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Zebrafish Assays of Ciliopathies

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Abstract

In light of the growing list of human disorders associated with their dysfunction, primary cilia have recently come to attention as being important regulators of developmental signaling pathways and downstream processes. These organelles, present on nearly every vertebrate cell type, are highly conserved structures allowing for study across a range of species. Zebrafish, in particular, have emerged as useful organisms in which to explore the consequences of ciliary dysfunction and to model human ciliopathies. Here, we present a range of useful techniques that allow for investigation of various aspects of ciliary function. The described assays capitalize on the hallmark gastrulation defects associated with ciliary defects as well as relative ease of visualization of cilia in whole-mount embryos. Further, we describe our recently developed assay for querying functionality of human gene variants in live developing embryos. Finally, a current catalog of known zebrafish ciliary mutant lines is included. The techniques presented here provide a basic toolkit for *in vivo* investigation of both the biological and genetic mechanisms underlying a growing class of human diseases.

I. Introduction

Primary cilia are highly conserved cellular structures, now understood to be present on the surface of most vertebrate cell types, at least during some point of their life cycle. In contrast to the multiciliated nature of cells bearing motile cilia, cells typically bear a solitary primary cilium that extends from the apical cell surface and is made up of a microtubule-based axoneme, anchored at the base by the basal body. Although the functions of the basal body are not fully understood, one of its roles is to regulate trafficking to the cilium (Jin *et al*., 2010), where proteins undergo transport along the axoneme. This mechanism, termed intraflagellar transport (IFT), is mediated by complexes of IFT and motor proteins that transport proteins between the basal body and the tip of the cilium (Pedersen and Rosenbaum, 2008).

While motile cilia have long been recognized as being of physiological importance in specialized ciliated cell types, such as those lining the lung epithelium, primary cilia (also known as sensory or immotile cilia) have only recently gained prominence as having an important role in the regulation of reception and transduction of cellular signaling (Berbari *et al*., 2009; Goetz *et al*., 2009; Satir *et al*., 2010). Two pathways with major roles in developmental specification, Sonic hedgehog (Shh) and Wnt, require intact functioning cilia for proper transduction (Corbit *et al*., 2005, 2008; Gerdes *et al*., 2007; Huangfu *et al*., 2003). There is some controversy over whether ciliary dysfunction in zebrafish produces defects in Wnt only, Shh only, or both. Although studies have shown Wnt-related defects in basal body suppressed embryos, including convergent extension phenotypes bearing the hallmark of defects in the noncanonical Wnt planar cell polarity (PCP) pathway (Ross *et al*., 2005) and a concomitant slight upregulation of canonical Wnt targets (Gerdes *et al*., 2007), a maternal

zygotic *ift88* mutant showed no sign of Wnt defects, even in the complete absence of cilia (Huang and Schier, 2009). The latter study showed Shh defects exclusively. A recent study, however, showed no sign of Hedgehog signaling defects in *ift57*, *ift88*, and *ift172* mutants (Lunt *et al.*, 2009). Although the exact role of cilia in *in vivo* regulation of these pathways remains elusive, the zebrafish has emerged as a useful model in which to query signaling defects associated with ciliary dysfunction.

A structure that has made this organism particularly useful in the study of cilia is the highly ciliated Kupffer's vesicle (KV), a transient spherical structure appearing at the 5-somite stage and persisting until Prim-5. KV, which plays a role in patterning akin to the roles of the mammalian node, is comprised of cells bearing cilia that beat to direct fluid flow across the structure and contribute to asymmetrical gene expression. Thus, KV offers a useful tool to investigate the extent of ciliary defects, both in the formation of cilia and in their functionality.

In addition to assays of motile ciliary beat function using KV flow as a model, recent efforts have focused increasingly on the use of this system to assay defects in primary cilia (Jaffe *et al.*, 2010). This has paved the way for the development of the zebrafish as a useful model for many of the disorders associated with ciliary dysfunction, the ciliopathies. The ciliopathies are a group of disorders often characterized by pleiotropic defects as a result of mutations in genes that affect ciliary function. Diseases of cilia range from relatively rare syndromes, such as Bardet–Biedl syndrome, Meckel–Gruber syndrome, and Joubert syndrome, to more common clinical entities, such as polycystic kidney disease. Although these disorders vary by their combinations of phenotypes, hallmark characteristics of ciliary dysfunction are often observed across them. These include renal dysfunction, retinal degeneration, obesity, mental retardation, and left–right patterning defects (Cardenas-Rodriguez and Badano, 2009). Many of these features, particularly kidney cysts, are found in zebrafish models of ciliopathies and have been used to identify ciliary mutants in forward genetic screens (Sullivan-Brown *et al.*, 2008; Sun *et al.*, 2004).

A useful feature in the development of zebrafish models for these disorders is the significant genetic overlap between ciliopathies, in which multiple disorders can be caused by mutations in the same gene or multiple genes can contribute to the same disorder. For example, the Meckel–Gruber syndrome genes, *MKS1* and *MKS3*, can contribute causative and modifying alleles to Bardet–Biedl syndrome, respectively, as does one of the nephronophthisis genes, *NPHP6/CEP290* (Leitch *et al.*, 2008). These data are likely representative of the mutual contribution of ciliary genes to similar biological processes that underlie overlapping disease phenotypes. As such, zebrafish ciliary mutants and morphants with defects in various genes often display similar phenotypes, such as the cystic kidneys and body curving defects observed in *ift* mutants (Sun *et al.*, 2004) or the gastrulation defects observed in *BBS* and *NPHP* morphants (Gerdes *et al.*, 2007; Leitch *et al.*, 2008; Zhou *et al.*, 2010); consequently, individual lines may serve as models for multiple ciliopathies.

Zebrafish models of human ciliopathies have been established in lines with null mutations in IFT or basal body genes as well as in wild-type embryos by suppressing protein expression of ciliary genes via injection of translation- or splice-blocking antisense morpholinos. For example, several mutant lines and a morpholino have been developed that target the zebrafish polycystic kidney disease ortholog, *polycystin-2* (Sun *et al.*, 2004). Morphant embryos develop pronephric cysts early in development and enlarged pronephric ducts (Obara *et al.*, 2006; Schottenfeld *et al.*, 2007; Sun *et al.*, 2004). Similarly, two lines have been identified through forward genetic screens with mutations in Joubert syndrome genes, *arl13b* and *cc2d2a* (Owens *et al.*, 2008; Sun *et al.*, 2004). These lines provide useful

developmental systems in which to investigate phenotypes resulting from the loss of these genes. In addition to ciliary mutants, zebrafish ciliopathy models have been generated using targeted gene knockdown via morpholino injection. Each of the 14 Bardet–Biedl syndrome genes, for example, has been investigated in this way with suppression resulting in early developmental phenotypes, including perturbed gastrulation movements (Gerdes *et al.*, 2007; Zaghloul *et al.*, 2010). In addition to providing insight as to the developmental ramifications of perturbing BBS gene expression, we have recently demonstrated the power of this system to model human BBS gene functionality. Using human BBS mRNA to rescue the morphant phenotypes, this system allows for interrogation of the functional consequences of any introduced variant (Leitch *et al.*, 2008; Zaghloul *et al.*, 2010). This provides amethod of "humanizing" the zebrafish to use it as a model for human disease gene function and variability. The ability of human variants to function in zebrafish represents a tremendous advantage of this system due to the possibility of exploring on a large scale the consequences of gene variation on developmental phenotypes in an *in vivo* vertebrate system with physiological relevance to humans. This method, described below, adds power of the zebrafish model.

II. Methods

A. Visualization of Cilia in Zebrafish Embryos

Visualization of cilia in ciliary morphant or mutant embryos is often a necessary first step in verifying a ciliary defect. Because ciliary structural proteins are highly conserved, reagents developed for use in mammalian systems can be readily applied to zebrafish. This includes common antibodies used for specific labeling of individual cilia.

1. Whole-mount Fluorescence Imaging of Cilia—The following protocol was developed for whole-mount immunostaining of cilia using a mouse anti-acetylated α-tubulin primary antibody (Sigma T6793):

1. Embryos cultured in embryo medium (Westerfield, 2000) should be fixed at the desired embryonic stage. We have found that use of Dent's fixative (80% MeOH, 20% DMSO) is more effective for labeling of cilia compared to 4% paraformaldehyde. Fix embryos at room temperature for 2 h to overnight. To dehydrate, transfer embryos to 100% MeOH for at least 2 h before proceeding and store up to several weeks at −20 °C.

   Rehydrate embryos sequentially for 10 min in each wash: 75% MeOH in PBS, 50% MeOH in PBS, 25% MeOH in PBS, and 100% PBS. Wash once in immunostaining buffer (1× PBS, 0.1% Tween 20, 1% BSA) for 10 min.

2. Transfer embryos to blocking buffer (10% sheep serum, 1% BSA, 1× PBS) for 1 h at room temperature. Incubate embryos in 1° antibody (1:500 in blocking buffer) solution overnight at 4 °C.

3. To remove 1° antibody solution, wash embryos in immunostaining buffer twice, 10 min each. Transfer embryos to 2° antibody solution – goat anti-mouse (Invitrogen A-10667) – at 1:1000 in blocking buffer. Incubate for 1 h at room temperature. Wash embryos in immunostaining buffer twice, 10 min each.

4. Embryos can be incubated in DAPI (1:5000) solution for 10 min, at this point, and then washed twice, 10 min each, in immunostaining buffer.

5. Following the protocol, embryos are stored at 4 °C in immunostaining buffer or 100% MeOH until imaging.

6. For imaging, place individual embryo in a depression slide, covered with Permount. Quickly, place a coverslip over the depression well and slide the coverslip over embryo until the desired orientation is attained (Fig. 1A). Embryos should be imaged using confocal microscopy (Fig. 1B).

2. Visualization of Kupffer's Vesicle—KV can be visualized in segmentation and early pharyngula stage embryos from five somites until prim-5 (24 hpf). This heavily ciliated spherical structure makes it ideal for visualization of the presence/absence of cilia, ciliary length, and ciliary orientation. KV is best observed by dual antibody staining of both cilia (via anti-acetylated α-tubulin antibody) and the apical surface of epithelial KV cells (via anti-atypical protein kinase C antibody; Amack *et al.*, 2007). This is carried out using the immunostaining protocol described above with two minor adjustments:

1. Include the second, anti-aPKC antibody (Santa Cruz Biotechnology SC-216) in the primary antibody solution for overnight incubation at 4 °C. The final concentration should be 1:100 while the anti-acetylated α-tubulin concentration remains at 1:500.
2. Include a second, goat anti-rabbit (Invitrogen A-11012) antibody in the secondary antibody solution. Both secondary antibodies should be used at 1:1000.

B. Phenotypic Assays of Ciliary Dysfunction

Though the molecular mechanisms underlying the early developmental steps giving rise to ciliopathy phenotypes have yet to be fully deciphered, assessment of zebrafish embryos early in development provides insight into defects in patterning and specification that may give rise to later organ-specific defects. The most prominent of these early defects are those associated with perturbed PCP signaling and resulting defects in convergent extension movements. Embryos with PCP defects exhibit characteristic phenotypes at the segmentation stages. We have developed a suite of assays to systematically assess these phenotypes to determine the extent of the defect conferred by ciliary dysfunction.

1. Live Embryo Phenotypes—Defective convergent extension, as a result of disrupted PCP signaling, produces aberrant movement of cells during gastrulation. The result is perturbed specification of anatomical structures during segmentation stages, which may give rise to later organogenesis defects. This assay of ciliopathy mutants and morphant phenotypes focuses on phenotypes established in PCP signaling mutants (Sepich *et al.*, 2000).

1. Inject wild-type embryos at the one- to two-cell stage with a morpholino targeting a ciliary gene of interest or, alternatively, collect embryos from matings of a ciliary mutant line.
2. Culture embryos in embryo medium for 11–14 h until the 8–10 somite stage.
3. Embryos can be scored directly in the culture dish, without dechorionation. We have devised a system of scoring and categorization into classes based on the extent and number of defects. Observed convergent extension defects are: shortening of the body axis (from lateral view), reduced head size (lateral view), broadening of the notochord (from dorsal view), kinking of the notochord (dorsal view), wide somites (dorsal view), and thin somites (dorsal view). Embryos considered "Class I" or moderately affected are characterized by the presence of one very severe defect or two moderate defects (Fig. 2). Embryos classed as "Class II" or severely affected are characterized by the presence of three or more defects of moderate or extreme severity (Fig. 2). An additional class, "Class Y," was introduced to denote embryos in which gastrulation epiboly movements were not completed.

2. Measurement of Convergent Extension Defects by ISH—To verify and quantify the extent of the convergent extension defect, we have developed a method by which to measure the most prominent convergent extension defect, shortening of the body axis.

1. Fix morphant or mutant embryos at 8–10 somites in 4% paraformaldehyde overnight at 4 °C. Transfer to 100% MeOH. Store at −20 °C for at least 2 h or as long as several months.
2. Perform whole-mount *in situ* hybridization according to standard protocol (Thisse and Thisse, 2008) using a riboprobe cocktail of *myoD*, *krox20*, and *pax2* to label adaxial mesodermal cells, somites, anterior neural structures, and pronephric mesoderm, respectively. Individual embryos must be flat-mounted and imaged with consistent magnification across all samples.
3. On each embryo image, measure the distance from the first to the last (anterior to posterior) appreciable somite. Measurements for embryos in each injection or mutant treatment, or controls, are normally distributed when plotted. Each curve can then be compared to others as a means of quantification of the average shortened body axis defect for each group (Fig. 3).

3. Epiboly Cell Tracking—Convergent extension movements occur during epiboly and are characterized by convergence of cells on the dorsal axis and extension along that axis. We have developed a method to track the movement of cells throughout gastrulation to observe the process of convergence and extension.

1. After injection of morpholinos into 1- or 2-cell stage embryos or collection of mutant embryos, culture embryos in embryomedium until the 16-cell stage. Inject a single marginal blastomere (see Fig. 4A) with 1 nL of 10,000-MW dextran-conjugated fluorescent lineage tracer (Invitrogen D-22910). *Note*: At the 16-cell stage, it is impossible to determine the dorsal from the ventral axis of the embryo. Therefore, only 50% of lineage-tracer-injected embryos will express fluorescence on the dorsal part of the embryo. This is easily determined at epiboly stages when the dorsal axis is easily distinguishable due to formation of the shield.
2. Culture embryos in embryo medium until 30% epiboly (4.67 hpf). Position embryos in embryo medium on an agarose imaging dish (5% agarose with semispherical wells indented in agarose, one per embryo) such that the lateral view of the embryo is facing up.
3. Image embryo using a fluorescence-capable dissecting microscope and camera at 30% epiboly. Without moving the embryo, continue to culture the embryos in the dish until 50% epiboly. Image the embryo again. Repeat at 65 and 75% epiboly (Fig. 4B).
4. On comparison to each other, control embryos at 30, 50, 65, and 75% epiboly will exhibit a distinct migration of fluorescent cells toward the dorsal axis and an elongation along the axis in a narrowing arc. Ciliary morphants and mutants exhibit a defect in this process characterized by a delay or failure of cells to migrate to and along the dorsal axis. This can be easily visualized by the failure of the distinctive fluorescent arc to form by 75% epiboly.

4. Assaying Human Gene Variation in Zebrafish—The zebrafish model has many benefits, particularly in the areas of functional genetics and disease. A considerable advantage is the functionality of exogenous human genes in zebrafish embryos. We have developed a protocol for suppression of endogenous embryonic genes and introduction of the homologous human gene to assay for altered functionality conferred by known variants.

1. Prepare cDNA of the human ciliary gene of interest by subcloning into the pCS2+ vector. For analysis of mutation effect, variants can be introduced by site-directed mutagenesis via PCR (Quick Change, Stratagene). Linearize the cDNA plasmid with the appropriate restriction enzyme and transcribe using SP6 polymerase (mMessage mMachine Kit, Ambion).
2. Prepare a range of concentrations for a ciliary morpholino of interest with known efficacy in zebrafish. It is imperative for a translation-blocking morpholino that a portion of the sequence is 3′ of the ATG start site. Otherwise, the morpholino may bind to and prevent translation of the co-introduced exogenous mRNA as well.
3. Starting with a morpholino concentration of moderate effect (i.e., approximately 50–60% of embryos exhibiting phenotypes), co-inject morpholino with three different concentrations of wild-type human mRNA (typically, 50, 100, and 125 pg/nL and 1 nL delivered per injection) at the one- to two-cell stage. Culture embryos until desired stage for phenotyping (8–10 somites for PCP phenotypes) in embryo medium. If scoring PCP phenotypes, the proportion of unaffected, Class I, and Class II embryos should be quantified for each injection combination, as well as morpholino alone and uninjected controls. On introduction of wild-type mRNA, a curve of efficacy for the differing mRNA concentrations can be determined based on the proportion of unaffected embryos. An increase in unaffected embryos with co-injection indicates a rescue of the morphant phenotype. Based on this curve, the optimal rescue concentration can be determined using either one of these concentrations or another concentration within this range.
4. Once the concentrations for both morpholino and mRNA have been determined for wild-type rescue, the same concentrations are used for subsequent co-injection of morpholino and mutant mRNA. Inject the morpholino/mRNA cocktail at the one- to two-cell stage and culture embryos in embryo medium until the desired stage for phenotyping. *Note*: This stage must be exactly the same for mutant mRNA rescue embryos and wild-type rescue embryos. Embryos should be categorized by phenotypic class with quantification of the proportion of unaffected, Class I, and Class II embryos.
5. Compare the quantification of the mutant rescue experiment to the wild-type rescue experiment to determine if mutant mRNA behaves similarly to wild type or causes a perturbed phenotype. Mutant mRNAs that rescue the morphant phenotype in a manner similar to wild type are deemed benign or wild type. Those that do not rescue the morphant phenotype, producing defects similar to morpholino alone, have probably lost all functionality and are, therefore, functionally null. An mRNA that partially rescues the phenotype is likely to be a hypomorphic or partial loss-of-function variant. Finally, those variants that do not rescue the phenotype and produce defects more severe than morpholino alone may be behaving in a dominant negative fashion, a possibility that can be verified by injection of mutant mRNA alone. Categorization of variants in this way can be used to assay a large series of variants and to assign functionality to ambiguous alleles identified in the context of human genetic disease.

III. Zebrafish Ciliary Mutant Lines

Although we lack mutant lines for most ciliary genes, 11 zebrafish lines with mutations in known ciliary genes and 1 transgenic reporter line have been developed. Most of these are readily available via the Zebrafish International Research Center (http://zebrafish.org/zirc/home/guide.php).

1. *oval* and *MZoval* (gene: *ift88*, allele(s): *tz288*): Both the mutant line bearing the point mutation (*ovl*) (Tsujikawa and Malicki, 2004) and the line with the maternal zygotic allele (MZ*ovl*) (Huang and Schier, 2009) display a loss of cilia but have intact basal bodies. The transduction of Shh signaling differs, however, in these lines with the former embryos showing no effect on Shh (Lunt *et al.*, 2009), whereas the maternal zygotic mutant embryos have dampened Shh (Huang and Schier, 2009). Embryos do display morphological defects associated with ciliary dysfunction including degeneration of photoreceptor outer segments (Tsujikawa and Malicki, 2004) and disrupted neural and somite patterning.

2. *fleer* (*ift70*, *m477*): Embryos produced with this point mutation exhibit hallmark pleiotropic features associated with ciliary defects, including kidney cysts, randomized left–right asymmetry, hydrocephalus, and rod outer segment defects (Pathak *et al.*, 2007). Further, this mutant exhibits shortened cilia with reduced beat amplitude, possibly caused by defects in tubulin polyglutamylation. The *fleer* locus is homologous to the *C. elegans* DYF1 gene.

3. *seahorse* (*lrrc6l*, *hi3308/fa20r/tg238a*): Kidney cysts are highly prevalent in these mutants, as well as the characteristic body curvature and left–right patterning defects often seen in ciliary mutants (Kishimoto *et al.*, 2008; Sun *et al.*, 2004). Cilia are present in *seahorse* embryos, although with reduced motility (Serluca *et al.*, 2009), which suggests that this gene is not necessary for ciliogenesis. However, canonical Wnt signaling is suppressed while noncanonical Wnt is upregulated, indicating an important role of the gene in the regulation of this pathway (Kishimoto *et al.*, 2008).

4. *Elipsa* (*traf3ip1*, *m649/tp49d*): *elipsa* embryos exhibit kidney cysts, body axis curving, and defects in retinal development, as well as defects in ciliogenesis (Malicki *et al.*, 1996; Omori *et al.*, 2008). The protein, homologous to the *C. elegans* DYF-11, localizes to basal bodies and axonemes in otic vesicle and olfactory epithelial cells, whereas in retina the protein is only found in basal bodies. The protein plays an important role in linking the IFT particle to the membrane-associated small GTPase Rab8 via its interaction with Rabaptin5 (Omori *et al.*, 2008).

5. *switch hitter* (*lrrc50*, *tm317/hu255*): *swt* mutant embryos develop downward body curving, left–right patterning defects, and severe pronephric cysts (Sullivan-Brown *et al.*, 2008). Although mutants exhibit cilia, *lrrc50* is necessary for ciliary motility in KV and thus gives rise to the patterning defect (van Rooijen *et al.*, 2008). This gene is mutated in primary cilia dyskinesia (PCD) patients (Loges *et al.*, 2009), which suggests the potential of this mutant as a model for the disorder.

6. *qilin* (*qilin*, *hi3959A*): Identified in a forward genetic screen, mutant embryos develop kidney cysts and body curving (Sun *et al.*, 2004). The *C. elegans* ortholog, DYF-3, undergoes IFT, thereby indicating a role for this gene in promoting formation of sensory cilia (Ou *et al.*, 2005).

7. *curly* (*ift57*, *hi3417*): This mutation results in loss of cilia (Lunt *et al.*, 2009) and produces renal cysts and body curving (Sun *et al.*, 2004) as well as shortened photoreceptor outer segments with decreased opsin and retinal degeneration by 5 dpf (Krock and Perkins, 2008). Since no defects in Shh signaling were observed in *ift57* mutants (Lunt *et al.*, 2009), the mutant phenotype is attributed to the role of *ift57* in regulating kinesin-II dissociation from the IFT particle (Krock and Perkins, 2008).

8. *larry* (*ift81*, *hi409Tg*) and *moe* (*ift172*, *hi2211Tg*): Although embryos exhibit kidney cysts and body curvature, neither cilia formation nor Shh signaling is affected (Lunt *et al.*, 2009; Sun *et al.*, 2004) in these mutants. However, outer segments of photoreceptors do not form, giving rise to a severe retinal defect (Sukumaran and Perkins, 2009).

9. *pkd2/curly up* (*polycystin-2*, *hi4166Tg/hu2173/tc321/tg226d/tp85a/ty30*): Although these mutants show body curvature and left–right patterning defects, no kidney cysts develop and ciliogenesis does occur (Sun *et al.*, 2004). However, morpholino suppression of the gene produces cysts and hydrocephalus, which suggests that the gene may also be involved in regulation of these phenotypes (Obara *et al.*, 2006; Schottenfeld *et al.*, 2007; Sun *et al.*, 2004).

10. *scorpion* (*arl13b*, *hi459Tg*): Amodel for Joubert syndrome, cilia do not develop in mutant embryos, resulting in characteristic ciliary phenotypes including kidney cysts, body curvature, and left–right patterning defects (Sun *et al.*, 2004). The protein localizes to the cilium, a feature that is required for its proper function (Duldulao *et al.*, 2009).

11. *sentinel* (*cc2d2a*, *w38*): Also an ortholog of a Joubert syndrome gene, *sentinel* mutants exhibit pronephric cysts and body curvature defects (Owens *et al.*, 2008). The mammalian ortholog localizes to the basal body and interacts with another ciliopathy-associated protein, CEP290 (Gorden *et al.*, 2008).

12. *Arl13b–GFP transgenic* (*Tg(βact::Arl13b–GFP)*): This line has been developed for visualization of fluorescent cilia in live embryos. Embryos produce an Arl13b–GFP fusion protein under the control of the β-actin promoter, which allows for strong expression across all tissues. GFP-expressing cilia are observed in the axonemes of motile cilia (KV, floor plate) and primary cilia (ectoderm, notochord, somites, and neural tube) (Borovina *et al.*, 2010).

IV. General Considerations/Future Development

Zebrafish have proven to be an extremely useful model for the study of ciliary biology due to the developmental ramifications of ciliary dysfunction. It is important to note that special consideration must be applied when assessing the defects associated with ciliary knockdown. For example, defective gastrulation movements are a hallmark of disrupted ciliary function and have been observed by morpholino-induced knockdown as well as in various mutant strains. However, this is also a common side effect of morpholino-associated toxicity, making imperative the need for verification of knockdown specificity by rescue. The use of gastrulation defects as a phenotypic readout for ciliary gene knockdown highlights the challenges of morpholinos in comparison with genomic manipulation. As more mutant lines become available, the concerns raised with morpholinos will be alleviated although other factors will remain as issues. Masking of a ciliary phenotype due to other genomic factors is a potential problem that may result in milder phenotypes or none at all. Morpholino injection, a more acute treatment, will therefore still remain a useful technique for knockdown as it can circumvent this problem.

As zebrafish tools for the study of ciliary dysfunction, such as mutant lines, antibodies, and cultured cells, continue to be developed, the advantages of this system for analysis of ciliary biology will expand. For example, zebrafish are not inbred strains and therefore exhibit considerable phenotypic variability. Considering the extent of variability across human ciliopathies and the complexity of those disorders, zebrafish present the opportunity to investigate ciliopathies in the context of genomic variability. Further, the continued development of transgenic reporter lines and transient reporter fusion proteins will allow for

direct visualization of ciliary processes *in vivo*. This type of manipulation is elegantly demonstrated by the recent observation of centrosomal movement during gastrulation *in vivo* through the use of GFP-labeled centrosomal proteins (Sepich *et al.*, 2011). Similar studies can track ciliogenesis, ciliary orientation, and movement of ciliary proteins through various stages of development. Detailed cellular studies such as these will capitalize on the many advantages of the zebrafish to make it an indispensable tool in the study of ciliary biology.

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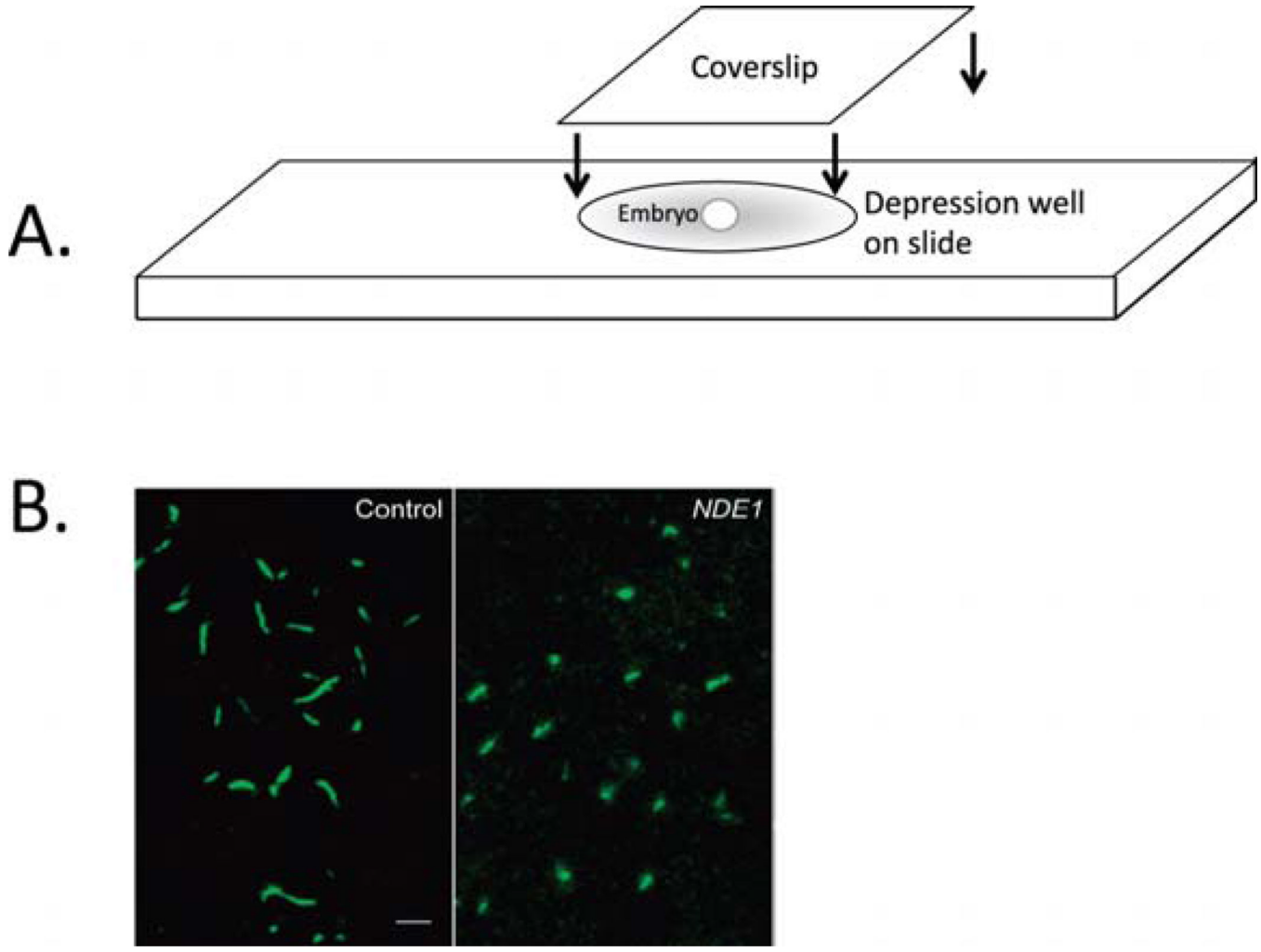

Fig. 1.

Visualization of cilia in whole-mount fixed embryos. (A) After immunostaining for acetylated α-tubulin,whole embryos are mounted in depression well slides and covered with a coverslip. Once placed in the well, the embryo is rotated by gentle rotation of the coverslip until the desired orientation for imaging is achieved. (B) Confocal imaging of cilia in Kupffer's vesicle in immunostained, mounted embryos. Cilia conformation and length can be easily observed. In this example, control embryos exhibit normal length cilia, whereas *NDE1*-overexpressing embryos exhibit shorter cilia (modified from Kim *et al.*, 2011). (Permission from Nat. Cell Biol., License #: 2721450638335.) (For color version of this figure, the reader is referred to the web version of this book.)

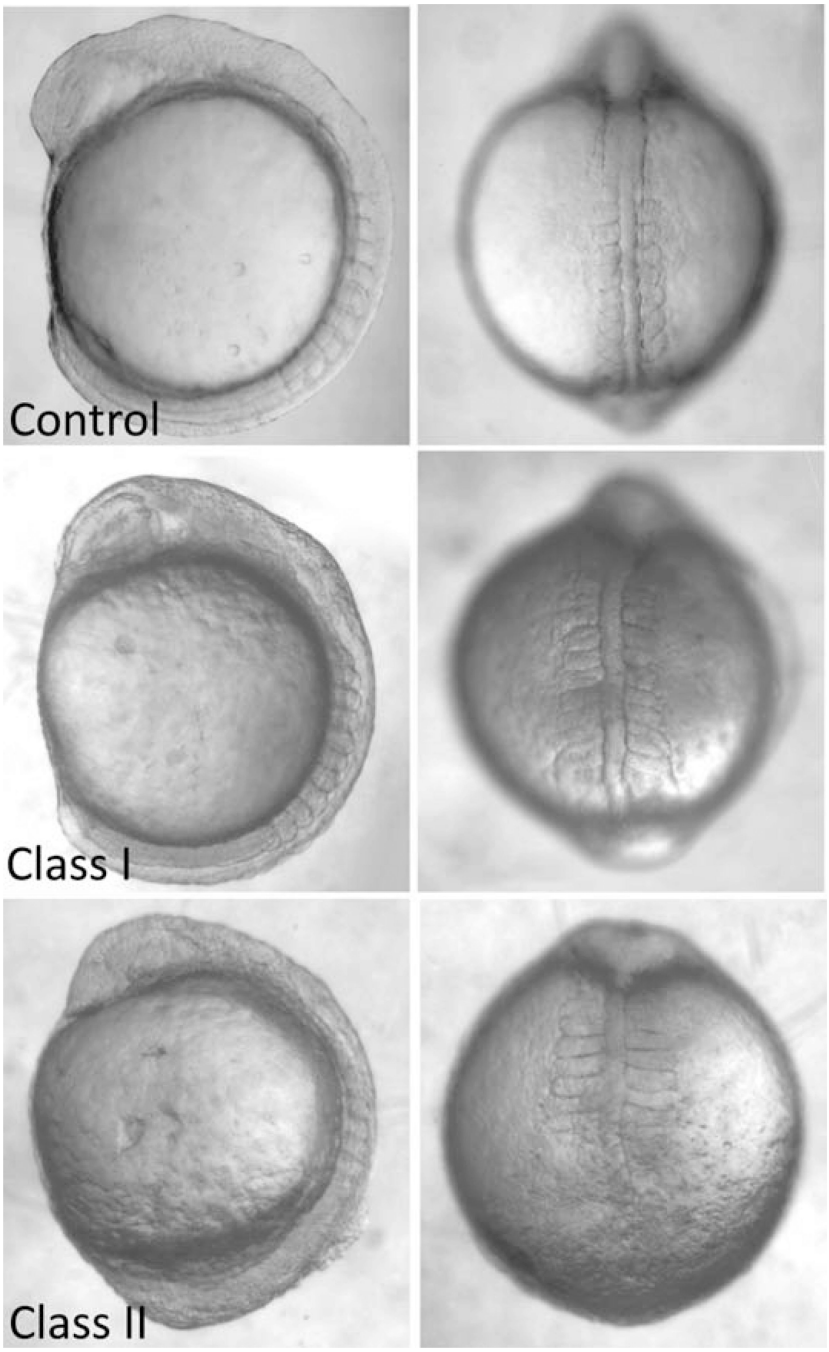

Fig. 2.

Gastrulation defect phenotypes associated with ciliary dysfunction. Embryos with disrupted ciliary gene expression (by morpholino suppression in this example) exhibit gastrulation defects that manifest at somitic stages as shortened body axes, widened and kinked notochords, and widened and thinned somites. Embryos can be categorized, based on the extent and severity of the phenotypes, into Class I (moderately affected) and Class II (severely affected).

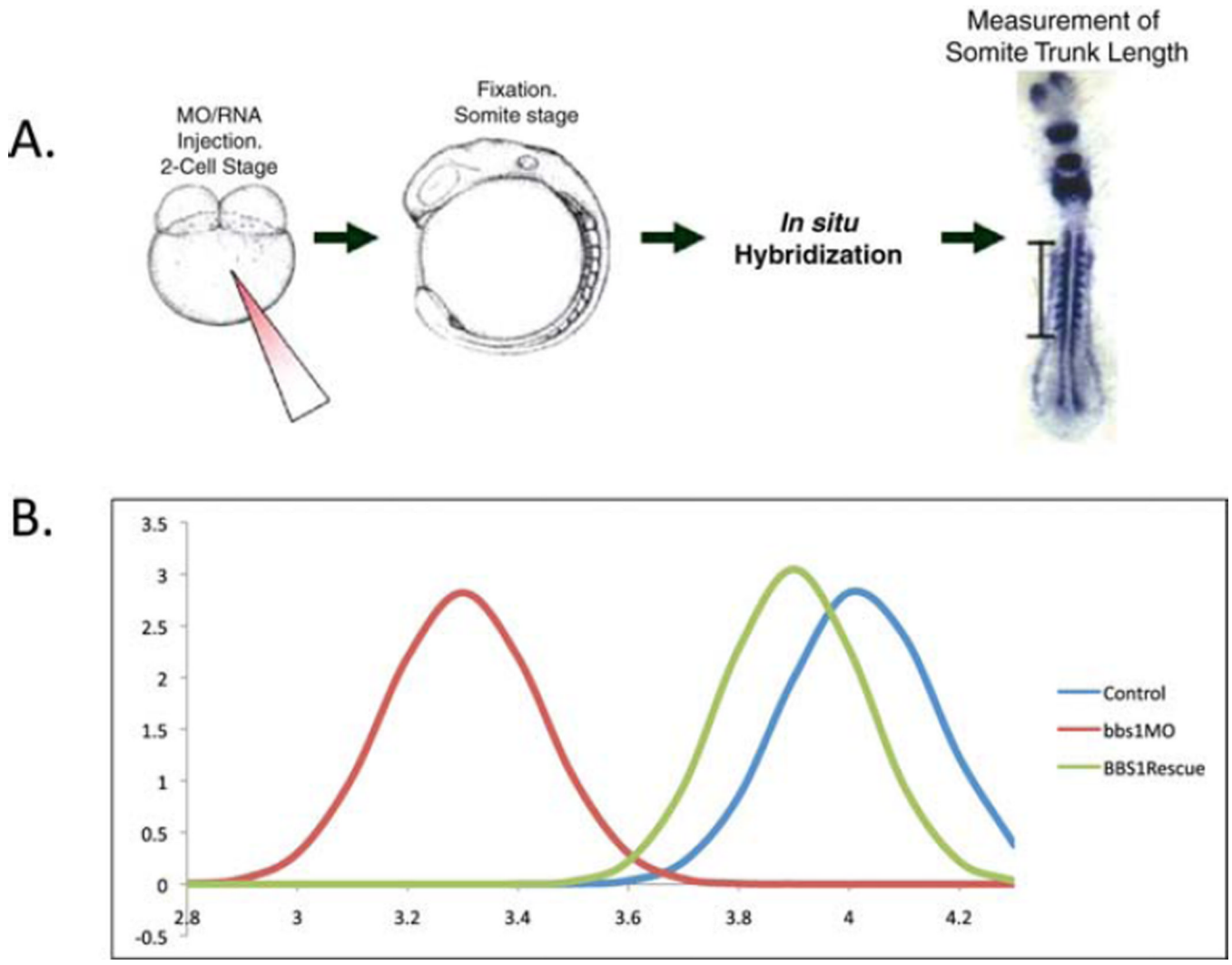

Fig. 3.

Measurement of somite-trunk length at somite stages. (A) Shortening of the body axis, the most prominent defect observed in ciliary mutant and morphant embryos at somitic stages, is quantified by labeling of somite stage embryos with markers of somites and notochord, and anterior/posterior markers. Embryo trunk length is measured as the distance from the first to the last somite (bar). (B) *BBS1* morpholino knockdown and rescue by wild-type mRNA. For each injection treatment, the trunk lengths of groups of embryos are measured; the observed lengths are normally distributed. Defects in ciliary function, shown here by treatment with *bbs1* morpholino, produce shorter body axes compared to controls, and the morphant phenotype can be rescued by co-injection of *BBS1* wild-type mRNA. (For color version of this figure, the reader is referred to the web version of this book.)

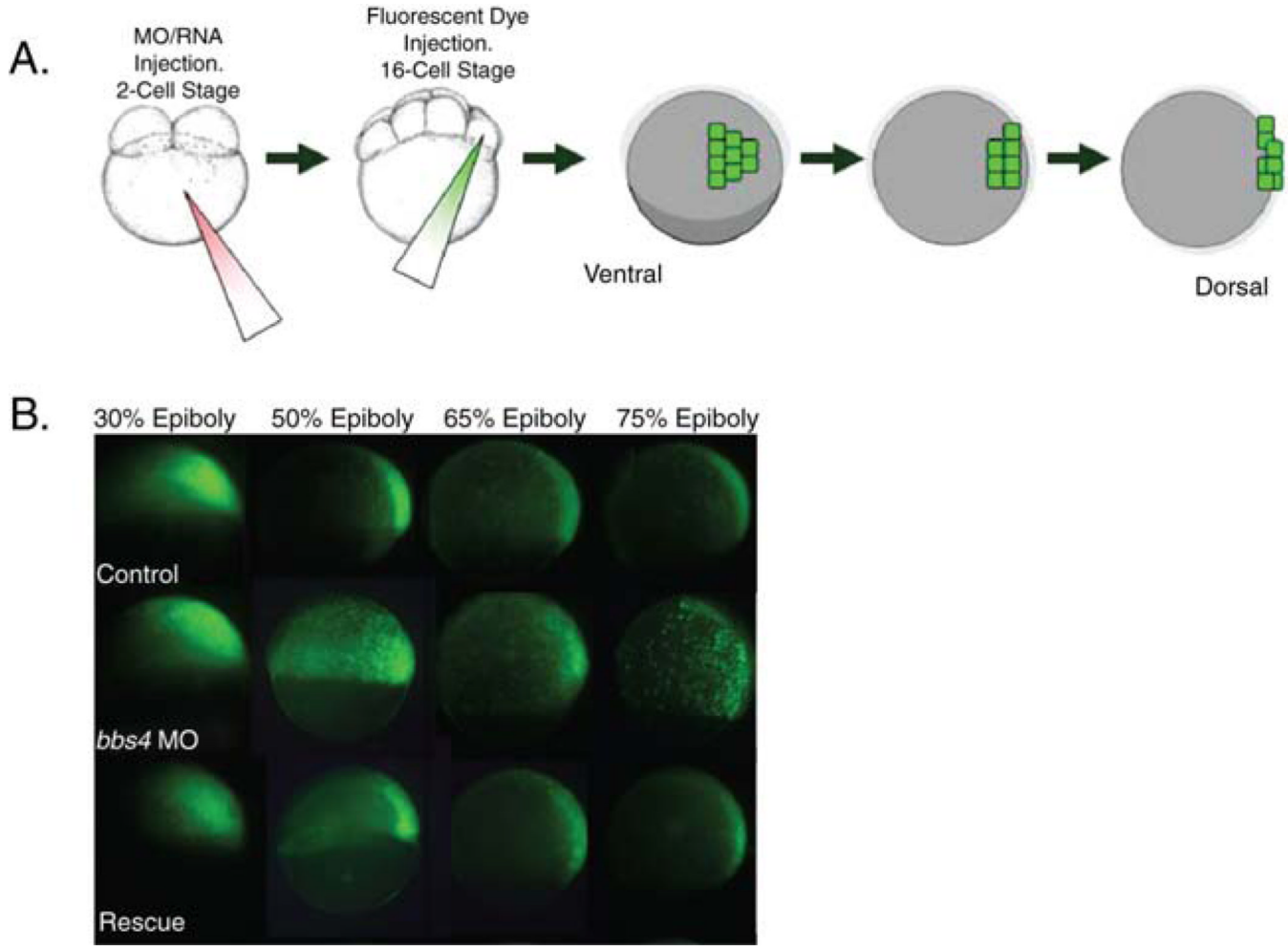

Fig. 4.

Labeling of cells and tracking of movements through gastrulation. (A) After embryo treatment with morpholino or RNA injection at the 1- to 2-cell stage, fluorescent dye is injected into a single marginal blastomere at the 16-cell stage. At gastrulation stages, fluorescing cells can be tracked to monitor movement. (B) Ciliary morphant embryos (*bbs4* shown here) exhibit defects in the characteristic movements whereby cells converge on and extend along the dorsal axis. This defect can be rescued by co-injection of wild-type mRNA (modified from Zaghloul *et al.*, 2010). (Permission from Nat. Cell Biol., License #: 2721450638335). (See color plate.)