New Methods in Cardiovascular Biology

A Method to Measure Myocardial Calcium Handling in Adult Drosophila


Rationale: Normal cardiac physiology requires highly regulated cytosolic Ca\(^{2+}\) concentrations and abnormalities in Ca\(^{2+}\) handling are associated with heart failure. The majority of approaches to identifying the components that regulate intracellular Ca\(^{2+}\) dynamics rely on cells in culture, mouse models, and human samples. However, a genetically robust system for unbiased screens of mutations that affect Ca\(^{2+}\) handling remains a challenge.

Objective: We sought to develop a new method to measure myocardial Ca\(^{2+}\) cycling in adult Drosophila and determine whether cardiomyopathic fly hearts recapitulate aspects of diseased mammalian myocardium.

Methods and Results: Using engineered transgenic Drosophila that have cardiac-specific expression of Ca\(^{2+}\)-sensing fluorescent protein, GCaMP2, we developed methods to measure parameters associated with myocardial Ca\(^{2+}\) handling. The following key observations were identified: (1) Control w\(^{1118}\) Drosophila hearts have readily measureable Ca\(^{2+}\)-dependent fluorescent signals that are dependent on L-type Ca\(^{2+}\) channels and SR Ca\(^{2+}\) stores and originate from rostral and caudal pacemakers. (2) A fly mutant, held-up\(^2\) (hdp\(^2\)), that has a point mutation in troponin I and has a dilated cardiomyopathic phenotype demonstrates abnormalities in myocardial Ca\(^{2+}\) handling that include increases in the duration of the 50% rise in intensity to peak intensity, the half-time of fluorescence decline from peak, the full duration at half-maximal intensity, and decreases in the linear slope of decay from 80% to 20% intensity decay. (3) Hearts from hdp\(^2\) mutants had reductions in caffeine-induced Ca\(^{2+}\) increases and reductions in ryanodine receptor (RyR) without changes in L-type Ca\(^{2+}\) channel transcripts in comparison with w\(^{1118}\).

Conclusions: Our results show that the cardiac-specific expression of GCaMP2 provides a means of characterizing propagating Ca\(^{2+}\) transients in adult fly hearts. Moreover, the adult fruit fly heart recapitulates several aspects of Ca\(^{2+}\) regulation observed in mammalian myocardium. A mutation in Drosophila that causes an enlarged cardiac chamber and impaired contractile function is associated with abnormalities in the cytosolic Ca\(^{2+}\) transient as well as changes in transcript levels of proteins associated with Ca\(^{2+}\) handling. This new methodology has the potential to permit an examination of evolutionarily conserved myocardial Ca\(^{2+}\)-handling mechanisms by applying the vast resources available in the fly genomics community to conduct genetic screens to identify new genes involved in generated Ca\(^{2+}\) transients and arrhythmias. (Circ Res. 2011;108:1306-1315.)

Key Words: Drosophila heart □ myocardial calcium □ GCaMP2

Cardiomyocytes require highly regulated Ca\(^{2+}\) handling to maintain normal contractile function and intracellular signaling.\(^1,2\) Abnormalities in Ca\(^{2+}\) handling are associated with cardiac hypertrophy, cardiomyopathies, and heart failure.\(^3-6\) Moreover, altered Ca\(^{2+}\) regulation can lead to loss of myocytes, dysregulated contractile function, and arrhythmias that lead to sudden cardiac death.\(^1,7-9\)

Although the major components involved in myocardial Ca\(^{2+}\) regulation are known, the ability to identify new molecules in Ca\(^{2+}\) cycling is limited to the availability of genetic models systems that allow for genetic screens. The fruit fly, Drosophila melanogaster, is an excellent genetic model that has vast resources to facilitate the identification and mapping of new genes that cause disease.\(^10,11\) Previously, we and others have demonstrated that the fly heart can recapitulate aspects associated with mammalian cardiac contractile dysfunction.\(^1,4,5,12-19\) Moreover, because many of the genes and signaling pathways are evolutionarily conserved among flies and mammals, studies of the fly cardiac system can provide insight into human heart disease.\(^17,20-25\) For

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example, receptor tyrosine kinase signaling pathways, Notch pathways, and many transcription factors have been identified in the fly, thus providing insights into how corresponding orthologs function in mammals. Therefore, we sought to develop a method to measure \( \text{Ca}^{2+} \) handling in the adult fly heart. This approach establishes the means to potentially identify new mutants previously not known to be involved in myocardial \( \text{Ca}^{2+} \) signaling.

The fly circulatory system consists of a single layer of \( \text{tinC} \)-expressing myocytes arranged as an open linear tube with the main cardiac chamber located directly underneath the dorsal cuticle in the proximal abdominal region. Additionally, a strap of non-\( \text{tinC} \)-expressing striated muscle cells, referred to as the ventral longitudinal muscle or dorsal diaphragm, is tightly associated with the ventral aspect of the abdominal circulatory system. Despite a simple circulatory system, many genes that are critical for cardiac function in the fly are conserved among mammals, including humans. For example, transcription factors such as NKX2.5, structural proteins such as \( \delta \)-sarcoglycan, dystrophin, myosins, and troponins, and receptor-mediated signaling pathways are required for normal cardiac function in the fly heart.

On the basis of the concept that genes and pathways necessary for \( \text{Drosophila} \) heart function are conserved among species, we developed a method to measure \( \text{Ca}^{2+} \) handling in the intact adult fly heart. Transgenic flies in a \( \text{w}^{1118} \) genetic background that expressed the \( \text{Ca}^{2+} \)-dependent fluorescent reporter GCaMP2, under the direct control of the 304-bp cardiac-specific \( \text{tinC} \) genomic element, allowed us to measure myocardial propagating \( \text{Ca}^{2+} \) transients with high fidelity in adult \( \text{Drosophila} \). We characterized myocardial \( \text{Ca}^{2+} \) transients in control \( \text{w}^{1118} \) flies and in a fly mutant, \( \text{hdp}^{2} \), that has an enlarged cardiac chamber and impaired contractile function similar to the functional abnormalities observed in mammalian dilated cardiomyopathy. Our results demonstrate that the adult fly heart has similar \( \text{Ca}^{2+} \)-handling properties in comparison with mammalian hearts and establish new methods to potentially identify fly mutants that have abnormalities in genes affecting the regulation of myocardial \( \text{Ca}^{2+} \).

### Methods

#### Materials

The \( \text{w}^{1118} \) flies were obtained from Bloomington Stock Center (Bloomington, IN). The \( \text{hdp}^{2} \) was provided by Dr James Vigoreaux. GCaMP2 vector was obtained from Addgene (Cambridge, MA). The pCasper5 and pGreenHPelican plasmids were obtained from the \( \text{Drosophila} \) Genome Resource Center (Bloomington, IN). Rhod-2-AM and Fluo-4 \( \text{Ca}^{2+} \)-sensitive dyes were obtained from Invitrogen (Carlsbad, CA). Cytochalasin D and cadmium chloride were purchased from Sigma-Aldrich (St. Louis, MO). Diltiazem, caffeine, thapsigargin, and tetrodotoxin (TTX) were purchased from EMD chemicals. Probes used for quantitative polymerase chain reaction were obtained from Applied Biosystems, Inc. (Carlsbad, CA).

#### Transgenic Fly Engineering

Transgenic flies harboring cardiac-specific GCaMP2 were generated in a \( \text{w}^{1118} \) genetic background as described in the Online Data Supplement at http://circres.ahajournals.org. To examine myocardial \( \text{Ca}^{2+} \) handling in \( \text{hdp}^{2} \), the transgenic GCaMP2 line was introduced into the \( \text{hdp}^{2} \) line that was previously backcrossed into the \( \text{w}^{1118} \) genetic background. Therefore, the differences in fluorescence were attributed to the troponin I mutation because the \( \text{w}^{1118} \) genetic background was used for all experiments.

#### Optical Coherence Tomography

The cardiac chamber sizes in awake, adult \( \text{Drosophila} \) were measured using optical coherence tomography as previously described. Adult female flies were collected between 5 and 7 days after eclosion, briefly anesthetized by use of carbon dioxide exposure, gently placed on soft gel plates, and allowed to awaken before imaging using a 1310-nm optical coherence tomography microscopy system (Bioptigen, Inc., Durham, NC). M-modes obtained from transverse oriented B-mode through the A1 segment of the \( \text{Drosophila} \) heart were used to calculate end-diastolic dimensions (EDD), end-systolic dimension (ESD), and heart rate. Fractional shortening was calculated as \( \frac{\text{EDD} - \text{ESD}}{\text{EDD}} \times 100 \).

#### Heart Dissections

Female flies were collected 48 hours after eclosion for myocardial \( \text{Ca}^{2+} \) fluorescence measurement experiments. Hearts were prepared according to previously described methods. Detailed methods are described in the Online Data Supplement. The heart remained attached to the dorsal cuticle and was readily identified as a beating structure along the midline from abdominal segments A1 to A4 (Figure 1). Dissected fly hearts remained beating for up to 2 hours at room temperature. Fly heart contractions were stopped by incubation in hemolymp buffer that contained 40 \( \mu \text{mol/L} \) cytochalasin-D. Multiple trials to stop fly heart contractions with blebbistatin were unsuccessful. In order to image fly hearts on an inverted microscope, each heart preparation was transferred to a confocal dish containing 100 \( \mu \text{L} \) of oxygenated hemolymph buffer. The heart specimen was oriented with the dorsal side up, and a glass cover slip was gently applied to avoid movement during imaging.

A Leica 165FC stereomicroscope equipped with an Andor iXon 860 EMCCD camera was used for experiments using cadmium, EGTA, diltiazem, TTX, caffeine, thapsigargin, and low [\( \text{Na}^{+}\) ] buffer. Images were analyzed using Andor Solis software (Andor Technologies, Inc., South Windsor, CT).

For experiments to determine the effects of motion artifact caused by cardiac contractions, \( \text{Ca}^{2+} \)-independent fluorescence was measured at baseline and after abolishing contraction by treatment with 40 \( \mu \text{mol/L} \) cytochalasin-D for 10 minutes at room temperature.

### Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>dF/dt(_{\text{max}})</td>
<td>slope of maximum fluorescence intensity increase</td>
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<tr>
<td>EDD</td>
<td>end-diastolic dimension</td>
</tr>
<tr>
<td>EMCCD</td>
<td>electron-multiplying charge-coupled device</td>
</tr>
<tr>
<td>ESD</td>
<td>end-systolic dimension</td>
</tr>
<tr>
<td>FDHM</td>
<td>full duration half-maximum</td>
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<tr>
<td>GCaMP2</td>
<td>circularly permuted enhanced green fluorescent protein fused with M13 helix of myosin light chain at the N terminus and calmodulin at the C terminus</td>
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<tr>
<td>hdp(^{2})</td>
<td>\textit{held-up} (^{2}) mutant fly</td>
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<tr>
<td>IP3</td>
<td>inositol-3-phosphate</td>
</tr>
<tr>
<td>LTCC</td>
<td>L-type calcium channel</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoendoplasmic reticulum calcium ATPase</td>
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<tr>
<td>(\text{tinC})</td>
<td>DNA regulatory region of the ( \text{Drosophila} ) ( \text{tin} ) gene</td>
</tr>
<tr>
<td>(\text{tinC-GCaMP2})</td>
<td>transgenic fly harboring GCaMP2 under the control of the ( \text{tinC} ) DNA element</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
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<tr>
<td>(\text{w}^{1118})</td>
<td>laboratory stock fly</td>
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Fluorescence Ca\(^{2+}\) Imaging

Fly hearts were imaged using a Nikon TE2000-U inverted fluorescence microscope equipped with an Andor iXon 860 EMCCD camera at a rate of 200 frames per second. The spatiotemporal analysis of Ca\(^{2+}\) signals was performed using Matlab software as follows. Ca\(^{2+}\) signals were detrended by fitting and subtracting a second-order polynomial and temporally filtered using 12-sample median and mean filters. Nonmyocardial pixels inside the recording field of view were excluded from further analysis by imposing a threshold on the peak-to-peak range of each pixel’s fluorescence intensity. Ca\(^{2+}\) transient activation times were defined along each wave of the heart. For each recording, the conduction velocity was calculated using a manually selected rostral and caudal subregion by dividing the distance between their centers of mass by the difference between their mean activation times.

Quantitative Polymerase Chain Reaction

Total RNA samples from the dissected hearts from groups of 60 female \(w^{1118}\) or \(hdp\) flies 48 to 72 hours after eclosion were prepared and used for quantitative (real time) polymerase chain reaction as described in detail in the Online Data Supplement.

Statistical Analyses

Statistical calculations used t tests or analyses of variance with Bonferroni corrections for multiple comparisons using GraphPad Prism statistical software (GraphPad Software, Inc., La Jolla, CA).

Results

To measure Ca\(^{2+}\)-handling properties in the adult fly heart, we engineered transgenic flies that harbored a non–His-tagged GCaMP2 construct under the control of tinC in a \(w^{1118}\) background. We designated this fly line as \(w^{1118};tinC-GCaMP2\). GCaMP2 is a circularly permuted enhanced green fluorescent protein that contains a calmodulin Ca\(^{2+}\) binding domain and the M13 helix of myosin light-chain kinase and has been previously used as a Ca\(^{2+}\)-sensing fluorescence indicator.\(^{43–45}\) Adult hearts isolated from \(w^{1118};tinC-GCaMP2\) had robust, cardiac-specific fluorescence (Figure 1 and Online Movie I). High-speed imaging (200 fps) of fluorescence signals along the heart tube in abdominal segments A2 and A3 were measured for all subsequent studies. Cytochalasin-D was used during the preparation of heart samples to stop cardiac contraction while maintaining Ca\(^{2+}\)-dependent fluorescence. Therefore, the measured fluorescence signals reflected changes in cytosolic Ca\(^{2+}\) and unlikely the result of motion artifact. Heart preparations that were not treated with cytochalasin-D demonstrated significant motion artifact during imaging that accounted for at least 50% of the observed changes in fluorescence intensity (Online Figure I). As an additional control, experiments using tinC-GFP fly hearts demonstrated a significant increase in fluorescence intensity during systole (Online Figure I). Of note, we also conducted experiments that used the application of Ca\(^{2+}\)-sensitive dyes but observed poor incorporation of dyes into the fly heart with significant nonspecific accumulation in noncardiac tissue, rendering us unable to acquire reproducible Ca\(^{2+}\) transients (data not shown).

We processed the raw image data from recordings of the fluorescence signals from the adult heart preparations using Matlab software to measure changes in pixel intensities. Each cardiac Ca\(^{2+}\) wave was represented by \(\approx 5000\) pixels per frame for 500 frames of image data corresponding to the abdominal A2 and A3 segments of the fly heart. This region of the heart tube is a single cell layer thick and contains approximately 8 cardiomyocytes on each side of the heart.\(^{46}\) Because the level of GCaMP2 that was present in each heart
can vary and GCaMP2 is not a ratiometric indicator, we normalized fluorescence intensity of each pixel to the maximum intensity at each pixel over all frames obtained during each recording. The changes in pixel fluorescence intensities in the hearts were analyzed from pseudocolored movies that represented changes at each pixel from 50% rise to 50% decay in normalized intensity (Online Movie II and corresponding isochrone map in Figure 1). Additionally, the isochrone pixel intensities for each recording were averaged along the vertical columns and represented as a line (Figure 1B). The vertical column averaging was performed because this processing served as an additional control for motion artifact due to possible subtle fly heart contraction after cytochalasin-D treatment. We therefore used the average pixel intensity traces for each heart beat to quantify the Ca\(^{2+}\)/H\(_{11001}\) transient in each heart (Figure 1C).

We examined 714 propagating Ca\(^{2+}\) transients from 32 \(w^{1118}\) flies and showed that 57.9% of generated Ca\(^{2+}\) transients were directed anterograde (from the caudal to the rostral direction) and 36.6% were directed retrograde (from the rostral to the caudal direction). Bidirectional generated Ca\(^{2+}\) transients occurred in 5.8% of heart preparations (Figure 2). These findings were consistent with the previous description of rostral and caudal pacemakers in the adult fly heart.\(^{31,47,48}\)

To characterize myocardial Ca\(^{2+}\) handling in \(w^{1118}\) flies, we measured parameters associated with the generated Ca\(^{2+}\) transients, reflecting changes in the intracellular Ca\(^{2+}\) concentration. These parameters included the maximal slope of increasing fluorescence intensity (dF/dt\(_{max}\)), the duration from the 50% rise in intensity to peak intensity, the half-time of fluorescence decline from peak, the FDHM, and the linear slope from 80% to 20% intensity decay (Figure 3A). Additionally, conduction velocities for propagating Ca\(^{2+}\) transients along the heart tube were determined. Because the majority of generated Ca\(^{2+}\) transients occurred in the anterograde and retrograde directions, the properties of the Ca\(^{2+}\)-induced fluorescence intensities and conduction velocities in both groups were compared. All parameters, including conduction velocities, were similar whether propagation was
To examine the contribution of voltage-dependent Na\textsuperscript{+} channel activity to Ca\textsuperscript{2+} transients (Figure 4C through 4E). Additionally, the generated Ca\textsuperscript{2+} transients were completely blocked by 200 \textmu mol/L Cd\textsuperscript{2+} (data not shown).

We examined the effects of intracellular Ca\textsuperscript{2+} stores depletion on generated Ca\textsuperscript{2+} transients by using thapsigargin, an inhibitor of sarcoendoplasmic reticulum calcium ATPase (SERCA).\textsuperscript{30,51} Fly hearts that were treated with the thapsigargin had reductions in generated Ca\textsuperscript{2+} transients with decreases in peak fluorescence and the slopes of fluorescence increase and decrease (Figure 4F through 4I). Last, we examined the effects of octopamine, a G-protein coupled receptor agonist, and observed a decrease in the FDHM and a more steep linear slope of intensity decay, suggesting that the model responds to agonist stimulation (Online Figure II).

Because dilated cardiomyopathies and heart failure have been associated with alterations in myocardial Ca\textsuperscript{2+} handling, we next tested whether a dilated heart in the fly would recapitulate the known abnormalities in generated Ca\textsuperscript{2+} transients in mammalian hearts. Previously, we identified a mutant, hdp\textsuperscript{2}, that has a point mutation in troponin I conserved among multiple species including Drosophila and humans and has a dilated heart phenotype.\textsuperscript{17} The hdp\textsuperscript{2};tinC-GCaMP2 flies were generated by genetic crosses. The cardiac-specific expression of GCaMP2 did not significantly affect cardiac function in w\textsuperscript{1118} or hdp\textsuperscript{2} as determined by optical coherence tomography measurements (Online Figure II).

We measured generated Ca\textsuperscript{2+} transients in hearts from adult, age-matched hdp\textsuperscript{2};tinC-GCaMP2 and w\textsuperscript{1118};tinC-GCaMP2 (Figure 5A). In comparison with w\textsuperscript{1118}, hdp\textsuperscript{2} mutant hearts had similar dF/dt\textsubscript{max} but significant differences in the duration of the 50% rise in intensity to peak intensity, the half-time of fluorescence decline from peak, FDHM, and the linear slope of decay from 80% to 20% fluorescence intensity (Figure 5B through 5F). The conduction velocities were not statistically different between w\textsuperscript{1118} and hdp\textsuperscript{2} hearts (0.76±0.04 versus 0.65±0.04 cm/s, P=NS for w\textsuperscript{1118} versus hdp\textsuperscript{2}, respectively). Additionally, the duration of the fluorescence decay was significantly different between w\textsuperscript{1118} and hdp\textsuperscript{2} hearts over a range of defined intensities (Online Figure III).

Abnormalities in mRNA transcript levels in key components of Ca\textsuperscript{2+} cycling have been associated with myocardial dysfunction. Therefore, we performed quantitative polymerase chain reaction analyses of several well-recognized components involved in intracellular Ca\textsuperscript{2+} cycling in hearts from w\textsuperscript{1118} and hdp\textsuperscript{2} mutants (Figure 6A). Interestingly, hdp\textsuperscript{2} mutants had significant reductions in ryanodine receptor transcripts in comparison with w\textsuperscript{1118}. There were no significant differences in SERCA, L-type Ca\textsuperscript{2+} channel, IP\textsubscript{3} receptor, or sodium-Ca\textsuperscript{2+} exchanger transcripts between hdp\textsuperscript{2} and w\textsuperscript{1118}. Additionally, hdp\textsuperscript{2} hearts had reductions in caffeine-augmented generated Ca\textsuperscript{2+} transients (Figure 6B through 6D). These findings are consistent with the known changes in a variety of vertebrate animal models and humans with dilated cardiomyopathy.\textsuperscript{6}

![Diagram](https://example.com/diagram.png)

Figure 3. Comparison of anterograde and retrograde directed Ca\textsuperscript{2+} transients in adult hearts from w\textsuperscript{1118};tinC-GCaMP2. A, Representative trace of averaged pixel intensities from a w\textsuperscript{1118};tinC-GCaMP2 heart. Definitions of dF/dt\textsubscript{max} (A), the duration of the 50% rise to peak fluorescence intensity (B), the duration of the peak to 50% decay in fluorescence intensity (C), FDHM (D), and the slope of linear decay from 80% to 20% fluorescence intensity (E) are shown. B, Comparison between parameters of pixel intensity traces and conduction velocity for anterograde (i.e., caudal to rostral) (n=25) and retrograde (i.e., rostral to caudal) (n=31) Ca\textsuperscript{2+} transients in w\textsuperscript{1118};tinC-GCaMP2 hearts. There were no statistically significant differences in the parameters measured between anterograde and retrograde Ca\textsuperscript{2+} wave propagations.

Anterograde or retrograde (P=NS) (Figure 3B). Additionally, the effects of motion on the generated Ca\textsuperscript{2+} transient parameters were determined by comparing fly hearts treated with cytochalasin-D versus vehicle alone. The beating fly heart preparations had significant delays, with the generated Ca\textsuperscript{2+} transient parameters suggesting that contraction artifact can alter interpretation of fluorescence measurements (Online Figure I).

Next, we addressed the ability to manipulate fly myocardial Ca\textsuperscript{2+} handling using pharmacological interventions to examine the relevance of our model to the mammalian heart. To examine the contribution of voltage-dependent Na\textsuperscript{+} channels to the generated Ca\textsuperscript{2+} transients, we examined the effects of low [Na\textsuperscript{+}]\textsubscript{o} buffer, TTX, or cadmium (Cd\textsuperscript{2+}). Low [Na\textsuperscript{+}]\textsubscript{o} buffer (2 mmol/L) caused a complete block of Ca\textsuperscript{2+} transients followed by a resumed Ca\textsuperscript{2+} transient, suggestive of Ca\textsuperscript{2+} waves (SR overload-induced spontaneous Ca\textsuperscript{2+} release) or Na\textsuperscript{+}-independent excitability under experimental conditions (Figure 4A). The resumed propagated Ca\textsuperscript{2+} transients had slower conduction velocities (0.71±0.05 cm/s for 110 mmol/L [Na\textsuperscript{+}]\textsubscript{o}, buffer versus 0.49±0.04 cm/s in 2 mmol/L [Na\textsuperscript{+}]\textsubscript{o}, buffer, P<0.05, n=9). The administration of TTX (100 \mu mol/L) did not block the propagating Ca\textsuperscript{2+} transients (data not shown).

To examine the contributions of extracellular Ca\textsuperscript{2+} and L-type Ca\textsuperscript{2+} channels, we performed experiments in the presence of EGTA or diltiazem. The chelation of free extracellular Ca\textsuperscript{2+} resulted in the complete inhibition of generated Ca\textsuperscript{2+} transients (Figure 4B). Next, we used diltiazem, an inhibitor of L-type Ca\textsuperscript{2+} channels.\textsuperscript{49} The administration of diltiazem resulted in attenuation and subsequent cessation of generated Ca\textsuperscript{2+} transients (Figure 4C through 4E). Additionally, the generated Ca\textsuperscript{2+} transients were completely blocked by 200 \mu mol/L Cd\textsuperscript{2+} (data not shown).
Discussion

We developed a new method based on the cardiac expression of GCaMP2, a genetically encoded Ca\(^{2+}\) indicator, to measure myocardial Ca\(^{2+}\) cycling in adult *Drosophila*. Genetically encoded Ca\(^{2+}\) indicators have been used to examine cardiac Ca\(^{2+}\) handling in other models, including the mouse and zebrafish.\(^{52-56}\) However, the genetic resources to map and identify mutants that affect myocardial Ca\(^{2+}\) handling in these genetic models can be quite laborious. The method that we describe has a number of significant advantages. First and foremost, *Drosophila* genetics and genomics offer unique resources for gene discovery that are not available in other model systems. These genetic resources facilitate the mapping and identification of gene mutations and have been used to identify a variety of genes that affect different aspects of *Drosophila* cardiac contractile function.\(^{13,19}\) Flies that have molecularly defined genomic deficiencies, have P-element insertions, or harbor RNAi for specific gene knockdown can be introduced into the tinC-GCaMP2 background to investigate how individual genes potentially affect myocardial Ca\(^{2+}\) handling. Alternative approaches that rely on applying Ca\(^{2+}\) indicator dyes can lead to nonspecific, extracardiac tissue distribution. For example, dye incorporation into the non-tinC-expressing striated ventral longitudinal muscle can prevent adequate labeling of the fly heart and lead to diminished specificity during cardiac Ca\(^{2+}\) measurements. Our method has the advantage of genetically targeted cardiac-specific Ca\(^{2+}\) measurements that circumvent this potential problem.

The circulatory system in flies is simple in comparison with that of mammals; however, the generated Ca\(^{2+}\) transients in adult *Drosophila* hearts share several characteristics with mammalian myocardial Ca\(^{2+}\) handling. First, the Ca\(^{2+}\)+-dependent fluorescence wave in tinC-GCaMP2 hearts moves along the heart tube in an organized manner. However, unlike mammalian hearts, the fly heart has 2 pacemakers that control anterograde and retrograde contractions.\(^{31,47,48}\) Second, the generated Ca\(^{2+}\) transients in fly hearts are dependent on extracellular Ca\(^{2+}\) via L-type Ca\(^{2+}\) channels and intracellular calcium stores. Third, the generated Ca\(^{2+}\) transients appear to require sodium channels because low [Na\(^{+}\)]\(_o\) causes a transient cessation of Ca\(^{2+}\) transients. Fourth, Ca\(^{2+}\) transient parameters undergo changes after stimulation with octopamine, an agonist of the octopamine G-protein coupled receptor. Fifth, the fly heart appears to have altered Ca\(^{2+}\) handling in the context of the *hdp\(^2\)* mutant. The *hdp\(^2\)* mutant
has a well-characterized deterioration of the indirect flight muscles, progressive myopathy of other muscle groups, and a dilated cardiomyopathic phenotype. In comparison with those from *w*1118, hearts from mutant *hdp2* have a prolonged duration of 50% rise to peak intensity, but no significant changes in dF/dtmax. This suggests that although the early rise in cytosolic Ca²⁺ concentration is similar, the late rise in cytosolic Ca²⁺ is delayed. Furthermore, in *hdp2* hearts, the half-time of fluorescence decline from peak is prolonged, and the linear slope from 80% to 20% of fluorescence decay is decreased. These findings suggest that Ca²⁺ reuptake into the SR and Ca²⁺ extrusion into the extracellular space are impaired in the hearts from *hdp2* in comparison with those from *w*1118.

The mechanisms that are responsible for the observed changes in cytosolic Ca²⁺ cycling and contractile dysfunction in *hdp2* hearts remain to be identified. Hearts from *hdp2* mutants had reductions in RyR transcript levels, consistent with the prolonged duration of 50% rise to peak intensity that we observed in the GCaMP2 flies. Additionally, *hdp2* hearts had reductions in caffeine-augmented increases in the generated Ca²⁺ transients, indicating a reduced SR Ca²⁺ load. These results are consistent with some previous studies in mammalian models of heart failure. However, the transcript levels for SERCA were not significantly different between *w*1118 and *hdp2*, although the duration of peak intensity to 50% decay is prolonged and the linear slope from 80% to 20% of fluorescence decay is decreased in *hdp2* hearts. A possible explanation for these findings is that alterations of Ca²⁺ reuptake in *hdp2* hearts may result from changes in the posttranscriptional level of SERCA or alterations in proteins that control SERCA function.

Alternatively, the troponin I mutation in *hdp2* flies may change the structure and Ca²⁺ binding properties of contractile protein complexes, thereby altering the buffering capacity of these complexes for cytosolic Ca²⁺. Recent studies using preparations of indirect flight muscle from *Drosophila* have demonstrated that the site of the *hdp2* troponin I mutation decreases the concentration of Ca²⁺ necessary for thin filament activation. Furthermore, on the basis of comparisons of the conserved site of the mutation in troponin I in *hdp2* flies and mammals, studies suggest that the change in Ca²⁺ binding to the troponin I/troponin C complex is an indirect effect of the *hdp2* mutation. Thus, changes in the interaction between contractile proteins and Ca²⁺ may also explain some of the changes in Ca²⁺ transients in *hdp2* mutants.

There are limitations to our approach to measuring myocardial Ca²⁺ handling in adult flies. First, GCaMP2 is a
nonratiometric Ca\textsuperscript{2+} indicator, similar to several nonratiometric Ca\textsuperscript{2+} indicator dyes.\textsuperscript{55,60,61} Additionally, the amount of GCaMP2 is variable in each fly heart. Therefore, the absolute changes in cytosolic Ca\textsuperscript{2+} concentration cannot be determined, and relative changes must be used for comparison among experiments. Second, the fluorescence intensity of GCaMP2 is lower than that of fluorescence dyes because the intensities of the fluorescence dyes depend on the loading conditions.\textsuperscript{45,55,56} Thus, GCaMP2 expression in the fly heart is not sufficient enough to permit imaging through the cuticle of the live insect. Therefore, the tissue surrounding the heart must be partially dissected away prior to imaging. The generation of newer genetic indicators with improved signal-to-noise ratios may circumvent the inability to directly measure Ca\textsuperscript{2+}-dependent fluorescence intensity through the cuticle. Third, although GCaMP2 and Ca\textsuperscript{2+} indicator dyes are nonratiometric, GCaMP2 has different affinities and kinetics regarding Ca\textsuperscript{2+} binding and unbinding in comparison with chemical dyes.\textsuperscript{45,55,56} The response of GCaMP2 to Ca\textsuperscript{2+} binding and unbinding is slower than that of chemical dyes and therefore will tend to prolong the Ca\textsuperscript{2+} duration. Fourth, the fly heart must be electromechanically dissociated prior to imaging to minimize changing optical properties in the tissue that occur during contraction. In fact, in the beating fly heart, approximately 50% of the change in fluorescence intensity is the result of motion. Additionally, the parameters associated with the generated Ca\textsuperscript{2+} transients from fly hearts that were electromechanically dissociated are significantly different from hearts that were contracting. Therefore, measurements of myocardial Ca\textsuperscript{2+} in our fly model require pretreatment with cytochalasin-D to uncouple true Ca\textsuperscript{2+}-dependent fluorescence from motion artifact. Unfortunately, blebbistatin did not stop fly heart contractions, and therefore the use of cytochalasin-D introduces another potential limitation because cytochalasin-D is known to affect intracellular Ca\textsuperscript{2+}.\textsuperscript{62}

Last, our studies suggest that the propagating Ca\textsuperscript{2+} transient is mediated by electric action potential propagation. The generated Ca\textsuperscript{2+} transients in fly hearts are transiently blocked by low extracellular Na\textsuperscript{+} buffer, suggesting that sodium-dependent action potentials are necessary for the observed Ca\textsuperscript{2+} transients. The generated Ca\textsuperscript{2+} transients are also sensitive to Cd\textsuperscript{2+}, consistent with a dependence on voltage-sensitive Na\textsuperscript{+} channels similar to that observed in mammalian hearts.\textsuperscript{63–65} However, the dose of Cd\textsuperscript{2+} required to block fly heart Ca\textsuperscript{2+} transients is in a range that can also inhibit L-type Ca\textsuperscript{2+} channels. The effects of TTX were modest and suggest that the fly heart may not be sensitive to TTX. Therefore, more definitive studies using voltage-sensitive dyes will be necessary to understand the contribution of the electric action potential to the Ca\textsuperscript{2+} transient in the fly heart.

Overall, our results demonstrate the potential utility of Drosophila as a model of myocardial Ca\textsuperscript{2+} handling. Future applications including an examination of fly mutants using this methodology have the potential to identify and characterize new genes that regulate intracardiac Ca\textsuperscript{2+} signaling and may lead to new insights into human cardiovascular diseases.

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Disclosures
None.

References
The study describes a new method to measure calcium transients in the adult fruit fly heart using flies that express a cardiac-specific, reversible calcium-dependent fluorescent protein reporter and a high-speed imaging system. By applying this technique, the propagating calcium transients were measured in adult fly hearts from controls and mutants that have dilated cardiomyopathic phenotypes. Drosophila myocardial calcium transients propagate in 2 directions and are similar to mammalian myocardial calcium transients in that they require extracellular sodium, extracellular calcium via L-type calcium channels, and intracellular calcium stores. These findings further support the fruit fly as a model of cardiovascular disease. Furthermore, this method suggests that the fly can serve as a genetically tractable system for identifying genes that affect myocardial calcium handling in mammals.
A Method to Measure Myocardial Calcium Handling in Adult Drosophila

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ONLINE SUPPLEMENTAL MATERIALS

A method to measure myocardial calcium handling in adult *Drosophila*
ONLINE SUPPLEMENTAL METHODS

Transgenic fly engineering:

Transgenic flies harboring cardiac-specific GCaMP2 were generated as follows. The 304 bp DNA element that specifies expression in the fly heart was obtained from $w^{1118}$ genomic DNA and subcloned using BamH I sites proximal to the fly hsp70 minimal promoter using BamH I sites in pGreenH-Pelican. Next, the tinC-hsp70 minimal promoter was subcloned from pGreenH-Pelican and engineered with Bgl II sites for insertion into pCasper5. This construct was used to create transgenic flies for the cardiac specific expression of GFP, designated $w^{1118};$ tinC-GFP.

Two forms of GCaMP2 were used to engineer transgenic flies. GCaMP2 that had the previously engineered histidine tag and GCaMP2 without the histidine tag were individually subcloned into the pCasper5 plasmid. Each construct was injected into $w^{1118}$ embryos using standard methods. The initial set of transgenic flies harboring His-tag-GCaMP2 had detectable fluorescence expression in the embryonic dorsal vessel but showed poor expression in the adult heart (Online Movie III). Transgenic flies that were engineered with GCaMP2 that lacked the His-tag were generated and seven independent transgenic lines were recovered and bred into a $w^{1118}$ genetic background. Each transgenic line was examined for cardiac specific GCaMP2 expression. The transgenic lines with the highest expression were identified, the chromosomal location of the transgenes were mapped by segregation analysis, and used for further experiments.
**Heart dissections:**

Female flies were collected 48 hours after eclosion for myocardial Ca\(^{2+}\) fluorescence measurements. Hearts were prepared according to previously described methods.\(^4\), \(^5\) Briefly, adult flies were placed on a soft gel plate dorsal side down and the thorax and legs were removed from the abdomen using iridectomy scissors. Next, the flies were bathed in oxygenated hemolymph buffer (108 mM NaCl, 5 mM KCl, 8 mM MgCl\(_2\), 1 mM NaH\(_2\)PO\(_4\), 4 mM NaHCO\(_3\), 5 mM HEPES (pH 7.1), 10 mM sucrose, 5mM Trehalose, and 2 mM CaCl\(_2\)) at room temperature. N-methyl-d-glucamine used in place of NaCl for experiments employing low sodium (2mM) buffer. The ventral abdominal cuticle was removed and abdominal organs were gently dissected away from the heart. All air was removed using finely drawn glass capillaries. Next, surrounding fat was removed by liposuction using glass capillaries. The heart remained attached to the dorsal cuticle and was readily identified as a beating structure along the midline from abdominal segments A1 to A4 (Figure 1). Dissected fly hearts remained beating for up to 2 hours at room temperature.

**Quantitative Polymerase Chain Reaction (qPCR):**

Total RNA samples from the dissected hearts from groups of 60 female \(w^{1118}\) or \(hdp^2\) flies 48-72 hours after eclosion were prepared using RNA-Bee (Tel-Test “B”). One \(\mu\)g of RNA was used for generation of cDNA using SuperScript II reverse transcriptase (Invitrogen, Inc.). Applied Biosystems Taqman Gene expression assays were used to perform quantitative (real time) RT-PCR (L-type
Ca\(^{2+}\) channel (Ca-α1D): Dm 0179379_g1; Ryanodine receptor (Rya-44F): Dm 01842306_m1; SERCA (Ca-P60A): Dm 01841898_m1; NCX (CalX): Dm 02136140_m1; Inositol 3-posphate receptor (Itp-r83): Dm 02147941_g1; and endogenous control (RpL32): Dm 02151827-g1). The following reaction components were used for each probe: 2 µL cDNA, 12.5 µl 2X TaqMan Universal PCRMaster Mix without Amperse (Applied Biosystems, Inc.), 1.25 µl of probe, and 9.25 µl water in a 25 µl total volume. Reactions were amplified and analyzed in triplicate using an ABI PRISM\(^{\text{TM}}\) 7000 Sequence Detection System. PCR reaction conditions were as follows: Step 1: 95°C for 10 minutes, Step 2: 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Expression relative to Rpl32 was calculated using 2\(^{-\Delta\Delta C_{T}}\) and levels were normalized to baseline. We performed five independent experiments in triplicate using different batches of flies for each experiment.
### Online Table I.
The effects of Octopamine on Ca2+ transients in the fly heart

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fold Change (Post-Octopamine/Pre-treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transient Rate (#/sec)</td>
<td>1.24 ± 0.12</td>
</tr>
<tr>
<td>Conduction Velocity (cm/sec)</td>
<td>1.16 ± 0.13</td>
</tr>
<tr>
<td>dF/dT\text{max} (%Fluorescence/msec)</td>
<td>1.04 ± 0.02*</td>
</tr>
<tr>
<td>Time from 50% rise to peak fluorescence (msec)</td>
<td>0.96 ± 0.02*</td>
</tr>
<tr>
<td>Half time of decline from peak fluorescence (msec)</td>
<td>0.88 ± 0.04</td>
</tr>
<tr>
<td>FDHM (msec)</td>
<td>0.90 ± 0.03*</td>
</tr>
<tr>
<td>Slope of linear recovery from 80% to 20% (%fluorescence/msec)</td>
<td>1.21 ± 0.1*</td>
</tr>
</tbody>
</table>

Comparison of changes in parameters of pixel intensity traces and conduction velocity for $w^{118}$; $tinC$-GCaMP2 heart before and after 1 minute exposure to 100 uM octopamine (n=16). Data are mean ± SEM; *,p<0.05 for pre-treatment vs. exposure to octopamine.
**Online Figure I. The effects of cardiac contraction on fluorescence intensity.**

A. Comparison of fluorescence intensity in hearts from *w^{118}; tinC-GCaMP2* before and after cytochalasin D treatment. In the pre-cytochalasin D group, baseline and peak fluorescence corresponded to diastole and systole, respectively. The difference between peak fluorescence before and after cytochalasin D treatment represents the contribution of cardiac contraction to the fluorescence intensity signal. Data is represented as the fold change in fluorescence intensity compared to baseline. *p<0.05 for peak vs. baseline
fluorescence intensity (n=3 flies per group). #p<0.05 for peak fluorescence in pre vs. post-cytochalasin D treatment. **B.** Comparison of fluorescence intensity in heart from *w*¹¹¹8; *tinC-GFP* during diastole and systole. Data is represented as the fold change in fluorescence intensity compared to baseline. *p<0.05 for peak vs. baseline fluorescence intensity (n=3 flies per group). **C.** Effects of cardiac contraction on the parameters of the generated Ca²⁺ transients. Comparison between parameters of pixel intensity traces of Ca²⁺ transients in *w*¹¹¹8; *tinC-GCaMP2* hearts that were beating (No cytochalasin-D) vs. treated with cytochalasin-D to stop contractions. *p<0.05 for No cytochalasin-D treatment vs. cytochalasin-D treatment (n=18 per group).
Online Figure II. Optical coherence tomography measurements of cardiac chamber sizes in adult flies. A. End diastolic dimensions measured in microns, B. End systolic dimension measured in microns, and C. Fractional Shortening calculated as a percentage in the A1 segment of hearts from awake, adult $w^{1118}$ (n=19) and $hdp^2$ (n=12) alone or $w^{1118}$ (n=9) and $hdp^2$ (n=11) harboring cardiac specific GCaMP2 ($tinC\text{-}GCaMP2$). Individual measurements (black circles) and the mean (open circles) with SEM are shown. *p<0.05 for $hdp^2$ and $hdp^2$; $tinC\text{-}GCaMP2$ compared to $w^{1118}$ and $w^{1118}$; $tinC\text{-}GCaMP2$ by ANOVA with Bonferroni correction for multiple comparisons.
Online Figure III. Comparison of the fluorescence intensity durations between $w^{1118}$; $tinC$-GCaMP2 and $hdp^{2}$; $tinC$-GCaMP2 as a function of percent intensity. The duration of fluorescence intensity from 50% increase in intensity to 50%, 60%, 70%, 80%, and 90% decay in fluorescence intensity in hearts from $w^{1118}$; $tinC$-GCaMP2 and $hdp^{2}$; $tinC$-GCaMP2. Individual measurements (black circles) and the mean (open circles) with SEM are shown. *$p<0.05$ for $w^{1118}$; $tinC$-GCaMP2 vs. $hdp^{2}$; $tinC$-GCaMP2 for the indicated duration of the fluorescence intensity.
ONLINE SUPPLEMENTAL MOVIES

Online Movie I. Cardiac-specific dynamic fluorescence measurement from a w^{1118}; tinC-GCaMP2 heart.

Online Movie II. Pseudocolored cardiac-specific dynamic fluorescence measurement from a w^{1118}; tinC-GCaMP2 heart. The movie shows three consecutive propagations of Ca^{2+} along the heart from an adult w^{1118}; tinC-GCaMP2. Blue (50% fluorescence intensity) to red (100% fluorescence intensity) at each pixel is shown. Retrograde (rostral to caudal), anterograde (caudal to rostral), and bidirectional (originating from the rostral and caudal ends) are shown.

Online Movie III: His-tagged GCaMP2 expression in the embryonic dorsal vessel. The dorsal vessel of a transgenic fly in w^{1118} genetic background harboring tinC-HisTag-GCaMP2.
References