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In vivo* investigation of cilia structure and function using *Xenopus

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Abstract

Cilia are key organelles in development and homeostasis. The ever-expanding complement of cilia associated proteins necessitates rapid and tractable models for *in vivo* functional investigation. *Xenopus laevis* provides an attractive model for such studies, having multiple ciliated populations, including primary and multiciliated tissues. The rapid external development of *Xenopus* and the large cells make it an especially excellent platform for imaging studies. Here we present embryological and cell-biological methods for the investigation of cilia structure and function in *Xenopus laevis*, with a focus on quantitative live and fixed imaging.

Introduction

The discovery of widespread roles for cilia in animal development and disease has motivated an explosion of cell biological studies of cilia assembly and function. Over the last decade, the frog *Xenopus* has emerged as a powerful model organism for ciliary biology, due to the epidermis of amphibians being covered by a mucociliary epithelium remarkably similar to that lining the mammalian airway. In fact, the first vertebrate cilia to be described in any detail were those of multiciliated cells on the amphibian epidermis (Sharpey, 1830; 1835), and this system has been used for cilia studies consistently for over 100 years (Assheton, 1895; König & Hausen, 1993; Nokhbatolfoghahai, Downie, Clelland, & Rennison, 2005; Twitty, 1928).

In general, *Xenopus* provides a powerful and versatile vertebrate model system that has been a cornerstone of cell and developmental biology research (Harland & Grainger, 2011). *Xenopus* provides ample biological material; rapid development and large embryos make it outstanding for developmental studies; its large cells make it outstanding for cell biological imaging; and manipulation of gene function is routine (Davidson & Wallingford, 2005; Harland & Grainger, 2011; Werner & Mitchell, 2012). Finally, as a tetrapod amphibian, it is closely related in evolution to mammals (Hellsten et al., 2010). Over the last decade, *Xenopus* has been central to wide array of discoveries in ciliary biology (Vincensini, Blisnick, & Bastin, 2011; Werner & Mitchell, 2012; Wessely & Obara, 2008).

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As an experimental platform, *Xenopus* has been used to study cilia-mediated embryonic patterning (Chung et al., 2012; S. K. Kim et al., 2010; Park, Haigo, & Wallingford, 2006), basal body docking to the apical cell surface prior to ciliogenesis (Brooks & Wallingford, 2013; Park, Mitchell, Abitua, Kintner, & Wallingford, 2008), assembly of ciliary axonemes (Hayes et al., 2007; S. K. Kim et al., 2010; Park et al., 2006), dynamics of IntraFlagellar Transport (Brooks & Wallingford, 2012; 2013; Chung et al., 2014), and planar polarization of ciliary beating (Mitchell, Jacobs, Li, Chien, & Kintner, 2007; Mitchell et al., 2009; Park et al., 2008; Werner et al., 2011). Importantly, results from *Xenopus* have consistently prefigured results of similar experiments in mammals, including in specification of multiciliated cells (Stubbs, Vladar, Axelrod, & Kintner, 2012; Tan et al., 2013), and in the biogenesis of cilia themselves (Gray et al., 2009; Heydeck, Zeng, & Liu, 2009; Park et al., 2006; Tissir et al., 2010; Vladar, Bayly, Sangoram, Scott, & Axelrod, 2012). Finally, *Xenopus* has also been recently used as an experimental model for studying human ciliopathic disease genes (Boskovski et al., 2013; Hoff et al., 2013; S. K. Kim et al., 2010).

Here, we present methods for the quantitative analysis of cilia structure and function by confocal microscopy in *Xenopus*. We begin with a discussion of general principles in fixed and live imaging and subsequently discuss some specific analytical paradigms for the study of ciliary localization, structure, and dynamics. A number of other methods papers have recently been written about *Xenopus* and they make excellent resources for some topics not thoroughly discussed here (Joshi, Kim, & Davidson, 2012; H. Y. Kim & Davidson, 2013; Werner & Mitchell, 2013). We stress, here, that the methods discussed below should be seen largely as examples. Each quantitative investigation is likely to require its own parameters, and these often need to be empirically determined. That said, the guidelines we discuss below should be helpful in thinking about and designing studies of cilia in *Xenopus*.

A brief introduction to *Xenopus* husbandry and manipulation

General protocols for *Xenopus* care and handling, as well as embryo acquisition can be found in (Sive, Grainger, & Harland, 2000) and on Xenbase (<http://www.xenbase.org>; (Bowes et al., 2008)). For most experiments, adult female *Xenopus* should be ovulated overnight by injection of human chorionic gonadotropin (HCG) and incubation at 15–18°C. Eggs can be obtained the next day by gently squeezing females, and these may then be fertilized by the application of homogenized male testis (Sive et al., 2000).

Injection of mRNA and antisense oligonucleotides

One commonly used method for visualization of cilia in *Xenopus* is labeling of ciliated tissues by the microinjection of mRNA (generated from Ambion mMessage mMachine kits) encoding fluorescent fusion constructs. Such injections can be targeted between 4-cell and 64-cell stages (Kieserman, Lee, Gray, Park, & Wallingford, 2010; Nieuwkoop & Faber, 1994). At the 4-cell stage, injection of the two dorsal blastomeres will label the tissues of the gastrocoel roof plate (analogous to the mammalian node) and neural tissues, whereas injection into the two ventral blastomeres will label the non-neural ectoderm, including the multiciliated cells of the epidermis. Finer targeting can be accomplished by waiting for embryos to reach subsequent stages and injecting into domains mapped at the 32-cell stage (Dale & Slack, 1987; Moody, 1987; Nieuwkoop & Faber, 1994).

Targeted injection can also be used for functional manipulation. For example, targeted knockdown of proteins of interest can be accomplished by injection of antisense morpholinos (Gene Tools) blocking either the splicing or translation of mRNA. Additionally, injection can be used for mRNA mediated overexpression or dominant negative studies. Such manipulations can be traced by the addition of a simple fluorescent dextran or an mRNA encoding a fluorescent construct. See the section on Live Cell analysis later in this chapter and also (Kieserman et al., 2010) for more information.

Explanting and dissection

One of the most powerful aspects of the *Xenopus* model system is the ability to perform explant manipulations, which allow for the visualization of internal tissues, as well as sophisticated cut-and-paste experiments. More on such techniques can be found in (Hamburger, 1960; Keller, 1991). Of particular interest in the study of cilia are explants of the gastrocoel roofplate (GRP), the *Xenopus* equivalent of the mammalian node. A discussion of this tissue and experimental methods for its isolation can be found in (Schweickert et al., 2007; Shook, Majer, & Keller, 2004).

In addition to explant manipulation of living embryos, fixed *Xenopus* tissues (see below), such as the ciliated neural tube can be exposed by dissection. This can be accomplished crudely, by simply cutting with a fresh razor blade, or more finely by the application of embedded sectioning techniques (e.g. vibrotomy, cryosectioning). See also (Davidson & Wallingford, 2005; Wallingford, 2010)

Imaging of cilia in fixed *Xenopus* embryos and tissues

One common method for analyzing cilia structure and function in *Xenopus* is via immunostaining for ciliary proteins. The following section discusses fixatives and details a protocol for preparing and staining embryos. Additionally, Table 1 provides a list of common antibodies that are useful in the study of cilia in *Xenopus*.

Discussion of common fixatives

Three common fixatives exist for the preparation of *Xenopus* tissues for imaging: 1) MEMFA, a formaldehyde fixative; 2) Dent's, a methanol fixative; and, 3) Trichloroacetic acid (TCA) fixation (formulations are given below). While we give some discussion general discussion of each fixative below, we firmly believe that empirical determination is the best way to understand which should be used for each particular case.

MEMFA is a standard fixative useful in a number of different *Xenopus* methods, including general morphological stereomicroscopy, *in situ* hybridization, and, in some cases, fluorescence approaches (both in stereo- and confocal microscopy). The standard MEMFA formulation as given by Sive et al (Sive et al., 2000), is:

0.1 M MOPS (pH 7.4)

2 mM EGTA

1 mM MgSO₄

3.7% Formaldehyde

A 10x preparation of the salts can be stored, and fresh formaldehyde added just before fixation. While MEMFA is a generally useful fixative, some caution should be taken when it is utilized for fluorescence imaging. First, as with most fixatives, MEMFA application leads to quenching of protein-encoded fluorophores (FP tags; e.g. GFP, RFP), and such quenching increases over time, and thus, the timing of fixation is a key concern. For examination of the multiciliated cells of the epidermis, ~30min fixation at room temperature should be sufficient. This short fixation will minimize quenching, often allowing for the direct visualization of expressed FP constructs. For internal tissues, the GRP and the neural tube, longer fixation is required to allow for fluid penetration. For these tissues fixation for 1–2hr at room temperature or, alternatively, overnight at 4°C is recommended. Given the longer fixation time for these tissues, immunofluorescent secondary amplification of encoded fluorophores (e.g. with an antibody targeted against the FP) is recommended (see below). As a final note, MEMFA fixation involves formaldehyde crosslinking of proteins, which can have deleterious effects on antibody-antigen binding in immunohistochemical preparations, especially in protein-dense structures such as basal bodies. If an antibody is not giving signal in MEMFA it should be tested in other fixative preparations as well.

Dent's is another common fixative that is especially useful for imaging microtubules and is made as a 4 MeOH : 1 DMSO (v/v) preparation (Sive et al., 2000). This is a considerably gentler fixative than MEMFA, and thus tissues are more prone to damage and care should be taken in embryo transfer and handling. Dent's is likely not a good fixative for embryos that one wishes to dissect later, but can be applied to explants. The methanol in this fixative (and alcohols generally) will severely quench the direct fluorescence of encoded FP tags and, therefore, visualization of these elements requires secondary immunofluorescent detection (see Table 1 for potentially useful antibodies for the detection of FPs). However, the lack of crosslinking in this preparation often makes it useful in cases where MEMFA fixation interferes with immunodetection (e.g. some basal body proteins (Werner & Mitchell, 2013)).

Finally, Trichloroacetic acid (TCA) can be used at 2% (w/v, in molecular-biology grade water) as a fixative. This is a relatively harsh treatment for tissues, and so fixation time should be as limited as possible. Often 15–20 minutes is sufficient, though some cases may require up to 30 minutes. If TCA fixative is left on too long, tissues will often be damaged and fail to stain. Even with appropriate fixation time, some antibodies will not work after TCA treatment. However, TCA fixation often results in better detection of phosphoproteins.

For uncharacterized antibodies or tissue contexts, it will be beneficial to test all three of the above fixation conditions, since there is as yet no way to successfully predict which method will return the best signal for any given condition. Finally, while some agitation (i.e. rotation or nutation) is beneficial for fixation, it should be noted that cilia, especially those of the epidermal MCCs, are prone to disassociation from cell bodies over long fixation times, or under heavy agitation, and thus both parameters should be minimized to the extent possible.

Immunostaining protocol

N.B. The following method is adapted from (Lee, Kieserman, Gray, Park, & Wallingford, 2008), and that reference contains additional discussion that the reader may find informative.

Reagents

- Fixative (see above)
- PTW buffer:
 - Dissolve 0.1% Tween in 1X PBS (phosphate buffered saline). Make up in liter quantities
- MeOH
- TBS buffer:
 - 155 mM NaCl and 10 mM Tris-Cl (pH 7.5) in molecular biology grade water
- TBS-T buffer:
 - Add 0.1% Triton X-100 to TBS
- Blocking solution
 - Add 10% Fetal Bovine Serum (FBS) and 5% DMSO to TBS
- 30% H₂O₂ (optional, Step 4)
- Autofluorescence reducing agent (optional, Step 5)
 - Add 100mM NaBH₄ to TBS, allow to stand in refrigerator for 48hr before use
- 5mL glass scintillation vials

Protocol—N.B. If embryos are expressing FP constructs, carry all steps out in containers protected from the light

- 1 Fix embryos in 5mL glass scintillation vials (see above discussion):
 - For MEMFA, fix for 30min (for epidermal staining) 2hrs (for deeper tissues) at room temperature on nutator or rotator.
 - For Dent's, fix for 4hrs-overnight on a nutator at 4°C
 - For TCA fix for ~15–30mins at room temperature
- 2 Remove fixative and wash embryos:
 - For MEMFA or TCA, wash 5 × 5 min with PTW, then 5 × 5 min with 100% MeOH
 - For Dent's, wash 5 × 5 min with 100% MeOH
- 3 Dehydrate embryos (unnecessary for direct visualization of encoded FPs):

- For staining epidermal MCCs, incubate embryos (in 100% MeOH) at -20°C for 30min–1hr.
- For other tissues, incubate (in 100% MeOH) at -20°C overnight

N.B. Steps 4 and 5 below are optional and largely apply to deeper tissues. If pigment is a problem, follow step 4, then proceed to step 7. If autofluorescence is a problem, follow steps 4–5, then proceed to step 7.

4 Bleaching pigment:

- Prepare a solution of 10% H_2O_2 in MeOH. Incubate embryos in this solution in a light box for 1–3h.
- Rehydrate embryos through a series methanol series, allowing the embryos to nutate gently for 10min in each of the stages below:
 - 50% MeOH/50 % TBS
 - 25% MeOH/75% TBS
 - 100% TBS

5 Reducing autofluorescence:

- Incubate embryos in autofluorescence reducing agent for 4h at $20\text{--}23^{\circ}\text{C}$

6 Rehydrate embryos (skip this if you have done step 4 above):

- Rehydrate embryos through a series methanol series, allowing the embryos to nutate gently for 10min in each of the stages below:
 - 50% MeOH/50 % TBS
 - 25% MeOH/75% TBS
 - 100% TBS

7 Wash 5×10 min in TBST

8 Incubate embryos or tissues in blocking solution at room temperature on a nutator:

- For epidermal multiciliated cells incubate 30min
- For other tissues incubate 1h

9 Remove blocking solution and replace with blocking solution containing primary antibody (see Table 1 for information about common antibodies for the study of cilia). For quantitative feature extraction, both control and experimental embryos should be stained with aliquots of the same antibody preparation.

10 Incubate in primary antibody solution (as little as $300\mu\text{L}$ can be used, depending on the number of embryos):

- For epidermal multiciliated cells incubate 30min–1h at room temperature on a nutator. (Some antibodies with weaker affinities may require overnight incubation at 4°C .)

- For other tissues incubate overnight at 4°C on a nutator
- 11** Remove primary antibody solution (this can be saved and reused 3–4x)
- 12** Wash embryos or tissues:
 - 5 × 20min after brief (1hr) primary antibody incubation
 - 5 × 1h in TBST after overnight primary antibody incubation
- 13** Remove TBST and add blocking solution with appropriate secondary antibody. Incubate as per step 10 above. From this step onwards, protect samples from ambient light to prevent photobleaching.
- 14** Wash 5 × 1hr in TBST (or 5 × 20min for epidermal cells)
- 15** Image (see discussions of mounting and imaging parameters below) or store. Embryos can be stored, for 1–3d in TBS in the dark. If the embryos need to be stored over longer time-periods they should be dehydrated by a 5 × 5m series of methanol washes. Note that long-term storage and methanol treatment both have negative impacts on total fluorescence.

Methods for live cell analysis of cilia in *Xenopus*

The following section details general principles for live cell analysis of cilia structure and function.

Visualization with fluorescent fusion constructs

The most common method for live cell imaging in *Xenopus* is the targeted injection of *in vitro* transcribed mature mRNA encoding an FP (e.g. GFP) tagged protein of interest. It should be remembered that this is an overexpression technique, with all of the caveats that implies. It is therefore important to perform some initial characterization of all newly generated constructs. First, a broad dose curve of mRNA injection should be performed (one common initial curve is 50–500pg by 50pg increments, though this varies significantly with the size and relative expression of the construct). Such a curve yields multiple important pieces of information. First, any toxic effects of higher mRNA doses can be discerned, often at the embryonic level, but also at the cellular level. Second, dose curves allow one to find the minimal dose of mRNA that can be injected to return a usable signal. If necessary, the initial dose curve can be further iterated upon, by shrinking the dose intervals until a truly optimal dose is found. This dose-curve approach is important in order to minimize the amount of exogenous expression, thus ensuring minimal perturbation of endogenous processes, a key concern for reporter and localization constructs.

The next step in validating fusion constructs is to test their functionality. In *Xenopus* one of the best ways to do this is to attempt to rescue morpholino knockdown phenotypes for the endogenous gene by injection of mRNA encoding the fusion construct. The first step in this process is to use a validated morpholino (see discussion of MOs below) to identify a defect, which is easier to do at the embryonic level but perhaps more compelling if done at the cellular level. For genes whose function is required for ciliogenesis or cilia signaling, injection of targeted morpholino into the presumptive dorsal tissue at the four-cell stage

often results in neural tube closure phenotypes (e.g. (Chung et al., 2012; S. K. Kim et al., 2010; Park et al., 2006)). This is a convenient and easily visualized embryo-level phenotype that can also be used to quantitatively examine rescue by mRNA injection. Though more compelling, analyzing rescue at the cellular level (e.g. imaging the cilia directly) is more difficult due to the mosaic nature mRNA and morpholino inheritance by daughter cells after injection. Therefore, cell level analysis requires careful quantification over a broad population of cells.

The collection of publically available *Xenopus laevis* cDNA sequences and the now fairly comprehensive genome sequence (which can be found at <http://www.xenbase.org>) (James-Zorn et al., 2013) is improving constantly, and it is recommended that fusion constructs be made to these sequences if possible. However, in some cases it has been demonstrated that cross species constructs can be used to assess function. Indeed, we have found that human IFT protein fusions to GFP report well in *Xenopus*, though not as well as *Xenopus* IFT proteins (E.R.B. unpublished). If such constructs are used, extra care in validation should be used.

A particularly useful alternative to mRNA expression involves injection of plasmid DNA containing the cDNA of interest driven by a cell-type specific promoter. It has long been known that this method results in highly mosaic expression (Vize, Melton, Hemmati-Brivanlou, & Harland, 1991), we and others have found it to be highly effective for imaging of Multiciliated cells (Chung et al., 2014; Werner & Mitchell, 2013).

DNA containing a fusion construct coupled to the *Xenopus* TUBA1A-B promoter (often referred to as the alpha-tubulin promoter) (Deblandre, Wettstein, Koyano-Nakagawa, & Kintner, 1999; Stubbs, Davidson, Keller, & Kintner, 2006) is injected at the 4–8 cell stage. This scheme allows for high-expression of constructs specifically in the epidermal multiciliated cells (though, as with most promoters, there is some leakiness). This method gives more variable expression (DNA is inherited very unequally by daughter cells) but does reduce background fluorescence and confounding effects from the surrounding goblet cells, and additionally can be used to more specifically drive dominant negative or overexpression constructs. It should be noted that many *Xenopus* promoters lead to off-target expression, and the alpha-tubulin promoter is one useful exception. For more details on this method see (Werner & Mitchell, 2013)

Many constructs exist for visualizing cilia or cilia related proteins in *Xenopus* and some of these are detailed in Table 2.

For routine visualization, some transgenic lines have been established and are often available from the National Xenopus Resource Center (<http://www.mbl.edu/xenopus/laevis-stocks/>) in the United States, and the European Xenopus Resource Center (<http://www.port.ac.uk/research/exrc/transgenicandmutantlines/>) (Pearl, Grainger, Guille, & Horb, 2012). Often these centers will work with you to create transgenic lines for your constructs of interest. Once a line has been established, transgenic male testis can be used to fertilize eggs from wild-type females to give uniform expression of reporter constructs from embryo to embryo.

Morpholinos and other gene-perturbation technologies

The most common method for gene-function perturbation in *Xenopus* is the targeted injection of antisense morpholino-oligonucleotides (MO) (Gene Tools). These oligomers of ~25 nucleotides have a DNase/RNase resistant backbone and persist for ~72hr at room temperature, though the effective dose in each cell is reduced with every division (Eisen & Smith, 2008). In practice, morpholinos are useful throughout the stages where the epidermis is ciliated. Morpholinos reduce the effective level of gene-product by one of two methods; translation blocking morpholinos base pair with mRNA transcripts near or across the translation start site, inhibiting the function of the translation machinery. Splice blocking morpholinos instead base pair at or near an exon-intron junction and inhibit mRNA splicing and maturation. In most cases, splice blocking morpholinos are preferable (see below), though they can lead to truncated partially functional proteins if splicing is not blocked early in the sequence.

As with mRNAs, it is important to extensively validate morpholinos, and this validation follows largely the same principles discussed above. A preliminary dose curve is important, as morpholinos can exhibit high cytotoxicity and their dose must be minimized. Additionally, it is critical to control for off-target effects with morpholinos. The best control is rescue of morpholino-induced phenotypes (e.g. open neural tubes for cilia related morpholinos) by co-injection of mRNA encoding the morpholino target. Care must be taken to ensure that this rescue mRNA cannot be targeted by the morpholino. For splice blocking morpholinos, this is not a concern; the morpholino is incapable of targeting the spliced, mature mRNA. For translation blocking morpholinos, the translation start site of the rescue mRNA should be altered to prevent base pairing (often 5' fusion of an FP tag in frame accomplishes this; alternatively the transcription start can be altered to generate significant mismatch, or the human cDNA can be used, as *Xenopus* and human DNA sequence diverge significantly even when protein sequences are similar). In some cases rescue cannot be achieved by mRNA co-injection and a second non-overlapping morpholino should be designed and assessed for phenotypic similarity to the first, though this is a somewhat less compelling control. Additionally, a 5-base mismatch morpholino may also be useful. A thorough review of MO controls is provided in (Eisen & Smith, 2008)

Other methods to perturb gene-function exist, including over expression of dominant negative constructs by mRNA injection, or alpha-tubulin driven expression from plasmid DNA. In addition, emerging technologies (TALENs (Suzuki et al., 2013), CRISPRs (Blitz, Biesinger, Xie, & Cho, 2013)) may soon allow for rapid and practical transgenic perturbation of gene function.

Mounting *Xenopus* embryos

Once manipulated embryos are at the proper stage, they should be mounted for imaging. The following sections detail mounting embryos for analysis of epidermal multiciliated cells.

Imaging chambers

For imaging *Xenopus* an inverted scope is highly recommended, as imaging requires a volume of liquid to maintain both living and fixed embryos in an intact state. Additionally, axonemes of epidermal multiciliated cells are easier to image in an inverted context.

For inverted imaging, it is important to have watertight imaging chambers capable of holding ~1–5mL of liquid. We have previously described high-quality reusable versions of these chambers elsewhere (Kieserman et al., 2010). Briefly, they consist of: 1) a metal base with a depression the size and depth of a single piece of round cover glass (25mm diameter) and a threaded collar; 2) a metal or silicone top-piece with complementary threading to the base and a depression for housing a nearly flush O-ring. When the O-ring containing top is screwed firmly (but not overly so) into the base, it creates a watertight seal. Plans for these imaging chambers are available on request and can be produced at most machine shops. Additionally, equivalent chambers are available commercially (Life Technologies # A-7816).

An alternative to permanent chambers is to create temporary chambers as needed. One method for creating these chambers is detailed below.

Protocol for creating temporary imaging chambers

Materials

- 35mm plastic petri dishes
- 25mm diameter round cover glass (preferably 0.17 thickness)
- Silicone grease gun (a syringe filled with silicone grease)
- American dime (10¢ piece) (or metal circle of similar diameter)
- Bunsen burner
- Tongs or hemostat
- Razor blade

Procedure—N.B. The following steps should be performed in a well-ventilated hood.

1. Disassemble the petri dish, and invert the bottom half
2. Heat a small coin (we use a US dime) in flame of Bunsen burner until hot.
3. Place hot dime in the middle of the petri dish bottom half, and allow to melt through, forming a hole in the surface
4. Allow dish and dime to cool
5. Carefully clean and smooth the outside rim of the hole in the dish with a razor blade (the melting will leave a flange that must be smoothed).
6. Apply a very thin ring of silicone grease or vaseline around the circumference of the hole

7. Gently press a piece of cover glass down into the silicon grease until it is flush with the petri dish surface
8. Test for water tightness by placing the petri dish cover glass down on a kimwipe and adding a small amount of liquid to the dish
9. This will take practice. Repeat as necessary.

Once these chambers have been made they can be reused by removing the cover glass, cleaning old grease with alcohol, and the replacing with new grease and cover glass. Be sure to check the seal after each replacement.

Mounting fixed embryos at stage 26/27

N.B. If embryos have been stored in methanol, rehydrate them as in Step 6 from the immunostaining protocol above. Once embryos are in TBST, proceed as below.

To mount fixed embryos, add a small volume of TBS to the imaging chamber, and then transfer two or three embryos to the center of the cover glass. Embryos can be induced to lie flat on their flanks by placing a small piece of square cover glass over them. A more rigid flattening scheme is to place a dab of silicone grease on each corner of a piece of cover glass, and then gently press it down to sandwich the embryos. However, fixed embryos are often quite fragile so care is required not to apply too much pressure. If none of these mounting methods are satisfactory, follow the steps for mounting live embryos (below) but be mindful that fixed embryos do not react well to even limited exposure to air.

Mounting live embryos at stage 26/27

Mounting living embryos to visualize multiciliated cells can be difficult, as at these stages embryos exhibit a random twitching motion, a touch-avoidance reflex, and a ciliary mediated gliding motility. However, undesired motility can be reduced by the following mounting protocol.

Materials

- Imaging chambers (see above)
- 0.8% (w/v) Low-melting point agarose (in embryo media, e.g. 0.3X MMR)
- Embryo transfer pipette
- P20 Micropipette
- Ice bucket and foil or 12°C incubator
- Embryo media

Mounting

1. Assemble the imaging chamber and wipe clean both sides of the cover glass with a kimwipe, being exceptionally careful not to smear the glass.
2. Transfer two or three embryos and place in minimal volume drop in the center of the cover glass in imaging chamber.

3. Allow embryos to sink, and then remove as much of the media as possible while still leaving the smallest possible drop of liquid containing the embryo. Media should be removed by unipolar suction at one end of the drop. This will allow axonemes to splay and become trapped between the embryo body and the cover glass.
4. Add ~10 μ L of cool but still liquid 0.8% LMP agarose, applying it in a gentle, circular pattern beginning in the middle of the embryos and radiating outwards
5. Embryos will float up in the agarose, so it is necessary to remove as much as possible to keep as much of the epidermis flat against the cover glass as is reasonable.
6. Once the excess agarose has been removed, place embryos on foil in ice bucket or in 12°C incubator for ~1min to allow the agarose to set
7. Retrieve the imaging dish and add a second layer of agarose, following the procedure from step 4. If the embryos do not bend up, allow the second layer of agarose to set as in step 6. If the embryos float or bend up, restart mounting with fresh cover glass.
8. Gently place a small volume of 0.3X MMR or other appropriate embryo media in the imaging chamber. This volume should be sufficient to cover the embryos. If long term imaging (i.e. more than 1–2h per chamber) experiments are planned, additional media should be added to prevent dehydration.

The above technique requires patience and practice, but allows for high-resolution imaging of trapped multiciliated cell axonemes, which due to their large size are quite attractive for dynamic studies (see below). Even if the above mounting is performed expertly, many regions of the curved embryo will not be flat against the glass, so some post mounting selection is required. Finally, embryos will still break free over time, due to twitching motility. For imaging over long time-scales, embryos should be pre-treated by bathing them in a 60mm petri dish full of embryo media containing 0.1% Tricaine or Benzocaine for 1–2min. Embryos should then be maintained in this media while imaging. However, care should be taken with such treatments, as they may impact embryo health and thus the process being observed. If truly long-term imaging is required, such that the embryo will elongate significantly, a less restrictive method is required. These methods have been described elsewhere (Joshi et al., 2012; Kieserman et al., 2010; Werner & Mitchell, 2013).

Image acquisition

Discussion of imaging parameters for *Xenopus*

As with other organisms and contexts, careful selection of imaging parameters is an important concern for *Xenopus* imaging. Given the aqueous environment where *Xenopus* are imaged, high-magnification water immersion lenses are the best choice for visualizing sub-cellular structures and events. However, oil immersion lenses will work as well, although with a small optical aberration. Most ciliary imaging will likely be done with a 40x, 63x, or 100x objective. 40x magnification is sufficient to capture a field of ~7–10 cell diameters in the *Xenopus* epidermis, and can crudely resolve the axonemes and basal bodies of a single

multiciliated cell (see below for more specific information on imaging these structures). A 63x objective gives the best balance between resolving sub-cellular structures (e.g. basal bodies) and returned signal. 100x objectives return significantly less light, and should be reserved for observation of quite small objects (e.g. axonemal intraflagellar transport trains, see below).

In all cases, laser power should be minimized as much as possible, as in addition to photobleaching effects higher laser power can increase background autofluorescence in *Xenopus* cells. This is especially true in epidermal cells which contain yolky platelets in the deeper cytoplasm (Kieserman et al., 2010). In extreme cases prolonged, high-intensity laser exposure can lead to cytotoxicity, therefore long-term imaging experiments should use minimal power and exposure times, and cells should be allowed to rest between acquisition windows. Other parameters, including gain and optical thickness should be adjusted accordingly (see (H. Y. Kim & Davidson, 2013) for a detailed discussion of this concern).

Image saturation is an important concern for quantitative analysis, and so acquisition parameters should be set so that few or ideally no pixels are saturated. Saturated pixels skew quantitative results, especially if they are a large percentage of the total pixel number. Additionally, they provide no relative fluorescence information and interfere with normalization. Saturation should be checked with the range-indicator tool in the acquisition window of the microscope software. If the area to be imaged contains both very dim and very intense regions, which would lead to saturation at low bit depth (e.g. 8-bit images) it may be beneficial to use a higher bit-depth (e.g. 16-bit), though this requires more disk space for image storage and computational resources for image processing, and is therefore not always an ideal solution for large 4D data sets. It is worth noting that post-processing approaches can bring up relative intensity, but cannot subtract from it in absolute terms, and therefore slight underexposure is preferred to overexposure or saturation.

Imaging axonemes and axonemal compartments

Axonemes from epidermal multiciliated cells can be visualized in a number of ways, the simplest are either staining with an acetylated tubulin antibody or, for live imaging, the mRNA driven expression of GFP or RFP conjugated to some kind of membrane insertion domain. Either of these methods is sufficient for analysis of gross axonemal structure, i.e. measuring length. Recent work has shown that axonemes are subdivided into compartments (Fliegauf et al., 2005; Kubo, Yuba-Kubo, Tsukita, Tsukita, & Amagai, 2008; Satish Tammana, Tammana, Diener, & Rosenbaum, 2013; Schröder et al., 2011). The long axonemes of *Xenopus* epidermal multiciliated cells make excellent platforms for the visualization of ciliary sub-compartments, and generally for the molecular organization along the length of axonemes (Brooks & Wallingford, 2012; 2013; Chung et al., 2014). To visualize the proximal domain of axonemes (e.g. the region closest to the cell body) we have used mRNA encoding the microtubule-binding domain of Enscosin (MAP7) fused to GFP (Brooks & Wallingford, 2012; 2013). Conversely, the distal portion of the axoneme can be visualized by FP-tagged EB3 or CLAMP/Spel1 (Brooks & Wallingford, 2012; Pedersen et al., 2005; Schröder et al., 2011). FP-CLAMP is particularly useful, as in addition to marking an enriched region at the distal tip it also decorates the entire length of the axoneme at a low

intensity. Thus, a simple two color labeling of MAP7 and CLAMP, will provide information about both the proximal and distal compartments, and about the total length of the axoneme (Fig. 2; see discussion of quantification below). These markers can also be used to delimit axonemal domains for the analysis of ciliary structural or motility components (e.g. Tektins, Ribbon proteins; (Chung et al., 2014)). This analysis can be performed either in living embryos (though it might be impaired by axonemal twitching) or after a brief fixation (~20–30min in MEMFA).

Imaging axonemal beating patterns

Axonemal motility is a fascinating process and the coordinated beating of cilia in multiciliated cells is required for fluid flow in many epithelial organs, including the airway, the ventricles of the brain, and the reproductive tracts (Lyons, Saridogan, & Djahanbakhch, 2006; Sawamoto et al., 2006; Wanner, Salathé, & O’Riordan, 1996). This beating can be readily observed in *Xenopus* multiciliated cells expressing one of the following: high doses of membrane-GFP (300pg or driven by the alpha tubulin promoter) (Chung et al., 2014); injection of 250pg of GFP-Xl.16654 (Werner et al., 2011); or low doses of Tau-GFP (50pg; higher doses are highly cytotoxic) (Park et al., 2008). The multiciliated cells analyzed in this approach should be positioned such that their axonemes can beat freely. In the mounting paradigm described above, these cells can be found in places where the epidermis naturally folds, or on the portions of the flank not located tight against the glass. Clearly resolving ciliary beating requires a high-speed approach (such as a spinning-disk or line scanning confocal) as cilia beat with a frequency of ~20 beats per second in control MCCs (Chung et al., 2014; Werner et al., 2011), and the ideal image acquisition time interval should be significantly less than the lifetime of the event under study. Therefore, acquisition speed should be at least 200 frames per second to quantify basic beat parameters such as frequency. This imaging paradigm can be used to examine both overall slower motility and losses in the coordination of ciliary beating. However, obtaining truly high-resolution beat information on single axonemes requires 1000fps. Methods for such high-speed imaging of axonemal beating have been well described for *Xenopus* (Werner & Mitchell, 2013).

Imaging axonemal IFT dynamics

Ciliary biogenesis and maintenance requires the active trafficking of proteins from the cell body to the distal tip. This transport is accomplished by the intraflagellar transport (IFT) pathway, a multi-protein complex of two subunits, IFT-B, which is thought to govern anterograde transport in cilia, and IFT-A, which appears to largely govern retrograde transport. These two complexes assemble into an IFT unit and multimerize to form cargo carrying trains that move along axonemal microtubules via kinesin and dynein motors (Ishikawa & Marshall, 2011; Rosenbaum & Witman, 2002). These trains are highly dynamic and move at a rate of ~0.8µm/sec in both the anterograde (i.e. base to tip) and retrograde (tip-base) direction in *Xenopus* axonemes (Brooks & Wallingford, 2012). These IFT trains can be visualized by injection of mRNA encoding FP tagged IFT proteins. In wild type axonemes, both IFT-B and IFT-A proteins are found in anterograde and retrograde trains, therefore only a single labeled IFT is required to visualize dynamics under normal conditions. GFP-IFT20, GFP-IFT80, and GFP-IFT43 work well as labels for trains when mRNA is injected at 100pg. Other IFT fusions are likely to work as well, though larger

constructs may require a change in the amount of mRNA injected. An example of labeled IFT trains is shown in Fig. 1

Imaging IFT transport in *Xenopus* axonemes requires a moderately high-speed approach, as acquisition speeds need to be 2–4 fps at a minimum; additionally such imaging requires a high magnification objective as the trains are quite small (100x is recommended, high-quality 63x objectives will work, and may provide more returned light, and thus better images). Another important consideration is returned signal. IFT trains labeled by this method are not terribly intense, especially as compared to the peri-basal body pools where the majority of IFT proteins localize within the cell. Therefore, it is important to find optical sections that largely exclude the apical surface of the cell, or where the axonemes are laying across unlabeled neighboring cells, in order to increase the signal to noise ratio to allow for better detection of IFT trains.

The long axonemes of *Xenopus* multiciliated cells allow for the identification of individual IFT trains. To date, tracking of these objects has been done manually, as the poor signal/noise ratio, rapid movement, and small size of these objects make automated trafficking error-prone. However, with advanced acquisition technologies and more advanced tracking algorithms these axonemes will be an exciting platform for automated approaches to understanding axonemal trafficking dynamics.

Imaging basal bodies and associated structures

Basal bodies are modified centrioles that anchor and nucleate the axonemal structure (Ishikawa & Marshall, 2011). In *Xenopus* multiciliated cells, these structures are generated *de novo* from cytoplasmic structures known as deuterosomes and undergo an apical migration (Klos Dehring et al., 2013). In mature multiciliated cells, these structures form an orderly array at the apical surface (Fig. 3), but in some cases where cilia are defective, there are also defects in the apical migration of basal bodies. These structures can be visualized by staining for γ -tubulin or by the injection of a small amount of mRNA encoding an FP-centrin2 construct (50–100pg).

Basal bodies have additional accessory structures that can be used to assess the polarity of cilia. For example the striated rootlet can be marked by low doses of FP-CLAMP. When FP-CLAMP is used in conjunction with FP-Centrin, the polarity of basal bodies can be assessed. The methodology for this has well described recently by Werner and Mitchell (Werner & Mitchell, 2013), to whom we refer you, and has been used to great effect in the study of multiciliated cell polarization (Mitchell et al., 2009; Park et al., 2008; Werner et al., 2011)

Finally, a number of ciliary proteins, including the IFT proteins localizes to the base of the cilia in what are known as peri-basal body pools. For IFT, these pools act as reservoirs of IFT proteins that can be assembled into cycling trains before being injected into the axoneme (Fig. 3; (Ishikawa & Marshall, 2011)). In cases like this, the basal body can often be used a fiduciary marker to examine changes in protein localization to the base of cilia. However, care should be taken to ensure that the basal bodies themselves are unchanged by the experimental manipulation (see discussion on quantification below).

Quantitative analysis of ciliary properties

This section details some approaches to extracting numeric features from confocal datasets. The methods below can be used as is but may also serve as examples of the development of quantification strategies targeted towards specific analytical needs.

Software

The quantification methods below rely upon an up-to-date installation of the Fiji redistribution of the NIH ImageJ software, which is freely available from (<http://fiji.sc/Fiji>). Fiji/ImageJ is also functionally extensible by the installation of a number of freely available plugins. If a specific quantitative method is required, it is often worth searching for a preexisting plugin.

If 3D (or 4D) visualization is required, ImageJ has a number of plugins available. Alternatively, Imaris (BitPlane) and Volocity (Perkin-Elmer) are popular and powerful commercial visualization suites.

Notes on post-processing

Some minimal post-processing is often desirable before publication of data (e.g. background subtraction), but be mindful that such processing must be applied uniformly and cannot obfuscate the data. Additionally, excessive filtering and smoothing should be avoided (Cromey, 2010). Aside from this being good scientific practice, even the appearance of such manipulation can delay publication, so care should be taken. (Cromey, 2010; Rossner & Yamada, 2004). In all cases, processing should only be applied to a copy of the image. The original image file must be kept intact and unprocessed. For quantitation, use unprocessed data.

Analysis of IFT dynamics

GFP Labeled IFT trains are readily apparent in *Xenopus* axonemes and can be manually tracked between frames from time-series taken at 0.5s intervals (though lower intervals are better). Such manual tracking is sufficient to provide basic dynamic information. As an example, the velocity of an IFT train can be determined as displacement (obtained by line measurement in Fiji) over time. However, trains often exhibit some minor variation in velocity between frames, either due to intrinsic rate variation or from acquisition artifacts. Therefore, it is desirable to give an average velocity of a train over many frames (Brooks & Wallingford, 2012). If each frame is measured individually for this calculation, the variation in these velocities can also be recorded, which can be one consideration in understanding alterations of dynamic behavior. Other properties of IFT trains can also be extracted by manual analysis as required, though such analysis can become time-consuming for large populations.

Axonemal sub-compartment analysis

To extract the length and mean intensity of an axoneme or a sub-compartment, use the freehand line tool in Fiji to draw a line along the desired length, and use the measure

function. Repeat for each non-overlapped axoneme or compartment that is clearly visible in the cell.

The percentage occupancy of a compartment can be measured at the level of individual axonemes by dividing the length of the compartment by the total length of the axoneme in which it is found. This is a good measure for comparing the length of compartments, as individual cilia of a multiciliated cell can vary in length by a few microns, and this measure is inherently normalized to reflect that.

In order to compare compartment intensity data across conditions, it is critical to normalize it against some fiduciary label (i.e. some co-injected marker that does not change between conditions). As an example RFP-CLAMP and EMTB-3xGFP (also sometimes called MAP7MT-GFP or MAP7-GFP) both label enriched compartments of the axoneme (distally and proximally respectively), but also additionally label the axoneme at a low level. In conditions tested thus far this low level enrichment is similar between controls and cases where the axoneme length is perturbed. Therefore, it is possible to normalize the mean intensity of the enriched compartment against the mean intensity of an equivalent length of non-enriched axoneme. This normalization allows for comparison across embryos and between control and experimental conditions (Brooks & Wallingford, 2012). Another marker that is usually largely fiduciary, even in cases where cilia are significantly perturbed, is membrane-RFP (or GFP) (Brooks & Wallingford, 2013). In all cases the potential fiduciary marker should be analyzed for significant differences between control and experimental conditions. If there is no significant change in the fiduciary label, these normalized intensity measures can then be directly compared

The two above measures can be used to test for differences in the molecular architecture or localization between conditions. In addition, the normalized intensity gives some proportional indication of how protein levels may differ between control and experiment, though if the measures are done for markers produced from exogenous mRNA it is important to remember that both control and experiment are over- and/or mis-expression contexts.

Analysis of IFT levels within axonemes

In some cases, it may be beneficial to measure the amount of IFT in axonemes. For near-absolute measurements biochemical approaches are required. However, fluorescence intensity can be useful as a proxy for this measure (Brooks & Wallingford, 2013). The mean intensity of IFT signal along an axoneme can be extracted using line intensity measurement as above. As before, this signal must be normalized to a fiduciary marker (likely membrane-RFP).

Assessment of basal body/peri-basal body protein co-localization

One important question in ciliogenesis is the localization of ciliary structure and transport molecules to the base of the cilium, prior to their injection. *Xenopus* multiciliated cells offer a unique platform to investigate these localization questions. Each multiciliated cell in a control embryo has ~150 apically docked basal bodies at stage 26/27 (Klos Dehring et al.,

2013), and in the case of IFT proteins, each basal body has its own associated pool (Brooks & Wallingford, 2012). Unfortunately, given the close-packed nature of these structures, the IFT pools are often too densely packed for automated analysis to yield a direct 1:1 relationship between a basal body and its associated pool. However, it is possible to normalize the intensity of all pools in the cell to all of the basal bodies, resulting in a single measure of localization at the cell level. This method is detailed below.

1. Open a raw unprocessed single-plane image containing one channel of labeled basal bodies (by, e.g., centrin-RFP) and one channel of peri-basal body pools (e.g. GFP-IFT20, 80, or 43) in Fiji.
2. Perform any necessary cropping while the channels are still merged, and then split the channels into individual images using the Split Channels function under the Image/Color menu.
3. For each channel independently run the 3D object counter (under the Analyze menu), set the minimum size to 20 (or adjust as appropriate to your image), and then empirically determine what threshold maximizes the detection of individual objects by moving the slider. Be aware that the best threshold will still likely have some conjoined pools.
4. Hit OK to run the detector.
5. Results will come up as a collection of object maps, and a statistics window, with several parameters per foci detected including surface area and mean intensity. (If these values do not appear, make sure that they are selected in the 3D OC Options menu under Analyze). Save these statistics by appropriate naming convention.
6. Using spreadsheet software (e.g. Excel) calculate the mean of the mean intensities of the centrin foci for a given cell, and independently the mean of the mean intensities for IFT pools for that cell. Divide the mean IFT intensity by the mean centrin intensity, place this value in a new sheet as the reported normalized intensity for that cell. The generalizable formula is the following:

$$\text{Normalized localization per cell} = \frac{\text{mean } \sum (\text{IFT foci mean intensities})}{\text{mean } \sum (\text{centrin foci mean intensities})}$$

7. Repeat as necessary, then use the normalized values to test for significant differences between experimental conditions

Note that this method can also be used to examine the size of apparent size of basal bodies and peri-basal body pools, as well as the number of objects between conditions. This automated method allows for rapid quantification of basal body localization. If more detailed quantification of individual basal bodies is required, this can be done via manual measurement.

When the above method is used, quantitative aspects of basal bodies should be compared between control and experimental conditions. While the number of centrin-positive basal bodies may change upon the perturbation of a gene, the size and average intensity of basal

bodies should remain closely aligned (Brooks & Wallingford, 2013). If this is not the case, then centrin may not be a suitable fiduciary mark for the given perturbation. If this is the case, it may be beneficial to immunostain for γ -tubulin as a secondary assessment of basal body quality.

Conclusion

Xenopus provides a powerful model system for studies of cilia structure and function. Here we have focused largely on quantitative imaging approaches, a powerful tool in understanding the dynamic cell biology underlying these organelles. However, many other methods are also available in this model system (Davidson & Wallingford, 2005; Joshi et al., 2012; Werner & Mitchell, 2013), and the rapid and tractable nature of embryological and molecular manipulations make *Xenopus* an exciting model for cell biology in general and studies of cilia in particular.

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Figure 1. IFT Analysis

(A) A single confocal slice of a *Xenopus* epidermal multiciliated cell expressing GFP-IFT20 by targeted injection of mRNA. Yellow dashed box indicates area magnified in A'. (A') A series of stills from a time series from the highlighted area in A. An example anterograde and retrograde train are labeled with blue and pink arrowheads respectively. Such data can be used to investigate dynamic properties of IFT.

Figure 2. Axoneme structure/compartiment analysis

(**A**) A single confocal slice of an epidermal multiciliated cell expressing RFP-CLAMP. The image has been overexposed in post-processing to visualize both the distal enrichments (compartments) and the faint signal along the length of the axoneme. (**B**) The same cell labeled with GFP-MAP7MT (the microtubule binding domain of MAP7). The image has been overexposed in post-processing to visualize both the proximal enrichments (compartments) and the faint signal along some of the more distal axonemes. (**A'** and **B'**) Enlarged images of a single axoneme from the yellow boxes in **A** and **B**.

Figure 3. Basal body localization analysis

(**A** and **B**) A single epidermal multiciliated cell co-expressing Centrin-RFP, which marks basal bodies, and GFP-IFT20, which localizes in peri-basal body pools. (**A'** and **B'**) Computed maps of Centrin and IFT foci respectively. These maps were generated by the 3D object counter plugin in Fiji. Each identified foci is associated with an intensity value that can be used to assess basal body localization at a cell level. Note, that due to the close spacing, some IFT pools are assigned as a single object by the detection algorithm, making this approach infeasible for single basal body analysis

Table 1

A list of some common primary antibodies for investigation of cilia in *Xenopus*.

Antibody (Host)	Company (Cat #)	Structure labeled	IF Usage notes*
Alpha-tubulin (rabbit)	Sigma DM1A (T6199)	Microtubules, including axonemes	1:1000(short protocol), 1:5000 (long protocol)
Acetylated alpha-tubulin (mouse)	Sigma 6-11-B (T6739)	Stable, acetylated microtubules (mainly axonemes in epidermal multiciliated cells)	1:500 (short protocol), 1:1000 (long protocol)
γ -tubulin (mouse)	Abcam (ab27076)	Centrioles/basal bodies	1:1000 (short protocol), 1:2500– 1:5000 (long protocol). Overstaining can lead to non-specific signal
γ -tubulin (rabbit)	Abcam (ab11321)	Centrioles/basal bodies	See notes on mouse γ -tubulin
GFP (chicken)	Abcam (ab13970)	Amplification of GFP signal (e.g. from mRNA injection)	1:500
GFP (rabbit)	Abcam (ab6556)	Amplification of GFP signal (e.g. from mRNA injection)	1:500
RFP (rabbit)	Abcam (ab62341)	Amplification of RFP signal (e.g. from mRNA injection)	1:500

* All concentrations should be empirically determined for experimental conditions. These concentrations are provided as a guide.

Table 2Some fluorescent fusion constructs for ciliary investigation in *Xenopus*

Construct	Structure(s) labeled	Injection dose (per blastomere at 4-cell stage)*
Membrane GFP (EGFP fused to the Ras farnesylation sequence)	Many cell membranes, including axonemes	100pg for standard confocal imaging. Up to 500pg for high-speed approaches
Membrane RFP (Farnesylation sequence fused to monomeric RFP)	Many cell membranes, including axonemes	100pg for standard confocal imaging. Up to 500pg for high-speed approaches
Centrin2-RFP	Centrioles/basal bodies	50–100pg, high doses can be mildly-moderately perturbing
RFP-CLAMP/CLAMP-GFP (Calponin homology and microtubule associated protein)	Distal axonemal compartments, polarized basal body associated rootlet	100pg for visualizing distal ciliary compartment, 50pg for visualizing basal body polarity. (At high doses CLAMP will mark microtubules in the cell body). High doses are cytotoxic
EMTB-3xGFP (also called MAP7MTB-GFP or MAP7-GFP; The microtubule binding domain of Enscinsin (Microtubule associated protein 7) fused to 3X concatemerized GFP)	Proximal axonemal compartment	100pg for distal compartment. This dose will also label cytoplasmic microtubules. High doses may be cytotoxic
GFP-IFT20 (anterograde IFT-B member)	Axonemal IFT trains and peri-basal body cytoplasmic pools of IFT	100–150pg for visualizing trains (can increase to 250pg with no obvious cell or embryo phenotypes)
GFP-IFT80 (anterograde IFT-B member)	Axonemal IFT trains and peri-basal body cytoplasmic pools of IFT	100–150pg for visualizing trains (can increase to 250pg with no obvious cell or embryo phenotypes)
GFP-IFT80 (retrograde IFT-A member)	Axonemal IFT trains and peri-basal body cytoplasmic pools of IFT	100–150pg for visualizing trains (can increase to 250pg with no obvious cell or embryo phenotypes)

* These doses are provided as a starting point and should be empirically tested before use