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Methods to assess *Drosophila* heart development, function and aging

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Abstract

In recent years the Drosophila heart has become an established model of many different aspects of human cardiac disease. This model has allowed identification of disease-causing mechanisms underlying congenital heart disease and cardiomyopathies and has permitted the study underlying genetic, metabolic and age-related contributions to heart function. In this review we discuss methods currently employed in the analysis of the Drosophila heart structure and function, such as optical methods to infer heart function and performance, electrophysiological and mechanical approaches to characterize cardiac tissue properties, and conclude with histological techniques used in the study of heart development and adult structure.

Keywords

Drosophila; heart; development; cardiomyopathy; congenital heart disease; genetics; optical heart beat analysis; electrophysiology; atomic force microscopy; immunohistochemistry; KCNQ; tinman

Introduction

The *Drosophila* heart as a tool for investigating cardiomyopathies

The *Drosophila* heart or dorsal vessel is a linear tube that is reminiscent of the primitive vertebrate embryonic heart tube. Although the final heart structure in *Drosophila* is very different from that in vertebrates, the basic elements for cardiac development, function and aging are remarkably conserved (Bodmer 1995, Cripps and Olson 2002, Ocorr et al. 2007a). Because of the simplicity in structure and availability of powerful genetic tools, the *Drosophila* heart has emerged as a pioneering model system for unraveling the basic genetic and molecular mechanisms of cardiac development, function and aging (Bodmer and Frasch 2010, Nishimura et al. 2011). The *Drosophila* heart model has proven to be a valuable asset to elucidate the etiology of human cardiac disease, including dilated and restricted

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cardiomyopathy, channelopathies, diabetic and congenital heart disease, as well as cardiac senescence (Birse et al. 2010, Cammarato et al. 2008, Melkani et al. 2013, Na et al. 2013, Ocorr et al. 2007b, Qian et al. 2008, Taghli-Lamallem et al. 2008, Wessells et al. 2004, Wolf et al. 2006). The *Drosophila* heart has also been used as a tool for the identification of novel genes and pathways potentially involved in heart disease (e.g. (Kim et al. 2010, Kim et al. 2004, Neely et al. 2010, Qian et al. 2011). Of note, certain important ion channel gene functions are conserved between *Drosophila* and humans to maintain a regular heart rhythm, such as KCNQ (Ocorr et al. 2007b). Interestingly, some of these ion channels do not play a significant role in the (faster beating) adult mouse heart (Nerbonne 2004). This suggests that in some regards the fly heart model may be more informative than the mouse model.

We will first discuss different methods to assess heart function that may change under different genetic and environmental conditions, as well as with age. Then we will summarize tools and markers for structural features of the heart during development, maintenance and aging. An overview of the different methods discussed in this chapter is presented in Table 1.

Optical-based analysis methods to measure heart function and performance

Heart Rate and Pacing

The *Drosophila* heart is a linear tube with a non-contractile aorta that extends from the posteriorly located heart to the head in both larva and adults. Rudimentary valve-like structures divide the heart into chambers and prevent back flow of hemolymph. In larvae the heart is suspended within the hemocoel and undergoes substantial remodeling during pupal stages prior to forming the four chamber abdominal heart of the adult fly (Molina and Cripps 2001; Zeitouni et al. 2007). Early efforts to examine heart function in intact *Drosophila* were performed on dissected larva (Gu and Singh 1995) and early pupa, where the cuticle is nearly transparent (Dowse et al. 1995). For larval preparations the heart rate was determined visually. For the pupal preparation, light is passed through the heart and detected by a phototransistor in the microscope eyepiece. Changes in overall light intensity could be recorded and displayed as linear traces. Using custom software, Maximum Entropy Spectral Analysis (MESA) the overall heart rate could be determined (Dowse et al. 1989, Dowse and Ringo 1991). In addition, heartbeat rhythmicity could be quantified by MESA as a correlation coefficient. Using genetic mutants and pharmacological manipulations these techniques provided evidence that the fly heartbeat is myogenic and that the cardiac action potential likely does not have a substantial Na^+ current since heartbeats were not affected by tetrodotoxin (TTX; Gu and Singh 1995, Johnson et al. 1998). These studies also showed that reduction of extracellular Ca^{2+} stopped heart function suggesting that the cardiac action potential is Ca^{2+} based, although each of these groups came to different conclusions concerning the specific type of Ca^{2+} channel that was involved. In addition a prominent role for K^+ channels was suggested in that channel blockers such as TEA caused dramatic reductions in heart rate (Gu and Singh 1995) and mutations in shaker, ether-a-gogo and slowpoke K^+ channels all affected heart rate albeit to different extents (Johnson et al. 1998).

A variation on these optical detection methods has been developed for monitoring the adult hearts expressing GFP (Dulcis and Levine 2005). The GFP signal permits the heart walls to be visualized through the cuticle; however, since the fluorescent signal is usually very low, high frame rate recordings require specialized equipment that is costly. Another approach for optically monitoring heart function in adult makes use of infrared light and an array of sensors oriented along the abdominal cuticle (Wasserthal 2007). Longer wavelengths are better able to penetrate the pigmented cuticle of the adult thus permitting heart rate and rhythmicity to be determined *in vivo* from tethered adult flies. However, the spatial resolution is limited and frame rate is still relatively low with this method.

In order to test cardiac performance, cardiac stress tests have been developed based on increasing or 'pacing' the resting heart rate. These assays can be used to compare the effects of different mutations as well as the effect of age on heart function. In one such assay, pacing of pupal or adult hearts to higher rates was achieved by increasing temperature (Paternostro et al. 2001, White et al. 1992). In another pacing assay, the head and abdomen of adult flies are placed to touch two strips of electrode jelly (Fig. 1) and a square wave current is passed through the animal to pace the heart to 6 Hz (the upper end of the unperturbed heart rate range, which normally is 4–5 Hz) for a set amount of time (30 sec; (Wessells and Bodmer 2004, Wessells et al. 2004). Immediately after pacing, heart function is monitored visually through the abdominal cuticle (A2–3) and flies are scored depending on whether the heart can still beat following the stimulus or whether it fails to contract or contracts in a spasmodic fashion. This assay has been used to show that insulin signaling plays a heart-autonomous role in cardiac senescence (Wessells et al. 2004) and KCNQ channels are critical for cardiac repolarization in *Drosophila* (Ocorr et al. 2007b).

Semi-Automated Optical Heartbeat Analysis (SOHA)

The movement detection methodologies described above all rely on detecting overall changes in the intensity of light passing through the heart while it is beating ('darkness' profiles, Fig. 2A). A more sensitive method that relies on the combined use of two different computer algorithms has been developed for use with high speed, high resolution digital cameras (Ocorr et al. 2007b). This Semi-automated Optical Heartbeat Analysis (SOHA; detailed method in Fink et al. 2009) uses two computer algorithms that were developed to combine information about overall darkness changes (Fig. 2A), which denote systolic periods, with a pixel-by-pixel analysis of intensity changes detecting only the regions in the movie frame that are moving (Fig. 2B). This combinatorial approach thus detects the individual inward and outward movements of the heart wall and uses the gross darkness changes to determine the "state" (i.e. contracted v. relaxed); permitting the quantification of large numbers of contractions in relatively long movie clips. This capability becomes significant with respect to rhythmicity measurements (see below). Camera setups that can record images at speeds of 150–500+ fps with pixel resolution in the 1 micron range allow very precise temporal and spatial measurements. In addition, the high spatial resolution enhances the ability to detect even subtle changes in contractility measured as Fractional Shortening (FS, e.g. decreased FS with age; Cammarato et al. 2008). Of particular usefulness, this program produces high-resolution qualitative records of heart wall movements (M-mode, kymograph) by excising a 1 pixel horizontal "slice" of pixels from

each frame of the movie and aligning them horizontally to provide an edge trace displaying heart wall movements in the X-axis and time along the Y-axis (Fig. 2C; Fink et al. 2009).

This analysis program can also be used with lower speed and lower resolution movies, such as of GFP fluorescent-labelled hearts (Lalevee et al. 2006, Monnier et al. 2012). However, due to the lower resolution and speed, the output will be limited to heart rate (see discussion below). This analysis method also works with movies of intact (*in vivo*) preparations of larval, pupal and adult *Drosophila* hearts (Ocorr et al. 2007b; Senatore et al. 2010), as well as larval zebrafish and day 8–9 embryonic mouse hearts (Fink et al. 2009). However, SOHA is most sensitive when using semi-dissected preparations to expose the beating heart and using direct immersion objectives. Although it takes some time to learn the dissection technique (Vogler and Ocorr 2009), the attainable speed of dissection is roughly equivalent to the time it takes to position the intact organisms and the light source for other optical methods, including OCT (see below). SOHA is not fully automatic because it is desirable to permit user input to verify and correct (when needed) the systolic and diastolic intervals detected by the combined algorithms. It also requires the user to manually mark the heart edges for the diameter and fractional shortening measurements (detailed in Fink et al. 2009, and illustrated in Ocorr et al. 2009).

Using the SOHA method, a number of disease models have been developed, including channelopathy models (Akasaka et al. 2006, Ocorr et al. 2007b), restricted and dilated cardiomyopathy models (Cammarato et al. 2008, Taghli-Lamallem et al. 2008, Cozhimuttam Viswanathan et al. 2013), diabetic cardiomyopathy models (Birse et al. 2010, Na et al. 2013), and a cardiac amyloidosis model (Melkani et al. 2013).

A more fully automated optical methodology for data collection has been also developed for intact adult flies (Feala et al. 2008). In this method the automation is achieved thanks to a customized fly deposition system and software that can recognize and orient the heart for the camera. An automated stage orients the flies such that heart wall movements can then be detected and a single line of pixels is recorded to produce a real time M-mode records from which heart rate can be determined. This methodology was used to characterize the response to acute hypoxia and could be used, with appropriate optimization, to screen for genetic modifiers of this response.

Optical Coherence Tomography (OCT)

Optical coherence tomography (OCT) uses optical wave lengths in the infrared light range to produce subsurface images. Light passing through the fly is scattered but the pattern of scattering can be used to build an image of structure lying 1–2mm below the surface using a technique referred to as interferometry. These images are referred to as A-scans and can be generated over time to provide a movie of the beating heart. These scans can also be used to generate a cross-sectional image called a B-scan. This technique has been used successfully to study the *Drosophila* heart in anaesthetized flies (Wolf et al. 2006). As proof of principle, the effects of mutations in muscle structural proteins on heart function were examined. Mutations in Troponin I, Tropomyosin 2 and δ-Sarcoglycan all produced a dilated cardiac phenotype in the fly model.

Because of the processing required, the image capture rate is relatively low compared to high speed optical cameras. However, higher speeds up to 100 fps have been recently reported (Tsai et al. 2011, Tsai et al. 2013, Zhou et al. 2013). The advantage of this method is that tissue structure and dynamics can be observed *in vivo*, however because resolution is limited to a 10 micron range (Yu et al. 2010, Zhou et al. 2013) this method is best suited to determinations of heart rate and cardiac dilation and less so for rhythmicity and contractility measurements such as fractional shortening. Finally, the equipment required for this analytical method is highly specialized and costly.

Comparisons between optical-based analysis methods

All of these optical methodologies have limitations that must be kept in mind. One is the speed of data capture. The adult fly heart beats *in vivo* at rates of 3–6 Hz while the denervated adult heart preparation as well as the larval heart both beat more slowly at 1–3 Hz. Unlike most other heart models, such as Zebrafish and mouse, the fly has distinct systolic and diastolic intervals (Figure 2C). In denervated hearts the heart contraction event (systolic interval) is typically shorter than the relaxation event (diastolic interval); the average systole is 0.2 – 0.25 sec, whereas the average diastole is 0.3 – 0.6 sec. Thus, the ability to precisely track the contraction event will be dependent on the rate at which the optical images are captured. For example a 30fps movie will have only 3–4 frames covering systole and 11–12 frames covering diastole, whereas a 150fps movie will have 5 times as many frames covering each interval. Thus data captured at low frame rates cannot be used to reliably quantify distinct intervals but will permit rate and crude rhythmicity measurements.

A second limitation is the image resolution. This limitation primarily comes into play when one is using images to determine the contractility based on the diameters of the heart wall during diastole and systole (fractional shortening). The heart tube in adult flies is on average 60 – 80 microns in diameter during diastole and 40 – 50 microns during systole. Ideally one would like to resolve images to at least the 1 micron level for this type of measurement. Even with adequate resolution this measurement could be affected by low frame rates which might make it difficult to identify a frame where the heart is in its most contracted state.

A related consideration concerns the ability to accurately mark the edges of the heart in intact animals for the purpose of measuring diameters. Since fat bodies are closely associated with the heart in most animals and due to the opacity of fat in light microscope, it is not possible to accurately identify the edges thus accurate fractional shortening measurements are not usually possible in intact preparations.

Finally a consideration that is unrelated to the method of data capture is that of rhythmicity measurements. In the fly heart there are two pacemaker regions that control the direction of contraction. One is located in the anterior conical chamber region; the other is located in the posterior terminal chamber. The pacemakers themselves have not been identified and may well be a “network” property of the myocardial cells themselves. Numerous studies of adult flies have documented spontaneous alterations in directionality of the contractions that are also associated with changes in beating frequency. The changes in beating frequency appear to be influenced by neuronal input because removal of the ventral nerve cord, and not just the brain, results in a more consistent and rhythmical beating pattern although directionality

can still alternate (Ocorr et al. 2007b). Thus a determination of rhythmicity in intact animals will have to contend with this inherent arrhythmicity. In addition, movement artifacts present in most *in vivo* manipulations could be interpreted as arrhythmic heart beats so these must be identified and controlled for.

Electrophysiological and mechanical assessments of heart function

Extracellular Electrical Recording

Extracellular signals have been recorded from the exposed fly heart using standard electrical recoding techniques. All electrical recording methodologies necessitate that the heart first be exposed by dissection. Suction electrodes have been used to record extracellular signals from the posterior end of the adult fly heart (Papaefthimiou et al. 2001). This heart preparation involved loosening the posterior tip of the heart from its attachment to the abdominal cuticle and gently sucking the detached portion into a small bore glass capillary. Extracellular recordings were relatively stable over an 8 hour period and the output was used to demonstrate an excitatory effect of octopamine on the heart rate, accompanied by a reduction in the amplitude and duration of the extracellular potential.

A Multi-electrode Array (MEA) apparatus has also been used to record extracellular field potentials from semi-intact fly heart preparations (Ocorr et al. 2007b). A typical MEA plate contains indium-tin oxide or titanium microelectrodes that are typically 10 microns in diameter and spaced in a variety of grid patterns at intervals of 30 or more microns. The dimensions of the adult fly heart are roughly 80–100 microns wide and 800–1000 microns in length. Thus for standard arrays only two or three electrodes are likely to contact the heart at the same time. Nevertheless it is possible to obtain extracellular field potentials. This technique was used to show prolongation of repolarizing potentials in hearts that were mutant the KCNQ K^+ channel that underlies cardiac repolarization in both flies and humans (Ocorr et al. 2007b).

Intracellular Electrical Recording

Intracellular recordings have been reported from both larval and adult heart preparations. In the larva the heart is not attached to the body wall and is relatively mobile, thus a floating electrode technique was employed to minimize damage to the heart by the impaled electrode during contractions (Lalevee et al. 2006). In this study, recordings from larval hearts were used to examine a role for the outwardly rectifying K^+ channel *ork1* in larval hearts. The data indicated that currents through Ork1 likely set the (resting) membrane potential and influenced the rate of spontaneous action potential generation in the heart.

Unlike in larva, the adult heart is somewhat fixed to the abdominal cuticle by the extracellular matrix and standard intracellular recording techniques have been used successfully to record from beating hearts (Dulcis and Levine 2005). In this study the authors state that the recordings were made from the ventral longitudinal muscle (VLM). This ventral layer of muscle, though not myocardial in origin, but made from progenitor cells from larval lymph glands, contains longitudinally oriented myofibrils and thus would not be expected to mechanically contribute to the heart tube contractions, which are preferentially circumferential. This notion is further supported by recent studies using

atomic force microscopy that have shown that the VLM layer is much softer than the myocardial layer (Kaushik et al. 2011; see below). Thus, it is likely that the electrode had actually penetrated through the VLM and that they were recording from myocardial cells.

In both larva and adult hearts, the recorded action potentials exhibit pacemaker potentials that would be expected of a myogenic heart. However, both preparations exhibited suspiciously high resting membrane potentials (≥2212;25 mV; Dulcis and Levine 2005, Lalevee et al. 2006). This could be due to un-optimized extracellular medium and recordings or could point to a basic difference between myocardial cells from flies and other organisms. Our unpublished observations and results from a recently published study (Magny et al. 2013) record resting membrane potentials of −40 mV and lower, suggesting that the former is the case. Finally, for all electrical recording methods, damage due to movement and movement based artifacts remain a problem when recording from beating cardiac muscle.

Assessment of Muscle Mechanics

Cardiac disease and aging is often accompanied by diastolic dysfunction that has been attributed to a stiffening of the cardiac tissue, due in part to damaging passive mechanical properties of the cardiomyocytes and their extracellular milieu (Kass et al. 2004). Diastolic dysfunction is also observed in *Drosophila* under mutant or aging conditions (Cammarato et al. 2008), suggesting that similar changes in mechanical properties could occur in flies as in humans. One method to detect changes in muscle tension has been applied to *Drosophila* hearts (Ocorr et al. 2007b). This methodology was adapted from a system used to measure tension in mammalian cultured myocytes and uses tiny carbon fibers attached to a force transducer to monitor tension generation across the fly heart tube, and used to show that the ability to generate tension was significantly altered in hearts from KCNQ mutant flies.

Recently, the use of atomic force microscopy (AFM) has been applied to monitor cardiac mechanical properties of the adult *Drosophila* heart (Kaushik et al. 2011, Kaushik et al. 2012, Cozhimuttam Viswanathan et al. 2013). Using this approach it has been shown that the heart's stiffness changes with age or with genetic manipulations that perturb the integrity of the contractile apparatus within cardiomyocytes. Interestingly, the age-dependent increase in cardiac stiffness observed in wildtype fly hearts was reversed with genetic manipulations that prolong lifespan and improve cardiac performance with age (Nishimura et al. 2014).

The principle of AFM and its application to the *Drosophila* heart (using a MFP-3D Bio Atomic Force Microscope) are described in detail in Kaushik et al. (2011). Briefly, AFM uses a nano-indentation approach to measure the stiffness of the *Drosophila* heart and requires a similar dissection procedure as for SOHA. A silicon nitride cantilever with a 2μm borosilicate sphere tip is placed directly on the heart and stepped down on the heart tube's centerline. Its deflection is measured with a laser, providing Hertzian plots of force versus indentation depth, whose linearized slope is a relative measure of stiffness. Remarkably, using this technique it has been possible to identify differences in stiffness between individual muscle layers, i.e. between the contractile myocardial layer and the non-cardiac ventral layer beneath the heart (Kaushik et al. 2012).

A caveat for both of these methods of heart mechanical analysis is that the equipment is highly specialized, requires considerable experience and the techniques themselves may be damaging to heart muscle function. Nevertheless, these protocols do provide valuable information concerning some mechanical properties of the cardiac tissue that cannot as of yet be obtained by other methods.

Histological methods to assess heart development and structural integrity

The *Drosophila* heart develops from cardioblasts (CB) and pericardial cells (PC), which derive from progenitors that are specified in the lateral mesoderm during mid-embryonic stages. From this region both cell types migrate towards the dorsal midline, where they assemble into a linear tube and differentiate into the beating dorsal vessel. Since the identification of the cardiac determinant Tinman, a homeobox transcription factor, additional transcription factors and signaling pathways that underlie cardiac specification have been characterized and have subsequently also been identified as relevant for vertebrate cardiogenesis (for review see Harvey and Rosenthal 2010). Specified heart precursor cells fall into 3 groups, which can be individually identified based on the combination of marker genes they express (Figure 3). Similarly, PCs can also be subdivided into different subtypes most of which likely undergo histolysis at the end of embryogenesis (Sellin et al. 2006); only ~40 PCs persist into larval stages (as nephrocyte-like cells). Antibodies and reporter lines with lacZ and GFP are available for many of the key cardiac transcription factors that control cardiac specification (see Table 2).

From stage 13 to 16 of embryonic development, the heart precursors facilitate two key steps of morphogenesis: the migration and alignment of the cardioblasts at the dorsal midline and the formation of the heart lumen. These steps require the activity of cell polarity and cell adhesion proteins and a number of genes that are involved in the formation of most or all tissues (such as Laminins, DE-Cadherin, see Haag et al. 1999, Yarnitzky and Volk 1995), as well as signaling pathways that have a more restricted, tissue-specific pattern of expression, such as Slit/Robo or Net/Unc5 (Albrecht et al. 2011, Macabenta et al. 2013, MacMullin and Jacobs 2006, Medioni et al. 2008, Qian et al. 2005b, Santiago-Martínez et al. 2006). At this stage, the cardioblasts appear columnar, with distinct localization of basement-membrane markers at the apical/dorsal and basal/ventral side as well as cell-junction markers present at the interface between neighboring CBs. Once the CBs have established the dorsal contact between the junctional domains, they undergo cell shape changes and form a second, ventral cell-cell contact that encloses a single lumen (as originally described in Rugendorff et al. 1994). During these steps, the shape, polarity and identity of all CBs can be determined using a battery of antibodies (summarized in Table 2). Finally, CBs differentiate into beating cardiomyocytes, a process that includes the onset of expression of muscle-myosin and its incorporation into myofibrils (Lehmacher et al. 2012).

For the immunohistochemical analysis of protein expression and localization, standard protocols of *Drosophila* embryo fixation can be applied (Sullivan et al. 2000). In general, embryos from overnight collections are fixed using Formaldehyde/PBS/Heptane/MetOH-based protocols, and embryos of the correct stage are selected during mounting of specimens (staging according to Hartenstein 1993). Alternatively, timed embryo collections can be

used to limit the embryos to relevant stages, but care has to be taken to carry over a sufficient number of embryos during all staining and washing steps. While standard fixation works for the majority of antibodies, there are several antibodies that do require an optimized protocol to preserve the localization pattern or epitope. To detect F-actin with fluorescence-labeled phalloidin exposure to methanol needs to be avoided; this can be accomplished by either by hand-devitellinization of embryos or by replacing methanol with 80–90% ethanol in the devitellinization step (Narashima and Brown 2006). Both methods will result in a limited number of usable embryos, which is disadvantageous when an embryo of a rare genotype and specific stage is to be selected. Heatfixation offers an alternative for some antibodies, since it allows for better visualization of cortical proteins such as Armadillo or DE-Cadherin (Müller and Wieschaus 1996).

Because the heart is localized to the dorsal surface it can easily be imaged from wholemounts. While the central part of the dorsal vessel is very close to the epidermis (8–10μm), high quality images can be obtained with high-NA objectives. The posterior heart proper descends another 25μm into the tissue, adding significantly more, but usually acceptable noise. Filleting the embryo along the ventral midline can improve the image quality (Lovato et al. 2002), but this also increases the risk of damaging the specimen. To bring the coverslip as close as possible to the embryo but without compressing it, a bridge can be built with No. 1-sized coverslips as spacers (No. 1.5 coverslips would increase the space between the embryo and the top coverslip, resulting in a reduced image quality at high resolution). This allows to reliably image embryonic hearts to depths of 35μm even when using laser confocal microscopes or epifluorescent microscopes in combination with structured illumination.

For the analysis of cardiac cell specification, migration and alignment whole-mount imaging along the D/V axis is usually sufficient; however the three-dimensional properties of heart assembly are not optimally captured with confocal microscopy due to the lower axial resolution. The diameter of the embryonic heart tube is only 2–5μm, and with a typical axial resolution of only about 0.7μm the luminal properties are poorly reflected by Z-scans. While gross-morphological lumen defects can be identified using whole-mounts, using cross-sections of embryos and imaging along the A/P axis results in images of much higher resolution (Harpaz and Volk 2011, Macabenta et al. 2013). These methods reveal clearer defects of cardioblast shape and heart lumen that would not have been resolved otherwise. However, since heart lumen formation is a dynamic process that does not occur synchronously along the heart but at different time points along A/P positions (aorta versus heart proper), and is presumably different depending on cardioblast cell type (ostia lumen versus cardiomyocyte lumen), it is important to register sections according to their position along the A/P axis for the interpretation of phenotypes.

Due to the dynamic nature of heart morphogenesis, live-imaging offers an alternative to follow cardiac development over time. In *Drosophila*, many fluorescent reporter lines such as Moesin-RFP/Actin-GFP exist and have been successfully applied to study aspects of heart tube assembly (Knox et al. 2011, Medioni et al. 2008, Vanderploeg et al. 2012). The dorsal, subcutaneous location of the heart allows easily obtainable high-quality confocal images of the heart as it undergoes morphogenesis.

The larval and adult heart can also be characterized using immunohistochemical methods (Alayari et al. 2009). In general, visualizing the structural proteins of the heart such as proteins of the sarcomere, (e.g. phalloidin staining of F-actin, Myosin or the Z-band marker α-Actinin (Mery et al. 2008)), allows reliable detection of cardiac defects in overall morphology and myofibrillar organization. In addition, antibodies against extracellular proteins like the collagen-IV Pericardin provide information about the extracellular matrix surrounding the heart (Na et al. 2013). GFP fusion proteins have been used to detect subcellular localization of sarcomeric proteins (Mery et al. 2008), human genes expressed in the fly heart such as α-B-crystalin (Xie et al. 2013) and amyloid aggregates (Melkani et al. 2013). Finally, electron microscopy has been used to examine the adult heart tube; especially noteworthy is the work from A. Paululat's group on the ultrastructure of the wildtype fly heart (Lehmacher et al. 2012).

Summary

The *Drosophila* heart model provides an accessible and easily manipulated system for the analysis of cardiac development as well as function. The ease of genetic manipulation, limited genetic complexity and conservation of gene function with vertebrates makes it an ideal system for the identification of gene candidates and mechanisms involved in vertebrate heart development, function and disease.

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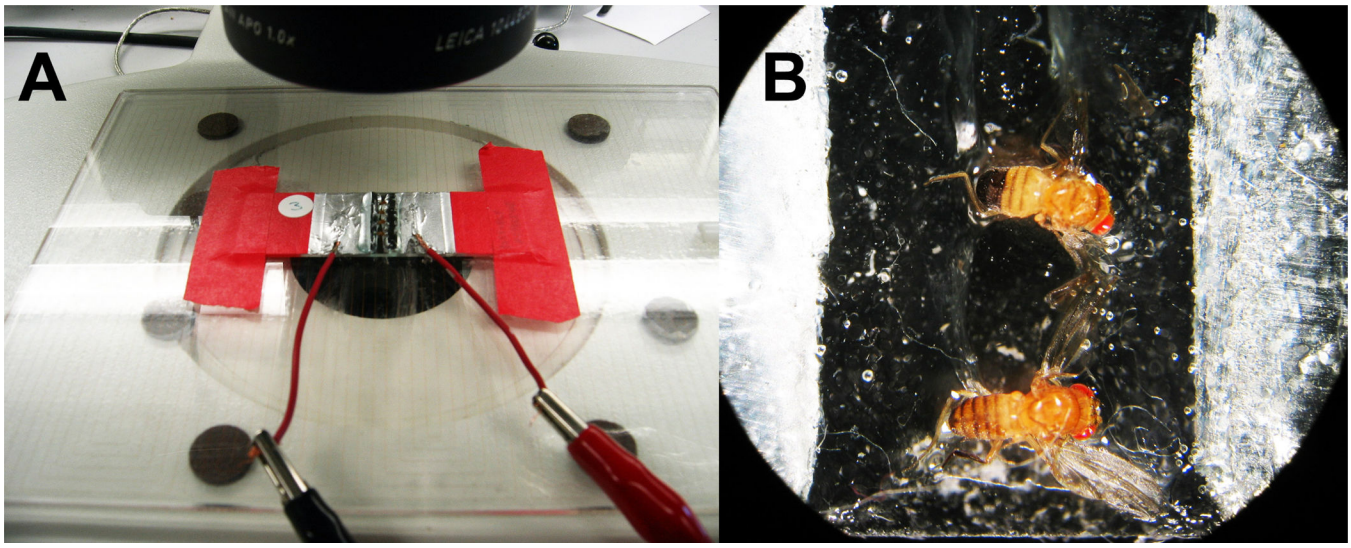

Figure 1. Pacing Assay of Cardiac Function

(A) Electrical pacing setup showing microscope slide with foil electrodes connected to two leads that deliver the square wave pulse from a stimulator (not pictured). **(B)** View through the microscope of two flies with their heads inserted into one line of electrode gel and the abdomen tip into the other. It is important that the two lines of gel do not touch.

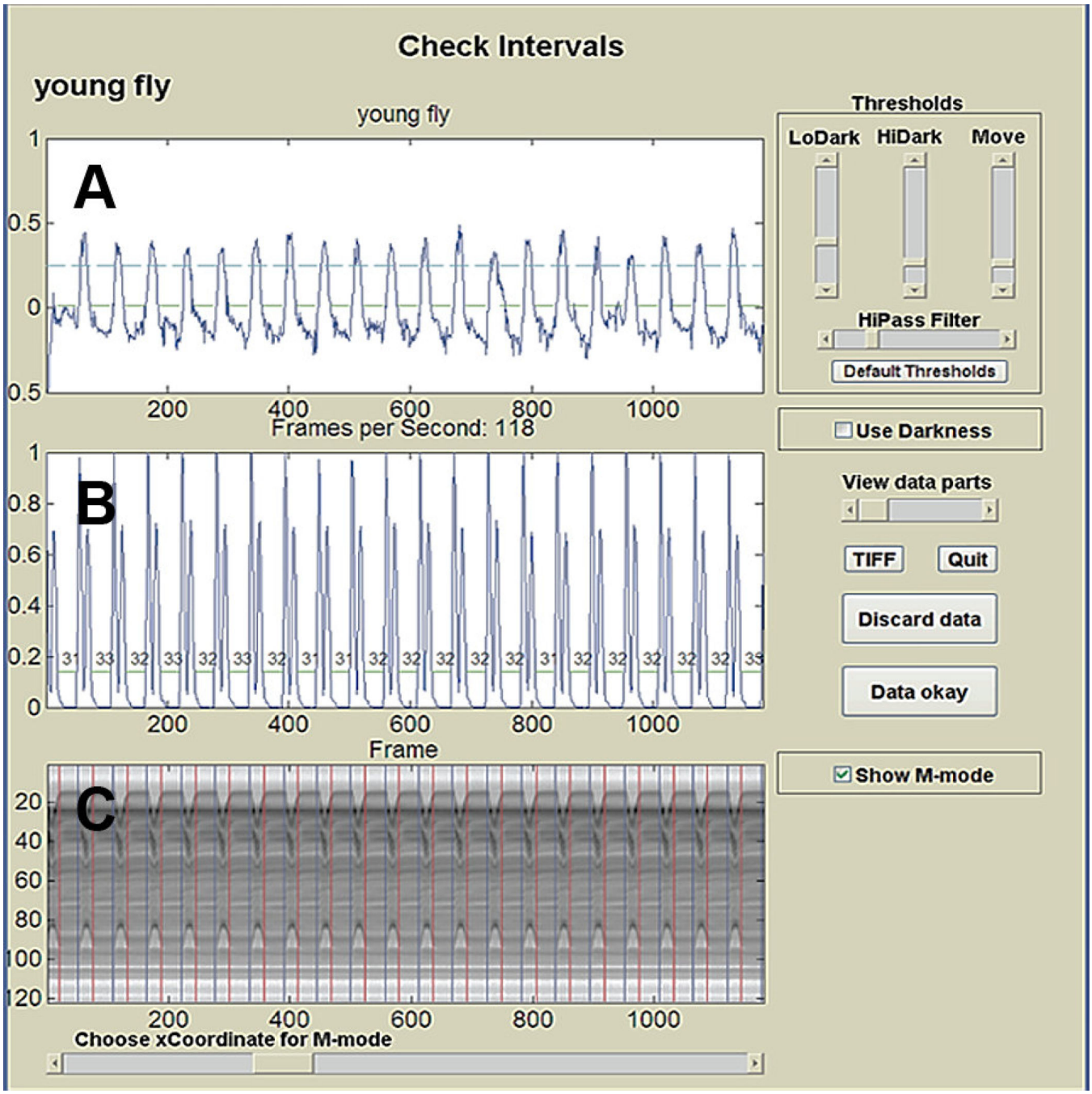

Figure 2.

SOHA output showing the gross darkness changes in **(A)** the top window, **(B)** the pixel-by-pixel intensity changes in the middle window, and **(C)** the corresponding M-mode in the bottom window. Adapted from Fink et al. 2009.

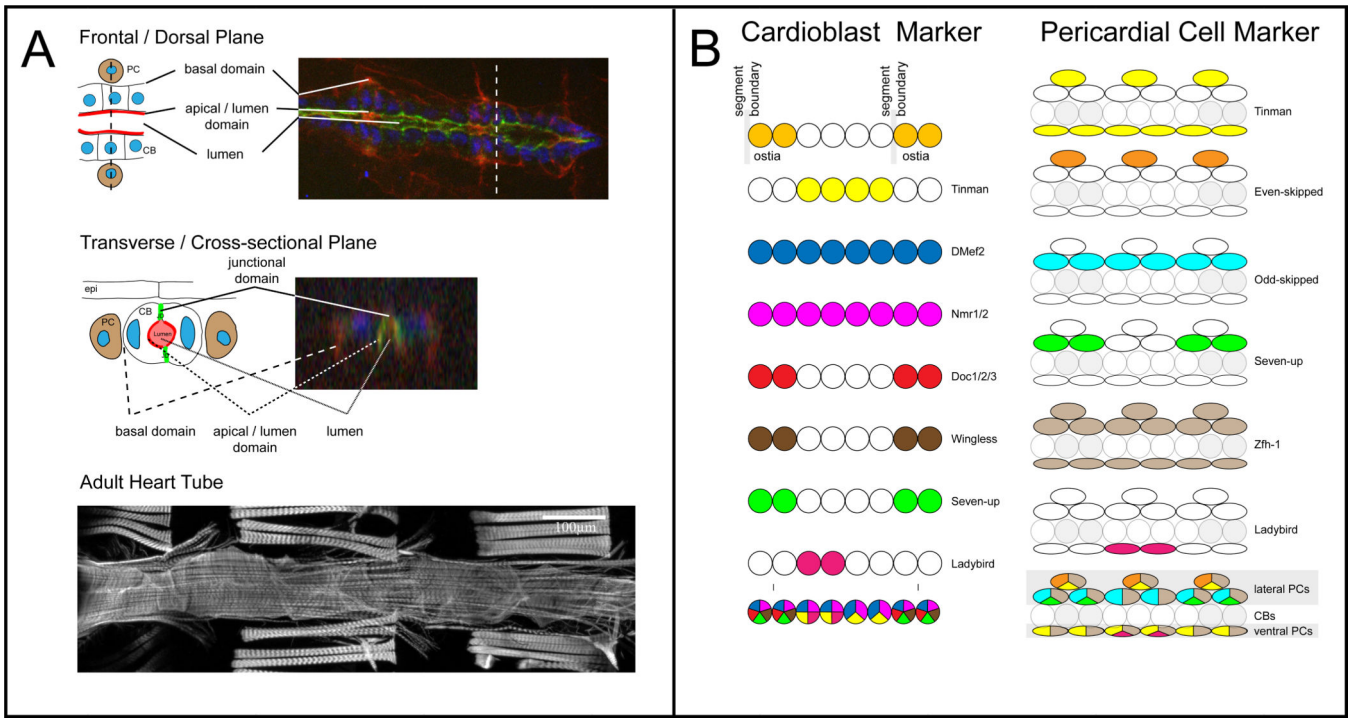

Figure 3. Heart Lumen Cross sections, Adult Heart Staining and Cardiac Marker genes

A. Schematic overview and fluorescent image of the embryonic heart tube (early stage 17), with domains highlighted. Cardiac nuclei are labeled with anti-Neuromancer1 (blue), basal + luminal domains are labeled with anti-Dystroglycan (red), and the luminal domain is marked with anti-Slit (green; for more markers see Table 2). Adult heart tube stained against F-actin. B. Combinatorial code of transcription factors and signaling pathways to identify embryonic cardioblast and pericardial cell types. See Table 2 for references.

Table 1

Comparison of the different methods for analyzing heart function in Drosophila.

Method	Effect on Animal	Heart Rate	Systolic & Diastolic Intervals	Systolic & Diastolic Diameters	Rhythmicity	Structure	Stiffness	Stress Response
Gross Δ Intensity Optical Method	Stressful / *in vivo*	+			+			
SOHA Optical Method	Invasive / *in situ*	+	+	+/− 1μ	+	+		
Atomic Force Microscopy	Invasive / *in situ*						+	
Optical Coherence Tomography	Stressful / *in vivo*	+		+/− 10μ	+	+		
Electrical Recordings	Invasive / in situ	+	+		+			
Immuno histochemistry / Electron Microscopy	*in situ*					++		
Electrical Pacing	Stressful / *in vivo*							+

Table 2

Overview of markers for heart specification, polarity and differentiation

Protein	cardiac cell types	subcellular localization	comment	cardiac reference	source (non-cardiac reference)
Transcription factors/Specification					
DMef2	CB	nucleus		Lilly et al. (1995)	
Dorsocross1/2/3 (Doc1/2/3)	CB	nucleus	Ostia cell marker, also in amnioserosa	Reim et al. (2003)	
Even-Skipped (Eve)	PC	nucleus		Su et al. (1999)	DSHB clone 3C10 Frasch et al. (1987)
Hand-LacZ	CB + PC	cytoplasm	lacZ reporter	Han and Olson (2005)	
Ladybird-early (Lbe)	CB + PC	nucleus		Jagla et al. (1997)	
Nmr1/H15	CB	nucleus		Leal et al. (2009)	
Odd-Skipped (Odd)	PC	nucleus		Ward and Coulter (2000)	
Seven-up-LacZ	CB	cytoplasm	lacZ reporter, Ostia cell marker	Lo and Frasch (2001)	
Tail-up	CB + PC	nucleus	in amnioserosa	Mann et al. (2009)	DSHB clone 40.3A4
Tinman (Tin)	CB + PC	nucleus		Venkatesh et al. (2000), Yin et al. (1997)	
Zfh-1	CB + PC	nucleus		Bodmer Rolf (1993)	Ruth Lehmann
Wingless (Wg)			Ostia cell marker	Lo et al. (2002)	DSHB clone 4D4
Polarity/Cell shape					
Actin			filopodia, myofilaments		Phalloidin (Life Technologies)
alpha-Spectrin			membrane marker	Ponzielli et al. (2002)	DSHB clone 3A9
Armadillo (Arm)			junctional domain marker	Medioni et al. (2008)	DSHB clone N2 7A1
beta-Integrin (beta-PS1)			apical+basal	Vanderploeg et al. (2012)	DSHB clone CF.6G11
beta3-Tubulin				Damm et al. (1998)	
DE-Cadherin			junctional domain	Haag et al. (1999)	DSHB DCAD2
Discs-large (Dlg1)			membrane marker	Qian et al. (2005a)	DSHB clone 4F3
Dystroglycan (Dg)			apical+basal	Qian et al. (2005a)	Deng et al. (2003)
Multiplexin			heart proper lumen (st.17)	Harpaz et al. (2013)	
NetrinB			luminal domain	Albrecht et al. (2011)	
Robo			luminal domain	Qian et al. (2005b)	DSHB clone 13C9
Robo2 (lea)			PC	Qian et al. (2005b)	Rajagopalan et al. (2000)
Slit			aorta + heart proper lumen	Rothberg et al. (1990)	DSHB clone C555.6D
Toll (Tl)			membrane marker	Wang et al. (2005)	Hashimoto et al. (1991)

Protein	cardiac cell types	subcellular localization	comment	cardiac reference	source (non-cardiac reference)
Unc5			luminal domain	Albrecht et al. (2011)	
Differentiation					
Pericardin (Prc)				Chartier et al. (2002)	DSHB clone EC11
Tropomyosin				LaBeau et al. (2009)	Abcam ab50567
Myosin-heavy chain (Mhc)				LaBeau et al. (2009)	Kiehart and Feghali (1986)
DSur			RNA probe	Akasaka et al. (2006)	