Video Article

Technique to Target Microinjection to the Developing *Xenopus* Kidney

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Abstract

The embryonic kidney of *Xenopus laevis* (frog), the pronephros, consists of a single nephron, and can be used as a model for kidney disease. *Xenopus* embryos are large, develop externally, and can be easily manipulated by microinjection or surgical procedures. In addition, fate maps have been established for early *Xenopus* embryos. Targeted microinjection into the individual blastomere that will eventually give rise to an organ or tissue of interest can be used to selectively overexpress or knock down gene expression within this restricted region, decreasing secondary effects in the rest of the developing embryo. In this protocol, we describe how to utilize established *Xenopus* fate maps to target the developing *Xenopus* kidney (the pronephros), through microinjection into specific blastomere of 4- and 8-cell embryos. Injection of lineage tracers allows verification of the specific targeting of the injection. After embryos have developed to stage 38 - 40, whole-mount immunostaining is used to visualize pronephric development, and the contribution by targeted cells to the pronephros can be assessed. The same technique can be adapted to target other tissue types in addition to the pronephros.

Video Link

The video component of this article can be found at https://www.jove.com/video/53799/

Introduction

The *Xenopus* embryonic kidney, the pronephros, is a good model for studying kidney development and disease. The embryos develop externally, are large in size, can be produced in large numbers, and are easily manipulated through microinjection or surgical procedures. In addition, the genes governing kidney development in mammals and amphibians are conserved. Mammalian kidneys progress through three stages: the pronephros, mesonephros, and metanephros[1], while embryonic amphibians have a pronephros and adult amphibians have a metanephros. The basic filtering unit of these kidney forms is the nephron, and both mammals and amphibians require the same signaling cascades and inductive events to undergo nephrogenesis[2, 3]. The *Xenopus* pronephros contains a single nephron composed of proximal, intermediate, distal and connecting tubules, and a glomus (analogous to the mammalian glomerulus)[1, 4-6] (**Figure 1**). The single, large nephron present in the *Xenopus* pronephros makes it suitable as a simple model for the study of genes involved in kidney development and disease processes.

Cell fate maps have been established for early *Xenopus* embryos, and are freely available online at Xenbase[7-11]. Here, we describe a technique for microinjection of lineage tracers to target the developing *Xenopus* pronephros, although the same technique can be adapted to target other tissues such as the heart or eyes. Lineage tracers are labels (including vital dyes, fluorescently labeled dextrans, histochemically detectable enzymes, and mRNA encoding fluorescent proteins) that can be injected into an early blastomere, allowing the visualization of the progeny of that cell during development. This protocol utilizes MEM-RFP mRNA, encoding membrane targeted red fluorescent protein[12], as a lineage tracer. The targeted microinjection techniques for individual blastomeres in 4- and 8-cell embryos described here can be utilized for injection with morpholinos to knock down gene expression, or with exogenous RNA to overexpress a gene of interest. By injecting into the ventral, vegetal blastomere, primarily the pronephros of the embryo will be targeted, leaving the contralateral pronephros as a developmental control. Co-injection of a tracer verifies that the correct blastomere was injected, and shows which tissues in the embryo arose from the injected blastomere, verifying targeting of the pronephros. Immunostaining of the pronephros allows visualization of how well the pronephric tubules have been targeted. Overexpression and knockdown effects can then be scored against the contralateral side of the embryo, which serves as a developmental control, and can be used to calculate the pronephric index[13]. The availability of cell fate maps allows this targeted microinjection technique to be used to target tissues other than the pronephros, and co-injection of a fluorescent tracer allows the targeted microinjection to each tissue to be verified prior to analysis.

During embryo microinjection, developmental temperature should be regulated tightly, given that the rate of *Xenopus* development is highly dependent upon it[14]. Embryos should be incubated at cooler temperatures (14 - 16 °C) for 4- and 8-cell injections because the development time is slowed down. At 22 °C, development time from stage 1 (1 cell) to stage 3 (4 cells) is approximately 2 hours, while at 16 °C development time

to stage 3 is approximately 4 hours. It takes approximately 15 minutes to go from a 4-cell embryo to an 8-cell (stage 4) embryo at 22 °C, but takes approximately 30 minutes at 16 °C. Similarly, at 22 °C, it only takes 30 minutes for an 8-cell embryo to progress to a 16-cell embryo (stage 5). This time is increased to 45 minutes at 16 °C. Therefore, it is useful to slow the development rate of the embryos to enable enough time for injections at the 8-cell stage before the embryos progress to the 16-cell stage. Additionally, growth temperatures can be modulated to speed or slow embryonic development until the kidney has fully developed.

The epidermis of tadpole-stage *Xenopus* embryos is relatively transparent, allowing for easy imaging of the developing pronephros without dissection or clearing of the tissue[15]. Due to the relative transparency of *Xenopus* embryos, live cell imaging is also feasible[16,17]. Whole-mount immunostaining to visualize the pronephros is possible with established antibodies that label the proximal, intermediate, distal and connecting tubules of stage 38 - 40 embryos that allow for assessment of pronephric development after targeted manipulation of gene expression in *Xenopus* embryos[18-20].

Protocol

The following protocol has been approved by the University of Texas Health Science Center at Houston's Center for Laboratory Animal Medicine Animal Welfare Committee, which serves as the Institutional Care and Use Committee (protocol #: HSC-AWC-13-135).

1. Identification and Selection of Blastomeres for Kidney-targeted Injections

1. Prior to generating embryos, use the Normal Table of *Xenopus* Development[21] to understand the orientation of the early cell divisions in the embryo. Alternatively, access diagrams of the early developmental stages of *Xenopus* on Xenbase[11].
2. Access the interactive *Xenopus* cell fate maps on Xenbase[11] to select which blastomere will be targeted for microinjection.
3. Observe that the single cell embryo has a darkly pigmented animal pole and a vegetal pole, which is white and yolky. Note that a protective membrane, known as the vitelline envelope, covers the embryo.
4. Notice that the first cleavage typically occurs between the left and right sides of the embryo. These cells contribute equally to the pronephric lineage.
5. Note that the second cleavage divides the dorsal and ventral halves of the embryo, leading to a 4-cell embryo. The dorsal cells are smaller and have less pigment than the ventral cells (**Figure 2A and Figure 3A**).
    1. Identify the ventral blastomeres (V; the large, dark cells) on the left and right sides, which contribute more to the developing kidney than the dorsal (D; small, light cells) blastomeres (**Figure 2A**).
    2. If injecting into a 4-cell embryo, inject the left ventral blastomere to target the left kidney (**Figure 3A** and Section 3).
6. The third cleavage bisects the animal and vegetal sides, resulting in an eight-cell embryo. At this point, there are four animal blastomeres [left and right ventral (V1) and dorsal (D1)] and four vegetal blastomeres [left and right ventral (V2) and dorsal (D2)] (**Figure 2B and Figure 3B**).
    1. Locate the ventral, vegetal blastomeres (V2). These blastomeres contribute more to the developing kidney any other cells at this stage (**Figure 2B**). To target the left kidney of an 8-cell embryo, inject into the left V2 blastomere (**Figure 3B** and Section 3).
7. Notice that the fourth and fifth cleavages bisect the animal and vegetal blastomeres. Two progeny are generated from each blastomere, resulting in a 16-cell embryo. The cells are named after their predecessor. For example, the V2 blastomere from the 8-cell stage gives rise to V2.1 and V2.2 progeny at the 16-cell stage (**Figure 2C**). The V2.2 cell at the 16-cell stage provides the majority of the cells contributing to the developing kidney.
8. Note the sixth and seventh cleavages result in a 32-cell embryo. Again, two progeny are generated from each blastomere, which are named following their predecessor. For example, the V2.2 blastomere from the 16-cell stage gives rise to V2.2.1 and V2.2.2 at the 32-cell stage. There is an alternative naming system at the 32-cell stage in which cells are identified in four rows as A, B, C, and D (from animal to vegetal), and in four columns as 1, 2, 3, and 4 (from dorsal to ventral). Thus, the V2.2.2 blastomere, which contributes the most to the developing pronephros, is called C3 under this alternative naming system (**Figure 2D**).

2. Preparation of Embryos

1. Prepare 50 ml of Dejelly Solution (2% cysteine, NaOH to pH 8.0).
2. Isolate both testes from a single male frog according to standard protocols[14]. Place the testes in a 60 mm Petri dish filled with 10 ml Testes Storage Solution (1x Marc's Modified Ringers [MMR; 0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES pH 8, 0.1 mM EDTA][12], 1% bovine serum albumin, 50 µg/ml gentamycin). Store the testes at 4 °C.
   Note: Testes can be stored for approximately 7 - 10 days at 4 °C, but fertilization efficiency will decrease the longer the testes have been stored.
3. Squeeze a female frog to obtain eggs according to standard protocols[14]. Collect the eggs in a 100 mm Petri dish. Pour off any excess water.
4. Cut off ¼ of a testis while it is in Testes Storage Solution using forceps and a razor blade. Transfer the piece of testis to the Petri dish containing eggs. Adjust the size of the testis portion used to account for the size of the testis, how long the testis has been stored, and how many eggs are to be fertilized. Generally use ¼ to 1/3 of a freshly dissected testis to fertilize one clutch of eggs.
5. Cut the testis portion into small pieces using forceps and a razor blade. Add enough 0.3x MMR + 30 mg/ml gentamycin to the Petri dish to cover the eggs. Swirl the MMR in the dish to mix.
6. Wait approximately 30 min for fertilization to take place at room temperature. Note that the animal hemisphere (the pigmented side of the embryo) will sit on top of the embryo upon effective fertilization. Then, remove the MMR from the Petri dish using a transfer pipette. Add enough Dejelly Solution to the dish to cover the embryos.
7. Over the next few minutes, gently swirl the dish intermittently. Vigorous shaking of the dish at this time can cause axis defects. The jelly coat on the embryos will dissolve, and the embryos will congregate in the center of the dish during swirling. Once the embryos are closely touching each other in the center of the dish, remove the Dejelly Solution with a transfer pipette. Do not leave embryos in the Dejelly Solution for longer than 5 minutes, or the embryos may be damaged.

8. Wash the dejellied embryos 3 - 5 times in 0.3x MMR + 30 mg/ml gentamycin by carefully pouring or pipetting off the MMR and filling the dish with new MMR. Do not remove all of the MMR from the dish, or the embryos may be damaged.
9. Remove any unfertilized eggs or pieces of testis from the Petri dish using a transfer pipette.
10. Incubate embryos between 14 and 22 °C.
    Note: Embryos grown at lower temperatures develop more slowly than embryos grown at higher temperatures. Timing of developmental stages can be found on Xenbase[22].
    1. To space out their development, place half of the embryos from a single fertilization in a Petri dish kept at 14 °C, and the other half of the embryos in a Petri dish kept at 18 °C. This allows for two sets of injections into 4-cell or 8-cell embryos from a single fertilization.

3. Preparation of Injection Solutions and Microinjection of Embryos

1. Prepare the injection solution containing 0.01 ng/nl membrane-bound red fluorescent protein (MEM-RFP) mRNA[9] while the embryos are developing to the 4-cell or 8-cell stage. Store the injection solution on ice until ready to inject.
2. Load a 7" replacement glass capillary tube into a needle puller, with the top of the replacement tube aligned with the top of the needle puller case. Set the heat #2 value to 800, and the pull value to 650. Press the "pull" button to pull the needle. This will create 2 needles from a single 7" glass capillary tube.
3. Snip off the tip of a pulled needle with a pair of Dumont forceps.
   Note: After pulling the needle, the tip is sealed shut and must be cut open. The closer to the point of the needle that it is cut, the smaller the diameter the needle tip will be. Although the diameter of the tip will not affect the injection volume with the microinjection system used here, a tip with a larger diameter is more likely to damage the embryo.
4. Slip the micropipette collet onto the back of the needle. Next, slip the large hole O-ring onto the back of the needle behind the collet.
5. Fill the needle with mineral oil using a 27 gauge hypodermic needle, being careful not to get air bubbles in the needle.
6. Slip the needle onto the plunger of the microinjector, seating the needle into the large hole of the white plastic spacer installed on the plunger. The plunger should have a small O-ring nearest to the body of the microinjector, followed by the white spacer, large hole O-ring, and collet. Secure the needle by tightening the collet. Pull gently on the needle to make sure that it is properly secured.
7. Press and hold the "empty" button on the microinjector control box until there are two beeps.
8. Pipette 3 µl of injection solution onto a piece of Parafilm. Insert the tip of the needle into the bead of injection solution on the Parafilm. Press and hold the "fill" button on the microinjector control box to draw the injection solution into the needle.
9. Fill a 60 mm Petri dish lined with 500 micron polyester mesh with 5% Ficoll in 0.3x MMR + 30 mg/ml gentamycin. Carefully pipette 20 - 30 4-cell or 8-cell embryos into the dish.
10. Using a hair loop[14], manipulate the embryos so that the blastomere to be injected is facing the needle. To target the left kidney, line up the embryos so that the left ventral blastomeres of 4-cell embryos or the left V2 blastomeres of 8-cell embryos face the needle.
11. Inject 10 nl of injection solution into the selected blastomere of each embryo in the dish.
    Note: The mesh at the bottom of the Petri dish stabilizes the embryos and prevents them from rolling, allowing them to be injected without the use of a hair loop for stabilization.
12. Transfer injected embryos into wells of a culture plate that have been filled with 5% Ficoll in 0.3x MMR + 30 mg/ml gentamycin. Incubate the injected embryos at 16 °C for at least one hour to allow the injected blastomeres to heal.
13. Transfer the healed embryos into wells of a new culture plate that have been filled with 0.3x MMR + 30 mg/ml gentamycin by stage 9 (prior to gastrulation).
14. Incubate the embryos at 14 - 22 °C until the embryos reach stage 38 - 40[21].

4. Fixation and Immunostaining of Embryos

1. Prepare 50 ml of MOPS/EGTA/Magnesium sulfate/Formaldehyde Buffer [MEMFA: 100 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% (v/v) formaldehyde].
2. Using a transfer pipette, put 10 - 20 stage 38 - 40 embryos in a glass vial. Add 10 µl 5% benzocaine in 100% ethanol to the vial and invert vial to mix. Wait 10 min to anesthetize the embryos.
3. Remove the MMR from the vial using a glass pipette. If processing multiple vials at the same time, the vials can be held upright in a 24-well cell culture plate.
4. With a glass pipette, fill the vial with MEMFA. Place the vial on a three-dimensional rocking platform for 1 hr at room temperature.
5. Remove the MEMFA from the vial using a glass pipette. Fill the vial with 100% methanol. Place the vial on a three-dimensional rocking platform for 10 min at room temperature. Repeat this wash step one more time, and store the embryos in 100% methanol overnight at -20 °C.
6. Prepare 1x Phosphate Buffered Saline-Bovine Serum Albumin-Triton (PBT): 1x PBS, 2 mg/ml bovine serum albumen, 0.1% Triton X-100.
7. Prepare the primary antibody solution: 1x PBT with 10% goat serum with a 1:5 dilution of mouse monoclonal 4A6 antibody (to label the membranes of the intermediate, distal and connecting tubules[20]), a 1:30 dilution of mouse monoclonal antibody 3G8 (to label lumen of the proximal tubules[20]), and a 1:250 dilution of rabbit polyclonal RFP antibody (to label the MEM-RFP tracer). Store at 4 °C.
8. Prepare the secondary antibody solution: 1x PBT with 10% goat serum, 1:500 Alexa 488 goat anti-mouse IgG (stock concentration 2 mg/ml; to label 4A6 and 3G8), and 1:500 Alexa 555 goat anti-rabbit IgG (stock concentration 2 mg/ml; to label the MEM-RFP tracer). Store at 4 °C, covering the tube in foil to protect from light.
9. Alternatively, collect the primary and secondary antibodies after staining, and save at 4 °C for reuse in future experiments. If the antibody is to be saved for reuse, add 0.01% sodium azide.
10. Immunostain the embryos using established protocols[18].

5. Visualization of Embryos and Analysis of Targeted Pronephric Tissue

1. Screen the immunostained embryos to verify that the correct blastomere was injected by viewing the fluorescence of the tracer under a fluorescent stereomicroscope at 1X (to view whole embryo) and 5X (to view kidney) magnification. Place embryos in a multi-well glass plate

with the wells filled with 1x PBT using a transfer pipette with the tip cut off. Manipulate the embryos with a hair loop. Use only embryos which have the co-injected tracer present in the pronephros on the left side of the embryo (**Figure 3C, F and Figure 4C, F**) for gene overexpression or knockdown analysis.

2. Alternatively, clear the embryos in Murray's Clear (2 parts benzyl benzoate: 1 part benzyl alcohol) by placing the embryos in a glass vial and filling the vial with Murray's Clear. Visualize the embryos using a glass well plate.
   Note: Murray's Clear is an organic solvent, and should be handled with care. Wear gloves, and only use glass vials and pipettes with Murray's Clear.
3. Store embryos at 4 °C in 1x PBT for 2 - 3 weeks. For long-term storage of embryos, dehydrate the embryos by washing two times in 100% methanol at room temperature for 10 min. Store the embryos at -20 °C in 100% methanol.

Representative Results

Microinjections of 4- and 8-cell *Xenopus* embryos with MEM-RFP mRNA show different levels of targeting to the pronephros. **Figure 4** shows stage 40 embryos with correct MEM-RFP mRNA expression patterns. Embryos were injected in the left ventral blastomere (**Figure 4A**), and sorted for the proper expression pattern of MEM-RFP mRNA. In addition to expressing MEM-RFP in the proximal, intermediate, distal and connecting tubules of the kidney, properly injected embryos are expected to show fluorescence in the epidermis of the head, trunk, and tail. They should also show fluorescence in the otocyst, cement gland, proctodeum, and somites (**Figure 4C**). The distribution of MEM-RFP fluorescence in the kidneys of 8 properly injected embryos was determined with a fluorescent stereomicroscope (**Figure 4B**). Embryos with high levels of MEM-RFP expression in the epidermis that blocked the detection of kidney regions were scored as "occluded". MEM-RFP expression was detected in the proximal, intermediate, distal and connecting tubules of all embryos that were not scored as occluded. Stereoscope (**Figure 4D-F**) and confocal microscopes (**Figure 4G-I**) were used to verify the co-localization of MEM-RFP and kidney tissue immunostained with 3G8 and 4A6. Confocal imaging verified the co-localization of MEM-RFP with the proximal, intermediate, distal and connecting tubules of the kidney, indicating that microinjection of MEM-RFP into the left ventral blastomere correctly targets the kidney.

Embryos injected with MEM-RFP mRNA at the 8-cell stage (**Figure 5A**) show a narrower range of tissues displaying fluorescence, indicating that 8-cell injections reduce the potential for secondary effects to the kidney. Like embryos injected at the 4-cell stage, embryos injected at the 8-cell stage show fluorescence in the proximal, intermediate, distal and connecting tubules of the kidney, as well as the somites and proctodeum (**Figure 5C-F)**. Unlike embryos injected at the 4-cell stage, the cement gland and otocyst are not labeled with MEM-RFP. Out of 10 properly targeted embryos, one embryo showed MEM-RFP in nearly all of the proximal tubules, and 3 showed MEM-RFP in nearly all of the intermediate and distal tubules of the kidney (**Figure 5B**). The connecting tubules of 7 embryos were partially labeled with MEM-RFP. In addition, the kidneys of embryos injected at the 8-cell stage were easier to visualize, and only one was scored as occluded. Co-localization of MEM-RFP and antibodies labeling the kidney (3G8 and 4A6) was determined using a stereomicroscope (**Figure 5D-F**), and verified using a confocal microscope (**Figure 5G-I**). The proximal, intermediate, distal and connecting tubules of the kidney were labeled with MEM-RFP in embryos injected at the 8-cell stage, demonstrating that targeted injection of the V2 blastomere labels the kidney while displaying fluorescence in fewer tissues than embryos injected in the left ventral blastomere at the 4-cell stage.

This assay makes use of a fluorescently tagged mRNA as a tracer to indicate which tissues were targeted by microinjection. It is important to screen embryos for consistent tracer localization prior to analysis, and any embryos that to not display the expected tracer distribution pattern should be discarded. **Figure 6** shows an example of a stage 40 embryo that was incorrectly targeted with MEM-RFP mRNA at the 4-cell stage. MEM-RFP mRNA expression is located on the right side of the embryo instead of the left side (**Figure 6A**). In addition, most of the mRNA expression is in the skin of the lower trunk and tail, with little expression in the somites. No mRNA expression is seen in the proximal, intermediate, distal and connecting tubules (**Figure 6B**), confirming improper targeting of the kidney. Therefore, this embryo should not be used for analysis.

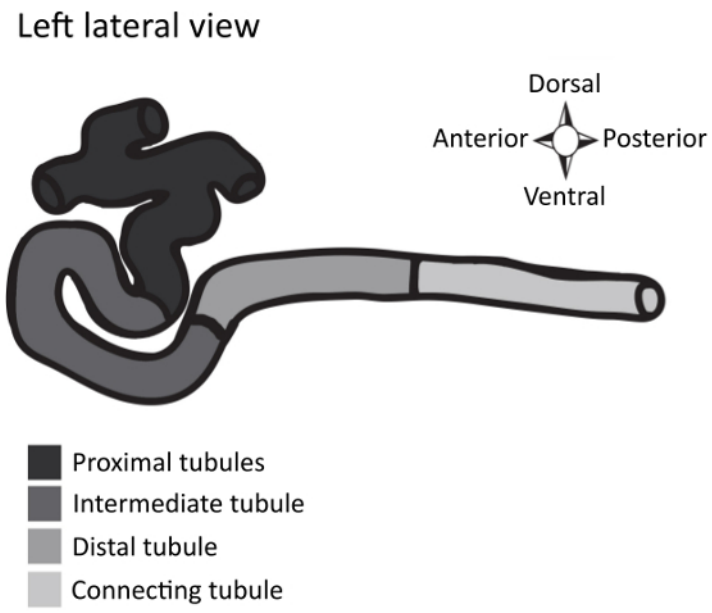

Figure 1: *Xenopus* Embryonic Kidney. Diagram of the kidney of a stage 35 *Xenopus* embryo, showing the proximal, intermediate, distal, and connecting tubules. Based on Raciti *et al*. (2008)[6]. Please click here to view a larger version of this figure.

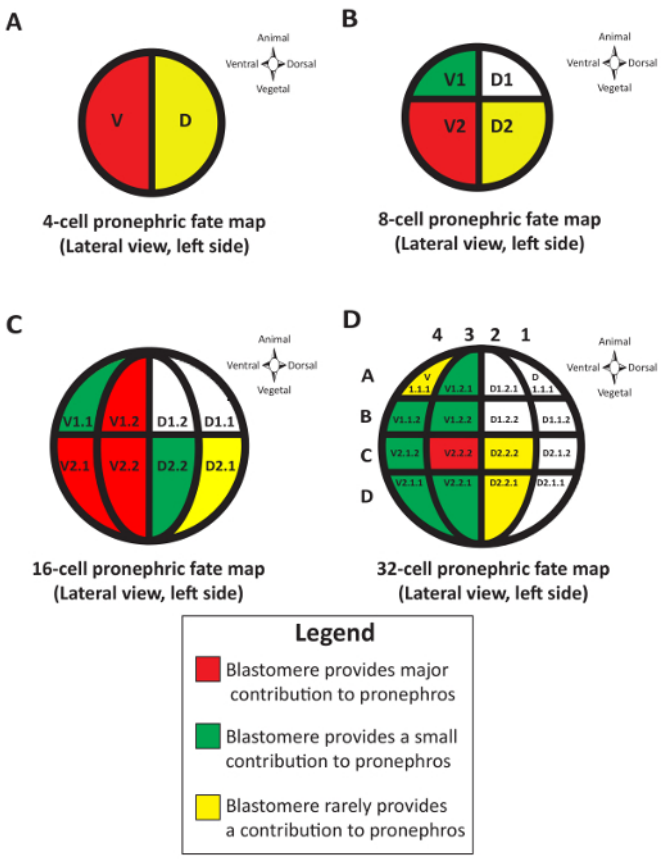

Figure 2: *Xenopus* Embryonic Fate Maps. Fate maps identifying and naming the blastomeres that contribute to the developing pronephros. A) Fate map of a 4-cell embryo. B) Fate map of an 8-cell embryo. C) Fate map of a 16-cell embryo. D) Fate map of a 32-cell embryo. Blastomeres of the 32-cell embryo are also labeled using an alternate blastomere naming system. Fate maps based on Moody (1987)[8, 9] and Moody and Kline (1990)[7]. Please click here to view a larger version of this figure.

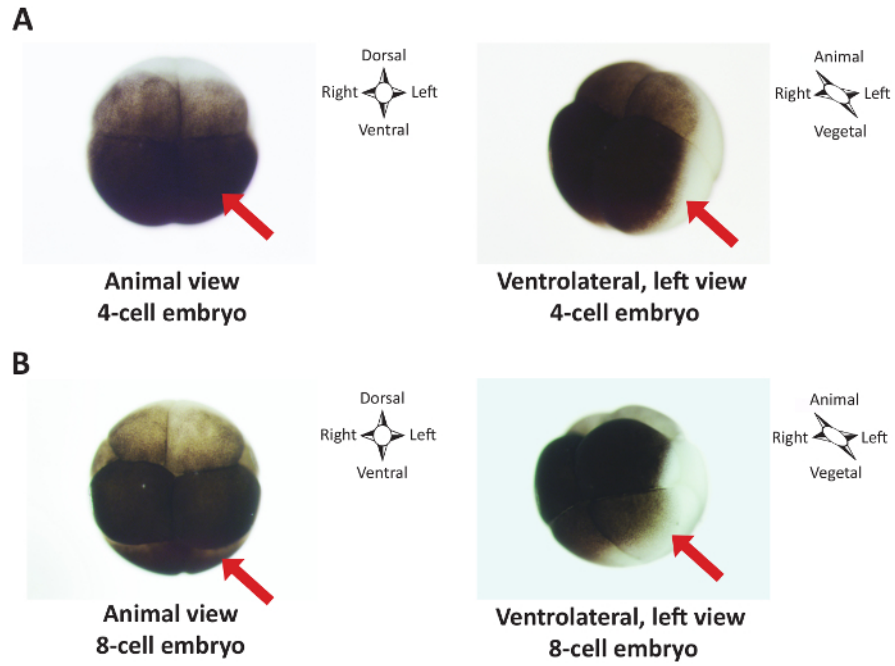

Figure 3: Injection Scheme for Targeting the *Xenopus* Pronephros. Red arrows indicate the cell to inject. A) Animal and ventral views of a 4-cell embryo showing injection of the left ventral blastomere to target the left pronephros. Red arrows indicate the left ventral blastomere. B) Animal and ventral views of an 8-cell embryo showing injection of the left V2 blastomere to target the left pronephros. Red arrows indicate the left V2 blastomere. A-B) Images of embryos taken on a stereoscope at 4X magnification. Injections based on the *Xenopus* fate maps developed by Moody (1987)[8, 9] and Moody and Kline (1990)[7]. Please click here to view a larger version of this figure.

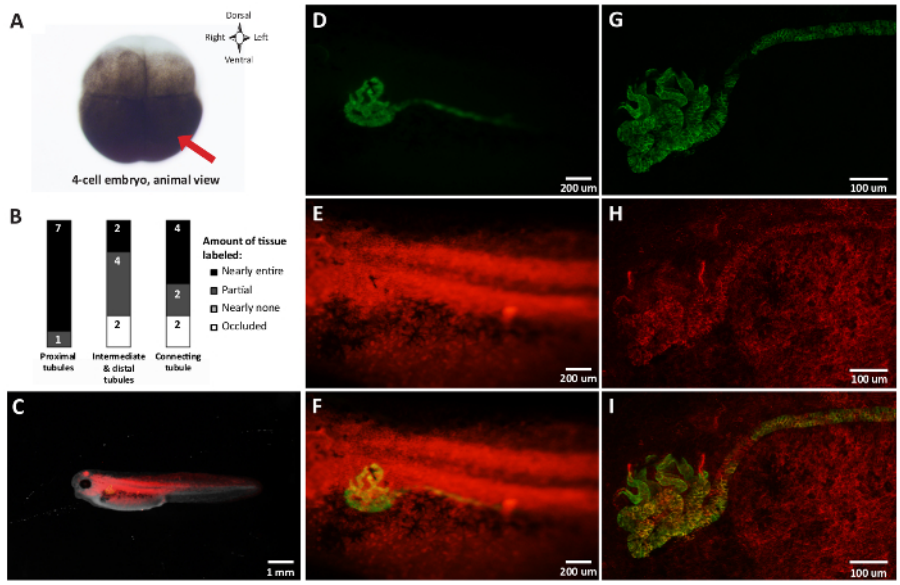

Figure 4: Examples of Embryos Targeted at the 4-cell Stage. Immunostained stage 40 *Xenopus* embryos showing targeted injection to the left pronephros at the 4-cell stage using MEM-RFP mRNA as a tracer. MEM-RFP labels cell membranes red. A) Schematic showing injection of MEM-RFP mRNA into the left ventral blastomere of a 4-cell embryo. B) Tissue localization of MEM-RFP fluorescence in the kidneys of properly injected embryos. C) Stage 40 embryo injected with MEM-RFP in the left ventral blastomere at the 4-cell stage. MEM-RFP localization is shown in red, while the kidney is labeled green. 1X magnification. D) Kidney stained with 3G8 to label the lumen of the proximal tubules, and 4A6 to label the membranes of cells in the intermediate, distal and connecting tubules. 5X magnification. E) Enlargement of the region over the kidney showing MEM-RFP localization. 5X magnification. F) Merged image showing co-localization of the MEM-RFP tracer with the kidney. 5X magnification. C-F) Images of embryos taken with an Olympus DP71 camera on a stereoscope. G) Confocal image of the kidney of a second embryo stained with 3G8 and 4A6. H) Localization of MEM-RFP in kidney and surrounding tissue. I) Merged image showing co-localization of MEM-RFP, 3G8, and 4A6 in the kidney. G-I) Confocal images using maximum projection at 20X magnification. Please click here to view a larger version of this figure.

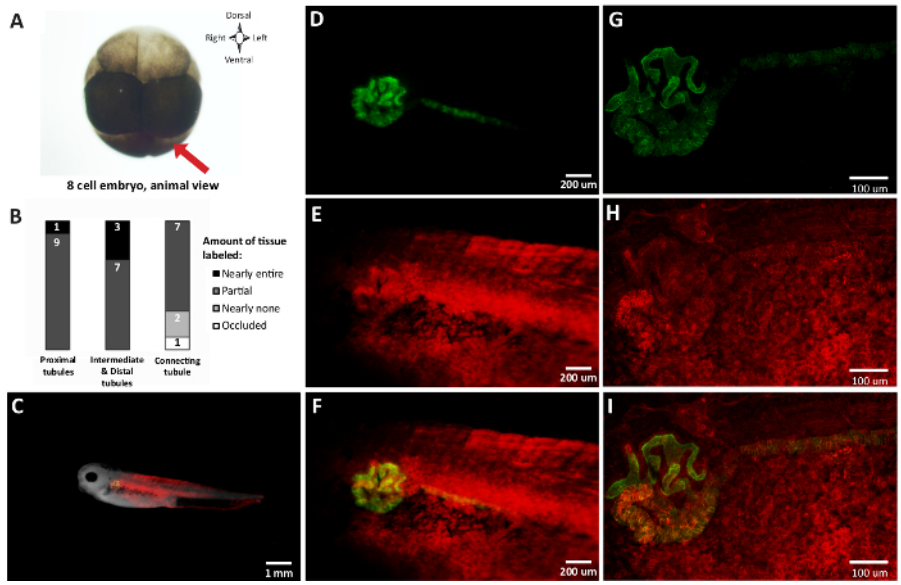

Figure 5: Examples of Embryos Targeted at the 8-cell Stage. Immunostained stage 40 *Xenopus* embryos showing targeted injection to the left pronephros at the 8-cell stage using MEM-RFP mRNA as a tracer. MEM-RFP labels cell membranes red. A) Schematic showing injection of MEM-RFP mRNA into the left V2 blastomere of an 8-cell embryo. B) Tissue localization of MEM-RFP fluorescence in the kidneys of properly injected embryos. C) Stage 40 embryo injected with MEM-RFP in the left V2 blastomere at the 8-cell stage. MEM-RFP localization is shown in red, while the kidney is labeled green. 1X magnification. D) Kidney stained with 3G8 to label the lumen of the proximal tubules, and 4A6 to label the membranes of cells in the intermediate, distal and connecting tubules. 5X magnification. E) Enlargement of the region over the kidney showing MEM-RFP localization. 5X magnification. F) Merged image showing co-localization of the MEM-RFP tracer with the kidney. 5X magnification. C-F) Images of embryos taken with an Olympus DP71 camera on a stereoscope. G) Confocal image of the kidney of a second embryo stained with 3G8 and 4A6. H) Localization of MEM-RFP in kidney and surrounding tissue. I) Merged image showing co-localization of MEM-RFP, 3G8, and 4A6 in the kidney. G-I) Confocal images taken using maximum projection at 20X magnification. Please click here to view a larger version of this figure.

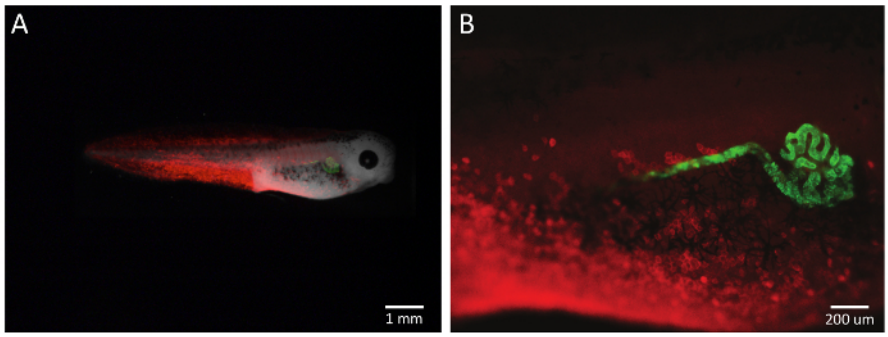

Figure 6: Example of embryo incorrectly targeted at the 4-cell stage. Immunostained stage 40 Xenopus embryo showing incorrect targeting of the left pronephros at the 4-cell stage using MEM-RFP mRNA as a tracer. MEM-RFP labels cell membranes red. A) Stage 40 embryo showing improper distribution of MEM-RFP indicative of injection into the incorrect blastomere. In addition to having incorrect tracer distribution indicating injection into the incorrect blastomere, this embryo was injected on the right side. 1X magnification. B) Merged image of the kidney showing a lack of co-localization of MEM-RFP with antibodies labeling the kidney (3G8, 4A6). 5X magnification. A-B) Images of embryo taken on a stereoscope. Please click here to view a larger version of this figure.

Discussion

Targeting the pronephros of developing *Xenopus* embryos relies on identifying and injecting the correct blastomere. Injection of the V2 blastomere of 8-cell embryos targets the left pronephros[18]. This leaves the contralateral right pronephros as an internal control. If morpholino knockdown or RNA overexpression is used to alter kidney development, the contralateral right pronephros can be used to compare the effects of gene knockdown or overexpression on the left pronephros. In this case, proper controls such as a mismatched control morpholino or dominant negative RNA construct should be used in addition to the contralateral internal control to analyze the results of alterations in gene expression. Due to the relative transparency of the *Xenopus* embryo, kidney defects can be easily quantified using multiple techniques. For example, gene knockdown or overexpression can be simply quantified by calculating the pronephric index of embryos immunostained with antibody 3G8[13], which compares the development of the proximal tubules on the injected and control sides of the embryo. In addition to studying pronephric development, this technique can be easily adapted to target other tissues by selecting the proper blastomere to inject using established fate maps[7-10]. In addition to targeting other tissue types, this protocol could also be used to study kidney development at different stages. This protocol looked at stage 40 embryos stained with antibodies 3G8 and 4A6, which detect differentiated kidney tissue. Younger embryos could

be immunostained with different kidney antibodies, or subjected to *in situ* hybridization[5, 6, 20]. For example, the antibody Lim1 can detect the developing pronephros[21, 22]. Likewise, *lim1, pax8*, and *hnf1-β* transcripts can be detected with *in situ* hybridization starting at stage 12.5[22-24]. Other later *in situ* hybridization markers can be used to detect different regions of the kidney at later developmental stages. For example, probes designed to detect expression of *nphs1*, *clckb*, *slc5a1*, and *atp1a1* in the glomus, distal and connecting tubules, proximal tubules, and the entire kidney, respectively, have been described[25, 5, 26, 27] . Assessing the kidney across multiple stages using different antibodies or *in situ* analysis would allow for a better understanding of how a treatment alters kidney development.

One critical aspect of this protocol is the correct selection and identification of the blastomere to be injected. In this protocol, we describe how to target the pronephros by injecting the left V2 blastomere of 8-cell embryos or the left ventral blastomere of 4-cell embryos. If the incorrect blastomere is injected, the pronephros will not be correctly targeted. Therefore, it is critical to become familiar with the development and cell cleavage planes of early *Xenopus* embryos prior to microinjection[28, 29]. Embryo cleavage does not always follow the established developmental stage charts, so it is helpful to only inject embryos in which the early cell cleavages look "normal" and the proper blastomere can be easily identified. This will decrease the number of embryos that have been improperly targeted. In addition, it is also important for the blastomeres of the embryo to be completely divided at the time of injection. For example, if the cleavage between blastomeres V1 and V2 of an 8-cell embryo is not complete before microinjection, the tracer may spread into V1 as well as V2. This would decrease the targeting efficiency of the microinjection, and the resulting embryo may show tracer distribution that looks more similar to that of an embryo injected at the 4-cell stage rather than the 8-cell stage.

Another critical part of this protocol is verification that pronephros was properly targeted by microinjection. This must be done prior to analyzing pronephric development, because embryos that have not had the pronephros properly targeted may skew the resulting dataset. To verify proper targeting, analyze the tracer distribution in each injected embryo. The injected (left) side of the embryo should display the vast majority of the fluorescent tracer. If the control (right) side of the embryo shows a substantial amount of fluorescence, then this embryo should be discarded. If this occurs, either the incorrect blastomere was injected, or the injected blastomere had not completed cleaved before injection. Second, fluorescence on the injected (left) side of the embryo must correctly target the pronephros. If the embryo was injected at the 8-cell stage, the dorsal and ventral somites, lateral plate, hindgut, proctodeum, skin of the trunk and neural crest in the trunk are expected to show fluorescence in addition to the pronephros[6, 29]. In addition to the tissues listed for the 8-cell injection, embryos injected at the 4-cell stage should have wider distribution of the fluorescent tracer in the skin and somites as well as targeting of the cement gland, otocyst, lens and olfactory placode. If the embryo does not display the proper tissue targeting, it should be discarded prior to analysis (**Figure 5**).

The targeted injections described in this protocol provide a means of manipulating gene expression in a desired tissue. A limitation of this technique is that although these injections are targeted, they do not influence only the developing kidney. The 4- and 8-cell injections described in this technique are more targeted than injections done at the single-cell stage, where gene expression will be altered in the entire embryo, and injections done at the two-cell stage, where half of the embryo displays altered gene expression. They do not, however, target only one tissue. Although injections in the ventral blastomeres of 4-cell embryos and the V2 blastomeres of 8-cell embryos alter gene expression in the pronephros, they also alter gene expression in other tissues. Other affected tissues include the skin, somites, gut, and neural crest. Therefore, it is important to understand that although 4- and 8-cell injections are more targeted than one- or two-cell injections, they will still alter gene expression in multiple tissues. In addition to injecting embryos at the 4- and 8-cell stages, injections can also be performed at the 2-, 16-, and 32-cell stages. Embryos injected at the 2-cell stage will have a wider range of tissues affected than embryos injected at later stages. Although more technically challenging, embryos injected at the 16- and 32-cell stages will have a narrower range of tissues affected than embryos injected at the 4- and 8-cell stages.

The MEM-RFP lineage tracer is used in this protocol to verify proper targeting after microinjection. MEM-RFP mRNA labels cell membranes after the cells have translated protein from the injected RNA. The concentration of MEM-RFP lineage tracer used in this protocol (0.1 ng of MEM-RFP mRNA in a 10 nl injection) should be modified as needed to account for embryo death and desired intensity of tracer fluorescence. In addition to modifying the amount of lineage tracer injected, a different lineage tracer, such as rhodamine-dextran, quantum dots, or the histochemically detectable β-galactosidase, may be used instead of MEM-RFP. Lineage tracers become more dilute as the cells divide, causing the levels of fluorescence to decrease as the embryo develops. An additional application of this technique is to co-inject an exogenous RNA or morpholino with the lineage tracer to overexpress or knock down the expression of a gene of interest. The lineage tracer is used to verify correct targeting of the pronephros, but not to indicate where co-injected exogenous RNA or morpholino localizes in the embryo. Exogenous RNA and morpholinos may not spread through the injected blastomere at the same rate as the co-injected tracer, potentially resulting in daughter cells that have the tracer but not the exogenous RNA or morpholino present[30]. In fact, we have observed that two different RNA constructs injected into a single cell do not always co-localize (unpublished data). Therefore, presence of the tracer in a cell does not necessarily indicate that the co-injected exogenous RNA or morpholino is always present in that cell.

This protocol details a procedure for targeting microinjection to the blastomeres that give rise to the pronephros of developing *Xenopus* embryos. Gene expression is often manipulated in *Xenopus* embryos through injection of morpholinos or exogenous RNA, both of which can prevent cause early developmental anomalies if injected in one- or two-cell embryos. Embryos injected at the single cell stage exhibit knockdown or overexpression in all of the cells of the developing embryo. If the gene is necessary for proper early developmental processes, the embryo may not develop a pronephros. Injections performed at later stages, such as the 8-cell injections detailed here, can result in embryos that undergo gastrulation and eventual pronephric development[18]. Therefore, the targeted injections discussed here can overcome the gastrulation defects caused by alteration of the expression of some genes, allowing the embryo to progress through pronephric development. In the future, this protocol may also be used to target CRISPR constructs to desired blastomeres.

Disclosures

The authors declare that they have no competing financial interests.

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