Published in final edited form as:

Methods Mol Biol. 2012 ; 886: 121–132. doi:10.1007/978-1-61779-851-1_11.

An Immunofluorescence Method to Analyze the Proliferation Status of Individual Nephron Segments in the *Xenopus* Pronephric Kidney

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Abstract

Organ development requires the coordination of proliferation and differentiation of various cell types. This is particularly challenging in the kidney, where up to 26 different cell types with highly specialized functions are present. Moreover, even though the nephron initially develops from a common progenitor pool, the individual nephron segments are ultimately quite different in respect to cell numbers. This suggests that some cells in the nephron have a higher proliferative index (i.e. cell cycle length) than others. Here we describe two different immunofluorescence-based approaches to accurately quantify such growth rates in the pronephric kidney of *Xenopus laevis*. Rapidly dividing cells were identified with the mitosis marker phospho-Histone H3, while slowly cycling cells were labeled using the thymidine analogue EdU. In addition individual nephron segments were marked using cell type specific antibodies. To accurately assess the number of positively stained cells, embryos were then serially sectioned and analyzed by immunofluorescence microscopy. Growth rates were established by counting the mitosis or S-phase events in relation to the overall cells present in the nephron segment of interest. This experimental design is very reproducible and can easily be modified to fit other animal models and organ systems.

Keywords

Cell Cycle; Kidney; Metanephros; Mitosis; Pronephros; *Xenopus*

1. Introduction

The mechanism determining the final organ or total body size is an intriguing, yet still poorly understood question. A multitude of pathways balancing both proliferation and apoptosis has to be tightly monitored to ensure proper size control (1). The core pathways governing proper organ size control were initially identified in the fruit fly *Drosophila melanogaster*. Genetic screens for mutations resulting in tissue overgrowth demonstrated that proteins involved in two signaling cascades, Insulin/Tsc/TOR and Hippo, were central players (2). These studies were subsequently extended to many vertebrate systems. Importantly, it has become evident that size control mechanisms not only balance proliferation and apoptosis, but integrate many intrinsic cellular mechanisms like control of

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mRNA translation, macromolecular synthesis or autophagy, but also extrinsic and environmental clues, such as nutrient availability (3, 4).

In humans, an imbalance in size control mechanisms can result in clinical conditions. One example is Autosomal Dominant Polycystic Kidney Diseases (ADPKD). This genetically inherited illness of the kidney is characterized by a cyst-forming tubule epithelium, which constantly expands radially (5). This expansion compresses the renal parenchyma and gradually compromises renal function, leading to end-stage renal failure at later stages of life.

The mammalian metanephric kidney is a complex organ consisting of up to several million functional units, the nephrons, and up to 26 different cell types (3, 6). In respect to size control it is a rather exceptional organ; not only is its initial size tightly controlled during development, but it is also readily adaptable to partial kidney loss by compensatory growth/hypertrophy (7, 8). However, the intrinsic mechanism and extrinsic clues controlling these processes are still unclear; e.g. it is unknown, whether the kidney *per se* is growing in a linear fashion or if there are distinct growth phases in different nephron segments. This lack of understanding is - in part - due to the fact that the individual nephrons of the metanephric kidney develop asynchronously. As a consequence, nephrons of different differentiation statuses are present at the same time and growth of individual nephrons is difficult to assess.

Here we provide an alternative approach towards this question, namely the use of the simpler pronephros. In contrast to the metanephros, the pronephric kidney is composed of only two bilateral nephrons (9). It has been successfully used to study early and late events in kidney development and provided results that are easily translated to the metanephric kidney (10–12). Among organisms with functional pronephric kidneys the African clawed frog *Xenopus laevis* is a powerful model organism. The female frog can lay large amounts of eggs, which develop into embryos with a functional kidney after 2 days. Importantly, the developmental timing is highly dependable and precisely documented (13). This allows the collection of embryos from multiple females at precise developmental stages and performing analyses that are highly reproducible and statistically significant.

One prerequisite to addressing the growth characteristics of different nephron segments is their visualization. Different nephron segments are functionally highly specialized and many proteins - in particular glucose-, solute- or salt- transporters - are specific to individual segments. This segment specificity of individual markers is conserved to the more primitive pronephros as was initially shown in *Xenopus* (14, 15) and subsequently confirmed in zebrafish (16). A simple tool to visualize these subdivisions in *Xenopus* are the two monoclonal antibodies 3G8 and 4A6 that label the proximal tubules or the distal tubules and the pronephric duct, respectively (17). In respect to analyzing proliferation antibodies designed to monitor cell cycle state (e.g. phospho-histone H3 for cells undergoing mitosis) have been instrumental to study rapidly cycling cells both *in vivo* and *in vitro* (18). Conversely, incubating tissues with the thymidine analogues Bromodeoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU) is commonly used to label slow cycling cells (19, 20). These analogues are incorporated into the DNA during chromosomal replication and the number of labeled cells is directly proportional to the length of the cell cycle and the exposure time to BrdU/EdU (21).

Combining these nephron segment- and cell cycle-specific detection systems provides an unprecedented accuracy in the determination of the different proliferation statuses present in the developing nephron. This method (Fig. 1) will provide the baseline for future studies on the underlying molecular mechanisms. In addition, even though we described the technique for the *Xenopus* pronephros, it is extendable to other tissues or organisms. Finally, the

method is not restricted to the detection systems described here, but can easily serve as a blueprint for experimental designs using other antibodies or labeling techniques.

2. Materials

2.1 Immunohistochemistry

1. DENT'S Fixative: 10 ml Dimethyl sulfoxide (DMSO), 40 ml methanol
2. 10× PBS: 80 g NaCl, 2 g KCl, 14.4 g Na₂PO₄, 2.4 g KH₂PO₄, 800 ml Millipore water. Mix and adjust pH of 7.4 with NaOH. Adjust to 1 l with Millipore water. Autoclave and store at RT.
3. PBSw: Prepare 1× PBS using 10× PBS and Millipore water, filter with 0.45 µm bottle top filter and add Tween-20 to a final concentration of 0.1%.
4. Prehybridization Buffer: 10% goat serum (heat-inactivated at 56°C for 1 hour), 3% (w/v) Bovine Serum Albumin (BSA, Fraction V) in PBSw. Filter using a 0.45 µm syringe filter (see Note 1).
5. Methanol series: Prepare methanol dilutions (25%, 50%, 75%, 100%) by diluting methanol in 1× PBSw.
6. Ethanol series: Prepare ethanol dilutions (25%, 50%, 75%, 100%) by diluting ethanol in 1× PBSw.
7. Monoclonal antibodies: the pronephros specific antibodies 3G8 and 4A6 can be obtained from the European *Xenopus* Resource Centre at the University of Portsmouth and the pan-kidney α-Na/K-ATPase antibody from the Developmental Studies Hybridoma Bank.

2.2 Embryo Embedding and Sectioning

1. Paraplast: Pellets are placed in a funnel lined with filter paper and melted into a glass bottle overnight using a convection oven at 68°C. Melted paraplast should not be stored longer than a couple of days.

2.3 EdU Labeling and Visualization

1. The components for the EdU (5-ethynyl-2'-deoxyuridine) labeling are part of the Click-iT[®] EdU imaging kit from Invitrogen, but components can also be obtained individually.
2. 10 mM EdU stock solution: Add 2 ml DMSO to Component A and aliquot into microcentrifuge tubes. Store at −80°C.
3. EdU reaction buffer: Dilute Component D to a 1× solution with Millipore water and store at 4°C.
4. Alexa Fluor[®] 594 azide: Add 70 µl of DMSO to Component B. Store at −80°C.
5. Buffer Additive: 10× stock solution is prepared from Component F by adding 2 ml Millipore water to the vial and mix well. Store at −80°C. 1× working solution is prepared fresh every time by diluting it with Millipore water.
6. EdU working solution: 2.2 ml EdU reaction buffer, 100 µl CuSO₄, 6 µl Alexa Fluor[®] 594 azide and 250 µl Buffer Additive. The components have to be added in the indicated order. The solution has to be made freshly each time and cannot be

¹We normally prepare the buffer fresh; it can be frozen, but when thawed do not reheat above 37°C, since precipitate will form.

stored. The solution is sufficient for five slides and the amount needs to be adjusted according to the number of slides.

7. 1× PBS: dilute 10× PBS (see 2.2.1) with Millipore water.
8. PBS-BSA: Dissolve 3% (w/v) Bovine Serum Albumin (Fraction V) in 1× PBS. Mix thoroughly and keep at RT.
9. 4% Paraformaldehyde (PFA) PFA: Dissolve 4 g of PFA in 100 ml in 1× PBS by heating to 65°C, vortex frequently and filter using a 0.45 µm syringe filter.

3. Methods

All procedures are performed at room temperature (RT) unless otherwise indicated. For the removal of solutions from vials containing embryos, we recommend using a vacuum aspiration device consisting of a vacuum Erlenmeyer flask attached on one side to a vacuum source and on the other side to a rubber tubing capped by a yellow tip. This allows easy aspiration of most of the liquid without disrupting the embryos.

3.1 Whole Mount Immunohistochemistry

1. *Xenopus* embryos are obtained using standard methods (22) and cultured until the desired stages (23).
2. Embryos are transferred to 3.7 ml glass vials using transfer pipets, which are then filled to the rim with DENT's Fixative (see Note 2).
3. Vials are placed onto a tube rotator and embryos are fixed at 4°C overnight or 4 hours at RT.
4. Dent's Fixative is removed, embryos are washed in 100% methanol for 5 minutes and replaced again with 100% methanol. Fixed embryos can be stored for prolonged periods at −20°C.
5. Rehydrate fixed embryos through methanol series (100%, 75%, 50%, 25%) to PBSw by removing the fluid and replacing it with the next dilution. Incubate each step at RT for 5 minutes on a test tube rocker.
6. Incubate embryos in PBSw twice for 1 hour each at RT on a test tube rocker.
7. Aspirate PBSw and exchange with 1 ml of freshly prepared Prehybridization buffer and incubate at 4°C on a test tube rocker for 2 hours.
8. At the same time prepare a primary antibody master mix by diluting the 3G8 (1:100) and phospho-Histone H3 (Ser10, pH3, 1:500) antibodies in the appropriate amount of Prehybridization buffer (you will need a minimum of 1 ml per vial). Pre-incubate the master mix for 2 hours at 4°C on a test tube rocker.
9. Aspirate Prehybridization buffer and replace with a minimum of 1 ml of primary antibody master mix.
10. Incubate at 4°C on a test tube rocker overnight.
11. Aspirate solution and exchange with 3.5 ml PBSw and incubate at RT for 1 hour on a test tube rocker.

²Embryos fixed in Dent's or 4% PFA are delicate. Thus, the use of glass vials (e.g. 15×45 mm screw thread vials with rubber-lined cap) is recommended. In addition, it is important to completely fill the vials with fixative to prevent embryos from touching the air/fixative interface.

12. Repeat step 11 three times.
13. Aspirate PBSw and exchange with 1 ml of freshly prepared Prehybridization buffer per vial and incubate at 4°C on a test tube rocker for 2 hours.
14. At the same time prepare a secondary antibody master mix by diluting Alexa Fluor® 488 anti-mouse (1:1000, detects 3G8,) and Alexa Fluor® 594 anti-rabbit (1:1000, detects pH3) in the appropriate amount of Prehybridization buffer (you will need a minimum of 1 ml per vial). Pre-incubate the master mix in the dark for 2 hours at 4°C on a test tube rocker (see Note 3).
15. Aspirate Prehybridization buffer and replace with a minimum of 1 ml of secondary antibody master mix.
16. Incubate in the dark on a test tube rocker at 4°C overnight (see Note 3).
17. Aspirate solution and exchange with 3.5 ml PBSw and incubate in the dark at RT for 1 hour on a test tube rocker.
18. Repeat step 17 four times.
19. Dehydrate embryos through methanol series (25%, 50%, 75%, 100%) by removing the fluid and replacing it with the next dilution. Incubate each step at RT for 5 minutes on a test tube rocker.
20. Store Embryos in 100% methanol or proceed directly to next section.

3.2 Embryo Embedding and Sectioning

1. Transfer the number of embryos to be sectioned into a glass vial filled with 3.5 ml 100% isopropanol and incubate at RT for 15 minutes (see Note 4).
2. Aspirate solution and exchange with 3.5 ml 100% isopropanol. Incubate at 68°C for 15 minutes using a convection heating oven (see Note 5).
3. Decant solution into a waste container and exchange with 3.5 ml of a premade mixture of isopropanol/paraplast (1:1). Incubate at 68°C for 30 minutes.
4. Decant solution into a waste container and exchange with 3.5 ml liquid paraplast. Incubate for 1 hour at 68°C.
5. Repeat step 4.
6. Repeat step 4, but incubate only for 30 minutes.
7. In parallel, prepare embedding molds (one per embryo) by filling them with liquid paraplast to the top. Incubate the molds for approximately 30 minutes at 68°C.
8. Transfer the embryos into the molds filled with liquid paraplast using a pre-warmed transfer pipette.
9. Move one mold at a time to RT. Adjust the position of the embryo so that the head is orientated towards the bottom of the mold and the tail to the top using a straightened paper clip.

³Since the secondary antibodies and the EdU working solution contain fluorphores and are therefore light sensitive we regularly cover the tubes, molds and sections with aluminum foil to minimize photo bleaching.

⁴We normally process as many as five embryos in one glass vial. This number is, however, dependent on the experience of the user and can be adjusted at will. The number should be based on the time needed to process the embryos and transfer them into the molds without premature solidification of the paraplast.

⁵All tools used for the embedding (pipets, droppers and paper clips) need to be kept at 68°C to allow easy handling of the paraplast solutions.

10. Let the paraplast solidify overnight at RT without moving the molds (see Note 3).
11. Section the embryos at 25 μ m thickness with a rotary microtome (see Note 6).
12. Cover pre-cleaned superfrost microscope slides with H₂O on a slide warmer adjusted to 45°C. Assemble sections consecutively on the slide and put as many sections as possible on a single slide.
13. Incubate slides on the slide warmer overnight to assure that the sections stretch out and completely attach to the glass surface.
14. Remove slides from the slide warmer, transfer them to Coplin jars and dewax them in xylene for two times 5 minutes each (see Note 7).
15. Rehydrate sections into PBSw using a series of ethanol (100%, 75%, 50% and 25%) by transferring the slides from one Coplin jar to the one with the next dilution. Incubate each step at RT for 5 minutes.
16. Wash sections three times with PBSw in Coplin jars.
17. Wash sections once with Millipore water in Coplin jars.
18. Mount the sections using a water-based embedding media containing DAPI (e.g. ProLong[®] Gold Antifade Reagent) using a cover glass and try to avoid any air bubbles.
19. Dry sections at least overnight (see Notes 3 and 8).
20. Analyze the sections with a fluorescence microscope.
21. Identify 3G8-positive proximal tubules (green) on the sections and count all the pH3-positive (red) as well as DAPI-positive cells (blue) in this area (see Note 9).

3.3 EdU Labeling and Visualization

1. *Xenopus* embryos are obtained using standard methods (22) and cultured until the desired stages (23).
2. Thaw 10 mM EdU stock solution at RT (see Note 10).
3. Anesthetize *Xenopus* embryos with 250 mg/l Tricaine methane sulfonate (MS222) solution.
4. Inject *Xenopus* embryos with approximately 8 nl of the EdU stock solution into the ventral region at the level of the pronephric tubules using a standard microinjection setup (see Note 11) (22).

⁶To maximize the number of sections that one can put on a single slide, the paraplast block is cut as close as possible to the embedded embryo. For the subsequent analysis it is important to recover all sections and place them on the slide consecutively. Thus, we do not recommend using a heated water bath to spread the sections. The thickness of 25 μ m for the sections has been established empirically. It reflects the size of the cells in the pronephros at the stages analyzed and assures that each section is approximately one cell layer deep. The thickness of sections from different stages, organs or organisms needs to be adjusted accordingly.

⁷The freshness of the xylene is important. While it is possible to reuse it for several times, one has to verify proper dewaxing by visual inspection.

⁸Care should be taken to analyze sections after 24 hours, since the mounting media has not yet dried completely. Shearing of the coverslip can result in disrupting the tissue integrity and prevent proper subsequent analysis.

⁹3G8 labels the apical membrane of the proximal tubules. The only cells that are counted are those sharing this apical domain. Interstitial cells are neglected.

¹⁰We prefer EdU to the other frequently used Thymidine analogue BrdU, since the methodology to visualize BrdU requires harsh treatments to denature the chromosomal DNA and increase accessibility for the anti-BrdU antibody. Due to the decreasing cell size of the developing embryos, the timing of the treatments for the BrdU staining needs to be adjusted for each individual embryonic stage. In addition, the denaturation step destroys the epitopes used for immunofluorescence making the double staining protocol more difficult.

5. Return embryos to 0.1× Barth and monitor embryos until they regained consciousness.
6. Transfer embryos to a new plate containing 0.1× Barth and culture for 12 hours (see Note 12).
7. Transfer embryos to 3.7 ml glass vials containing 4% PFA using a transfer pipet (see Note 2).
8. Vials are placed onto a tube rotator and embryos are fixed at 4°C overnight or 4 hours at RT.
9. 4% PFA is removed and embryos are dehydrated through methanol series (25%, 50%, 75%, 100%) by removing the fluid and replacing it with the next dilution. Fixed embryos can be stored for prolonged periods at −20°C.
10. Embryos are embedded and sectioned as described above (section 3.2 steps 1–15).
11. Permeabilize and re-fix slides in 4% PFA for 15 minutes at RT using a Coplin jar.
12. Wash the slides in PBS-BSA twice for 5 minutes each using a Coplin jar.
13. Wash slides in PBSw for 20 minutes using a Coplin jar.
14. Prepare the EdU working solution. The slides are laid flat into a moist chamber and 500 µl of the EdU working solution is added onto each slide. It is important that the sections never dry out during the procedure, since this will result in background staining. A cover glass is gently put on the solution to ensure that the solution completely covers the slide and does not evaporate. Incubate for 30 minutes in the dark (see Note 3).
15. Remove EdU working solution (including the cover glass). Wash the slides in PBS-BSA for 5 minutes using Coplin jars.
16. Wash the slides three times in PBS for 5 minutes each in Coplin jars.
17. Transfer slides back into the moist chamber and add 500 µl of freshly prepared Prehybridization buffer to each slide and use a cover slide to prevent evaporation. Incubate in the dark at 4°C for 2 hours.
18. At the same time prepare a primary antibody master mix by diluting anti-α-Na/K-ATPase antibody (1:50, see Note 13) in the appropriate amount of Prehybridization buffer (you will need 500 µl per slide). Pre-incubate the master mix for 2 hours at 4°C on a test tube rocker.
19. Aspirate Prehybridization buffer, replace with 500 µl of primary antibody master mix and use a cover slide to prevent evaporation.
20. Incubate in the dark at 4°C overnight.
21. Wash the slides briefly in PBSw using Coplin jars.
22. Wash the slides five times for 1 hour each in PBSw using Coplin jars.

¹¹In our experience injection of the EdU into the *Xenopus* embryo is essential for the sensitivity and reproducibility of the assay. Adding the EdU stock solution directly to the culture medium did not reliably work even at higher concentrations of EdU. This seems to be a penetration problem of embryos at stages 38 and higher, since in “bathed” embryos EdU staining was primarily restricted to the epidermis.

¹²A 12-hour chase is sufficient to allow one mitosis event in the pronephric duct. In other tissues the timing of the EdU chase may need to be adjusted according to the respective cell cycle length.

¹³α-Na/K-ATPase labels epithelial structures including the entire pronephros. The 4A6 antibody can be used if one only wants to label the pronephric duct and distal tubules.

23. Transfer slides into a moist chamber, cover each slide with 500 μ l of freshly prepared Prehybridization buffer and use a cover slide to prevent evaporation. Incubate in the dark at 4°C for 2 hours.
24. At the same time prepare a secondary antibody master mix by diluting Alexa Fluor® 488 anti-mouse (1:1000, see Notes 3 and 14) in the appropriate amount of Prehybridization buffer (you will need 500 μ l per slide). Pre-incubate the master mix for 2 hours in the dark at 4°C on a test tube rocker.
25. Aspirate Prehybridization buffer, replace with 500 μ l of secondary antibody master mix and use a cover slide to prevent evaporation.
26. Incubate in the dark at 4°C overnight.
27. Wash the slides briefly in PBSw using Coplin jars.
28. Wash the slides in the dark five times for 1 hour each in PBSw using Coplin jars.
29. Wash slides briefly with Millipore water.
30. Mount the sections using a water-based embedding media containing DAPI (e.g. ProLong® Gold Antifade Reagent) using a cover glass and try to avoid air bubbles.
31. Dry sections at least overnight (see Note 8).
32. Analyze the sections with a fluorescence microscope.
33. Identify α -Na/K-ATPase-positive pronephric tubules on the sections (green) and count all the EdU-positive (red) as well as DAPI-positive cells (blue) in this area.

Acknowledgments

We would like to thank U. Tran and V. Kumar for critically reviewing the manuscript. D.R. is a recipient of a DFG postdoctoral fellowship (RO4124/1-1). This work was supported by NIH/NIDDK (5RO1DK080745-03) to O.W.

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¹⁴The Click-iT® EdU imaging kit from Invitrogen can be obtained with different fluorescence labels. The protocol here uses the Alexa Fluor® 594 azide, but any other fluorophore combination can be used.

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Fig. 1.
 Experimental Workflow using the 3G8 and Phospho-Histone H3.