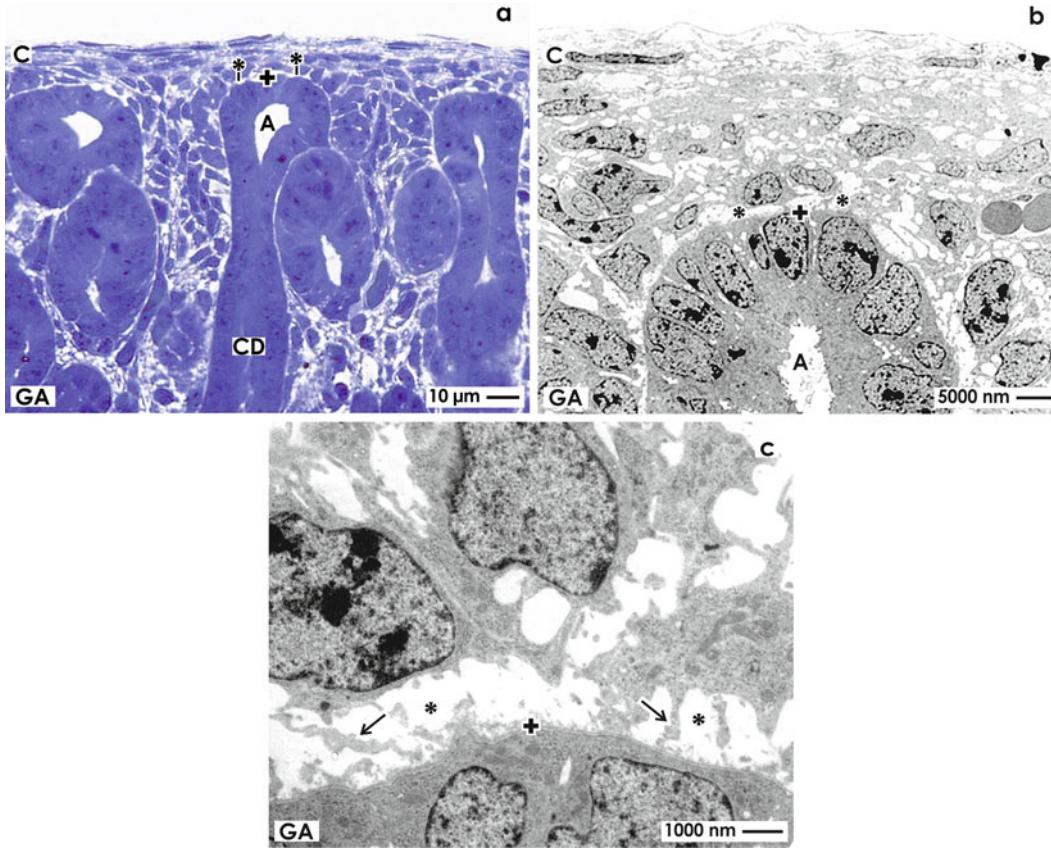

Fig. 3 Light microscopy of a semithin section and transmission electron microscopy of the renal stem/progenitor cell niche in the outer cortex of neonatal rabbit kidney after fixation in GA. (**a**) A semithin section depicts that epithelial stem/progenitor cells are enclosed by the basal lamina at the basal aspect of the CD ampulla (A) tip. The soma of mesenchymal stem/progenitor cells is separated from epithelial cells by the interstitial interface (*asterisk*). (**b**) Low magnification in transmission electron microscopy shows that epithelial and mesenchymal stem/progenitor cells keep always a distinct distance. The interstitial interface appears bright. (**c**) Higher magnification in transmission electron microscopy illuminates that between mesenchymal and epithelial stem/progenitor cells a bright interstitial interface is established. Extracellular matrix is barely visible. Single cell projections (*arrow*) from mesenchymal cells cross the interstitium to contact a CD ampulla (A). The basal aspect at the tip of a CD ampulla is marked by a cross (*plus*)

(Fig. 4a). A clearly visible lamina rara (L.r.), lamina densa (L.d.) and tiny fibers of the lamina fibroreticularis (L.f.) can be seen. An astonishingly bright interstitial interface (asterisk) keeps the soma of mesenchymal stem/progenitor cells in distinct distance. Few projections (arrow) of mesenchymal cells and tiny strands of extracellular matrix cross the interstitial interface to contact the basal lamina at the tip of a CD ampulla.

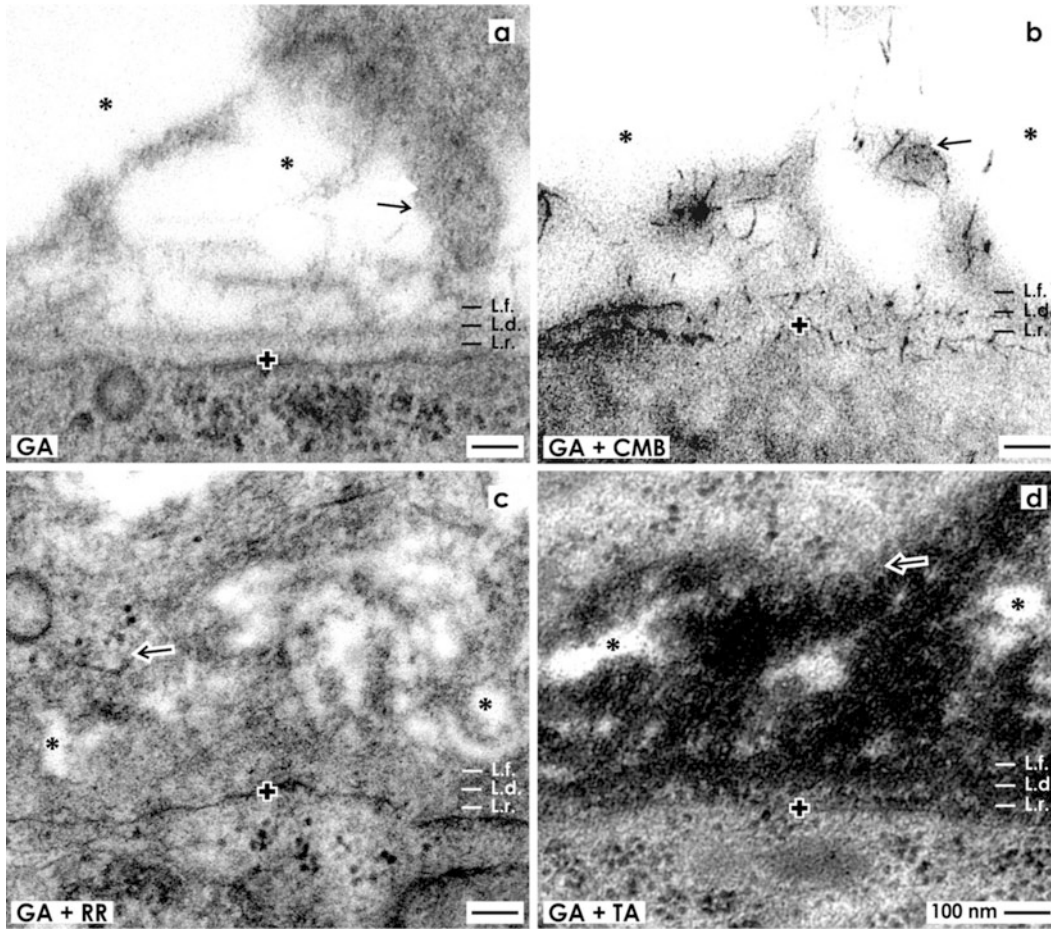

Fig. 4 Transmission electron microscopy of the renal stem/progenitor cell niche. The basal plasma membrane of epithelial stem/progenitor cells at the CD ampulla tip is labeled by a cross (*plus*). An astonishingly wide interstitial interface (*asterisk*) keeps mesenchymal stem/progenitor cells in distance. (**a**) Specimens fixed by GA show a basal lamina consisting of a lamina rara (L.r.), a lamina densa (L.d.) and a lamina fibroreticularis (L.f.) with some filigree fibers of extracellular matrix. The interstitial interface appears bright and does not show extracellular matrix. (**b**) Samples fixed by GA including cupromeronic blue exhibit buckles of proteoglycans that are incorporated in the basal lamina. Further lining projections (*arrow*) from mesenchymal stem/progenitor cells are covered by proteoglycans. (**c**) Specimens fixed by GA including ruthenium red illustrate that a broad band of heterogeneously composed label is established along the basal lamina. Further numerous strands of extracellular matrix labeled by ruthenium red can be seen within the interstitial interface. (**d**) Specimens fixed by GA including tannic acid demonstrate that a broad band of heterogeneously composed extracellular matrix is present along the basal lamina and in form of intense bundles within the interstitial interface. Thus, fixation by GA containing cupromeronic blue (**b**), ruthenium red (**c**), or tannic acid (**d**) unpacks extracellular matrix, which cannot be recognized after traditional fixation in GA (**a**)

2. Specimens fixed by **glutaraldehyde including cupromeronic blue** (GA + CMB) show that numerous buckles of proteoglycans are incorporated in the basal lamina at the tip of a CD ampulla (Fig. 4b). Especially at the basal plasma membrane of epithelial stem/progenitor cells and at the lamina fibroreticularis

(L.f.) proteoglycans are present. Further single projections (arrow) of mesenchymal stem/progenitor cells covered by buckles of proteoglycans cross the interstitial interface (asterisk). It can be seen that buckles of proteoglycans consist of a lengthwise body, a more or less round head and can exhibit a length of more than 100 nm. At their endings they can be linked to the next one so that longitudinal chains arise. Also buckles of proteoglycans are observed, which are linked on different sites to the next one so that a three-dimensional network arises. Finally, the interstitial interface between the lamina fibroreticularis and mesenchymal cell projections appears bright and does not reflect further fibers of extracellular matrix. This result speaks in favor for a specific reaction of cupromeronic blue on sites containing proteoglycans.

3. Specimens fixed by **glutaraldehyde including ruthenium red** (GA + RR) show that the basal lamina bordering the interstitial interface (asterisk) is intensively labeled (Fig. 4c). The typical three-laminar structure (Fig. 4a) of the basal lamina at the CD ampulla tip consisting of a lamina rara (L.r.), lamina densa (L.d.) and lamina fibroreticularis (L.f.) cannot be recognized anymore after ruthenium red label. Instead a broad band with cloudy substructure can be recognized lining along the basal lamina. Further bundles of extracellular matrix labeled by ruthenium red are traversing the interstitial interface. In all of the cases ruthenium red reveals a heterogeneous composition and shows abundant but fine-grained extracellular matrix.
4. Specimens fixed by **glutaraldehyde including tannic acid** (GA + TA) exhibit an intense label at the basal lamina of the CD ampulla tip (Fig. 4d). In this case the complete basal lamina is covered by a broad but more or less electron-dense coat of tannic acid label. Further at the interstitial interface (asterisk) broad strands with intense label are found exhibiting a remarkable microarchitecture in form of fine grains. In between distinct areas of the interstitial interface are bright and consequently not labeled by tannic acid. This special distribution speaks in favor for a stain-specific contrast and not for an unspecific background signal made by tannic acid.

In summary, the presented data exhibit that fixation of specimens with traditional glutaraldehyde solution does not show all the contained morphological structures hidden in the interstitium of the renal stem/progenitor cell niche (Figs. 3b, c and 4a). In contrast, fixation of specimens by glutaraldehyde including either cupromeronic blue (Fig. 4b), ruthenium red (Fig. 4c), or tannic acid (Fig. 4d) depicts that abundant extracellular matrix can be unveiled for analysis in transmission electron microscopy.

4 Notes

1. Important hint: For preparing and handling of the individual solutions and resins observe the relevant safety regulations of your country. Further use safety glasses, a white coat, and gloves! Work under a safety cabinet!
2. Storage of sodium cacodylate solution is performed in a sealed glass bottle at room temperature.
3. Storage of ethanol solutions can be performed in a sealed glass bottle at room temperature.
4. Storage of Epon™ resin is possible in a plastic syringe at −20 °C.
5. Storage of Pioloform solution is performed in a dark glass flask fitted with a ground-glass stopper at 4 °C.
6. After preparation of uranyl acetate the solution is stable for 2 month at 4 °C.
7. The Richardson staining solution is stable for months in a sealable glass bottle.
8. During all steps of embedding one has to take care that the tissue is not drying out.
9. Storage of coated slot grids is possible over month under atmospheric air in a petri dish.
10. Before and after use the grid is stored in a special box (B 801003080; Plano).
11. To avoid precipitation the lead citrate solution has to be centrifuged before use at $10 \times 1{,}000\ \text{min}^{-1}$ for 5 min.

Acknowledgement

This work was supported by the University of Regensburg, D-93053 Regensburg, Germany.

References

1. Minuth WW, Denk L (2012) Cell projections and extracellular matrix cross the interstitial interface within the renal stem/progenitor cell niche: accidental, structural or functional cues? Nephron Exp Nephrol 122:131–140
2. Patel SR, Dressler GR (2013) The genetics and epigenetics of kidney development. Semin Nephrol 33:314–326
3. Hendry C, Rumballe B, Moritz K, Little MH (2011) Defining and redefining the nephron progenitor population. Pediatr Nephrol 26:1395–1406
4. Carroll TJ, Das A (2013) Defining the signals that constitute the nephron progenitor niche. J Am Soc Nephrol 24:873–876
5. Faa G, Gerosa C, Fanni D, Monga G, Zaffanello M, van Eyken P, Fanos V (2012) Morphogenesis and molecular mechanisms involved in human kidney development. J Cell Physiol 227:1257–1268

6. Trueb B, Amann R, Gerber SD (2013) Role of FGFRL 1 and other FGF signaling proteins in early kidney development. Cell Mol Life Sci 70:2505–2518
7. Minuth WW, Denk L (2012) Illustration of extensive extracellular matrix at the epithelial-mesenchymal interface within the renal stem/progenitor cell niche. BMC Clin Pathol 12:16
8. Minuth WW, Denk L (2013) The interstitial interface within the renal stem/progenitor cell niche exhibits an unique microheterogeneous composition. Int J Mol Sci 14:13657–13669
9. Minuth WW, Denk L (2013) Structural links between the renal stem/progenitor cell niche and the organ capsule. Histochem Cell Biol 141:459–471
10. Hasko JA, Richardson GP (1988) The ultrastructural organization and properties of the mouse tectorial membrane matrix. Hear Res 35:21–38
11. Rothenburger M, Völker W, Vischer P, Glasmacher B, Scheld HH, Deiwick M (2002) Ultrastructure of proteoglycans in tissue-engineered cardiovascular structures. Tissue Eng 8:1049–1056
12. Kneissler U, Harendza S, Helmchen U (2003) Support film in transmission electron microscopy: experiences with polyvinyl butyral Pioloform BM 18. J Electron Microsc 52:355–357