

# The Zebrafish Heart as a Model of Mammalian Cardiac Function

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**Abstract** Zebrafish (*Danio rerio*) are widely used as vertebrate model in developmental genetics and functional genomics as well as in cardiac structure-function studies. The zebrafish heart has been increasingly used as a model of human cardiac function, in part, due to the similarities in heart rate and action potential duration and morphology with respect to humans. The teleostian zebrafish is in many ways a compelling model of human cardiac function due to the clarity afforded by its ease of genetic manipulation, the wealth of developmental biological information, and inherent suitability to a variety of experimental techniques. However, in addition to the numerous advantages of the zebrafish system are also caveats related to gene duplication (resulting in paralogs not present in human or other mammals) and fundamental differences in how zebrafish hearts function. In this review, we discuss the use of zebrafish as a cardiac function model through the use of techniques such as echocardiography, optical mapping, electrocardiography, molecular

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investigations of excitation-contraction coupling, and their physiological implications relative to that of the human heart. While some of these techniques (e.g., echocardiography) are particularly challenging in the zebrafish because of diminutive size of the heart (~1.5 mm in diameter) critical information can be derived from these approaches and are discussed in detail in this article.

**Keywords** Cardiac electrophysiology • Echocardiography • Electrophysiology • Excitation-contraction coupling • Optical mapping • Phylogeny

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## 1 Introduction to the Zebrafish as a Model

The teleostian zebrafish (*Danio rerio*) is a widely used non-mammalian vertebrate model in developmental genetics and functional genomics as well as in cardiac structure-function studies (Bakkers 2011; Verkerk and Remme 2012; Asnani and Peterson 2014). Zebrafish hearts are increasingly used as a model of mammalian cardiac function, in part, due to the similarities in heart rate and action potential duration and morphology with respect to human hearts (Arnaout et al. 2007, p. 126; Nemtsas et al. 2010). The zebrafish is a compelling model of mammalian cardiac function due to the clarity afforded by its ease of genetic manipulation, the wealth of developmental biological information, and inherent suitability to a variety of experimental techniques. However, in addition to the numerous advantages of the zebrafish system are also caveats and fundamental differences in how zebrafish hearts function. In this review, we discuss the use of zebrafish in techniques such as echocardiography, optical mapping, electrocardiography, molecular investigations of excitation-contraction coupling, and their physiological implications to the human heart.

Historically, the zebrafish has been an important developmental model in part due to its transparency in its larval stages that allows not only for discerning developmental checkpoints but also for direct observation of cardiac function (Granato et al. 1996; Kane et al. 1996). More recently the role of zebrafish as a model organism has been expanded to include the adult zebrafish due to the

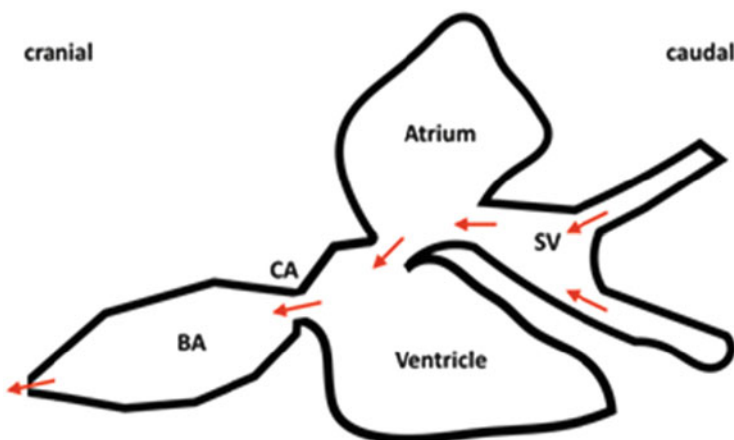
relative ease of genetic manipulation and a genome that bears similarity to humans (Bakkers 2011). Furthermore, zebrafish produce large numbers of offspring with short generation times and their upkeep is cost effective. This tropical fish is small, robust and survives well at temperatures close to room temperature (24–28°C) (Spence et al. 2008; Sidhu et al. 2014). The zebrafish genome is fully sequenced, publicly available, and was the third high-quality genome sequenced after those of mouse and human (Howe et al. 2013). Human Genome Wide Association Studies (GWAS) are limited to statistical association between a particular genetic variant and a given disease. Genetic variations associated with human disease often have a relatively minor phenotype, necessitating large amounts of sequencing data or testing in an alternative model system to elucidate the mechanism. Zebrafish as a vertebrate are closer genetically to humans than invertebrate models such as *Drosophila* or *C. elegans*. Hence they can be used as an animal model for mutations that have a strong phenotypic effect, and providing insight into the disease phenotype in humans.

The genetic similarity between humans and zebrafish enables the use of targeted mutagenesis techniques to systematically model human disease genes (Arnaout et al. 2007). Morpholino oligonucleotides (MOs) were the original standard technique for the generation of anti-sense knockdown mutations in zebrafish due to their time and cost effectiveness (Nasevicius and Ekker 2000; Bill et al. 2009); however, MOs were also susceptible to off-target inhibition (Law and Sargent 2014; Kok et al. 2015). Recently, MOs have been superseded for the most part by the application of clustered regularly interspaced palindromic repeats (CRISPRs) for targeted genetic modifications (Hwang et al. 2013). CRISPRs recently have been established as a powerful reverse genetic screening strategy in the zebrafish model (Shah et al. 2015) and are commonly used for both knock-outs and knock-ins. Through these gene screening and modification strategies, zebrafish can efficiently be used for the verification of candidate disease genes and functional characterization in human disease models. Further, high throughput sequencing technologies have also been used in zebrafish, including RNAseq for characterization of the transcriptome (Collins et al. 2012). Transcriptomic analysis provides the link between genotype and phenotype in zebrafish to better understand the underlying mechanisms of physiological responses (Qian et al. 2014). The combination of genomic, transcriptomic, and proteomic information available in the zebrafish provides a well-characterized platform for the exploration of many biological processes. An extensive freely available online database, ZEBRAFISHIN, integrates genetic, genomic, and developmental data for zebrafish as a model organism (Howe et al. 2013). This is closely linked to ZIRC, an NIH research facility that provides access to zebrafish lines, probes, and health services. These easily accessible services contribute to the appeal of the model.

There are important similarities between zebrafish and human cardiac physiology, including action potential morphology and basic contractile dynamics, making the zebrafish a robust model for cardiac function in experiments that focus on channelopathies and cardiomyopathies. The similarities in action potential (AP) morphology (Arnaout et al. 2007; Brette et al. 2008) contrast with other

model systems such as mice that possess fundamental differences from human hearts in terms of heart rates and repolarization characteristics. The similarities between mammals and zebrafish have made the zebrafish an ideal model, in some respects, for the study of channelopathy-causing mutations and the functional effects of electrophysiological abnormalities (Langheinrich et al. 2003; Langenbacher et al. 2005; Arnaout et al. 2007; Brette et al. 2008). Basic excitation-contraction (E-C) coupling is also conserved between teleosts and mammals with respect to fundamental features such as  $\text{Ca}^{2+}$  sparks and the ability of  $\text{Ca}^{2+}$  waves to trigger after-depolarizations (Llach et al. 2011; Bovo et al. 2013). This similarity is dictated by the conservation of key domains in several sarcomeric proteins (Fu et al. 2009). The use of transgenic zebrafish as models for cardiac function permits the manipulation of molecular components of the contractile apparatus. Transgenic studies allow the direct exploration of the genetic factors and molecular modifications that underlie human disease states. The application of these techniques makes the zebrafish particularly attractive for the study of human cardiac muscular diseases such as dilated or hypertrophic cardiomyopathies (Dahme et al. 2009).

Despite the many similarities, the zebrafish is a phylogenetically distant species from human. Fish are ectothermic with a morphologically distinct heart composed of two main contractile chambers working in series rather than the mammalian four-chambered heart as seen in Fig. 1. The two-chamber physiology as well as a lack of pulmonary circulation makes it difficult to completely integrate cardiovascular development and function. The end diastolic volume in the fish ventricle is greatly determined by atrial contraction rather than central venous pressure seen in mammals (Cotter et al. 2008). Fish typically show an early to late ventricular filling



**Fig. 1** Schematic of the chambers of a typical fish heart. SV is the sinus venosus; BA is the bulbus arteriosus; CA is the conus arteriosus. The heart outflow tract (OFT) of fish is formed by two portions: a proximal conus arteriosus and a distal bulbus arteriosus. Although the form and function of the CA in teleosts such as the zebrafish remain somewhat controversial

ratio around 0.2, much lower than the typical healthy human and indicative of differential hemodynamics (Ho et al. 2002). For this reason, direct comparison of some parameters of whole heart function in zebrafish cannot be made with that of humans.

While the basics of cardiac excitation-contraction (E-C) coupling are conserved across vertebrates, differences between teleost and mammalian  $\text{Ca}^{2+}$  sources, myofilament  $\text{Ca}^{2+}$  sensitivities and/or spatial arrangement of the contractile element are essential for function in the environmental conditions in which teleosts exist. Ventricular myocytes from zebrafish more closely resemble myocytes from neonatal mammals rather than adults in terms of structure and function (Iorga et al. 2011). They are around 100  $\mu\text{m}$  in length, thin (around 5  $\mu\text{m}$ ) (Brette et al. 2008) with decreased diffusion distance to the center of the cell and generally lacking T-tubules (trout (Farrell and Jones 1992; Vornanen 1998); neonatal rabbit (Huang et al. 2005)). Critical processes such as cardiac E-C coupling must be adaptable to allow for the necessary cardiac scope due to the broad environmental conditions to which ectothermic teleosts are exposed. The evolution of variability in cardiac contractility is an important consideration in ectotherms. The underlying evolutionarily mechanism for producing these contractile changes involves differential expression of paralogs, particularly, key proteins guiding the  $\text{Ca}^{2+}$  signaling of sarcomeric function. The understanding of both conservation and modification of electrophysiology and contractile components is essential in the appropriate utilization of the zebrafish model.

About 71% of human genes have at least one corresponding gene in the zebrafish genome, known as an ortholog (Howe et al. 2013). Due to gene duplication events there may be two orthologs in the zebrafish genome for a given human gene. The zebrafish genome has several thousand unique genes without a corresponding ortholog in the mammalian genome. Gene duplication is thought to be one of the major driving forces in evolutionary innovation as it introduces the ability to have novelty without loss of the original function (Ohno 1993). While most genes are lost after a duplication event, tissue specific expression may lead to the retention of multiple copies of a given gene, known as paralogs. These copies can then divide ancestral functions (sub-functionalization) or develop new functions (neo-functionalization) (Ohno 1993; Meyer and Schartl 1999). Gene duplication has led to multiple copies of genes that code for sarcomeric proteins and ion channels, consequently there may be multiple genes in zebrafish that are orthologous to a given human gene. This is an evolutionary adaptation by the zebrafish that allows them to regulate cardiac contractility through the differential expression of functionally similar isoforms and paralogs (Karasinski et al. 2001; Sehnert et al. 2002; Rottbauer et al. 2006; Seeley et al. 2007; Zhao et al. 2008; Ohte et al. 2009; Sogah et al. 2010; Alderman et al. 2012; Genge et al. 2013, 2016; Zou et al. 2015; Singh et al. 2016). Certain channels that produce cardiac ion currents are regulated by non-orthologous genes in zebrafish and humans, for example the major repolarizing ( $I_{K1}$ ,  $I_{Kr}$ ) and depolarizing ( $I_{Na}$ ,  $I_{CaL}$ ) currents (Leong et al. 2010a, b; Vornanen and Hassinen 2016). While variation in zebrafish gene content may be a method of coping with varying environmental conditions, the

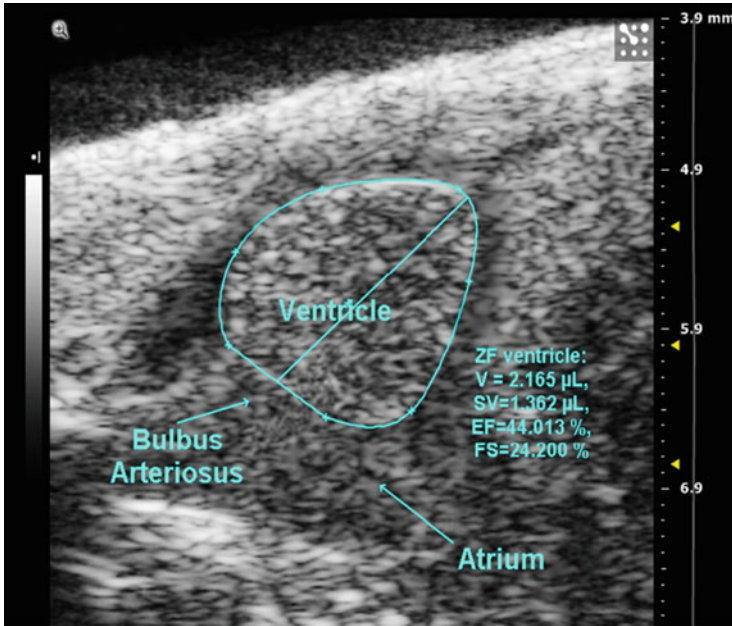
resulting unique gene content and expression in fish creates challenges in assigning a given function to a gene product that may be different between species.

Variation in ion currents due to genetic divergence between fish and humans due to this fish specific genome duplication can confound the selection of candidate molecules for medicinal drug development or in toxicological testing. With the increasing use of zebrafish as a model of vertebrate cardiac physiology (Milan et al. 2003; Arnaout et al. 2007; Scholz et al. 2009; Leong et al. 2010a, b; Nemtsas et al. 2010; Bovo et al. 2013; Dvornikov et al. 2014), the variation in paralog use must be considered due to the increased number of teleost-specific paralogous genes that code for cardiac proteins. Shared cardiac functions need to be distinguished as either derived or novel in zebrafish and humans. The discrepancies in gene content and cardiac function between humans and ectothermic models often originate in molecular adaptation to the native environment. Mammalian hearts function normally at a specific narrow temperature range (~37–38°C) while ectotherms must tolerate a broad range of environmental temperatures. Zebrafish must be able to maintain cardiac function between 6 and 38°C (Spence et al. 2008). The function of orthologous enzymes is conserved when the orthologous enzymes are at their typical physiological temperature (Somero and Hochachka 1969; Fields and Somero 1998; Somero 2005). As such there is variation in the biophysical properties of key proteins in the contractile element that are preferentially expressed by zebrafish at different temperatures. The substantial variation in cardiac function based on divergence in genetic background complicates the use of the zebrafish as a model. Even where commonalities are shared in whole-heart physiological measurements, the underlying mechanism may be divergent in teleosts. In this review, we will discuss both the conserved similarities in teleost and human hearts as well as the basis of variation that must be acknowledged when using this comparative model. While this can make the study of the ectothermic zebrafish difficult, zebrafish can be an excellent model for the study of mammalian cardiac systems when care is taken in the selection of appropriate techniques and questions.

## 2 In Vivo Function of the Zebrafish Heart

Visualization of *in vivo* heart function is important to identify the correlation between genotype and phenotype, to understand heart disease progression and/or treatment applied in longitudinal studies (Gemberling et al. 2015; Nair et al. 2016). High-resolution echocardiography has been used to examine cardiac structure and function of the *in vivo* adult zebrafish (Gerger et al. 2015; Lee et al. 2016). This accurate and non-invasive method allows real-time imaging *in vivo* for investigation of the many cardiac mutations available for zebrafish (Sun et al. 2008).

In echocardiographic imaging, two-dimensional (2D) bright (B)-mode image is considered the basic guide mode (Fig. 2). The zebrafish ventricle, atrium and bulbus arteriosus can be seen from a long-axis B-mode view. Functional parameters including stroke volume (SV), ejection fraction (EF), fractional shorting (FS),



**Fig. 2** Echocardiographic long-axis B-mode of ZF ventricle. 2D B-mode long-axis view of a zebrafish ventricle. Zebrafish ventricle, atrium and bulbus arteriosus can be seen from this view. Tracing on the edge of the ventricle throughout diastole (d) and systole (s) allows an assessment of cardiac functional parameters. *V* volume, *SV* stroke volume, *EF* ejection fraction, *FS* fractional shortening

and fractional area change (FAC) can be calculated from this view (Gerger et al. 2015; Lee et al. 2016). Cardiac volumes can be calculated from 2D B-mode images, using the Simpson method, which has proved to give the best prediction for calculating fish ventricular volumes (Coucelo et al. 2000). The end diastolic volume (EDV) of zebrafish is typically around 2.3  $\mu\text{L}$  using these methods (Lee et al. 2016).

One-dimensional (1D) Motion (M)-mode images are acquired by a rapid sequence of B-mode scans along a single line and displayed as a function of time. This view is challenging to obtain in zebrafish due to the small heart size ( $\sim 1.5$  mm); also the compact layer and the sponge layer in the zebrafish heart are difficult to distinguish. However, Liu et al. developed a method of ultrahigh frame-rate (UHFR) echocardiography which can record M-mode images and measure zebrafish ventricular internal diameter (Liu et al. 2013). They reported the fractional shortening (FS) to be  $42 \pm 4\%$  (mean  $\pm$  SD) and  $60 \pm 13\%$  for the long axis and short axis of the ventricle, respectively, and fractional area change (FAC) was  $77 \pm 9\%$  (Liu et al. 2013). FS calculated from 1D M-mode is much higher than the 2D B-mode calculation, which is around 17–24% (Hein et al. 2015; Lee et al. 2016).

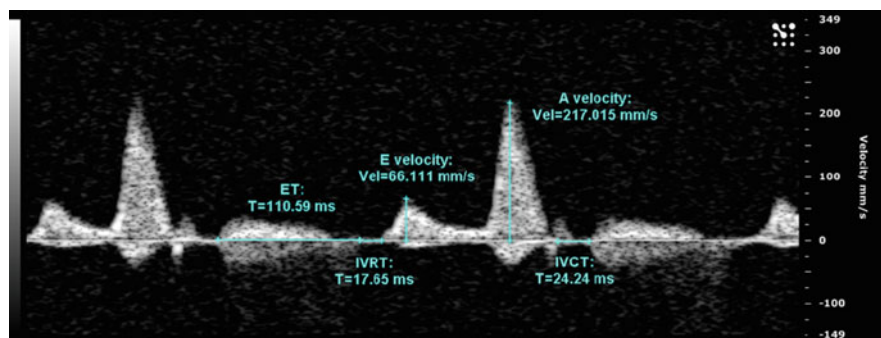
In the ectothermic zebrafish, acute temperature can be used as a perturbation on the whole animal model to quantify cardiac responses to stress, and thermal acclimation to alter cardiac function and morphology in similar-sized teleosts



(Johnson et al. 2014). In work done by our group, echocardiography with a high frequency probe (70 MHz) was used to observe cardiac function for warm-acclimated (WA) and cold-acclimated (CA) zebrafish at 28 and 18°C (Lee et al. 2016). Heart rate (HR) is the critical factor modulating the cardiac response to acute temperature change as it increases from  $78 \pm 6$  bpm at 18°C to  $162 \pm 10$  bpm at 28°C regardless of acclimation state. Stroke volume did not change in response to acute temperature change or acclimation ( $1.06\text{--}1.17 \mu\text{L}$ ). A similar lack of change in relative stroke volume measured with laser Doppler blood flow has been seen in other zebrafish undergoing the same temperature shift (Little and Seebacher 2013).

Doppler mode echocardiography is an important tool for studying hemodynamics, which is extremely useful for zebrafish heart imaging to identify the direction and velocity of blood flow from B-mode images (Ho et al. 2002). Parameters including heart rate (HR), ventricular inflow, ventricular outflow, the velocity time integral (VTI), isovolumic relaxation time (IVRT), isovolumic contraction time (IVCT), ejection time (ET), and myocardial performance index (MPI) can be clearly measured from the pulse wave (PW) Doppler mode (Sun et al. 2008; Lee et al. 2016).

One important parameter that can be determined in zebrafish heart by PW Doppler is the E/A ratio (Fig. 3). As there are two components of ventricular inflow: early (E) filling peak velocity during ventricular relaxation (passive filling) and the atrial (A) filling peak velocity that is the result of atrial contraction. Unlike mammals, the E velocities are significantly lower than the A velocities in fish hearts (Ho et al. 2002). This is consistent with the notion that the end diastolic volume in the fish ventricle is determined in large part by atrial contraction rather than central venous pressure as seen in mammals (Cotter et al. 2008). The increased importance of late ventricular filling by atrial contraction rather than the passive early filling of the ventricle is reflected in the early to late filling ratio (E/A ratio) of teleosts. Fish typically show an E/A ratio around 0.2, much lower than that of the healthy human



**Fig. 3** Zebrafish ventricular inflow velocity obtained by using pulse wave Doppler. The pulse wave Doppler mode of ventricular inflow. The velocity (in mm/s, y-axis) is shown over time (in ms, x-axis). Early filling peak velocity (E velocity), the atrial filling peak velocity (A velocity), ejection time (ET), isovolumic contraction time (IVCT) and isovolumic relaxation time (IVRT) can be measured from this view



( $1.5 \pm 0.4$ ) (Ho et al. 2002; Nagueh et al. 2009) under resting conditions. In our zebrafish study, E/A ratio was found to be 0.28 regardless of the acute or acclimated temperature state (Lee et al. 2016). This value corroborates previous zebrafish studies, which have shown similar E/A ratios at 15°C and 25°C (Ho et al. 2002; Sun et al. 2008). End systolic volume (ESV) of the fish heart is very small (Coucelo et al. 2000) relative to that of the mammalian heart even when normalized by body weight. This results in species such as zebrafish having relatively low systolic pressure generation ( $\sim 2.5$  mm Hg) in the ventricle (Hu et al. 2001).

One of the challenges for echocardiography in the zebrafish is the appropriate use of anesthetic. Tricaine methane sulfonate (MS-222) has been widely used as an anesthetic agent for zebrafish studies. It is known that the MS-222 doses normally used (100–200 ppm or mg/ml) are potent cardiac depressants and can result in heart rates less than 100 bpm or even death (Huang et al. 2010). A reasonable solution was proposed by Huang et al. in which they used a modified anesthetic protocol that was the combination of pH-adjusted MS-222 (45 ppm) and isoflurane (45 ppm), which showed significantly less impact on zebrafish cardiac function (Huang et al. 2010). We have found that using this drug combination, the zebrafish heart rate can be kept above 160 bpm at 28°C and achieved full recovery after long term exposure (up to 40 min) (Lee et al. 2016). Other anesthetics have been used that can also maintain cardiac function, or specifically for lacking a deleterious effect on SV, such as AQUI-S (Little and Seebacher 2013).

The zebrafish is an interesting model of cardiac regeneration that has been studied using *in vivo* echocardiography. It has been reported that zebrafish can remove scar tissue and regenerate its heart even after 20% ventricular resection (Poss et al. 2002). Non-invasive echocardiography is the most suitable tool for longitudinally observing the cardiac structure and function during recovery of adult zebrafish (Hein et al. 2015). Evaluation of ventricular recovery after cryo-cauterization and myocardial motion during heart regeneration has been done by Doppler imaging (Lee et al. 2014; Huang et al. 2015). Regeneration is of interest in the zebrafish model due to the insight it might offer for novel approaches to cardiac regeneration in humans.

Further advanced echocardiographic techniques have been used in zebrafish regeneration models, such as strain analysis using speckle-tracking applied on high-resolution B-mode images to assess the regional myocardial motion and deformation. It has been used to evaluate cardiac regeneration after myocardial cryoinjury (Hein et al. 2015). The imaging modalities evaluated both regional and global zebrafish ventricular functional changes and contribute to establish further adult zebrafish as cardiac disease model and regeneration process.

Amongst the numerous advantages of echocardiography and its hemodynamic measurements of the *in situ* heart are also significant limitations including a significant upfront investment in equipment and need for a highly skilled sonographer. By the very nature of making recordings from an intact organism, imaging quality is marred by the presence of movement artifact. Since bone is highly reflective to acoustical waves, there are a very limited number of probe positions and angles that give a clear view of the zebrafish heart. The combination of these

factors often limits recording lengths to several seconds and prevents the quantification of infrequent events such as errant beats by echocardiography. However, the use of whole animal *in vivo* cardiac function to characterize mutant phenotypes and to be able to do this non-invasively and longitudinally is invaluable, especially when coupled with other measures of cardiac function.

### 3 Whole Heart Measurements

One of the attractive elements of the zebrafish model is the similarity between the zebrafish and human ECG recordings, in large part, due to the similarities in heart rate and ventricular action potential durations. Developmentally, the transparency of zebrafish embryos allows heart rates to be manually observed (Granato et al. 1996). Additionally, embryonic ECGs are available from microelectrode recordings as early as 3 dpf, in which there can be diffusional drug delivery (Dhillon et al. 2013). In adult zebrafish, needle electrodes must be used and require anesthetic, which may be limited by the same cardiodepressive effects influencing echocardiographic measurements if appropriate anesthetic protocols are not employed (Milan et al. 2006; Chaudhari et al. 2013).

While hemodynamic measurements are invaluable, the absence of electrical information can make interpretation difficult. In particular, *in vitro* ECG recordings in the adult heart can show alteration of cardiac cycle components in response to drugs that exert electrophysiological effects on cardiomyocytes (Tsai et al. 2011). The addition of concurrent electrocardiography with hemodynamic measurements, which requires minimal additional equipment, gives a valuable physiological context and is ideally suited towards longer recordings. However, the insertion of the ECG electrodes greatly increases the invasiveness of the procedure, nullifying one of the key advantages of echocardiography. An additional limitation of ECG recordings in intact adult zebrafish is imprecise drug dosages with indirect delivery but this can be improved with intraperitoneal injections (Chaudhari et al. 2013). As well, continued gill movement in anesthetized fish contributes significant movement artifacts and signal noise. Averaging the ECG waveform over numerous cardiac cycles increases the signal integrity and further improvements in signal quality are possible with arrested gill movement and external perfusion but can introduce confounding effects (Milan et al. 2006).

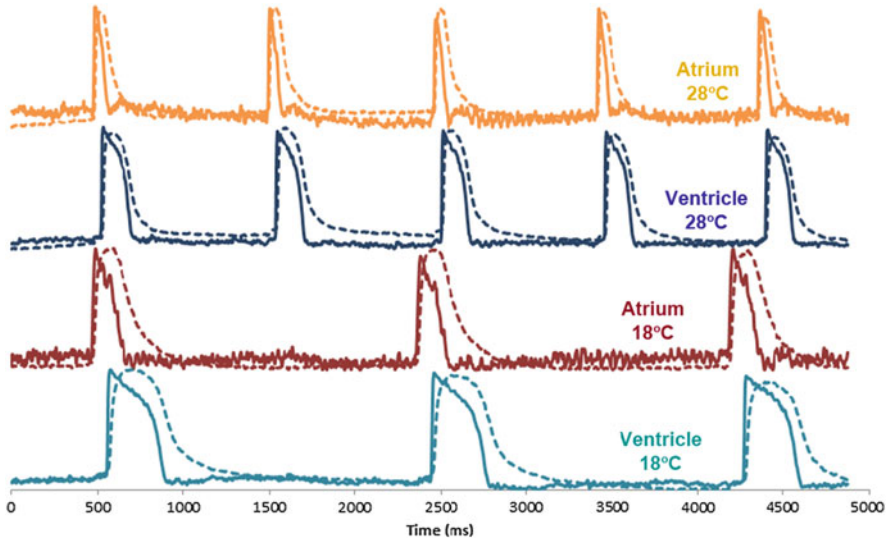
An alternative approach to intact recordings is to excise the heart, which circumvents the complexities of anesthetization and motion artifacts, with the additional advantage of diffusional drug delivery (Tsai et al. 2011). In the excised preparation, neurohormonal regulation is lost and there is an increased risk of physical damage. However, peri-cardiac incisions can be made on a euthanized zebrafish to allow direct visualization of the heart prior to full heart isolation (Lin et al. 2015). Since rate and rhythm are observable by eye and rough handling of the heart has instantaneous deleterious effects, the health of the zebrafish heart can be continuously monitored to assess any possible damage during extraction. While the

excision process can initially be technically challenging, the result is an exceptionally stable preparation in which heart rate and rhythm can be stable ex-vivo for 12–24 h (Lin et al. 2014).

ECGs can be recorded using microelectrodes (Tsai et al. 2011) or by field-electrodes in which the heart is positioned within 1 mm, of two 0.5 mm silver-chloride electrodes (Lin et al. 2015). Embedding the electrodes into the bottom of a vinyl-terminated PDMS (e.g., Sylgard)-coated Petri dish allows for relatively high throughput screening since each heart only needs to be positioned between the recording electrodes for a short duration. One of the limitations of these arrangements is the difficulty in performing solution changes without changing the relative positioning of the heart and electrode positioning may contribute to variability observed in p- and t-wave polarity (Tsai et al. 2011). Since relative positioning can change the apparent conduction axis (Dhillon et al. 2013), cannulation of the zebrafish heart through the aortic-equivalent bulbus arteriosus facilitates precise relative positioning and the rotation of the heart can be manipulated to optimize the ECG recording (Lin et al. 2015). Cannulation also allows for superfusion with minimal movement artifact to improve signal quality.

The primary information available in an ECG recording is the temporal relationship of atrial depolarization, ventricular depolarization, and ventricular repolarization from which clinically relevant parameters such as heart rate, atrioventricular delay, and ventricular action potential duration can be derived (Tsai et al. 2011; Dhillon et al. 2013). Since ECG electrodes directly measure electrical activity, the length of an ECG recording is effectively unlimited making ECG especially well suited to investigating rare electrical events. Complementary techniques such as glass microelectrodes and optical mapping techniques are better suited to determining intermediate voltage kinetics. Impaled microelectrodes are one of the few techniques that can give absolute membrane voltages and rates of membrane depolarization and repolarization. The discrete nature of a microelectrode recording allows electrical signals to be precisely localized. However the size of a zebrafish heart makes multiple simultaneous recordings, such as for conduction velocity measurements or for assessing cell-to-cell variability, difficult (Lin et al. 2015).

Optical mapping recordings lack the ability to measure absolute membrane potentials but are able to capture relative changes from the atria and ventricles simultaneously, making it especially well suited to conduction velocity measurements and for investigating arrhythmogenicity (Efimov et al. 2004; Herron et al. 2012). Two common potentiometric dyes, di-4-ANEPPS (Sedmera et al. 2003; Tsai et al. 2011) and RH-237 (Lin et al. 2014) have been used in adult zebrafish hearts. While both dyes are prone to photobleaching, extended imaging using di-4-ANEPPS has been reported to have phototoxic effects (Sedmera et al. 2003) whereas no changes in rhythm were observed using RH-237 (Lin et al. 2014, 2015). However, it is unclear whether this result was due to the properties of the potentiometric dyes or due to other experimental parameters such as differences related to the use of cytochalasin D versus blebbistatin as the excitation-contraction uncoupler. Tsai et al. (2011) also used di-4-ANEPPS but



**Fig. 4** Fluorescent voltage and calcium recordings as a function of temperature. Atrial and ventricular recordings were made at 28°C (*top*) and 18°C (*bottom*). Voltage signals (*solid lines*) have a fast upstroke and rapid repolarization, resulting in a prominent peak. The upstroke of the calcium transient (*dotted lines*) is initially fast and then slows as it approaches peak calcium. The slow phase of the calcium upstroke coincides with early repolarization such that  $\text{Ca}^{2+}$  levels are increasing while voltage levels are decreasing. The slow approach to peak calcium followed by gradual relaxation results in a rounded calcium transient peak. Observable effects of changing temperature include changes to heart rate, atrioventricular delay, and voltage and calcium transient durations

made no mention of rhythm changes related to imaging. In rat hearts, effects of di-4-ANEPPS on AV conduction have been observed (Nygren et al. 2003) but no changes to AV conduction were reported in zebrafish (Sedmera et al. 2003).

Optical mapping can be extended to include simultaneous calcium measurements and the combination of the potentiometric dye RH-237 and the calcium-sensitive dye Rhod-2 is a powerful technique which has been used in zebrafish (Lin et al. 2015) (Fig. 4). As many indicator dyes are sensitive to the cell-specific microenvironment, it should be noted that the RH-237/Rhod-2 combination used in zebrafish contained spectral cross-talk (Lin et al. 2015) whereas no cross-talk was reported when used in guinea-pig (Choi and Salama 2000) or in rat myocyte cultures (Fast 2005). The species specific background may necessitate the use of possible alternative dye combinations (reviewed in Fast (2005) and Jaimes et al. (2016)).

Optical mapping recordings are considerably data rich. Thus, in one voltage-calcium recording of the zebrafish heart, measurable parameters include: heart rate, atrial action potential durations, atrial calcium transient, atrial conduction velocity, atrioventricular delay, ventricular action potential duration, ventricular calcium transient, and ventricular conduction velocity but can require extensive

data analysis. MATLAB-based processing and analysis tools are published and freely available (Laughner et al. 2012) as are additional methods of cycle averaging to increase signal quality (Ding et al. 2014; Lin et al. 2015).

In optical mapping experiments, larger hearts are associated with larger signal intensities and the advantages of working with a heart smaller than 1 mm<sup>2</sup> may appear to be counterintuitive. One advantage of the zebrafish model, that leverages its diminutive size, is its compatibility with common fluorescence microscopes. As the dyes used in optical mapping utilize common excitation and emission wavelengths, many fluorescence microscopes can be readily converted to optical mapping applications with the addition of a sufficiently fast digital camera. With care, confocal and spinning disk microscopes can also be used.

Conduction velocities in the zebrafish are relatively fast, with 20 ms and 25 ms conduction times in the atrial and ventricular compartments, respectively (Sedmera et al. 2003). Therefore, high-resolution conduction velocity measurements benefit from high speed cameras upwards of 500 fps. However, speed requirements for accurately measuring action potential and calcium transient kinetics is surprisingly low, with fast-Fourier analysis indicating few frequency components above 100 Hz (Mironov et al. 2006). Continuous developments in camera technology make high speed sensors increasingly affordable. A potential difficulty with using a microscope-based optical mapping system with lower-cost digital cameras is the smaller sensor sizes which limits the viewable area. This can be circumvented with lower magnification objectives and/or the addition of a demagnifying transfer lens in front of the camera sensor. Alternatively, a custom optical pathway can be constructed with simple lens (Lin et al. 2015) in a custom fashion and can be done more cost effectively.

The small size of the zebrafish heart has many pragmatic advantages for use in a simpler optical mapping set-up worth noting. The size of the zebrafish heart contributes to its ability to maintain cardiac function for many hours without external perfusion or oxygenation. This, in combination with the flexibility of the zebrafish heart to thrive under ambient temperature conditions, means that zebrafish optical mapping experiments can be conducted in the absence of perfusion pumps, gas bubblers, and temperature controllers. In mammalian optical mapping experiments, the stability of the preparation is heavily dependent on maintaining adequate coronary flow and pulsatile flow from the perfusion systems can contribute to movement artifact during image acquisition. As 37°C is significantly higher than ambient, maintaining sufficient warmth throughout the imaging system is also a non-trivial concern. Conversely, zebrafish hearts can function comfortably between 18 and 28°C (Spence et al. 2008) and do not require precise temperature regulation since cardiac cycles separated by 1°C are not statistically different. The relative indifference of zebrafish hearts to these rather large variations in temperature allows consumer aquarium-grade water heaters and coolers, which are typically accurate to ±1°C, to be used to good effect.

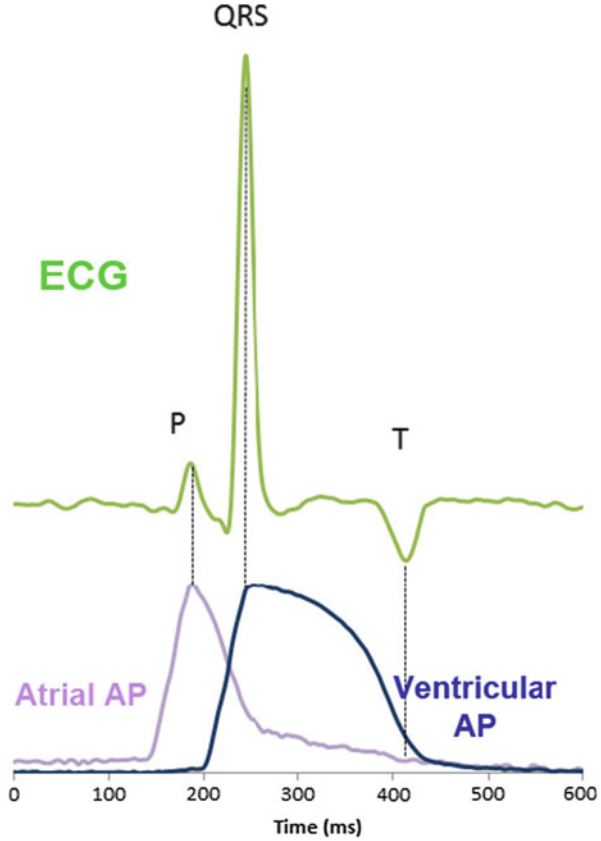
Further, the size of the zebrafish heart and the lack of an oxygenated perfusion system greatly decrease the rate of consumption of RH-237, Rhod-2 and, most importantly, blebbistatin per experiment. Blebbistatin, as a contractile inhibitor, is

necessary in modern optical mapping experiments to prevent muscle contraction, which would otherwise obscure the voltage and calcium dynamics recordings. As blebbistatin is typically supplied to mammalian hearts through the coronary circulation, the blebbistatin solution must be oxygenated and warmed prior to circulation, resulting in a circulating volume of hundreds of mL. As it is this recirculating volume that is used to deliver drugs to the coronary circulation, its volume can have a significant effect on the cost of each experiment. In a typical zebrafish optical mapping experiment, with no recirculating perfusion system, the imaging chamber holds 1.5 mL and RH-237 and Rhod-2 loading steps occur in 250  $\mu$ L chambers. These considerations all make the zebrafish a much more cost-effective model to work with.

In mammals, hypothermia reduces cardiac output primarily through negative chronotropic effects with complex effects on stroke volume (Wood and Thoresen 2015). Moderate hypothermia ( $37^{\circ}\text{C} - >29^{\circ}\text{C}$ ) is strongly inotropic due to changes in action potential duration and calcium handling (Shattock and Bers 1987) while more severe temperature reductions ( $>15^{\circ}\text{C}$ ) result in calcium overload and contracture (Liu et al. 1990; Wang et al. 1997). In zebrafish, reduced temperatures also reduce cardiac output primarily through negative chronotropic effects in which the transition from  $28^{\circ}\text{C}$  to  $>18^{\circ}\text{C}$  is associated with a 40% decrease in heart rate (Lin et al. 2014) while stroke volume is maintained (Lee et al. 2016). At  $28^{\circ}\text{C}$ , zebrafish heart rates vary across a wide range of values, from 50 bpm to over 200 bpm, and the average atrial and ventricular action potential duration ( $\text{APD}_{50}$ ) is 33 and 98 ms, respectively (Lin et al. 2014). In spontaneously beating hearts, the rate dependence of atrial and ventricular action potential durations was sufficiently shallow at  $28^{\circ}\text{C}$ , that the heart rate effect was comparable to inter-heart variability. Overall, there was a weakly correlated heart rate effect of 7 ms per 100 bpm for atrial  $\text{APD}_{50}$  and 14 ms per 100 bpm for ventricular  $\text{APD}_{50}$  at  $28^{\circ}\text{C}$ . The heart rate effect was also examined within hearts using field stimulation with similar effects (Lin et al. 2015).

Temperature strongly affects atrial and ventricular action potential durations, with  $Q_{10}$  values of 2.7 and 2.0, respectively, with additional effects on the action potential morphology. Because temperature has such a strong effect on heart rate, temperature-induced changes combine both the direct temperature effect together with indirect effects of reducing heart rate. For individual hearts, faster heart rates at  $28^{\circ}\text{C}$  would be expected to experience a larger indirect temperature effects due to the larger absolute change in heart rate. For example, a heart beating at 200 bpm at  $28^{\circ}\text{C}$  would experience a four-fold greater indirect temperature effect than a heart beating at 50 bpm upon cooling to  $18^{\circ}\text{C}$ . The average atrioventricular delay in zebrafish is 1 ms, with shallow rate dependence at  $28^{\circ}\text{C}$  but with a 70% increase in AV delay by cooling from  $28^{\circ}\text{C}$  to  $18^{\circ}\text{C}$ . Similar to mammalian hearts, zebrafish atrioventricular delays have decremental conduction, in which faster heart rates have longer atrioventricular delays. The absolute change experienced by a given heart will depend on the both on the starting heart rate at  $28^{\circ}\text{C}$  and the rate-dependence of AV delay at  $18^{\circ}\text{C}$ .

**Fig. 5** Simultaneous ECG and optical mapping. Silver chloride wire electrodes (0.5 mm) were placed 1 mm from the lateral aspects of the atria and ventricle and the ECG signal was acquired concurrently with optical mapping (RH-237). Average ECG (*green*), atrial AP (*lilac*), and ventricular AP (*blue*) cycles were created using the peak of the peak of the QRS-complex



Although no additional information is present in the ECG recording beyond what is available in the optical mapping recordings, ECG can have an important role in optical mapping experiments. Simultaneous recordings of ECG and optical mapping indicate that the peak of the P-wave aligns with the peak of the atrial action potential while atrial repolarization is absent from the ECG recording as is typically observed in the mammalian heart. For the ventricle, the exact relationship between the QRS complex and the ventricular action potential can vary with the AV delay and the peak of the R-wave roughly aligns with peak voltage while the end of the t-wave aligns with complete repolarization. However, even though rates of photobleaching and phototoxicity are limited even with 6 min of accumulated image acquisition time per heart (Lin et al. 2014), continuous illumination of the heart for several minutes results in movement artifact due to photo inactivation of blebbistatin activity. Therefore, the optical mapping approach is not as well suited for continuous monitoring as in ECG. Combining continuous ECG monitoring with episodic optical mapping recordings may be the ideal arrangement for imaging sporadic electrical events (Fig. 5).



Simultaneous voltage and calcium recordings using RH-237 and Rhod-2 can quantify the temporal differences in calcium handling between the atrial and ventricular compartments of the zebrafish heart (Lin et al. 2015). Atrial and ventricular calcium transients have distinctly separate relationships with respect to their voltage dynamics and changes in voltage kinetics produce parallel changes in the calcium dynamics. A variable rate stimulation protocol results in linear rate-dependent shortening of the action potentials. In the atrium, the calcium transient duration is 30 ms longer than the action potential duration over a 150 bpm difference in rate. In the ventricle, the calcium transient duration is 20 ms longer over the same range.

Another attractive feature in optical mapping technology is the potential use of genetically encoded voltage and calcium indicators, often referred to as optogenetic sensors, which can be brighter and inherently more photo-stable than fluorescent dyes, giving improved signal quality reviewed in Looger (2012) and Cohen (2016). The kinetics of optogenetic sensors may be slower than fluorescent dyes (Rose et al. 2014; Cohen 2016) but the high signal-to-noise ratio may yield higher effective temporal resolution. Optogenetic sensors are also not prone to leakage or compartmentalization and are more suitable for long imaging protocols (Broussard et al. 2014) and longitudinal studies. For example, one can conduct a time course study in tracking the development of the heart from embryo stage to adulthood and monitoring arrhythmogenesis in a mutant line. Perhaps more importantly, optogenetic sensors can have targeted expression opposed to the diffuse labeling associated with the incubation of fluorescent dyes, leading to far lower background signals, higher imaging contrast, and spatial resolution (Broussard et al. 2014). This targeted expression in the heart was recently demonstrated with a transgenic line carrying a dual function genetically encoded voltage and calcium indicators in which the reporter gene expression was driven by the cardiac specific promoter *cmlc2* (Hou et al. 2014). As zebrafish embryos are translucent and can be imaged under a conventional epifluorescent microscope, targeted expression in the heart also permits for ease in identifying potential founders that carry the optogenetic sensor of choice (Hou et al. 2014).

Numerous variants of optogenetic sensors have been developed in recent years. Typically, these sensors comprise a sensing domain tethered to one or two reporter fluorescent proteins (FPs), resulting in fluorescence readout either via Förster resonance energy transfer (FRET) or conformational change in monomeric FP. FRET-based sensors are ratiometric in nature, thus, less prone to motion artifact, making it the appropriate tool to image a moving heart (Lutcke et al. 2010). However, the signal-to-noise ratio of monomeric FP is higher than FRET-based indicators, leading to increased spatial and temporal resolution (Tian et al. 2009). For monomeric FP-based sensor, GFP is commonly used, although indicators with red-shifted fluorescent proteins are increasingly favored due to less phototoxicity (high wavelength excitation), deeper tissue penetrance, and less background autofluorescence (Shen et al. 2015; Abdelfattah et al. 2016).

Genetically encoded voltage indicators based on GFP include ArcLight and ASAP1. ArcLight reports action potential with high sensitivity ( $\Delta F/F = -35\%$  in

response to 100 mV depolarization) but with relatively slow upstroke (Jin et al. 2012). Comparatively, ASAP1 reliably reports AP with similar sensitivity ( $\Delta F/F = -30\%$  in response to 100 mV depolarization) and has faster on/off kinetics ( $\sim 2$  ms) (St-Pierre et al. 2014). For genetically encoded calcium indicators, extensive protein engineering in the GCaMP family has produced a whole line of indicators, with the most recent permutation GCaMP6 claimed to have highest sensitivity,  $\text{Ca}^{2+}$  binding affinity, larger dynamic range, and fastest kinetics (Chen et al. 2013). Furthermore, a derivative of GCaMP called GECO has been developed with a variety of FPs (Wu et al. 2014a, b; Zhao et al. 2014; Shen et al. 2015) resulting in the flexibility of choosing a calcium indicator with a range of  $K_d$  values for  $\text{Ca}^{2+}$  and spectral separation. This flexibility in the selection of fluorescent spectra enables multiplex imaging to determine the voltage and calcium dynamics using green- and red-shifted optogenetic sensors.

## 4 Isolated Cardiomyocytes

### 4.1 Electrophysiology

Zebrafish cardiac electrophysiology is comparable to mammals as they share major inward and outward currents resulting in similar action potential duration (APD) and morphology (Nemtsas et al. 2010). While optical mapping provides tangible advantages for exploring electrophysiology at multicellular and whole organ levels, it is complemented well by electrophysiological measurements on the individual cell. The use of patch clamping techniques on isolated zebrafish cardiomyocytes (Nemtsas et al. 2010; Zhang et al. 2011) or in heterologous expression systems expressing cloned ion channels (Scholz et al. 2009; Vornanen and Hassinen 2016) records individual channel activity critical for understanding the mechanism of function. Increased understanding can help to better predict the action of pharmacological agents when using the zebrafish heart as a model and is reviewed extensively elsewhere (Leong et al. 2010a, b; Alday et al. 2014; Vornanen and Hassinen 2016). However, as noted previously, due to whole genome duplication, as well as multiple orthologs and isoforms, the molecular mechanisms, function, and response to pharmacological agents of individual ion channels can be significantly different between the hearts of zebrafish and mammals (Verkerk and Remme 2012; Vornanen and Hassinen 2016). The following outlines current understanding of zebrafish cardiomyocyte ion channel currents with the view to highlight necessary caution in interpreting the results of APD and morphology when using zebrafish as a model of mammalian cardiac electrophysiology.

Similar to mammals, inwardly rectifying current ( $I_{k1}$ ) maintaining the cardiac resting membrane potential (phase 4) is present in both atria and ventricle of the zebrafish, along with higher  $I_{k1}$  density in the ventricle than atria (Nemtsas et al. 2010). However, six isoforms of Kir2 channels are expressed in zebrafish,

consequently driving  $I_{k1}$  mainly through orthologs of Kir2.4 and Kir2.2a which are not the main mammalian isoforms (Szuts et al. 2013; Hassinen et al. 2015), suggesting that  $I_{k1}$  is produced by divergent ion channels to those of mammals.

In zebrafish, the upstroke and the plateau phase of the AP are created by the depolarizing inward currents of  $\text{Na}^+$  ( $I_{\text{Na}}$ ) and  $\text{Ca}^{2+}$  ( $I_{\text{Ca}}$ ), respectively, (Nemtsas et al. 2010). The upstroke (phase 0) of the AP is driven by fast  $I_{\text{Na}}$  but with slower upstroke velocity than mammals (Warren et al. 2001). A more negative voltage dependence of inactivation seen in both cultured embryonic and freshly isolated adult zebrafish ventricular myocytes has been suggested to result in lower  $I_{\text{Na}}$  density and consequently the slower upstroke velocity (Warren et al. 2001; Nemtsas et al. 2010). Interestingly, whereas Nav1.5 channels, which underlie  $I_{\text{Na}}$  in mammalian cardiac tissue, are relatively TTX-insensitive compared to neuronal and skeletal muscle isoforms, zebrafish cardiac Nav1.5 channels display three times higher sensitivity to TTX than their mammalian counterpart (Nemtsas et al. 2010). It is intriguing as to how this is advantageous to the zebrafish, but it is due to the preservation of an aromatic residue, which is critical for TTX binding in mammalian neuronal and skeletal muscle channels, but which is substituted for cysteine in mammalian cardiac Nav1.5 channels (Haverinen and Vornanen 2007; Nemtsas et al. 2010). Another interesting discovery is the presence of two zebrafish orthologs (scn5Laa and scn5lab – identified as scn12aa and scn12ab on Ensembl) that share 60–65% amino acid identity with the human SCN5A gene encoding cardiac Nav1.5 channel (Novak et al. 2006; Chopra et al. 2010). Both of these genes are expressed in zebrafish hearts (Novak et al. 2006) and produce typical voltage-gated sodium currents when expressed in CHO cells (Chopra et al. 2010). The additional SCN5 gene is likely the result of the teleost-specific whole genome duplication based on linkage analysis (Novak et al. 2006).

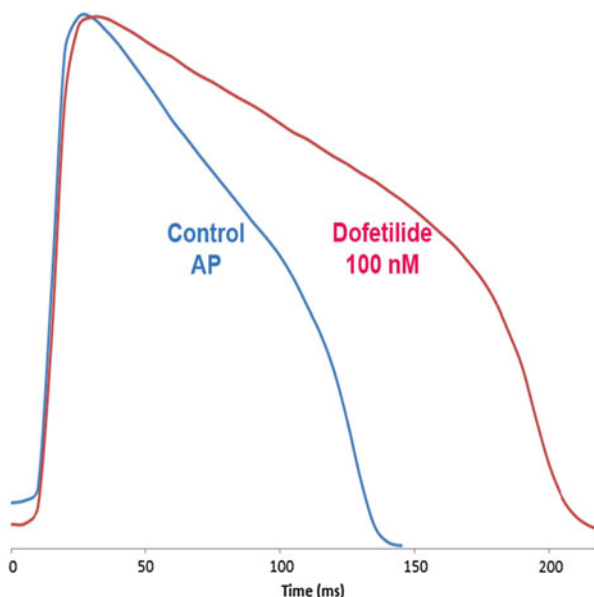
The L-type  $\text{Ca}^{2+}$  current ( $I_{\text{Ca,L}}$ ) is the primary contributor to the long plateau phase (phase 2) of the ventricular action potential in adult mammals, while T-type  $\text{Ca}^{2+}$  current ( $I_{\text{Ca,T}}$ ) is limited to pacemaker cells and immature cardiomyocytes (Mesirca et al. 2014). Careful pharmacological dissection of  $I_{\text{Ca,T}}$  and  $I_{\text{Ca,L}}$  using nifedipine and nickel showed that both currents are present in zebrafish atrial and ventricular myocytes (Nemtsas et al. 2010). L-type  $\text{Ca}^{2+}$  channel gene expression and robust  $I_{\text{Ca,L}}$  density was found in zebrafish ventricles, which contributes to the long plateau phase of AP and the resemblance of the mammalian AP phenotype (Rottbauer et al. 2001; Brette et al. 2008; Nemtsas et al. 2010). The dominant L-type  $\text{Ca}^{2+}$  channel  $\alpha 1$  subunit expressed in human hearts is  $\alpha 1\text{C}$  (Cav1.2), which when mutated in zebrafish, abolishes ventricular contraction (Rottbauer et al. 2001). However,  $\alpha 1\text{D}$  (Cav1.3) is also expressed in the adult zebrafish heart (Sidi et al. 2004). Furthermore, L-type  $\text{Ca}^{2+}$  channels are heteromeric protein complexes, which include a cytoplasmic  $\beta$  subunit that modulates electrophysiological activity. Two genes have been identified in zebrafish as orthologs for the primary  $\beta$  subunit expressed in human cardiac tissues, but the function of each is unclear based on morpholino studies in zebrafish embryos (Zhou et al. 2008). The presence of robust T-type  $\text{Ca}^{2+}$  current in mammals typically occurs in the developing heart, suggestive of a more immature phenotype of zebrafish cardiomyocytes.

The functional relevance of  $I_{Ca,T}$  in adult zebrafish myocytes is yet unknown (Verkerk and Remme 2012), but in embryos  $I_{Ca,T}$  blockade by NNC55-0396 reduces action potential generation (Alday et al. 2014). The T-type  $Ca^{2+}$  channels expressed in zebrafish hearts display properties likely to be consistent with the Cav3.1 subunit (Nemtsas et al. 2010), although immunofluorescence experiments suggest the additional presence of Cav3.2 (Alday et al. 2014). The genetic basis for these channels is unknown.

In mammals, the transient outward current ( $I_{to}$ ) constitutes the rapid repolarization (phase 1) of the AP, but the current is thought to be absent in the adult zebrafish heart (Nemtsas et al. 2010). The slow delayed rectifier  $K^+$  current ( $I_{ks}$ ), which accounts for the mammalian repolarization reserve that is necessary to adapt to stress conditions (Schmitt et al. 2014) may also not be expressed in zebrafish cardiomyocytes (Nemtsas et al. 2010; Alday et al. 2014). However, orthologs of mammalian KCNQ1 and KCNE1 are expressed at the transcript level in zebrafish (Tsai et al. 2011; Wu et al. 2014a, b), and in intact whole heart organ optical mapping experiments the  $I_{Ks}$  blocker chromanol 293B prolonged AP duration (Tsai et al. 2011). It is unclear why these studies differ in their reporting as to the presence of a functional  $I_{Ks}$  current. Given the prominent dependence of  $I_{Ks}$  current on stimulation rate and adrenergic tone (PKA levels), one possible reason is the difference between measurements made in isolated cells compared with intact hearts.

Analogous to mammals, the rapid delayed rectifier  $K^+$  current ( $I_{kr}$ ) is a dominant repolarization current in the zebrafish heart (Nemtsas et al. 2010). Cloned zebrafish ether-a-go-go-related gene (zERG) channels display comparable biophysical properties to human  $I_{kr}$  producing hERG channels (Scholz et al. 2009). Moreover, loss of function of  $I_{K_r}$  in zebrafish hearts recapitulates perturbed repolarization as observed in the human long QT syndrome (LQTS) leading to significant interest in the utility of zebrafish hearts as a model for studying and screening arrhythmogenic  $I_{K_r}$  gene mutation phenotypes (Arnaout et al. 2007). In zebrafish embryos Jou et al. knocked down the KCNH2 gene with morpholinos and then injected the wild-type or mutant hERG1 gene. The authors observed a high success rate for detecting LQTS causing mutations to produce repolarization disorders and bradycardia in the zebrafish embryos. In addition, Milan et al. applied a wide variety of pharmacological agents known to cause acquired LQTS to zebrafish embryos and demonstrated that the majority produced repolarization disorders, suggesting the system may be used as a high throughput screening tool (Milan et al. 2003). These studies present an exciting potential utility of the zebrafish model, but one key issue is that in zebrafish  $I_{kr}$  is not generated by the orthologous channel of hERG1, which is found in mammalian cardiomyocytes; but rather is produced by an ortholog of hERG2 (KCNH6) (Leong et al. 2010a, b). This presents a potential confounding factor when investigating hERG1 mutations that cause electrical disturbances such as LQTS as it is currently unclear whether mutations in the hERG1 ortholog of zebrafish fully recapitulate the disease (Arnaout et al. 2007). Further, the similarity between zebrafish orthologs of hERG may require further characterization of the appropriate gene to be used for mutation. Optical mapping of

**Fig. 6** Voltage recording of dofetilide effect using optical mapping. Observable 70 ms prolongation in  $APD_{50}$  45 min after application of 100 nM of the HERG (ZERG) channel blocker dofetilide (RED)



adult zebrafish hearts revealing AP and calcium handling information will also likely aid in the development of the zebrafish heart as a model for human electrical disorders.

In terms of pharmacological screening, understanding the molecular correlate is essential as different paralogs of zebrafish may produce ion channels with varying drug sensitivity (Abi-Gerges et al. 2011; Hassinen et al. 2015). However, functional relationship in pharmacology can still be established using optical mapping if appropriate care is taken given the caveats described above. Part of the importance of using zebrafish in pharmacological model is in monitoring the effects of drugs on the interdependence of ion channels, i.e. the entire depolarization and repolarization currents (Pugsley et al. 2015). Preliminary work using 100 nM dofetilide showed that the  $APD_{50}$  is elongated by 75 ms (Fig. 6) mimicking acquired long-QT syndrome. This work corroborates what has been done on individual channels with many compounds prolonging QT intervals in zebrafish (Langheinrich et al. 2003; Milan et al. 2003; Mittelstadt et al. 2008), suggesting that zebrafish can still be used in gross pharmacological screening.

Evidently, cardiac electrophysiology of zebrafish is quantitatively different from mammals, but the qualitative similarities allow the zebrafish heart to serve as a useful tool in modeling mammalian cardiac excitation, arrhythmogenesis, and pharmacological screening. With appropriate attention paid to characterization of the wild-type zebrafish, electrophysiological techniques will be important for understanding disease state models of mutants as well.

## 4.2 Excitation-Contraction (E-C) Coupling

The basic process of cardiac E-C coupling, converting the electrical signal of an action potential to the mechanical response of contraction, is conserved between humans and fish (Bovo et al. 2013). However, specifics of the mechanism are different. In mammalian cardiac excitation-contraction coupling, membrane depolarization activates voltage-gated L-type  $\text{Ca}^{2+}$  channel (LTCC or  $\text{Ca}_v1.2$ ) which, in turn, activates the opening of ryanodine receptors (RyR2) leading to a much greater calcium release from the sarcoplasmic reticulum (SR) via  $\text{Ca}^{2+}$ -induced calcium release (CICR). Proportionally, SR calcium release accounts for between 70 and 90% of the calcium necessary to activate contraction and extensive membrane invaginations of the transverse-tubule network allow this calcium release to occur deep within the cell.

In zebrafish cardiac E-C coupling, membrane depolarization also activates voltage-gated calcium channels, which in turn activates SR calcium release. However, the relative contribution of SR calcium release is thought to be far more limited, contributing to around 20% of the calcium transient and the majority of the calcium necessary to activate contraction comes directly through transsarcolemmal influx from extracellular sources (Zhang et al. 2011; Bovo et al. 2013). As in most fish, zebrafish cardiomyocytes lack an extensive T-tubular network (Brette et al. 2008; Di Maio and Block 2008), such that an apparent spatial separation exists between RyR and LTCC possibly explaining the reduction in SR  $\text{Ca}^{2+}$  release (Bovo et al. 2013). Interestingly, RyR phosphorylation by PKA shows that it acts similarly to the mammalian system (Bovo et al. 2013) but the RyR are apparently organized into release clusters with low cytosolic calcium sensitivity (Bovo et al. 2013). However, calcium sparks have been demonstrated in zebrafish that are quite similar in properties and frequency to trout, mammalian and human cardiomyocytes. Although zebrafish hearts may have similar heart rates and action potential durations with respect to humans, the underlying mechanisms of E-C coupling still need to be resolved.

These differences in E-C coupling have profound differences in the way that contractility is physiologically regulated in each system. Due to the dominant role of SR calcium release in mammalian systems, contractility is primarily controlled by modulating the amount of SR calcium release with lesser effects through increasing calcium influx. Since SR calcium release is affected by the SR calcium load, contractility is indirectly affected by the activity of SR-ATPases (SERCA).

In zebrafish myocytes, contractility is less reliant on CICR and is primarily regulated by modulating calcium entry, which occurs mainly through L-type and T-type calcium channels with secondary effects through reverse-mode sodium-calcium exchange (NCX) activity. In zebrafish ventricular myocytes,  $I_{\text{Ca,L}}$  accounts for 50% of calcium influx (Zhang et al. 2011). Zebrafish also maintain mammalian-like systems of response to  $\beta$  adrenergic receptor stimulation through LTCC-mediated increased cytosolic  $\text{Ca}^{2+}$  transient amplitude upon addition of the PKA activator forskolin (Bovo et al. 2013). Just as in mammalian hearts, increased heart

rate increases diastolic calcium and increases calcium-dependent inactivation. Increased diastolic calcium levels would also be expected to increase SR calcium loads in the zebrafish heart. However, because SR calcium release is unlikely to be a dominant source of activating calcium, the increase in SR calcium release is unable to compensate for the decreased calcium entry and zebrafish hearts exhibit a negative force-frequency response (Bovo et al. 2013). However, this interpretation is complicated by the fact that the murine heart, in which SR  $\text{Ca}^{2+}$  release plays a dominant role, often exhibits a negative force-frequency response. The apparent lesser importance of the SR for E-C coupling is reflected in a lack of arrhythmias with morpholino-knockdown of SERCA activity but defects in contractility in zebrafish embryos (Ebert et al. 2005).

Increased frequency directly reduces  $I_{\text{Ca}}$ , by limiting the time to recovery from voltage-dependent inactivation and indirectly reduces  $I_{\text{Ca}}$ , by increasing calcium-dependent inactivation through rising diastolic calcium levels. In combination, increasing the effective heart rate in single ventricular myocytes, from 30 bpm to 120 bpm, reduces  $I_{\text{Ca}}$  by 45% (Zhang et al. 2011). Yet, the magnitude of the calcium transient is relatively unaffected suggesting to some investigators that an alternative mechanism of calcium influx must be in place. However, this notion remains controversial in light of the comparable properties of the RYR2 and the  $\text{Ca}^{2+}$  sparks in zebrafish.

Contractility in mammalian cardiomyocytes varies with voltage and the relationship closely follows the voltage-dependence of activation of LTCC which peaks near 0 mV, reflecting an optimal balance between channel activation and driving force for calcium entry. However, in zebrafish myocytes, contractility continues to increase at more depolarized membrane potentials. In mammalian hearts the primary role of NCX is in relaxation, exchanging cytosolic calcium for extracellular sodium, which is favored by hyperpolarized membrane potentials and by high cytosolic calcium levels. However, due to the stoichiometry of 3  $\text{Na}^+:\text{Ca}^{2+}$ , strong depolarization can induce reverse-mode NCX activity resulting in calcium influx.

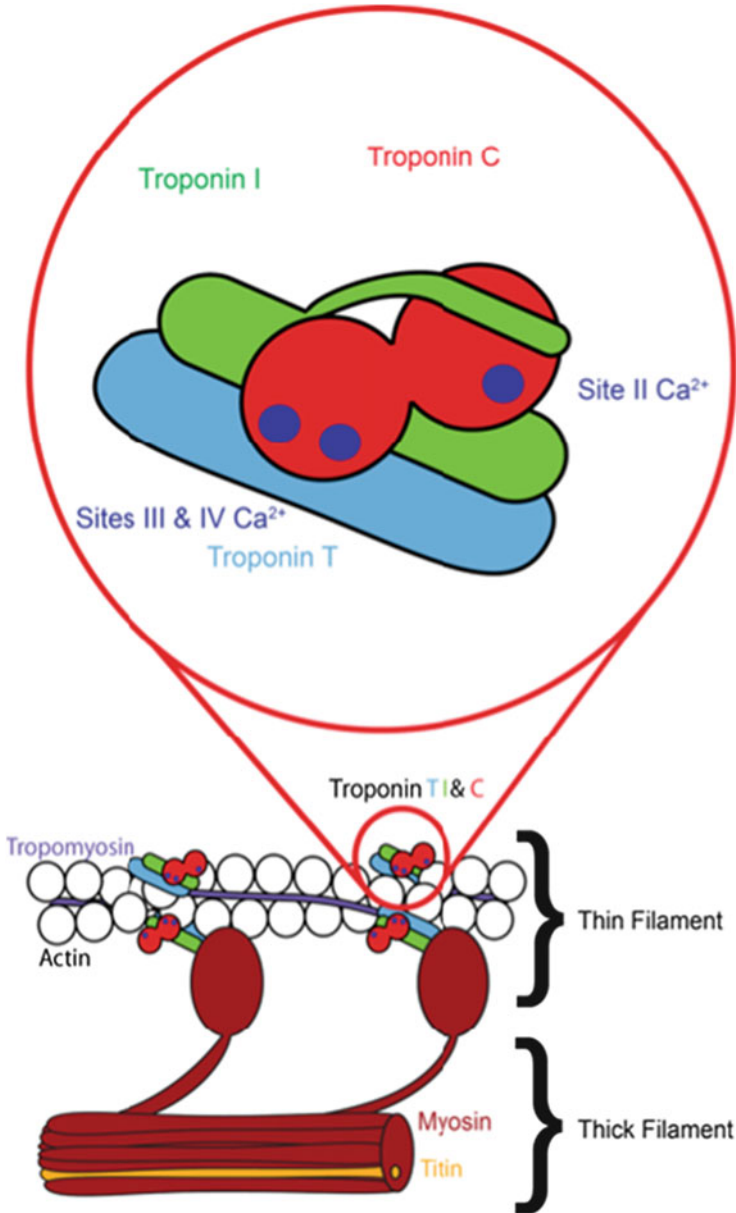
In fish, both  $I_{\text{Ca}}$  and reverse-mode NCX are capable of triggering  $\text{Ca}^{2+}$  release from the SR (Hove-Madsen et al. 2003). Reverse-mode NCX activity is most favorable when there is strong membrane depolarization, high cytosolic sodium concentration, and low cytosolic calcium – conditions that are present at the upstroke of the action potential (Birkedal and Shiels 2007). This is augmented by the fact that fish typically have a higher density of NCX than adult mammals. At high stimulation rates,  $I_{\text{Ca}}$  is reduced and  $[\text{Na}^+]_i$  may further increase, favoring increasing amounts of reverse-mode NCX. In general, a higher NCX activity is observed in teleost cardiomyocytes relative to mammalian (Vornanen 1999; Hove-Madsen et al. 2000; Zhang et al. 2011). Therefore, high NCX activity may contribute to the zebrafish heart's ability to maintain contractility at high heart rates despite the strong suppressive effects on  $I_{\text{Ca}}$ . Defective NCX activity leads to abnormal  $\text{Ca}^{2+}$  transients and de-synchronized contractions in the tremblor (tre) mutant, indicating the importance of NCX for proper rhythmicity of the zebrafish heart (Langenbacher et al. 2005).



It is likely that to some degree, the small volume and elongated shape of zebrafish ventricular myocytes (and the commensurate high surface-to-volume relationship) compensates for lack of T-tubules as in mammalian atrial cells (Blatter et al. 2003). This could relate to the impact of temperature on CICR. Mammalian hearts can induce rapid elevations in cytosolic  $\text{Ca}^{2+}$  via CICR, but it appears that zebrafish must rely primarily on direct  $\text{Ca}^{2+}$  influx (Verkerk and Remme 2012) as a slower but more robust mechanism of E-C coupling in a temperature variable environment. However this conclusion must be reconciled with the following facts: (1) the SR  $\text{Ca}^{2+}$  load and uptake rate (temperature adjusted) in many teleosts is comparable to or greater than that of mammals; (2) caffeine contractures are comparable in magnitude; (3) calcium can be released spontaneously from the SR and (4) the properties of sparks are similar to mammals. In some respects one could view the zebrafish as a model of immature human cardiac myocytes in many ways with the lack of t-tubules and reliance on extracellular  $\text{Ca}^{2+}$  (Huang et al. 2005). While this may not be the ideal model for E-C coupling to compare to mature humans, the similarities with immature human cardiomyocytes and overall developmental pathways allow zebrafish to be used in recapitulating disorders that occur at these stages of development.

### 4.3 Contraction

Work characterizing myofilament mechanical function is critical to understanding contractile dynamics of many zebrafish cardiomyopathy models. Several of these studies have concluded that cardiac myofibrils from zebrafish, like those from mice, are suitable contractile models to study cardiac function at the sarcomeric level (Iorga et al. 2011; Dvornikov et al. 2014). The proportions of the zebrafish cardiomyocyte make cardiac myofibrils ideally suited for force and sarcomere length measurements in response to changes in  $[\text{Ca}^{2+}]$ , as the entire cell can be perfused simultaneously (Stehle et al. 2002, 2009; Lionne et al. 2003). The fundamental mechanism of cardiac contraction is conserved across vertebrates. The basic contractile unit of the sarcomere is composed of thick and thin filaments, which form the characteristic striated pattern of cardiac muscle and regulate cardiac contraction and relaxation. As the cytosolic  $\text{Ca}^{2+}$  concentration increases, the regulatory  $\text{Ca}^{2+}$  binding site II of cTnC becomes occupied, leading to a conformational change that exposes a hydrophobic patch on cTnC. This hydrophobic patch then interacts with TnI and allows the TnI inhibitory peptide to withdraw from the actin filament (Parmacek and Solaro 2004). The cTnC- $\text{Ca}^{2+}$  binding interaction also interrupts the TnT-Tropomyosin (Tm) interaction, and permits actin/myosin cross-bridge formation and initiates muscle contraction. The modulation of twitch force by both extracellular  $[\text{Ca}^{2+}]$  and sarcomere length (SL), myofilament length-dependent activation, is an intrinsic property and critical mediator of contractile function of both mammalian and zebrafish cardiomyocytes (Dvornikov et al. 2014) (Fig. 7).



**Fig. 7** Schematic of the role of troponin in muscle contraction

Similarities exist between the mammalian and zebrafish system in regulation of contractile force, namely a direct proportionality between extracellular  $[\text{Ca}^{2+}]$  and cytosolic  $\text{Ca}^{2+}$  transients (Dvornikov et al. 2014). As has been established in the mammalian system (Allen and Kurihara 1979; Janssen and de Tombe 1997) cell

length does not affect the magnitude of the cytosolic  $\text{Ca}^{2+}$  transient in the first twitch following cell stretch, just as in zebrafish (Dvornikov et al. 2014). The appearance of ventricular myofibrils from zebrafish is similar to mouse papillary muscles (Iorga et al. 2008, 2011). These myofibrils often branch just as in mammals (Iorga et al. 2011). The resting SL recorded in zebrafish (1.85  $\mu\text{m}$  in (Dvornikov et al. 2014); 2.12  $\mu\text{m}$  in (Iorga et al. 2011)) is comparable to that in all teleost species studied to date (Shiels et al. 2006; Patrick et al. 2011) as well as murine models ((SL =  $2.03 \pm 0.03 \mu\text{m}$ ) (Iorga et al. 2008)).

$\text{Ca}^{2+}$  sensitivity in cardiac myocytes is influenced much more by SL than skeletal myocytes (Hanft and McDonald 2010). In zebrafish,  $\text{Ca}^{2+}$ -dependent force of myotomal myofibrils is almost one order of magnitude lower (Iorga et al. 2011) than skeletal muscle of other teleosts (Altringham and Johnston 1982) and mammals (Edman 2005; Poggesi et al. 2005). The contractile element and thus cardiac TnC have an increased sensitivity for  $\text{Ca}^{2+}$  at longer sarcomere lengths (Granzier et al. 1991; de Tombe et al. 2010). Extracellular  $\text{Ca}^{2+}$ , cytosolic  $\text{Ca}^{2+}$ , and consequently twitch force are directly proportion in single cardiac myocytes isolated from adult zebrafish hearts (Dvornikov et al. 2014). The kinetics of force development in zebrafish were found to be dependent on  $[\text{Ca}^{2+}]$  but the relaxation was independent, neither of which was affected by SL (Dvornikov et al. 2014) in agreement with findings on the mammalian sarcomere (Poggesi et al. 2005). The rate constant of force development after  $\text{Ca}^{2+}$  activation, isometric force at maximal  $\text{Ca}^{2+}$  activation,  $\text{Ca}^{2+}$  sensitivity of force, and force response to an external stretch are all comparable in the zebrafish and mice models (Iorga et al. 2011). In mammalian systems, persistent stretch is concurrent over minutes with a slow increase in twitch force (Cingolani et al. 2011) known as the slow force response (SFR), which is poorly understood. This property is not present in either trout (Patrick et al. 2011) or zebrafish (Dvornikov et al. 2014), therefore the study of the mechanisms of SFR may not be possible in zebrafish.

The mammalian force–SL relationship is curvilinear and depends on extracellular  $[\text{Ca}^{2+}]$  (ter Keurs et al. 1980; Kentish et al. 1986). The slope of this relationship increases proportionally with stretch as does the twitch force in mammals (ter Keurs et al. 1980; Kentish et al. 1986; de Tombe and ter Keurs 1991). This forms the basis of the Frank-Starling response of the heart, in which the heart ejects a greater stroke volume at greater end diastolic volumes. This is an essential property of the myocardium which differentiates it from skeletal muscles and this response is intact in the zebrafish heart and individual myocytes. The force–pCa relationship is comparable in mice and zebrafish myofibrillar bundles, though maximal force may be slightly higher in the latter (Iorga et al. 2011). Both zebrafish skinned myofibrils and ventricular cells display a length-dependent force generation that is similar in magnitude to the mammalian sarcomere (Mateja and de Tombe 2012; Dvornikov et al. 2014). Increased sensitivity of the zebrafish sarcomere is thought to be causal as the changes occur at constant cytosolic  $[\text{Ca}^{2+}]$  (Dvornikov et al. 2014). The zebrafish is a good model for studying length dependent activation (LDA) as it shares this property as expected with other teleosts such as trout (Patrick et al. 2010) as well as mammals (Mateja and de Tombe 2012).

Passive stiffness is similar in zebrafish and mice myofibrils with low stretch (Iorga et al. 2011). Passive force at low stretch in rainbow trout and rats also shows similar levels of passive stiffness (Patrick et al. 2010). However, with extensive stretch (beyond  $\sim 2.5 \mu\text{m}$ ), passive forces in zebrafish and mice are very different (Iorga et al. 2011). Force decay upon  $\text{Ca}^{2+}$  removal is biphasic in zebrafish (Steffen et al. 2007; Iorga et al. 2011) and numerous species of mammals (Stehle et al. 2002; Poggesi et al. 2005). The initial slow decay of force can be used to estimate cross-bridge detachment, this phase occurs while SL is constant (Poggesi et al. 2005; Stehle et al. 2009); the rate constant corresponding to this phase is similar in zebrafish and mice when measured at  $10^\circ\text{C}$  (Iorga et al. 2011). A delay then occurs which is longer in zebrafish than that seen in mice at this common experimental temperature (Iorga et al. 2011). Following the delay a faster force decay occurs as the sarcomeres lengthens accelerating cross-bridge detachment (Poggesi et al. 2005; Stehle et al. 2009); the rate constant corresponding to this phase of relaxation is smaller in zebrafish than in mice (Iorga et al. 2011). The overall effect is that zebrafish myofibrils relax more slowly than those isolated from mice, this makes sense in light of murine ( $\sim 600$  bpm at  $37^\circ\text{C}$ ) and zebrafish ( $\sim 150$  bpm at  $28.5^\circ\text{C}$ ) resting HR at their respective physiological temperatures (Iorga et al. 2011). While the molecular basis is currently unknown, it has been speculated that this is due to different titin isoforms and/or differential phosphorylation states of the contractile proteins in fish (Seeley et al. 2007; Patrick et al. 2010; Iorga et al. 2011; Zou et al. 2015) based on the weak contractile phenotype of the *pickwick* mutation (Driever et al. 1996) in zebrafish. A further benefit in using zebrafish myofibrils is their slower rate of contraction and relaxation compared to murine models such that kinetic parameters can be determined above  $10^\circ\text{C}$  (Iorga et al. 2011).

Variation in paralogs of contractile proteins can create variation in the mechanics of cardiac contraction present in zebrafish studies of cardiac myofibrils (Iorga et al. 2011; Dvornikov et al. 2014). In mammals, many of these sarcomeric proteins have muscle-specific paralogs that are expressed such that a single paralog is uniquely found in each muscle type (Huang and Jin 1999). Mutant zebrafish have been used to study cardiac specific orthologs of human sarcomeric proteins such as troponin T (Sehnert et al. 2002), troponin C (Ohte et al. 2009; Sogah et al. 2010), tropomyosin (Zhao et al. 2008), myosin light chain (Rottbauer et al. 2006), and titin (Seeley et al. 2007; Zou et al. 2015). However both the expression patterns and function of these same sarcomeric proteins may vary from humans due to retention of additional duplicated products in the zebrafish genome. Hence the phenotype of these mutants may not be directly translated to humans without knowledge of the fish-specific molecular basis. While the overall mechanism of contraction is conserved, the molecular basis of contraction in fish provides an interesting platform to understand the consequence of genome duplication in the zebrafish model.

## 5 Molecular Basis of Cardiac Function

With numerous mutations of zebrafish genes serving as both cardiomyopathy and channelopathy models, it becomes increasingly important to ensure that the correct zebrafish equivalent to the disease-causing human gene is found. Despite the fact that zebrafish have additional copies of many genes that are important to cardiac function, the wealth of knowledge available on the genomic level allows us to translate the physiological and biological conclusions that can be gleaned from zebrafish-based experiments effectively to mammalian systems. By addressing questions on the molecular level, one can better design experiments that are biologically appropriate to the model. An example of this can be seen in using genomic and phylogenetic analyses in conjunction with transcriptomic data, and integrating these findings with protein structure and functional data (e.g., molecular dynamics simulations and isothermal titration calorimetry) for exploration of troponin (Tn) paralogs (Genge et al. 2016; Stevens et al. 2016).

In mammalian cardiac cells co-expression of multiple variants of proteins composing the Tn complex can result in modified protein interactions and variation in  $\text{Ca}^{2+}$  sensitivity leading to non-uniform contraction (Wei and Jin 2011). Co-expression for Tn isoforms is often indicative of a diseased state of the mammalian heart (Anderson et al. 1991; Wei and Jin 2011). For example, the co-presence of multiple TnT splice variants in adult mammalian hearts reduces cardiac efficiency by desynchronizing the  $\text{Ca}^{2+}$  activation of the thin filaments (Jin et al. 2008). While mixed isoform usage of both TnT and TnI is found during fetal cardiac development, co-expression results in less contractile force (Fentzke et al. 1999; Yin et al. 2015). Many sarcomeric proteins also have multiple homologs expressed in the zebrafish heart (Alderman et al. 2012; Genge et al. 2013; Shih et al. 2015). Paralogs for transcripts of both TnC and TnI are expressed in a chamber- and temperature-specific manner in cardiac tissue of zebrafish (Sogah et al. 2010; Alderman et al. 2012; Genge et al. 2013, 2016). The presence of multiple fish-specific gene products of important components of the cardiac contractile element may be indicative of how fish have evolved mechanisms to thrive in variable environments. Incorporating the chamber-specific variation with temperature response reveals the complexity of these mechanisms in achieving survival, but may also be important to understanding variability in results in a zebrafish model.

TnC is a highly conserved protein across phylogeny, with few sequence changes maintained in any vertebrate ortholog. The cardiac contractile element in all species studied to date is less sensitive to  $\text{Ca}^{2+}$  when the temperature is decreased (Harrison and Bers 1989), therefore it is understandable that a higher relative TnC sensitivity is needed for teleosts to maintain proper cardiac function in colder environments (Gillis et al. 2005, 2007). The role of paralog usage likely creates functional differences in the atrium and ventricle through the use of chamber-specific isoforms of many contractile proteins (e.g., myosin heavy chain and myosin light chain (Karasinski et al. 2001)). In zebrafish, TnC1b and TnC1a transcripts are expressed

in a chamber-specific manner (Genge et al. 2013). This could possibly lead to changes in the  $\text{Ca}^{2+}$  affinity of the entire Tn complex.

The consequences of many gene products for Tn have been explored in some detail by our group on the protein function level (Genge et al. 2013, 2016; Stevens et al. 2016). These paralogs have very similar functions, but the differences between them involve the subtle manipulation of the energetic landscape of the transition between closed and open conformations. This has an effect on  $\text{Ca}^{2+}$  binding properties that is related to  $\text{Ca}^{2+}$  sensitivity, yet still maintains limited structural deviation between the structural models of each TnC paralog (Stevens et al. 2016). The expression of multiple TnC paralogs with minimal differences in  $\text{Ca}^{2+}$  sensitivity may allow flexibility in response to variable environmental conditions while still maintaining uniform cardiac contraction. While two paralogs of the cardiac TnC protein are not present in humans, the mechanism of initiation of cardiac contraction is conserved. Many disease-causing mutations are expected to increase  $\text{Ca}^{2+}$  sensitivity in a manner similar to the naturally occurring mutations in fish TnC (Gillis and Tibbits 2002), as a result, great care must be taken in the generalization of biological data. Additionally a more complete understanding of the molecular details of the contractile apparatus machinery across species will allow us to more accurately and efficaciously interpret and translate experimental designs and results between the zebrafish model and human disease.

Interaction sites that mediate important functions by binding regulatory proteins have strong evolutionary constraints on amino acid substitutions (Guharoy and Chakrabarti 2005). The conformation of TnC increases in degree of hydrophobic patch opening upon interaction with the switch region of TnI in mammalian models (Gagne et al. 1995; Li et al. 1999). The TnI switch peptide (TnI<sub>SW</sub>) will bind to and stabilize the open conformation of TnC, while simultaneously occluding the hydrophobic cleft (Li et al. 1999). To fully understand the dynamics between the open and closed state of TnC, TnI is necessary to stabilize the open conformation, which results in higher sensitivity. Having multiple paralogs in combination to form the Tn complex in teleost heart will change strength of interaction. A lower strength of interaction between mammalian cardiac TnC/TnI is responsible, at least in part, for the dependence of cardiac contractility on the formation of strong cross-bridges for full activation to occur. The increased dependence of strong cross-bridge formation has been suggested to be partially responsible for the steeper length dependence of force generation. Variability between contacts and residue distances between the TnI switch and TnC paralogs may promote faster sarcomeric relaxation and lower  $\text{Ca}^{2+}$  sensitivity (Palpant et al. 2010), as has been found in mammalian TnI3 (Genge et al. 2016). Correlated mutations suggest that compensatory changes that occur between interacting residues are crucial to maintain protein function (Ovchinnikov et al. 2014). Utilizing the teleost Tn complex thus provides an example of how compensatory substitutions to prevent loss of function and provide an important capacity to adapt, as well as emphasize the importance of looking at mutations in one protein in context with interacting proteins. An understanding of the interactions with other proteins is necessary for full understanding of variability in

function of Tn or any other sarcomeric protein in not only the zebrafish, but also all vertebrate models.

The work done on the evolution of paralogs of the contractile unit can contextualize the variation seen in zebrafish as a cardiac model. Certain paralogs may be utilized to recapitulate a mammalian model under certain conditions despite over differences in gene and protein structures. The co-evolution of Tn subunits can thus be used for the model of variation in contractile function as well as understanding the consequences of gene duplication on contractile function in the zebrafish. This work is by no means in isolation. Another example can be seen in work focused on the importance of transcriptional regulation for paralog usage and splice variation in titin in producing diseased phenotype in zebrafish cardiomyopathy models (Seeley et al. 2007; Zou et al. 2015). It may be necessary to understand the impact of retained duplicate gene products on the molecular level in order to accurately generate biological questions as well as explain a number of physiological mechanisms that occur in the zebrafish.

## 6 Conclusions/Summary

Although numerous caveats exist in using zebrafish to directly model human cardiac physiology, for specific questions the advantages of the zebrafish model are obvious. The relative ease of genetic manipulation and the conservation of many components of E-C coupling make the adult zebrafish an attractive model for cardiac function studies. This requires accurate knowledge of the molecular basis of the components of cardiac ionic currents and the contractile unit. Despite variation in the molecular basis, the conservation of the structure of critical domains of several ion channels and sarcomeric proteins does not preclude this model if appropriate attention is paid to evolutionary divergence. The assumption cannot be made that an orthologous gene in zebrafish will have the same function as it does in human so appropriate phylogenetic studies coupled with functional assays should be used.

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