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Using *Xenopus* Skin to Study Cilia Development and Function

Michael E. Werner and Brian J. Mitchell¹

Department of Cell and Molecular Biology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA

Abstract

Cilia are prevalent biological structures that are important for cell signaling and for generating fluid flow (or motility). Cilia are found throughout biology from single-celled organisms to vertebrates, and many model systems have been employed for their analysis. Here, we describe the use of *Xenopus* larval skin as a system for the study of ciliogenesis and ciliary function. In particular, we describe basic molecular and embryological manipulations and imaging techniques that have proven particularly useful for understanding the polarized beating of cilia and the generation of directed fluid flow (Werner & Mitchell, 2012). However, these same tools have the potential to benefit a large number of cilia-related biological questions.

1. INTRODUCTION

The skin of amphibian embryos was among the first observed examples of a tissue with cilia-driven directed fluid flow (Sharpey, 1836). Since these initial observations, the experimental tools available in *Xenopus* have provided a fertile model system for the study of ciliated cell differentiation, radial intercalation, ciliogenesis, and cilia polarity, all of which are required and culminate in the generation of directed fluid flow (for review, see Werner & Mitchell, 2012). In particular, the molecular tools of morpholino oligos (MO)-based protein depletion and injection of mRNA encoding fluorescently tagged proteins allow for detailed visualization of cilia formation and function in an intact living tissue. This, coupled with the embryological tools of skin transplantation, allows one to address how wild-type multiciliated cells (MCCs) function in a mutant tissue and conversely how mutant MCCs function in a wild-type tissue. Many of the tools employed to study cilia are “standard” *Xenopus* techniques. We reiterate some of these methods here, and more importantly, we highlight the specific reagents and manipulations that have proven particularly useful (and unique) to *Xenopus* for the study of cilia.

2. MOLECULAR MANIPULATION OF *XENOPUS* CILIATED EPITHELIA

2.1. Expression of cilia-related proteins containing fluorescent tags from exogenous mRNA

The ease with which one can induce protein expression via targeted injection of mRNA is one of the primary strengths that has allowed *Xenopus* to maintain relevance as a model

organism. While this method can be used to modulate cell fate or developmental progression, it can also be used to introduce fluorescently tagged proteins for detailed microscopic analysis.

2.1.1 mRNA injection into blastomeres—The use of mRNAs encoding cilia-related genes fused to fluorescent markers is an extremely powerful approach to visualize and characterize detailed aspects of cilia formation and function. Table 10.1 summarizes some common markers useful for labeling different cilia-related structures. Detailed protocols for harvesting, fertilizing and injecting *Xenopus* embryos have previously been published (Mimoto & Christian, 2011; Sive, Grainger, & Harland, 1998, 2007b, 2007c, 2007f, 2010). Additionally, the *in vitro* synthesis of mRNAs is well established (Sive et al., 1998) and is also feasible using established optimized kits (e.g., Ambion mMessage mMachine Cat # AM1340). However, to ensure effective protein localization in the embryonic skin and in MCCs, four separate injections are performed, one in each of the four quadrants of the animal pole of the embryo. The ciliated epithelium derives from the ectoderm, therefore injections should be targeted away from the midline and more laterally toward the center of the cell on the animal side of each blastomere, which enriches the ectodermal lineage in mRNA (Sive et al., 1998). Embryos are then allowed to develop until they reach the desired developmental stage for investigation. MCCs begin to express MCC specific genes at low levels as early as stage 11.5, with strong expression typically evident by stage 14. MCCs begin radial intercalation as early as stage 14 (ideally visualized between stages 17 and 18), begin to undergo ciliogenesis around stage 20 (ideally visualized between stages 20 and 22), and have mature oriented cilia by stage 28 (visualized between stages 23 and 28). Fluorescently tagged proteins can be visualized in live embryos (see Section 3.2.2) or fixed for further processing (e.g., antibody labeling, see Section 3.1).

2.1.2 Restricted expression to ciliated cells using the α -tubulin promoter—Targeted injection of mRNA allows for some spatial control of protein synthesis. However, for many applications, temporal control and precise localization of proteins specifically to differentiating MCCs is required to avoid off-target developmental defects. There are several approaches to achieve this that rely on the use of the α -tubulin promoter (Stubbs et al., 2006). This promoter drives gene expression specifically in MCCs starting at stage 13 when MCCs begin to form and continues to drive expression in MCCs during ciliated cell differentiation and throughout development. Simply, one can inject 10–50 pg of plasmid DNA containing the α -tubulin promoter driving expression of one's gene of interest in a similar manner to the mRNA injections described above. For many cell biological analyses, the injection of plasmid DNA is sufficient; however, it has the drawback that it can result in mosaic and variable levels of expression that is problematic for tissue level analysis. For more consistent expression, it is preferable to make “transient transgenics,” a method that relies on injecting sperm nuclei along with the plasmid DNA into unfertilized eggs and achieving an F1 transgenic animal for immediate analysis. While this method requires slightly more effort, it will generate a more consistent level of expression across the tissue. There are currently numerous methods to achieve “transient transgenics,” and proper methods are beyond the scope of this chapter (Amaya & Kroll, 1999; Loeber, Pan, & Pieler, 2009).

2.2. Analysis of knockdown phenotypes

While limited success with RNAi-dependent knockdown of protein levels by injecting siRNAs or shRNAs into *Xenopus* embryos has recently been reported, most efforts to generate strong RNAi-mediated knockdown in whole embryos have been unsuccessful (Lund, Sheets, Imboden, & Dahlberg, 2011). Instead, MOs can be injected that prevent translation or proper splicing of mRNAs, thereby blocking the formation of a functional protein (Gene Tools, Inc.; Nasevicius & Ekker, 2000). MOs are chemically stable ~25 nucleotide long antisense oligos typically designed to bind to target mRNAs and interfere with protein translation. Since MOs are not recognized by the endogenous RNAi machinery, they are less likely to cause unwanted off-target effects; however, controls and rescue experiments are critical for validating a phenotype (Summerton, 1999).

2.2.1 Injection of antisense MOs to knockdown protein levels—MOs are usually designed to bind in close proximity to or overlapping with the mRNA start codon to prevent initiation of translation. Alternatively, MOs that are designed to overlap with splice sites interfere with proper pre-mRNA splicing (Draper, Morcos, & Kimmel, 2001). Splice MOs often yield a stronger reduction in the full-length protein and have the advantage that, in the absence of a good antibody, the extent of reduction of the full-length mRNA can be determined using qRT-PCR for mis-processed mRNA. However, splice MOs often generate partially truncated protein, as a result of alternative splicing. The mis-spliced protein may have residual biological functions or may act as a dominant negative or a dominant active form of the targeted protein. Generally, the use of more than one MO that cause the same phenotype as well as rescue experiments is advisable to minimize the risk that the observed phenotype is due to off-target effects of the MO (for a detailed review of the use of MOs in vertebrate embryos, see Eisen & Smith, 2008; Summerton, 2007). When working with an untested MO, it is necessary to empirically determine the optimal concentration. Most MOs seem to be effective at amounts ranging from 1 to 20 ng with doses of up to 60 ng used successfully in some reports. For coinjection with mRNA, it is critical that the mRNA be purified and resuspended in RNase-free water that is not treated with DEPC. Qiagen's RNeasy MinElute Cleanup kit (Cat # 74204) results in mRNA that is suitable for coinjecting with MOs.

2.2.2 Using mosaic embryos to circumvent potential lethality—Organism-wide knockdown of certain proteins using MOs may lead to broad developmental defects preceding formation of the ciliated epithelium. To circumvent such potential defects, MO injection can be restricted to a single cell at the 4-, 8-, or even 16-cell stage, thereby restricting the knockdown to only a subset of cells in the animal. This will often allow the development of a largely healthy embryo in which only a subset of cells are affected by the knockdown. In this case, it is necessary to label the cells that have received the MO either by using fluorescently labeled MOs or by coinjecting MOs with a marker mRNA such as membrane or nuclear targeted Red Fluorescent Protein. Injection of the MO with 0.1% phenol red can also be used to track the injection. This approach also allows the comparison of morphant and control tissue within the same embryo.

2.2.3 Rescue experiments—In order to validate the specificity of a phenotype associated with a MO-dependent protein knockdown, rescue experiments should be performed. Since MOs are often designed to target either parts of the 5'UTR when interfering with translation initiation or parts of an intron at intron–exon boundaries for splice MOs, in most cases, the MO phenotype can be rescued by simply coinjecting *in vitro* transcribed mRNA of the gene in question lacking the 5'UTR. In cases where the coding sequence is targeted, a rescue construct can be designed using alternate codon usage to maintain amino acid sequence but not DNA sequence.

3. VISUALIZATION OF CILIA IN *XENOPUS* EMBRYOS

3.1. Antibody staining for cilia-related proteins

In addition to the use of fluorescently tagged proteins, detailed aspects of ciliogenesis and cilia function can be visualized using immunofluorescence (IF) (see Table 10.1). Several common fixatives can be used to prepare whole embryos for IF of MCCs and will have to be optimized for each individual antigen:

1. Fixation of embryos:
 - a. For standard IF that maintains green fluorescent protein (GFP) fluorescence's fix for 2 h in 3–4% paraformaldehyde (PFA) in 1× phosphate-buffered saline (PBS). This can be followed by a dehydration step in 100% ethanol (EtOH, embryos for IF can be stored at –20 °C indefinitely but GFP fluorescence fades after several days).
 - b. Cilia and basal body markers often yield better results when fixed for 48 h in methanol (MeOH) or Dents (80% MeOH, 20% DMSO).
 - c. The combination of a 2 h 3–4% PFA fix followed by 48 h in Dents has also proved optimal for certain antibodies (Dammermann et al., 2009).
 - d. Alcohol-based dehydration over time diminishes GFP fluorescence and prevents visualization of the actin cytoskeleton using fluorescently labeled phalloidin; therefore, for these applications, 2 h in 3–4% PFA alone is necessary. (With this fixative skip dehydration with alcohol and proceed to step 3.)
2. Following dehydration with alcohol-based fixations such as EtOH and MeOH embryos must be rehydrated in steps to maintain tissue integrity. Rehydrate embryos by sequential washes in alcohol diluted in PBS (95% alcohol→90%→75%→50%→25%→ and finally several washes with 100% PBS).
3. Wash for 30 min in PBS-T (PBS+0.1% Triton).
4. Block with 10% heat-inactivated goat serum (HIGS) in PBS-T.
5. Incubate 2 h at room temperature (RT) or overnight (ON) at 4 °C in primary antibody in PBS-T with 5% HIGS.
6. Wash at RT for 2–5 h with multiple changes in PBS-T.

7. Incubate 2 h at RT or ON at 4 °C in secondary antibody in PBS-T with 5% HIGS.
8. Wash for 2–5 h at RT with multiple changes in PBS-T.
9. The samples are now ready for mounting (see Section 3.2.1).

3.2. Mounting and microscopy of *Xenopus* ciliated epithelia

3.2.1 Mounting of fixed embryos—Several different techniques have been described to mount whole embryos for fluorescent microscopy (Kieserman, Lee, Gray, Park, & Wallingford, 2010; Lee, Kieserman, Gray, Park, & Wallingford, 2008). For relatively flat fixed embryos, one can also sandwich the embryos between two large pieces of coverglass (Fig. 10.1A). Electrical tape is used as a spacer placing the embryos into holes cut into the tape using a scalpel or a razor blade. Generally, three layers of electrical tape are used for embryos stage 22 and younger, while two pieces are used after stage 22. This technique has the advantage that both sides of the embryos can be visualized, which is particularly important when performing mosaic or transplant experiments (see Sections 2.2.2 and 4.2):

1. Cut two or three 60-mm long pieces of electrical tape and place them one on top of the other on a 48 × 60 mm or 28 × 60 mm coverglass (Goldseal coverglass, VWR Inc., Cat # 48404 and 63770).
2. Using the edge of a sharp razor blade cut out an approximately 5 × 8 mm rectangle of electrical tape toward the middle of the coverglass to create a pocket that can accommodate up to three embryos. Multiple pockets can be cut into a single stack of labeling tape to accommodate additional samples per slide.
3. Transfer embryos to the pocket using a transfer pipette making an effort to minimize the amount of liquid that is transferred with the embryos.
4. Aspirate most of the liquid and quickly embed embryos in one drop of Fluorogel (EMS Cat # 17985-10) or Mowiol 4-88 (Polysciences, Inc., Cat # 9002-89-5).
5. Use forceps to position embryos within the pocket and carefully place a second 48 × 60 or 28 × 60 mm coverslip on top aligning the edges of both pieces coverglass.

3.2.2 Mounting of embryos for live imaging—Various mounting techniques for live imaging of *Xenopus* embryos have been described and can vary depending on the developmental stage of the embryo as well as the time period over which the embryo is to be imaged. Kieserman et al. have recently published a very comprehensive review covering several of these techniques, and we will refer to them for a detailed description (Kieserman et al., 2010). The key feature for mounting embryos for live imaging is keeping the embryos pressed against the coverglass for imaging while maintaining access to media and allowing for growth (for longer time course imaging). Additionally, a significant advantage for using *Xenopus* is the ability to culture explanted tissue (see Section 4.1) which can alleviate issues with three-dimensional growth and movement. One can use custom-made half-rose chambers (Wadsworth, 2007) attached to a 48 × 60 mm coverglass by applying a ring of silicone grease on the bottom of the chamber. Embryos can then be placed in the chamber and held in place by a smaller piece of coverglass with dabs of silicon on the sides (Fig. 10.1B). Customized microscope chambers as described here can be used for most

applications and yield reliable results using extended imaging up to several hours. For imaging of shorter time intervals especially when high spatial resolution is required, such as imaging microtubule dynamics or cilia beating, it is preferable to use the mounting technique described in Section 3.2.1; however, this is not suitable for longer time periods especially if the embryo undergoes drastic shape changes during the imaging time frame. Additionally, for imaging of tailbud stage or older embryos, it is often advisable to immobilize embryos using anesthetics such as 0.02% tricaine (ethyl 3-aminobenzoate methanesulfonate salt, Sigma–Aldrich Inc., Cat # A5040) made up in 0.1 × Marc's Modified Ringers (MMR, 1 × is 110 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 2 mM NaHCO₃, 5 mM HEPES, pH 7.8) to prevent embryos from twitching during imaging.

3.3. Visualizing cilia-dependent flow and live imaging of cilia beating

The cilia of MCCs beat in a coordinated manner that leads to the establishment of a directed fluid flow toward the posterior ventral side of the embryo. This flow is initially weak at stage 22 when the orientation of individual cilia is still poorly organized (Mitchell, Jacobs, Li, Chien, & Kintner, 2007). However, as the cilia become more coordinated and polarized, the flow velocity over the surface of the embryo increases until stage 28 when it reaches homeostasis. Defects in fluid flow are a consequence of ciliogenesis defects or defects in cilia polarity (Mitchell et al., 2007; Werner et al., 2011). Furthermore, strength and directionality of the fluid flow correlate with the extent of polarization of the ciliated epithelium. Hence, visualization of fluid flow is an important tool to identify and visualize defects in cilia function. While the visualization of fluid flow on the skin of embryos allows the identification of defects in cilia function or cilia polarity, it does not allow one to distinguish between these two processes. In order to achieve this, high-speed imaging of individual cilia beating is necessary (see Section 3.3.2). Since cilia on the skin of *Xenopus* embryos beat at an average frequency of about 20 Hz (beats per second), acquisition speeds of many conventional cameras or point scanning confocals are not sufficient. Instead, high-speed cameras (e.g., Phantom 7.2 CMOS) or high-speed line scanning confocal (e.g., Zeiss LSM 7-live) or resonance scanning confocal (e.g., Nikon A1R) systems are used to obtain sufficient temporal resolution for visualizing the stroke of individual cilia. While the beating of single cilia can be resolved reasonably well at 100 frames per second (fps), this is not sufficient to resolve the complexity of the approximately 100 cilia that beat on the surface of MCCs. In order to resolve individual beat dynamics, a range of 1000–10,000 fps is ideal (Werner et al., 2011). However, one can easily quantify beat frequency with imaging in the 200 fps range (Werner et al., 2011).

3.3.1 Visualization of fluid flow using fluorescent or dyed microspheres—

Visualization of fluid flow can be performed on most upright microscopes at fairly low magnification of 4–10×. A simple dissecting scope equipped with an eyepiece mounted digital camera (e.g., Scopetek DCM130E) is the most economical possibility. The velocity of fluid flow is generally measured indirectly by the displacement of microbeads along the surface of the embryo (Mitchell et al., 2007). If the microscope is equipped with a fluorescent light source, it is preferable to use fluorescently labeled microspheres since the recorded signal is brighter with minimal background making the recordings more amenable to particle tracking software:

1. Transfer 20 μ l of resuspended fluorescent (Invitrogen Inc., Cat # F8836) or dyed (Polysciences Inc., Cat # 24294) microspheres to an eppendorf tube and dilute 1:20 with $0.1 \times$ MMR.
2. Spin down for 1 min at 5000 rpm and aspirate off most of the liquid. Repeat the wash step to remove any remaining toxins and detergent that is contained in the storage buffer of the beads.
3. Resuspend beads in 100 μ l of $0.1 \times$ MMR with 0.5% glycerol. Beads tend to float in the media; therefore, 0.5% glycerol is added to the beads to accelerate their sinking onto the embryos when added to the Petri dish.
4. Using vacuum grease, form several small open arrowheads in a Petri dish (see Fig. 10.1C) and fill dish with $0.1 \times$ MMR. For analysis of older embryos, 0.02% tricaine can be added to the $0.1 \times$ MMR to anesthetize the embryos and prevent them from swimming away.
5. Transfer embryos into the Petri dish and using forceps or a hair loop push individual embryos into the open arrowheads with the anterior side facing the inside tip of the arrowhead (Fig. 10.1C). This should prevent the embryo from gliding across the dish due to ciliary beating.
6. Start imaging and add beads dropwise on top or slightly anterior of the embryo using a pipette. Beads should be displaced along the embryo when sinking into close proximity of the skin.
7. Velocity of fluid flow can be determined by tracking the displacement of individual beads either manually or automatically using simple particle tracking software (e.g., ImageJ). Because many cameras are equipped with rather basic acquisition software, it may be necessary to calibrate the acquired recordings when performing particle tracking since this information may not be contained in the acquired movie or may not be compatible with the tracking software. The careful use of uniformly consistent magnifications in combination with a micrometer allows one to overcome such calibration issues. The amount of displacement is a factor of both cilia-driven fluid flow and the mass of the beads. Therefore, it is critical to treat the velocities as relative rather than absolute and identically sized beads should be compared from control and experimental animals in each experiment.

3.3.2 Imaging of coordinated cilia beating using high-speed microscopy—To visualize beat frequency and beat form using nonfluorescent light microscopy, one needs a fast camera that captures >1000 fps (e.g., Vision Research Phantom class cameras or Casio EX-F1). However, visualization of the surface of the embryos requires some considerations. First, to obtain good cilia waveforms, it is important to visualize cells on the side of the embryo that are projecting into the media. This allows ample light to pass from the microscope to the camera, which is the primary challenge with high-speed imaging. Conversely, one can image explanted tissue (see Section 4.1) from the top. However, for this application, it is important to use either albino or very lightly pigmented embryos to allow a significant amount of light to pass through the tissue.

3.3.3 Visualization of cilia beating for quantification of beat frequency using fluorescent confocal microscopy

—An alternative to high-speed cameras is the use of high-speed confocal microscopy. In contrast to high-speed cameras, confocal microscopy requires that individual cilia are fluorescently labeled (e.g., Fig. 10.2A). We have previously described the use of a fluorescently tagged protein (Xl.16654) which strongly and specifically labels cilia. However, any other ciliary protein that associates with the ciliary axoneme or the tip of cilia and does not interfere with cilia function when overexpressed can be used as a fluorescent label. In general, this approach requires a relatively bright signal and light-sensitive objective with high numerical aperture. While it is difficult to obtain high-resolution waveforms of cilia beating, it is quite easy to generate kymographs that allow quantification of beat frequency (Fig. 10.2B):

1. Inject embryos with 0.1–1 ng of mRNA encoding a fluorescently tagged ciliary protein at the two- or four-cell stage. If testing the effects of MO knockdown or overexpression of a certain protein, MOs or experimental mRNA can be coinjected with the cilia marker.
2. Allow embryos to develop to the appropriate stage (preferably stage +28).
3. Prepare pockets of electrical tape on large coverslips as described above (see Section 3.2). The working distance of the objective dictates the number of layers of electrical tape to be used, and the ideal mounting condition must be determined empirically.
4. Transfer an embryo into the pocket and overlay with a second 48 × 60 mm coverslip.
5. Image individual ciliated cells at the side of the embryo away from the coverslip. Optimally, a subset of cilia toward the side of the cell give the best results for quantification, as it is easier to distinguish individual beat patterns. In our experience, acquisition of at least 200–250 fps for 5 s is necessary to obtain reliable and quantifiable recordings for scoring of beat frequency (Werner et al., 2011).
6. To quantify beat frequency, use kymographs aligned along the axis of beating of individual cilia. In polarized ciliated cells, this leads to a periodic pattern of lines in the kymograph representing each individual stroke of multiple cilia (Fig. 10.2B). Beat frequency can then be calculated from the amount of lines (strokes of cilia) over a given time period. When cilia polarity or cilia function is compromised, it is essential to image cilia at the side of a ciliated cell that are the least likely to come into contact with neighboring cilia which will interfere with the ciliary stroke. Statistically significant data can be obtained from analyzing five cilia per cell, on five cells from five embryos obtained from at least two independent experiments (Werner et al., 2011).

3.4. Imaging cilia orientation to study ciliated cell planar cell polarity

The skin of *Xenopus* embryos is an excellent system to study the establishment of cilia polarity. Robust oriented fluid flow along the skin of *Xenopus* embryos is dependent on proper orientation of individual cilia within ciliated cells and also coordination of orientation

between cells. Cilia orientation is dependent on both hydrodynamic forces and planar cell polarity (PCP) signaling cues (Mitchell et al., 2007, 2009; Park et al., 2008). Additionally, the skin of *Xenopus* embryos is a very good system to study PCP using cilia orientation as a readout for the polarization state of the cell or by extension of the entire tissue. Cilia polarity on live and fixed samples can be assayed using fluorescently tagged proteins to score the relative position of the striated rootlet relative to the basal body (Park et al., 2008).

3.4.1 Visualizing and scoring polarity of individual cilia—The axonemes of motile cilia are anchored within the cell via basal bodies and the associated striated rootlet. The rootlet is well characterized by electron microscopy; however, little is known about its molecular composition and function. Additionally, an appendage of the basal body, the basal foot, is known to project away from the basal body in the direction of cilia beating and has been used via TEM to score cilia orientation (Mitchell et al., 2007). Importantly, the rootlet projects 180° opposite from the basal foot (Park et al., 2008). It has been proposed that the rootlet not only helps anchor individual cilia in the cytoplasm, but it may also help ensure proper temporal coordination of the ciliary stroke between neighboring cilia by connecting their basal bodies via the cortical actin cytoskeleton (Werner et al., 2011). It has been shown that the orientation of individual rootlets relative to the associated basal body is guided by polarity cues during establishment of cilia polarity (Mitchell et al., 2009). Fluorescently labeled rootlet markers (e.g., GFP-CLAMP and GFP-Mig12) and basal body markers (e.g., centrin2-RFP, RFP-HYLS1, and RFP-SAS6) can be used to visualize the orientation of cilia in both live and fixed embryos (Fig. 10.2C):

1. Coinject embryos with 200 pg of GFP-CLAMP and 35 pg centrin-RFP mRNA per blastomere at the two- or four-cell stage. Add any other mRNA or MO to the injection for experimental analysis.
2. Let embryos develop to the desired stage and fix with 3–4% PFA for > 2 h to preserve the fluorescent tag.
3. Mount embryos as described in Section 3.2. Use forceps or hair loops to align the embryos along the long axis of the coverslip. It is advisable to mount embryos in the same orientation in each experiment to allow for a direct comparison of each experimental condition. This is particularly important if the confocal scan head or camera is not perfectly aligned relative to the XY axis of the microscope stage.
4. Image individual ciliated cells at magnifications of 40× or higher, acquiring several Z-planes on the surface of a ciliated cell to capture both the basal body and the rootlet which extends ~ 0.5 μm into the cytoplasm.
5. To score cilia orientation use maximum intensity projections to create a single image from each Z-stack (Fig. 10.2C). Nikon's Elements software or Metamorph can be used to score cilia orientation by measuring the relative angle composed of the vector pointing from the basal body along the associated rootlet and a reference vector aligned along the anterior–posterior (A–P) axis. Alternatively, it is possible to determine the relative XY coordinates of each basal body and associated distal tip of striated rootlet using ImageJ and then calculate the individual vector relative to the A–P axis using the atan2 function in Microsoft excel. Oriana 2.0 (Kovach

Computing Services) is a useful program for the analysis of circular vector data sets and can be used to generate circular standard deviation, variance, and mean direction (Mitchell et al., 2007).

4. EMBRYOLOGICAL MANIPULATIONS USEFUL FOR THE ANALYSIS OF CILIATED EPITHELIA

4.1. Using embryonic explants to study the role of fluid flow in establishing cilia polarity

Embryonic explants consist of groups of cells removed from the embryo and grown in culture. Because of the size of the embryo, it is relatively easy to microdissect and isolate individual tissues and culture them in a Petri dish. Explants such as Keller explants, which combine dorsal mesendoderm and ectoderm, have been particularly useful for the study of cellular interactions during convergent extension (Sive, Grainger, & Harland, 2007d, 2007e, 2007g). For the study of the ciliated epithelium, explants have been useful for determining the timing of the establishment of tissue polarity and for visualizing the process of MCC intercalation (Mitchell et al., 2007; Stubbs et al., 2006). MCCs differentiate within a sublayer of the ectoderm and undergo radial intercalation to join the outer epithelia. Ectodermal explants from embryos at stage 10 or older will form a functional ciliated epithelium in which MCCs properly intercalate and undergo ciliogenesis. However, explants initiated prior to stage 14 lack tissue level polarity cues and generate chaotic fluid flow. While explants can be grown directly in media to create a 3D sphere (see Section 4.3), for many applications, it is beneficial to attach the explant to a coverglass to create a flat surface for imaging or for use in a flow chamber (Mitchell et al., 2007).

The day before cutting explants prepare fibronectin-coated coverslips:

Day 1

- 1 Prepare a 20 mg/ml dilution of fibronectin in $1 \times$ PBS (stock solution aliquots of 1 g/ml can be stored at -20°C).
- 2 Place a sheet of wet Whatman paper in a 15-cm Petri dish for moisture and place a large glass plate on top.
- 3 Flame 22×22 mm pieces of coverglass after immersing in 100% EtOH to sterilize and place on the glass plate.
- 4 Place three to four small spacers cut from a sheet of plastic (1 mm thick) on the edges of each piece of coverglass (Fig. 10.3A).
- 5 Add 200 μl of fibronectin to the center of the coverglass (Fig. 10.3A).
- 6 Place a second coverslip on top of spacers. This evenly distributes the fibronectin and generates two usable coverslips with a smaller volume of fibronectin (Fig. 10.3A).
- 7 Leave at RT for 30–45 min and then place at 4°C ON.

Day 2

- 8 Place each coverslip individually in a 35-mm Petri dish making sure that the surface exposed to fibronectin is up.
- 9 Wash coverslips for 5 min in 1× PBS.
- 10 Block coverslips for 1 h in 1× PBS+1% bovine serum albumin (BSA).
- 11 Wash coverslips for 5 min in 1× PBS.
- 12 Remove PBS and fill Petri dish with Danilchik's for Amy (DFA) buffer+0.1% BSA+antibiotic (50 µg/ml gentamicin).

Preparation of explants

1. Transfer embryos into Petri dishes containing DFA buffer+0.1% BSA+antibiotic.
2. At the desired stage, carefully remove the vitelline membrane.
3. Using an eyebrow knife, remove a part of the ectoderm near the animal pole that will give rise to the ciliated epidermis (Fig. 10.3B) (Sive et al., 1998).
4. Make sure to trim away any nonectodermal tissue. With explants cut from stage 10 to 16 embryos, it is easy to separate the ectoderm from the underlying mesoderm and endoderm. In later explants, these tissues are more intimately associated and it is significantly more difficult to obtain pure ectodermal tissue.
5. Transfer the explant to the 35-mm Petri dish containing a fibronectin-coated coverslip using a wide bore transfer pipette. It is important to prevent the explant from coming into contact with the air–water interface as this will cause it to quickly disintegrate.
6. Carefully move the explant to the center of the coverslip with the outer ectodermal layer facing up.
7. Use a diamond knife to cut a small glass bridge from a 22×22 mm coverslip.
8. Add silicone grease to the ends of the glass bridge and carefully place the glass bridge over the explant. Carefully press down on the coverglass until the explant is held in place by the glass bridge (Fig. 10.3B).
9. Set aside control embryos that are the same developmental stage as the embryo from which the explant was obtained and keep them at the same temperature as the explant. These control embryos can later be used to determine the developmental stage of the explant.
10. Allow explants to attach to the coverslip for several hours at RT or 12 h at 16 °C.
11. Carefully remove the glass bridge using forceps without putting direct pressure on the explant.
12. When control embryos have reached the desired stage, explants can be fixed or used for live cell imaging.

Fixed explants can be imaged after IF staining by simply inverting the coverslip on which the explant is attached onto a second larger coverslip using dots of vacuum grease to hold the coverslip in place. Similarly, for live imaging of cilia or ciliated cell-associated proteins, coverslips can be inverted onto larger coverslips with a half-rose chamber (e.g., Fig. 10.1B) using dollops of vacuum grease to control the distance between the two coverslips. Fluid flow along explants can be visualized directly in Petri dishes using labeled microbeads (see Section 3.3.1). Alternatively, explants can be attached to glass bottom Petri dishes and displacement of fluorescent beads can be imaged through the relatively thin explant using an inverted microscope. Finally, the explants are amenable to studying the effects of external hydrodynamic forces using a customized flow chamber (Mitchell et al., 2007).

4.2. Skin transplants for the analysis of ciliated epithelia

One of the primary strengths of *Xenopus* as a model system is the ease with which one can perform embryological manipulations. In fact, skin transplantation performed in the early part of the nineteenth century established that the skin tissue of amphibians is polarized right after gastrulation and before the generation of motile cilia (Tung & Tung, 1940; Twitty, 1928). More recent work has built on these experiments, and we will describe the use of “full” transplants and “outer” transplants (Mitchell et al., 2009). In a full transplant, both the inner and outer layers of the epithelium are dissected away from one embryo and placed into an equal sized hole cut into a second embryo. This allows one to address the specific timing of the generation of tissue polarity since one can transplant tissue at different developmental stages, allow the embryos to develop, and compare them to the neighboring host tissue. The outer transplants, while technically more challenging, allow one to ask more detailed questions such as what happens to a wild-type MCC that intercalates into or next to a field of mutant cells, or vice versa (Fig. 10.4). This is a powerful approach that has allowed for the description of the domineering noncell autonomous effects of PCP components in directing the polarity of MCCs (Mitchell et al., 2009). However, this approach promises to be useful for numerous other questions:

1. Inject two pools of fertilized embryos (host and donor, Section 2.1.1), one with membrane-bound RFP and the other with a marker for the cellular process you are interested in assaying (e.g., centrin-RFP and GFP-CLAMP).
2. Let embryos develop to the desired stage. Full skin transplants are best performed between stages 13 and 16. Beyond stage 16, it is difficult to tease apart the individual germ layers making transplants more challenging. For “outer” transplants, the window is even smaller with stages 9–10.5 being optimal for teasing apart the outer epithelium from the inner presumptive MCC layer (Fig. 10.4B).
3. Place embryos in DFA with antibiotic (50 µg/ml gentamicin) and dissect out a small fragment (0.25 µm²) of the donor embryo and place that into an equally sized excision from a host embryo. The molecular manipulation being tested can be incorporated into either the outer layer (donor) or the intercalating MCC (host).
4. While the transplanted tissue is healing into the host, it is best to keep it immobilized in a Petri dish with a small broken piece of coverglass as a bridge

pressing down on the transplanted tissue. It is convenient to perform both the transplant and the healing on a bed of modeling clay.

5. After 1 h, at RT, the tissue should be healed and the coverglass can be removed.
6. Allow the tissue to develop to the desired stage and fix for microscopy (Fig. 10.4C) (see Section 3).

4.3. Studying the mechanism of intercalation using skin spheres

During radial intercalation, MCCs of *Xenopus* embryos must intercalate into the outer epidermal layer in a highly regulated manner. Interestingly, radial intercalation in the ciliated epithelium is not only restricted to MCCs but also includes the ion transport cells termed ionocytes (Stubbs et al., 2006). Additionally, radial intercalation has been extensively studied during gastrulation, where it plays an evolutionary conserved role that is essential for embryonic development (Damm & Winklbauer, 2011). The molecular mechanisms underlying radial intercalation are still largely unknown, suggesting that MCCs could be a useful model. Whole embryos are amenable for visualization of intercalation as are the ciliated epithelial explants described in Section 4.1. However, time-lapse imaging of the intercalating cell itself is challenging due to the fact that intercalation generally occurs perpendicular to the coverglass, resulting in significantly decreased image quality when imaging at increasing distances away from the coverglass. Additionally, the need for deep stacks increases the amount of bleaching that occurs during long time course experiments. We have recently developed a relatively simple system to overcome some of these limitations (Fig. 10.5). This system takes advantage of the fact that explanted tissues from the ectodermal cell layer of *Xenopus* embryos continue to develop in culture forming ciliated epithelia. When not attached to a fibronectin-coated surface such explants quickly ball up and form epithelial spheres in which MCCs intercalate from the inside of the sphere into the outer epithelium (Fig. 10.5B). These spheres can be much smaller than whole embryos making them significantly more amenable for mounting and imaging (Fig. 10.5A). More specifically, this system is very suitable for imaging of an intercalating cell at the side of the sphere allowing for a better three-dimensional image with significantly less bleaching:

1. Similar to cutting embryonic caps (Sive, Grainger, & Harland, 2007a) or performing tissue explants, remove part of the ectoderm from embryos at stage 10.
2. Using an eyebrow knife cut several small pieces of ectoderm of approximately 200–500 μm^2 .
3. Transfer small tissue explants into a fresh dish containing 0.5 $\times$ MMR+antibiotic using a wide bore transfer pipette avoiding any contact with air. Explants will naturally ball up into spheres.
4. Incubate in suspension until control embryos from the same developmental stage have reached stages 16–20.
5. To mount embryos for imaging, prepare agarose pads: melt 2% agarose (in 0.1 $\times$ MMR) in a heat block at 90 °C. Add a drop of agarose to a glass microscope slide positioned between two other slides affixed to the bench with two layers of electrical tape (Fig. 10.5A). Quickly place another glass slide on top of the agarose

drop perpendicular to the other three slides. The layers of electrical tape will create a buffer that will allow the agarose to harden at the right thickness.

6. Remove the slide with the agarose pad by sliding it along the other slide. Frequently air bubbles will have formed on the agarose pad leaving little holes after the agarose has cooled. These holes are ideal for mounting the skin spheres (Fig. 10.5A). Alternatively, little holes can be cut into the agarose using forceps to accommodate the spheres.
7. Transfer skin spheres to agarose pad and carefully drop a coverslip on the agarose pad and seal using melted petroleum jelly.
8. The spheroid cultures are now ready for live imaging (see Section 3.2).

4.4. Using reversible deciliation to study ciliogenesis

Cilia in various tissues are remarkably regenerative. As in many other systems, it is possible in *Xenopus* to reversibly deciliate embryos. Under these conditions, the embryos are nearly entirely stripped of cilia, while basal bodies remain at the apical surface. Cilia are capable of regenerating within 3–5 h restoring directed fluid flow (Fig. 10.2A). This method should prove useful to study the mechanism of ciliogenesis and cilia regeneration:

1. Transfer embryos to a 35-mm Petri dish containing 3 ml of 75 mM calcium, 0.02% NP40 in $0.1 \times \text{MMR}$ +antibiotic.
2. Incubate embryos for approximately 1–2 min until cortical fluid flow on most embryos has completely stopped. Constantly monitor embryos under the dissecting scope. It is sometimes helpful to add cell and yolk debris from a crushed embryo to the dish to better visualize the displacement of debris particles along the skin of the embryos as a measure of cilia movement. In older embryos, the presence of cilia can also be monitored by observing the gliding of the embryos along the surface of the dish due to the robust beating of cilia. Prolonged exposure to calcium and detergent will cause the skin of embryos to peel off, so it is critical to stop the treatment as soon as cilia-driven flow is lost.
3. When fluid flow on the surface of embryos has ceased quickly transfer embryos to a 60-mm Petri dish containing $0.1 \times \text{MMR}$ with antibiotic.
4. Wash embryos several times with fresh $0.1 \times \text{MMR}$ +antibiotic to remove all traces of detergent.
5. Immediately fix several control embryos in ice-cold MeOH. These embryos should be stained with the acetylated tubulin antibody to confirm that deciliation was successful.
6. Allow cilia to regenerate by incubating remaining embryos in $0.1 \times \text{MMR}$ +antibiotic. Partial cilia regrowth can be observed as early as 30 min to 1 h after deciliation as well as the onset of a weak fluid flow shortly thereafter (Fig. 10.2A). Full regeneration is complete within 3–4 h (Fig. 10.2A).

5. CONCLUSION

Not unlike the resurgence of cilia-related research in the past 15 years, *Xenopus* embryos are finding increasing popularity as a model system to study cilia and cilia-dependent processes. In fact, the combination of well-established cell and molecular biological techniques combined with high-resolution imaging makes *Xenopus* an excellent system to study cilia in the context of a developing organism. The applications for *Xenopus* as a model system for ciliated tissues extend beyond those described in this chapter and stretch from motile cilia in MCCs of the epidermis to a variety of other cilia, in particular, the primary cilia of numerous developing tissues, the motile cilia of the neural tube (Chung et al., 2012), and the single motile cilia in the gastrocoel roof plate (Schweickert et al., 2007). Additionally, a recent study has described cilia in the skin as a promising new model system to study intraflagellar transport (Brooks & Wallingford, 2012). Finally, *Xenopus* egg extracts have been successfully used to study centriole duplication and amplification which is essential for the formation of MCCs (Hatch, Kulukian, Holland, Cleveland, & Stearns, 2010). Taken together, these various applications point to a bright future for *Xenopus* as a model system for studying ciliated tissues.

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Figure 10.1.

Mounting of *Xenopus* embryos for microscopy. (A) A simple method for mounting embryos using electrical tape as a spacer between two large pieces of coverglass. (B) Mounting embryos for live imaging using a half-rose chamber mounted on a large coverglass. The embryo is held in place by a small piece of coverglass with two dollops of silicone grease. (C) Mounting embryos for imaging fluid flow. Embryos are held in place by a V-shaped barrier of silicone grease and the microscopic beads are dropped onto the embryos for imaging. An example of overlaid flows generated by this method (inlay C).

Figure 10.2.

Various tools for studying cilia in *Xenopus*. (A) Time course of chemical deciliation followed by regeneration. (B) Kymograph of cilia beating used to score the rate of beats per second. (C) MCC expressing centrin-RFP to mark the basal body and GFP-CLAMP used to mark the striated rootlet. Together, these markers are used to score the orientation of individual cilia (inlay).

Figure 10.3. Generating ciliated epithelial explants. (A) Making fibronectin-coated coverglass. (B) Cutting ectodermal tissue for generating explants. Explants are held in place by small pieces of coverglass with two dollops of silicone grease.

Figure 10.4.

Performing skin transplants. (A) Model for the development of ciliated epithelia, where MCCs are initially in a distinct sublayer of the epithelium and undergo radial intercalation to join the outer epithelium. (B) “Outer” skin transplants involve removing just the outer epithelium from one acceptor embryo (green) and replacing it with outer epithelium from a donor embryo (red). (C) Transplant recipient after developing to a stage when the MCCs have intercalated and undergone ciliogenesis. MCCs intercalate into the transplanted tissue and adjacent to the transplant (inlay).

Figure 10.5. Generating skin spheres. (A) Skin spheres are mounted in agarose pads. (B) Model for intercalation in the developing skin spheres.

Table 10.1Useful reagents for *Xenopus* multiciliated cells

Marker name	Description	Labeled structure in ciliated tissue and application	Fixatives	Suggested concentration
<i>Ciliary axoneme</i>				
Acetylated tubulin	Antibody (Sigma clone 6-11B-1 #T-6793)	Strongly labels ciliary axoneme (Deblandre, Wettstein, Koyano-Nakagawa, & Kintner, 1999)	Works: 100% MeOH, Dents, 3% PFA/EtOH Variable: 3% PFA alone	1:500
GFP-Xl.16654	Tagged Xl.16654	Strongly labels ciliary axoneme. Used for live imaging of cilia beating (Werner et al., 2011)	Works: 3% PFA or 3% PFA/EtOH Variable: 100% MeOH, Dents	250 pg mRNA 20 pg DNA
Beta tubulin	Antibody (Hybridoma bank E7c)	Strongly labels cilia and all cytoplasmic microtubules (Werner et al., 2011)	Works well in all fixatives	1:300 of concentrate
<i>Basal bodies</i>				
Gamma tubulin	Antibody (Sigma clone GTU-88 #T-6557)	Labels basal bodies in fixed samples (Park, Mitchell, Abitua, Kintner, & Wallingford, 2008)	Works: 100% MeOH, Dents Variable: 3% PFA/EtOH	1:500
Centrin-RFP	Tagged centrin2 (Xl.50473)	Strong basal body marker (Mitchell et al., 2009; Park et al., 2008)	Works: 3% PFA or 3% PFA/EtOH Variable: 100% MeOH, Dents	40 pg mRNA
GFP-HYLS	Tagged HYLS (Xl.13199)	Strong basal body marker (Dammermann et al., 2009)	Works: Dents, 3% PFA/EtOH, 3% PFA	250 pg mRNA 20 pg DNA
<i>Rootlets</i>				
GFP/RFP-CLAMP (Spef1)	Tagged CLAMP (Xl.26316)	Strong striated rootlet marker. Used to score cilia orientation (Mitchell et al., 2009; Park et al., 2008)	Works: 3% PFA or 3% PFA/EtOH Variable: 100% MeOH, Dents	250 pg mRNA 20 pg DNA
GFP-mid1ip1 (Mig12)	Tagged Mig12 (Xl.47645)	Strong striated rootlet marker. Used to score cilia orientation (Park et al., 2008)	Works: 3% PFA or 3% PFA/EtOH Variable: 100% MeOH, Dents	250 pg mRNA 30 pg DNA
<i>Other useful markers</i>				
EMTB-3 × GFP	Microtubule binding domain of ensconsin fused to 3 × GFP	Labels base of cilia, transition zone, and cytoplasmic microtubules (Brooks & Wallingford, 2012; Werner et al., 2011)	Works: 3% PFA or 3% PFA/EtOH Variable: 100% MeOH, Dents	200 pg mRNA 20 pg DNA
RBD-GFP	RhoA binding domain of Rhotekin	Transient localization to basal bodies. Biosensor	Best used on live embryos	200 pg mRNA

Marker name	Description	Labeled structure in ciliated tissue and application	Fixatives	Suggested concentration
		for RhoA activity (Park et al., 2008)		
Membrane-RFP	Membrane binding domain of GAP-43	Strong membrane localization. Used to mark cell boundaries and as a tracer (Stubbs, Davidson, Keller, & Kintner, 2006)	Works: 3% PFA or 3% PFA/EtOH Variable: 100% MeOH, Dents	100 pg mRNA
ZO-1 antibody	Rabbit antibody (Zymed #61-7300)	Labels tight junctions and marks cell boundaries and apical membranes (Merzdorf & Goodenough, 1997)	Works well in all fixatives	1:200
Phalloidin-Alexa568/647	Conjugated phalloidin (Invitrogen Inc.)	Labels cytoplasmic actin. Used to visualize cortical actin cap, subapical actin network, and cell boundaries	Works: 3% PFA in K-PIPES	1:500 (568) 1:250 (647)
GFP-utrophin	Actin binding domain of utrophin	Used for live imaging of cytoplasmic actin (Burkel, von Dassow, & Bement, 2007)	Works: 3% PFA in K-PIPES	175 pg mRNA 15 pg DNA
Lifeact-GFP	17 amino acid peptide. Binds filamentous actin	Used for live imaging of cytoplasmic actin	Works: 3% PFA in K-PIPES	175 pg mRNA 15 pg DNA
H2B-GFP	Histone H2B	Labels cell nuclei and mitotic chromosomes	Works: 3% PFA or 3% PFA/EtOH Variable: 100% MeOH, Dents	150 pg mRNA 15 pg DNA
<i>Other useful reagents</i>				
hGR-MCI	Inducible form of multicilin	Ectopic expression drives ectopic MCC formation (Stubbs, Vladar, Axelrod, & Kintner, 2012)	n.a.	40 pg mRNA
Notch-ICD	Intracellular domain of notch	Overexpression reduces or completely eliminates ciliated cells (Deblandre et al., 1999)	n.a.	175 pg mRNA
GFP-Dsh-C1	C1 domain of disheveled	Overexpression prevents migration of basal bodies to the apical surface of ciliated cells (Park et al., 2008)	Works: 3% PFA or 3% PFA/EtOH Variable: 100% MeOH, Dents	175 pg mRNA