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A Guide to Analysis of Cardiac Phenotypes in the Zebrafish Embryo

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Abstract

The zebrafish is an ideal model organism for investigating the molecular mechanisms underlying cardiogenesis, due to the powerful combination of optical access to the embryonic heart and plentiful opportunities for genetic analysis. A continually increasing number of studies are uncovering mutations, morpholinos, and small molecules that cause striking cardiac defects and disrupt blood circulation in the zebrafish embryo. Such defects can result from a wide variety of origins including defects in the specification or differentiation of cardiac progenitor cells; errors in the morphogenesis of the heart tube, the cardiac chambers, or the atrioventricular canal or problems with establishing proper cardiac function. An extensive arsenal of techniques is available to distinguish between these possibilities and thereby decipher the roots of cardiac defects. In this chapter, we provide a guide to the experimental strategies that are particularly effective for the characterization of cardiac phenotypes in the zebrafish embryo.

I. Introduction

Cardiac birth defects are found in as many as 1 in 100 infants (Hoffman and Kaplan, 2002). The prevalence of these defects provides a strong motivation for studies of the molecular mechanisms responsible for normal cardiac form and function. Although it is suspected that many congenital heart diseases have a genetic basis (Pierpont *et al.*, 2007), only a modest number of genes have been identified as disease loci (Bruneau, 2008; Ransom and Srivastava, 2007). Therefore, an increasingly growing community of investigators is eager to employ effective methods for determining the precise roles of essential genes during cardiogenesis.

In this regard, the zebrafish is an ideal model organism for studying the genetic regulation of heart development (Schoenebeck and Yelon, 2007). The zebrafish embryo is externally fertilized and transparent, allowing easy visualization of the heart and its contractility. The simple architecture of the zebrafish heart also aids analysis: it is composed of two major chambers, a ventricle and an atrium, each of which contains an inner layer of endocardium and an outer layer of myocardium. The particular characteristics of each cardiac tissue can be readily visualized with cellular resolution, facilitating distinctions between normal and aberrant phenotypes. In addition, since the zebrafish embryo does not require a functional cardiovascular system for its survival (Pelster and Burggren, 1996), cardiac defects can be analyzed in detail throughout embryogenesis.

To date, hundreds of studies have taken advantage of these benefits of the zebrafish for identifying crucial cardiac genes. Classical genetic screens have unearthed a prodigious number of mutations causing a variety of defects in the embryonic zebrafish heart (Alexander *et al.*, 1998; Beis *et al.*, 2005; Chen *et al.*, 1996; Stainier *et al.*, 1996; Warren *et al.*, 2000). Notably, genetic screens have been particularly effective at identifying two large categories of mutant phenotypes: mutations that disrupt the initial formation of the midline heart tube (Alexander *et al.*, 1998; Chen *et al.*, 1996; Stainier *et al.*, 1996) and mutations

that disrupt cardiac function (Chen *et al.*, 1996; Stainier *et al.*, 1996; Warren *et al.*, 2000). Additionally, knockdown of gene function via injection of gene-specific morpholinos has been instrumental in revealing the functions of numerous cardiac genes (Bill *et al.*, 2009; Eisen and Smith, 2008). Small molecule screens have also contributed to the identification of essential cardiac pathways (Kaufman *et al.*, 2009; Peal *et al.*, 2010): for instance, several compounds have been shown to disrupt cardiac valve morphogenesis (Scherz *et al.*, 2008), and others have been shown to influence cardiac action potential (Milan *et al.*, 2003, 2009).

Whether caused by mutations, morpholinos, or small molecules, cardiac defects typically manifest as pericardial edema and faulty blood circulation that are evident by 48 h postfertilization (hpf), if not earlier. This common phenotype can result from a wide variety of origins including defects in the specification or the differentiation of cardiac progenitors (Fig. 1A,B); errors in the morphogenesis of the heart tube, the cardiac chambers, or the atrioventricular canal (AVC) (Fig. 1C–E); or problems with establishing proper cardiac function. Recent studies have pioneered a variety of experimental strategies for distinguishing between these possibilities and thereby deciphering the roots of cardiac defects. In this chapter, we provide a guide for investigators who aspire to use state-of-the-art techniques to characterize cardiac phenotypes in zebrafish embryos.

II. Defects in Heart Size

Heart size defects can readily be assessed in the zebrafish embryo: an enlarged heart, a shrunken heart, or an apparent absence of cardiac tissue can easily be detected even on a dissecting microscope (Chen *et al.*, 1996; Stainier *et al.*, 1996). The perceptible size of the organ is a product of the number of cardiomyocytes and the morphology and arrangement of those individual cells. This section focuses on the possible causes of production of an inappropriate number of cardiac cells.

To determine whether heart size defects are a function of cell number defects, it is useful to employ a reporter transgene, *Tg(cmlc2:DsRed2-nuc)* (Mably *et al.*, 2003), that expresses DsRed in the nuclei of all differentiated cardiomyocytes. Nuclear localization of the fluorescent signal facilitates counting of cardiomyocytes in individual embryos; combining this technique with the use of an antibody recognizing an atrium-specific myosin heavy chain (S46; anti-atrial myosin heavy chain (Amhc) (Berdougo *et al.*, 2003)) allows quantitative analysis of the number of cells in each chamber (Schoenebeck *et al.*, 2007). However, nuclear DsRed expression is most robust and resolvable in embryos that are 48 hpf or older, since the DsRed protein requires approximately 24 h to properly fold and localize after initial *cardiac myosin light chain 2 (cmlc2)* promoter activation (Baird *et al.*, 2000; Bevis and Glick, 2002; Lepilina *et al.*, 2006). To count cells at earlier stages, an alternate approach visualizes cardiomyocyte nuclei with an antibody recognizing the transcription factor Mef2, which is detectable in cardiomyocytes as early as 24 hpf (Targoff *et al.*, 2008).

Increased or decreased numbers of cardiomyocytes could be the result of defects in the specification of cardiac progenitor cells. Fate maps of the late blastula have demonstrated that cardiac progenitors arise from bilateral multipotential zones within the embryonic margin (Fig. 1A) (Keegan *et al.*, 2004). During gastrulation, the cardiac progenitors migrate to reside in bilateral fields in the anterior lateral plate mesoderm (ALPM) (Fig. 1B) (Keegan *et al.*, 2004; Schoenebeck *et al.*, 2007). Gastrula fate maps have shown that the dimensions of these heart fields correspond well with the expression pattern of *hand2* in the ALPM (Schoenebeck *et al.*, 2007). Differentiation of progenitors into cardiomyocytes begins around the 14 somite stage, as indicated by the expression of myocardial markers such as *cmlc2* (Yelon *et al.*, 1999). Ventricular and atrial progenitors are spatially organized both

within the lateral margin and the ALPM (Fig. 1A,B) (Keegan *et al.*, 2004; Schoenebeck *et al.*, 2007). Once they differentiate, ventricular and atrial cardiomyocytes can be distinguished by molecular markers, such as *ventricular myosin heavy chain (vmhc)* and *amhc* (Fig. 1C) (Berdougo *et al.*, 2003; Yelon *et al.*, 1999). Thus, a failure to produce the right numbers of differentiated ventricular or atrial cardiomyocytes could reflect an alteration in the number of cardiac progenitors selected from within a multipotential zone, a change in the size of the heart fields within the ALPM, or a modification of the effective production of differentiated cells by the heart fields.

Fate mapping techniques can be employed to distinguish between these possibilities. Construction of a fate map in mutant, morphant, or drug-treated embryos can reveal alterations in the size of the cardiac progenitor pool, the organization of the progenitors, and their productivity in terms of the number of cardiomyocytes generated (Keegan *et al.*, 2004, 2005; Schoenebeck *et al.*, 2007; Thomas *et al.*, 2008; Waxman *et al.*, 2008). In these experiments, embryos are injected at the one-cell stage with a caged fluorescein dextran lineage tracer that can subsequently be photoactivated in selected cells using a laser (Keegan *et al.*, 2004; Schoenebeck *et al.*, 2007). The locations of the labeled cells are noted, and their contributions to the myocardium are assessed at later stages. In addition to determining whether the progeny of selected cells become ventricular and/or atrial cardiomyocytes, it is possible to count the number of cardiomyocytes derived from the labeled cells, particularly when using anti-fluorescein immunohistochemistry to detect the lineage tracer. This sensitive technique can resolve differences from the wild-type fate map and thereby indicate the origin of a defect in heart size: cardiac progenitors might be missing from their usual locations or might be found in atypical locations, ventricular and atrial progenitors might be disorganized, and individual progenitors might give rise to fewer or more cardiomyocytes than usual.

Several studies have utilized cell counting and fate mapping techniques to characterize heart size defects and their origins in zebrafish embryos (Schoenebeck *et al.*, 2007; Thomas *et al.*, 2008; Waxman *et al.*, 2008). In one example, this combination of experimental strategies was used to show that hedgehog (Hh) signaling promotes the specification of cardiac progenitor cells (Thomas *et al.*, 2008). In zebrafish *smoothened* mutants, Hh signaling is disrupted by the loss of function of the Smoothened receptor (Chen *et al.*, 2001; Varga *et al.*, 2001). These mutant embryos exhibit misshapen hearts that appear smaller than their wild-type counterparts (Fig. 2A,B) (Thomas *et al.*, 2008). Cell-counting experiments demonstrated that the shrunken appearance of the *smoothened* mutant heart is a consequence of a reduced number of cardiomyocytes in both the ventricle and atrium (Fig. 2C) (Thomas *et al.*, 2008). These results suggested that Hh signaling could modulate chamber size either through regulating specification of cardiac progenitors or proliferation of cardiomyocytes. Fate mapping in embryos treated with cyclopamine, a Smoothened antagonist, detected cardiac progenitor cells in the lateral margin only half as often as in wild-type control embryos (Fig. 2D), indicating inadequate specification of the cardiac progenitor population (Thomas *et al.*, 2008). In contrast, the productivity of successfully specified progenitor cells remained normal in cyclopamine-treated (CyA) embryos (4.6-labeled cardiomyocytes per experiment in wild-type embryos and 4.5-labeled cardiomyocytes per experiment in cyclopamine-treated embryos), indicating that Hh signaling is not essential for cardiomyocyte proliferation at the stages examined (Thomas *et al.*, 2008). These data, together with additional results, led to the conclusion that Hh signaling plays an important role in directing multipotential cells into the myocardial lineage.

In addition to resulting from altered progenitor specification or cardiomyocyte production, defects in heart size could be a consequence of inappropriate timing of myocardial differentiation. A recent study has shown that the zebrafish myocardium forms during two

distinct phases of myocardial differentiation (de Pater *et al.*, 2009). The first phase creates the primitive heart tube, beginning with the differentiation of the ventricle and progressing to the atrium. During the second phase, a separate population of late-differentiating cardiomyocytes forms the outflow tract at the arterial pole of the ventricle (Fig. 3). Therefore, defects in the number of cardiomyocytes could reflect ineffective execution of the timing of myocardial differentiation; for example, loss of Fgf signaling has been shown to inhibit the second phase of differentiation and thereby lead to an inadequate number of ventricular cardiomyocytes (de Pater *et al.*, 2009).

Two types of transgenic assays have been developed to monitor the timing of myocardial differentiation in zebrafish embryos, and both can be effective for detecting defects in timing. One strategy uses a pair of independent reporter transgenes (de Pater *et al.*, 2009): a transgene that expresses GFP in differentiated cardiomyocytes under the control of the *cmlc2* promoter, *Tg(cmlc2:egfp)* (Huang *et al.*, 2003), and a transgene that expresses DsRed under the control of the same promoter, either *Tg(cmlc2:DsRed2-nuc)* (Mably *et al.*, 2003) or *Tg(cmlc2:dsRed)* (Kikuchi *et al.*, 2010). This assay takes advantage of the difference in GFP and DsRed protein-folding kinetics to distinguish early-differentiating and late-differentiating populations of cells (de Pater *et al.*, 2009; Lepilina *et al.*, 2006). Since DsRed requires more time to mature and fluoresce than does GFP, early-differentiating cardiomyocytes express both GFP and DsRed at timepoints when the late-differentiating cells express only GFP (de Pater *et al.*, 2009). Thus, confocal imaging of DAPI-stained double transgenic embryos facilitates counting of both early-differentiated and late-differentiated cardiomyocyte nuclei; for example, analysis of double transgenic embryos at 48 hpf has been used to examine whether heart size defects are due to an altered number of late-differentiating cells at the arterial pole of the ventricle (de Pater *et al.*, 2009).

A second, complementary strategy uses a reporter transgene, *Tg(cmlc2:kaede)* (de Pater *et al.*, 2009), that expresses the green-to-red photoconvertible fluorescent protein Kaede under the control of the *cmlc2* promoter. Prior to photoconversion, differentiated cardiomyocytes exhibit green fluorescence but not red fluorescence (Fig. 3A,B). Upon exposure to UV light, the Kaede protein is cleaved and converts from its green form to its red form (Fig. 3C,D). At later stages, any cells exhibiting green fluorescence, but not red fluorescence, are interpreted as having initiated differentiation after the time of photoconversion. For example, photoconversion of embryos at 32 hpf, followed by the examination of fluorescence at 48 hpf, reveals green fluorescent cardiomyocytes at the arterial pole of the ventricle (Fig. 3E,F). Thus, this assay can be used to identify embryos with defects in the late differentiation of arterial pole cardiomyocytes.

III. Defects in Heart Shape

In order for the heart to be an effective pump, the cardiac chambers and the atrioventricular valve (AVV) need to acquire specific morphologies that are crucial for their function. Because of the dynamic nature of cardiac morphogenesis, defects in heart shape can have a variety of origins, ranging from complete failure of cardiomyocyte migration at early stages to subtle displacement of atrioventricular cushions at later stages. In this section, we discuss a series of experimental strategies for determining the possible causes of a misshapen heart in a zebrafish embryo.

A number of zebrafish mutations have been shown to cause dramatic defects in cardiomyocyte migration during early steps of heart morphogenesis (Kikuchi *et al.*, 2000, 2001; Kupperman *et al.*, 2000; Reiter *et al.*, 1999). Instead of exhibiting a single heart at the midline, these embryos have a pair of separate hearts in bilateral positions, a condition known as cardia bifida. During wild-type development, differentiated cardiomyocytes move

from their lateral positions in the ALPM toward the embryonic midline, where they merge to assemble the heart tube through a process called cardiac fusion (Fig. 1C) (Glickman and Yelon, 2002). In embryos with cardia bifida, cardiac fusion fails because cardiomyocytes fail to migrate to the midline; this phenotype is typically evident in the aberrant expression patterns of myocardial markers, such as *cmlc2*, between 18 and 22 hpf. Analyses of cardia bifida mutants have highlighted two different requirements for medial cardiomyocyte movement. One group of cardia bifida mutations (e.g., *casanova*, *bonnie and clyde*, *faust*, *miles apart*) disrupt the formation of the anterior endoderm that is adjacent to the migrating myocardium (Alexander *et al.*, 1999; Kikuchi *et al.*, 2000, 2001; Kupperman *et al.*, 2000; Reiter *et al.*, 1999), and a second group of mutations (e.g., *natter*, *hands off*) disrupt the organization of the extracellular matrix (ECM) that surrounds the cardiomyocytes (Garavito-Aguilar *et al.*, 2010; Trinh and Stainier, 2004). Together, these phenotypes indicate the importance of myocardium–endoderm and myocardium–ECM interactions during cardiac fusion. Therefore, when investigating new cardia bifida phenotypes, it is valuable to examine the specification and morphogenesis of the anterior endoderm, using appropriate markers (e.g., *Tg(-0.7her5:EGFP)*, *axial*, *sox17* (Kawahara *et al.*, 2009; Kupperman *et al.*, 2000; Osborne *et al.*, 2008; Tallafuss and Bally-Cuif, 2003)), as well as the deposition and composition of the ECM, using immunofluorescent detection of relevant components (e.g., fibronectin and laminin (Arrington and Yost, 2009; Garavito-Aguilar *et al.*, 2010; Trinh and Stainier, 2004)).

Subtle errors in cardiomyocyte movement do not necessarily result in a cardia bifida phenotype but can still interfere with the shape of the heart tube. Time-lapse confocal microscopy has been instrumental in elucidating the wild-type patterns of directed cardiomyocyte movements that underlie heart tube dimensions (Baker *et al.*, 2008; de Campos-Baptista *et al.*, 2008; Holtzman *et al.*, 2007; Smith *et al.*, 2008). Wild-type cardiac fusion begins with medially directed movements of the cardiomyocytes that recruit the bilateral populations toward each other (Fig. 4A,B) (Holtzman *et al.*, 2007). Then, anterior and posterior subsets of cardiomyocytes change to an angular direction of movement in order to construct a ring-like configuration of myocardium at the midline, referred to as the cardiac cone (Figs. 1C and 4C,D) (Holtzman *et al.*, 2007). Finally, the cardiac cone tilts to the left, due to the differential leftward migration of cardiomyocytes: cells in the posterior region of the cone exhibit a greater overall leftward displacement compared with cells in the anterior region of the cone (Fig. 5) (Smith *et al.*, 2008). Continued leftward and anterior migration of cardiomyocytes completes the elongation of the heart tube (Fig. 1D). Thus, heart tube morphology depends on the coordination of carefully choreographed patterns of cell movement, and disruption of these patterns can result in defects in heart shape.

To distinguish whether a dysmorphic heart originates with problems during cardiac fusion or tube elongation, it is sufficient to use myocardial markers, such as *cmlc2*, *vmhc*, and *amhc*, at a series of timepoints between 16 and 30 hpf to identify the first signs of abnormal cardiac morphology. Additional information about the cellular mechanisms underlying morphological defects can come from examination of markers that exhibit apicobasal polarity in cardiomyocytes, such as aPKC, β -catenin, and ZO-1 (Rohr *et al.*, 2006; Trinh and Stainier, 2004); apicobasal polarity is disrupted by mutations in *heart and soul* (*has*; *prcki*) and *nagie oko* (*nok*; *mpp5*) (Horne-Badovinac *et al.*, 2001; Peterson *et al.*, 2001; Rohr *et al.*, 2006), both of which block heart tube elongation. Finally, to determine the specific type of cell movement defects disrupting cardiac cone or heart tube formation, it is necessary to conduct appropriate time-lapse analyses. Several studies have utilized the transgene *Tg(cmlc2:egfp)* (Huang *et al.*, 2003) to track individual cardiomyocyte movements through time-lapse confocal microscopy (Baker *et al.*, 2008; de Campos-Baptista *et al.*, 2008; Holtzman *et al.*, 2007; Rohr *et al.*, 2008; Smith *et al.*, 2008). Such analyses can ascertain multiple parameters related to cell movement including direction of cell displacement

(medial, angular, leftward, anterior, etc.), rate of cell displacement, and degree of meandering (Figs. 4 and 5). For example, time-lapse analysis of cardiomyocyte movements during cardiac fusion in *cloche* mutant embryos revealed normal medial movement toward the midline but failure to execute angular movements, resulting in a dysmorphic cardiac cone (Holtzman *et al.*, 2007). Since *cloche* mutants lack endocardium, these data suggested that myocardium–endocardium interactions play an important role in directing cardiomyocyte behavior. In other examples, reduction of BMP signaling and reduction of Nodal signaling were shown to alter the direction, speed, and coherence of cardiomyocyte displacement during tube elongation, demonstrating the importance of these signaling pathways for driving leftward cell movement (de Campos-Baptista *et al.*, 2008; Smith *et al.*, 2008).

Although many types of heart shape defects originate during heart tube assembly, dysmorphic phenotypes can also appear during the stages of cardiac chamber emergence. Between 24 and 48 hpf, the heart tube transforms into morphologically evident cardiac chambers that exhibit distinct curvatures: a bulging concave curvature designated as the outer curvature (OC) and a recessed convex curvature called the inner curvature (IC) (Fig. 1D,E) (Auman *et al.*, 2007). Regional changes in cardiomyocyte size and shape create the differences between the chamber curvatures. In the linear heart tube (LHT), ventricular cardiomyocytes appear relatively small and round. During chamber emergence, cells in the ventricular OC become enlarged and elongated, creating a bulging morphology, whereas cells in the ventricular IC retain a rounded morphology and exhibit a smaller increase in size (Fig. 6) (Auman *et al.*, 2007). Errors in the execution of these cell shape changes can result in abnormal ventricular shape: a compact and narrow ventricle if cells fail to expand normally or a dilated and round ventricle if expansion is excessive (Auman *et al.*, 2007).

To determine if a dysmorphic chamber phenotype is caused by defects in cardiomyocyte morphology, it is necessary to visualize and assess cardiomyocyte size and shape using a marker that outlines cell boundaries (e.g., phalloidin or an anti-Dm-grasp antibody) together with a myocardial reporter transgene like *Tg(cmlc2:egfp)* (Auman *et al.*, 2007; Deacon *et al.*, 2010). Size is typically expressed in terms of cardiomyocyte surface area, and shape is typically evaluated using a circularity index that quantifies divergence from a perfectly circular morphology (Fig. 6D, E). For example, morphometric analysis has shown that knockdown of the microRNA *mir-143* results in ventricular OC cells that are small and circular, having failed to undergo the expansion and elongation seen in wild-type embryos; these results indicate an important role of this microRNA in modulating the expression of determinants of cardiomyocyte morphology (Deacon *et al.*, 2010). When evaluating the origins of chamber emergence defects, it is also important to keep in mind that defects in cardiac function, such as reduced blood flow and/or reduced contractility, can have a significant impact on cardiomyocyte morphology (Auman *et al.*, 2007). In this regard, it is valuable to consider whether an observed dysmorphic chamber could be a secondary consequence of a primary defect in heart function.

In addition to being caused by defects in heart tube assembly and chamber emergence, dysmorphic hearts can result from specific errors in the development of the AVC, a constriction of the heart tube found at the boundary between the atrium and the ventricle (Fig. 1E). In wild-type embryos, specification of the AVC is made evident by the restricted expression of markers such as *bmp4* and *versican* in the myocardium and *notch1b* and *has2* in the endocardium (Hurlstone *et al.*, 2003; Walsh and Stainier, 2001). As AVC differentiation proceeds, the adhesion molecule Dm-grasp is detectable in the AVC endocardial cells, but not in the remainder of the endocardium (Beis *et al.*, 2005). This is accompanied by distinct cell morphology changes in both the endocardium and myocardium: endocardial cells undergo transition from a squamous shape to a cuboidal

shape, while myocardial cells undergo apical constriction and extend their basolateral surfaces (Beis *et al.*, 2005; Chi *et al.*, 2008a). The AVC endocardial cushions will subsequently be remodeled into the leaflets of the AVV (Scherz *et al.*, 2008).

Errors in AVC formation frequently lead to a failure of constriction at the atrioventricular boundary that is morphologically apparent by 48 hpf. Additionally, AVC defects often result in retrograde blood flow, visible as blood vacillating in between the chambers (Beis *et al.*, 2005; Stainier *et al.*, 1996). To determine whether these phenotypes indicate an error in AVC specification or differentiation, it is appropriate to use the AVC molecular markers described above, as well as fluorescent reporter transgenes (such as *Tg(cmlc2:ras-gfp)* and *Tg(flkl:ras-cherry)*) to mark myocardial and endocardial cell borders and thereby visualize AVC cell shapes (Chi *et al.*, 2008a). For example, one group of mutations (e.g., *jeekyll*, *slipjig*) have been shown to disrupt the initial specification of the AVC; these mutants fail to exhibit restricted expression of AVC molecular markers and fail to undergo AVC-specific myocardial and endocardial cell shape changes (Chi *et al.*, 2008a; Walsh and Stainier, 2001). Conversely, *adenomatous polyposis coli* (*apc*) mutants fail to restrict AVC differentiation to the proper location: the cardiac chambers in *apc* mutant embryos exhibit expanded expression of AVC markers and excessive endocardial cushions that extend beyond their normal boundaries (Hurlstone *et al.*, 2003).

Subtle errors in valve function can cause retrograde blood flow even when initial AVC specification and differentiation occur normally. The use of specialized fluorescent microscopy techniques such as selective plane illumination microscopy (SPIM), which can capture optical sections in thick samples rapidly and with minimal photobleaching (Huisken and Stainier, 2009; Huisken *et al.*, 2004), has been instrumental in visualizing valve function defects in live embryos. SPIM analysis of wild-type embryos expressing *Tg(flkl:egfp)* (Jin *et al.*, 2005) in endocardial cells and *Tg(gata1:dsRed)* (Traver *et al.*, 2003) in erythrocytes has revealed key features of AVV maturation and function (Fig. 7) (Scherz *et al.*, 2008). At 55 hpf, the AVV undergoes three phases of motion: myocardial contractions bring the sides of the AVC together (Fig. 7A), then the juxtaposed sides of the endocardium engage in a rolling motion (Fig. 7B), and, finally, the AVC relaxes (Fig. 7C). At this stage, the relatively immature AVV is unable to prevent retrograde blood flow. Over time, the increasing thickness of the AVV begins to occlude the AVC lumen, preventing retrograde blood flow upon relaxation (Fig. 7D–F). Thus, examination via SPIM can reveal regulators of specific aspects of AVV morphology and behavior. For example, treatment of embryos with Cox2 inhibitors has been shown to displace the AVV toward the ventricle and thereby interfere with AVV rolling and lumen occlusion, indicating the importance of the Cox2 signaling pathway for the prevention of retrograde blood flow (Scherz *et al.*, 2008).

IV. Defects in Cardiac Function

The heart must regulate circulation efficiently with appropriately robust contractions triggered by propagated electrical impulses. Defects in cardiac function can range from absence of heartbeat to subtle arrhythmia, and these problems can be caused by abnormalities in the contractile apparatus or the conduction system. In this section, we discuss several experimental techniques that are suitable for determining the causes of defective heart function.

It is convenient to monitor heart function in the zebrafish embryo, since the contracting heart and flowing blood are readily visible on a dissecting microscope. The heart tube begins to contract by 24 hpf, and robust, serial contractions of the atrium and ventricle are apparent by 48 hpf. Correspondingly, sarcomere assembly begins within the primitive heart tube, and mature sarcomeres are organized by the time of chamber emergence (Huang *et al.*, 2009).

The cardiac conduction system matures over the same timeframe. Within the heart tube, electrical activity travels unidirectionally and smoothly from the venous pole to the arterial pole (Chi *et al.*, 2008b; Milan *et al.*, 2006). Once the cardiac chambers form, a cardiac conduction delay separates the atrial and ventricular rhythms; this delay is a consequence of the formation of specialized conduction tissue at the AVC (Fig. 8) (Chi *et al.*, 2008b; Milan *et al.*, 2006).

Qualitative assessment of heart function can rapidly discern the presence of severe defects such as failure of chamber contraction or lack of blood flow through the dorsal aorta. Quantitative methods can provide a more refined understanding of less dramatic phenotypes. Assessment of heart rate is straightforward and can elucidate even subtle alterations in the speed and rhythm of contraction. Additionally, degree of contractility can be quantified using high-speed video microscopy to determine ventricular fractional shortening, a measurement of systolic contractile function normalized to the diameter of the heart (Fink *et al.*, 2009; Rottbauer *et al.*, 2005, 2006). Finally, dynamics of blood flow can be measured by tracking movement of erythrocytes or fluorescent molecules introduced into circulation (Hove *et al.*, 2003; Schwerte and Pelster, 2000); alterations in the pace or pattern of blood flow can provide a complementary indication of subtle aberrations in cardiac function.

To determine whether functional defects originate with contractile apparatus abnormalities, it is effective to use transmission electron microscopy to inspect sarcomere structure directly. Examination of sarcomere formation between 24 and 48 hpf can distinguish between errors in myofibril assembly and maintenance. For example, in *silent heart* mutants, which lack the cardiac troponin T gene *tnt2*, myofibrils fail to assemble, as the thick filaments are disorganized in the absence of normal thin filaments (Sehnert *et al.*, 2002). In contrast, in *pickwick* mutants, which lack *titin*, nascent myofibrils form normally but higher-order sarcomeric structures are absent, suggesting a key role of Titin in sarcomere organization and maintenance (Xu *et al.*, 2002).

For heart function phenotypes that are not associated with defects in the contractile apparatus, it is logical to investigate whether cardiac conduction is aberrant. Electrical currents can be assayed *in vivo* by electrocardiography, and similar patch clamp techniques can be used to stimulate the heart to test its excitability (Rottbauer *et al.*, 2001). For optical mapping of cardiac conduction, calcium flux can be monitored using fluorescent dyes (Ebert *et al.*, 2005; Langenbacher *et al.*, 2005; Milan *et al.*, 2006) or with a fluorescent calcium indicator transgene (*Tg(cmlc2:gCaMP)*) (Chi *et al.*, 2008b), and transmembrane action potential can be evaluated using voltage-sensitive dyes (Panáková *et al.*, 2010). This combination of techniques can detect a wide variety of conduction abnormalities, ranging from cell-intrinsic defects in calcium handling to failure to develop specific types of conduction tissue. For example, the arrhythmia observed in *tremblor* (*ncx1h*) mutants is caused by defects in calcium extrusion that result in elevated intracellular calcium in cardiomyocytes (Ebert *et al.*, 2005; Langenbacher *et al.*, 2005). In a different case, *slipjig* (*foxn4*) mutants fail to specify the AVC and therefore do not develop specialized AVC conduction tissue; as a consequence, they exhibit an absence of atrioventricular conduction delay (Chi *et al.*, 2008a).

V. Summary

A wide variety of techniques are available to investigators seeking to determine the origins of defects in heart size, shape, and function in the zebrafish embryo. Whereas some of the applicable techniques require specific reporter transgenes or specialized microscopes, most are readily accessible and should facilitate characterization of cardiac phenotypes in a broad range of laboratories. Experimental strategies will continue to evolve as more molecular

markers, new transgenes, and advances in microscopy enhance the resolution of our inspection of cardiac development. These future prospects, together with the always increasing number of relevant mutations, morpholinos, and small molecules, promise a strong continuation of the utility of the zebrafish for illuminating the molecular mechanisms responsible for cardiogenesis.

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Fig. 1.

Phases of heart formation in the zebrafish embryo. (A) Schematic lateral view, dorsal to the right, of the late blastula. Ventricular (red) and atrial (yellow) myocardial progenitor cells are spatially organized within bilateral marginal zones at this stage. (B–D) Schematic dorsal views, anterior up, depicting the locations of ventricular (red) and atrial (yellow) cardiomyocytes during cardiac morphogenesis. (B) Myocardial progenitors migrate to form bilateral heart fields within the ALPM. (C) The process of cardiac fusion recruits cardiomyocytes to the embryonic midline, where they form a cardiac cone. (D) Continued migration of cardiomyocytes elongates the cardiac cone to create the heart tube. (E) Schematic frontal view, anterior up, depicting the morphologically distinct ventricle (red) and atrium (yellow) that are separated by a constriction at the atrioventricular canal following chamber emergence. Adapted from Schoenebeck and Yelon (2007).

Fig. 2. Hedgehog signaling promotes the specification of myocardial progenitor cells. (A, B) Lateral views of wild-type (A) and *smoothened* mutant (B) hearts at 48 hpf. Immunofluorescence with MF20 and S46 antibodies allows visualization of the ventricle (red) and atrium (yellow), both of which are misshapen in *smoothened* mutants. (C) Quantification of cardiomyocyte number at 52 hpf in wild-type and *smoothened* mutant hearts. Values represent the mean cell number (± standard deviation) for each category, and asterisks indicate statistically significant differences from wild type. (D) Fate maps of myocardial progenitors at 40% epiboly in wild-type and cyclopamine-treated embryos. Longitude coordinates of the first tier of the embryonic margin are depicted with dorsal as

the origin (0°) and ventral as 180° . Each dot represents an individual labeling experiment. Red dots represent embryos in which labeled blastomeres contributed to ventricular myocardium, and black dots represent embryos in which the labeled cells did not become cardiomyocytes. Adapted from Thomas *et al.* (2008).

Fig. 3. Photoconversion of Kaede provides an assay for the timing of cardiomyocyte differentiation. Lateral views of *Tg(cmlc2:kaede)* embryos, ventricle to the left. (A, B) Prior to photoconversion, the heart exhibits green, but not red, Kaede fluorescence. (C, D) After photoconversion, green Kaede fluorescence has been converted into red Kaede fluorescence. (E, F) Sixteen hours later, red Kaede fluorescence persists in the cells that were expressing *Tg(cmlc2:kaede)* at 32 hpf. These cells also contain newly generated green Kaede fluorescence. In contrast, newly differentiated cells in the outflow tract at the arterial pole (arrow) exhibit only green, and not red, Kaede fluorescence. Adapted from de Pater *et al.* (2009).

Fig. 4. Tracking the paths of individual cardiomyocytes during cardiac fusion. Selected images from a time-lapse of cardiac fusion in a wild-type embryo expressing *Tg(cmlc2:egfp)* between the 16 and 20 somite stages; dorsal views, anterior to the top. Arrows indicate paths traveled by individual cells from their starting positions (A, C) to their ending positions (B, D). Red arrows indicate medial movement, and yellow arrows indicate angular movement. (A, B) Cardiac fusion initiates with medial movement toward the midline. (C, D) Cardiomyocytes in anterior and posterior regions and then transition into angular patterns of movement that create the cardiac cone. Adapted from Holtzman *et al.* (2007).

Fig. 5.

Patterns of cardiomyocyte movement during heart tube elongation. (A) Selected images from a time-lapse of heart tube elongation in a wild-type embryo expressing *Tg(cmlc2:egfp)* beginning at the 23-somite stage; dorsal views, anterior to the top. The cardiac cone was divided into four regions (I–IV), and the movements of individual cardiomyocytes from each region were tracked. (B) Bar graphs indicate the mean displacement rate and meandering index for tracked cardiomyocytes; error bars indicate standard errors. Comparisons of the displacement rate (displacement/time) and meandering index (displacement/track length) for each quadrant indicate that posterior cardiomyocytes exhibit higher rates of displacement than anterior cardiomyocytes, whereas the meandering index is comparable for each subset of cardiomyocytes. Adapted from Smith *et al.* (2008).

Fig. 6. Regionally confined cell shape changes underlie the emergence of chamber curvatures. (A–C) Confocal projections of live hearts expressing *Tg(cmlc2:egfp)* with mosaic expression of *Tg(cmlc2:dsRed)*. Arrows point to representative cells expressing both *dsRed* and *egfp*. Ventricular cells in the LHT at 28 hpf (A) and in the IC at 52 hpf (C) are relatively cuboidal, whereas cells in the OC at 52 hpf (B) are flattened and elongated. (D, E) Bar graphs indicate the mean surface area and circularity index of cells in the LHT, IC, and OC. Error bars indicate standard error, and asterisks indicate statistically significant differences from LHT values. OC cells exhibit an increase in surface area with an accompanying decrease in circularity. Adapted from Auman *et al.* (2007).

Fig. 7.

Time-lapse imaging of atrioventricular valve function during development. Selected images from SPIM analysis of embryos expressing *Tg(flk1:egfp)* and *Tg(gata1:dsRed)* at 55 hpf (A–C) and 72 hpf (D–F), with accompanying schematics. (A–C) At 55 hpf, the AVC initially closes as a consequence of myocardial contraction (A), then closes further through a rolling motion (B), and finally relaxes in order to reopen (C). (D–F) Similar motions take place at 72 hpf, but the increased thickness of the AVC endocardium occludes the lumen and prevents retrograde blood flow during the steps of rolling and relaxation. Adapted from Scherz *et al.* (2008).

Fig. 8. Optical mapping of cardiac conduction. (A) Sequence of images depicts calcium activation initiating in the sinus venosus (top) and concluding in the ventricle (bottom) of a live heart expressing *Tg (cmlc2:gCaMP)* at 48 hpf. (B) Optical map of the pattern of calcium excitation shown in (A); isochronal lines represent every 60 ms. Images 5–10 (A) and isochronal lines 5–10 (B) indicate conduction delay at the AVC. Adapted from Chi *et al.* (2008).