



ELSEVIER

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Method Article

Improved cardiac contraction imaging in live *Drosophila* embryos



Hayley Milner, Scott J. Nowak*

Department of Molecular and Cellular Biology, Kennesaw State University, United States

A B S T R A C T

Drosophila melanogaster is a powerful model organism in which to address the genetics of cardiac patterning and heart development. This system allows the pairing of live imaging with the myriad available genetic and transgenic techniques to not only identify the genes that are critical for heart development, but to assess their impact on heart function in living organisms. There are several described methods to assess cardiac function in *Drosophila*. However, these approaches are restricted to imaging of mid- to late-instar larval and adult hearts. This technical hurdle therefore does not allow for the recording and analysis of cardiac function in embryos bearing strong mutations that do not hatch into larvae. Our technical innovation lies in transgenically labeling the cells of the *Drosophila* heart and using line scan-based confocal imaging to repeatedly image the walls of the heart. By plotting this line scan as a kymograph, heart contractions can be visualized and assayed, thereby allowing for quantification of physiological defects. This method can be used to obtain physiological data from known mutations that affect cardiac development yet are incapable of hatching into larvae for conventional analysis.

- Use transgenic methods to label heart proper walls
- Use high-speed line scanning to capture position of heart proper walls
- Create X vs. time plot to visualize and quantify contractions over imaging period.

© 2020 The Authors. Published by Elsevier B.V.

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

A R T I C L E I N F O

Method name: Line-scanning to image *Drosophila* embryonic heart contraction

Keywords: Dorsal vessel, Embryonic heart beat, Line scanning confocal analysis

Article history: Received 8 September 2020; Accepted 2 November 2020; Available online 5 November 2020

DOI of original article: [10.1016/j.ydbio.2020.09.001](https://doi.org/10.1016/j.ydbio.2020.09.001)

* Corresponding author.

E-mail address: snowak@kennesaw.edu (S.J. Nowak).

<https://doi.org/10.1016/j.mex.2020.101130>

2215-0161/© 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

Specifications table

Subject Area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	Heart contraction
Method name:	Line-scanning to image <i>Drosophila</i> embryonic heart contraction
Name and reference of original method:	Akirin is critical for early tinman induction and subsequent formation of the heart in <i>Drosophila melanogaster</i> . Howard et al., 2021. https://doi.org/10.1016/j.ydbio.2020.09.001

Method details

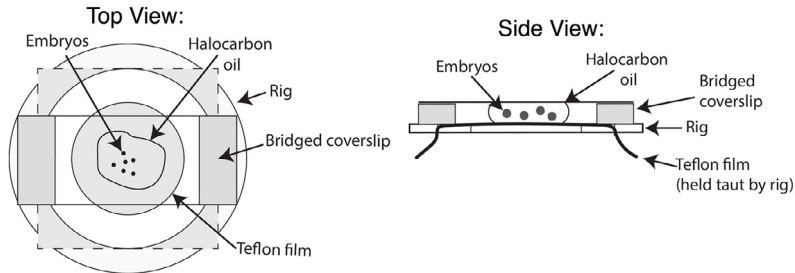
The *Drosophila melanogaster* heart, or dorsal vessel, has been extensively studied due to its high conservation of organogenesis and genetics found in higher phyla [1–4]. The wide variety of available genetic techniques that allow for ease of manipulation of the heart together with forward genetic screening methods widely used permit the rapid identification of previously unknown or unidentified genetic loci that are crucial for the proper organogenesis and function of the *Drosophila* heart [2,3,5]. Beyond simple imaging of fixed preparations, the use of live imaging has expanded the available toolkit for examining cardiac physiology in *Drosophila* [3,6,7]. However, the use of these techniques has been primarily optimized and developed for visualizing cardiac contraction in late instar larvae or adult *Drosophila* [6,7]. Live imaging-based studies of the embryonic *Drosophila* heart have been largely focused on dorsal vessel and aorta assembly rather than contraction [8]. One main reason for this is that the dorsal vessel is difficult to visualize in prehatching embryos, and embryonic hearts do not fully display continuous uninterrupted cardiac contractions until shortly prior to hatching [1]. Despite the lack of continuous heart contractions, a high level of coordinated cardiac contraction can be observed in late Stage 16 and Stage 17 embryos [4].

In an effort to quantify contractions in embryos bearing mutations in the gene *akirin*, which fails to hatch following Stage 17, we developed the following imaging protocol to assay and quantify cardiac contractions. The *akirin* locus encodes a 201-residue nuclear protein of unknown function that is over 70% conserved among nonfungal eukaryotes [9]. Akirin has no classifiable domains except for two divergent nuclear localization sequences [9,10]. One possible function for Akirin is as an adaptor protein that links transcription factor activity with chromatin remodeling complexes to facilitate proper levels of transcription factor activity in a wide variety of cellular and developmental contexts [10–13]. Recent work has identified Akirin as critical for proper patterning and morphogenesis of the *Drosophila* heart (Howard et al, 2020). The heart does form in *akirin* mutants but exhibits morphological defects and a significant lack of contractility. However, given that *akirin* mutants die prior to hatching, no currently described methods for quantifying and analyzing heart contractions are widely available. One notable feature of our method is that it obviates the need for specialized camera equipment [7] or specialized microscopic imaging rigs that are not widely available [14]. Rather our approach uses a published transgenic insect line that fluorescently labels the cells of the heart proper [15] and utilizes the line-scanning feature on standard and even low-feature affordable confocal microscope rigs to assay and quantify cardiac contraction in the prehatching embryo. This technique is amenable to high-throughput analysis, and it will be a useful tool for genetic screening and identification of previously unidentified loci critical for cardiac function in *Drosophila*.

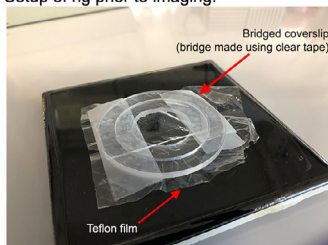
Materials and Methods

1. Labeling: To label the heart proper, we utilized insects bearing a transgene consisting of the *toll* promoter driving cytoplasmic GFP [15]. This transgene was recombined into an *akirin*³ mutant background [10] to create a *w; toll-cGFP,akirin*³/*TM3-sqh::mCherry* line [17]. The original *w; toll-cGFP* line, as well as wild-type siblings (*w; toll-cGFP,+/TM3-sqh::mCherry*) were used for comparison in imaging experiments. Assays were also performed in a similar manner in other *akirin* allelic backgrounds [17].
2. Embryos were collected on grape juice agar plates (Genesee Scientific), dechorionated in 50% bleach as per [10] and transferred to a drop of halocarbon oil on a sheet of taut Teflon film For

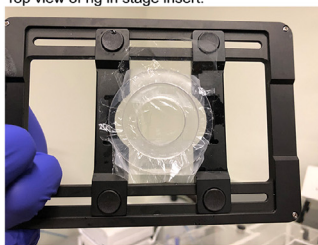
1.1 Live imaging rig design and setup:



1.2 Setup of rig prior to imaging:



1.3 Top view of rig in stage insert:



1.4 Rig mounted on inverted confocal microscope:

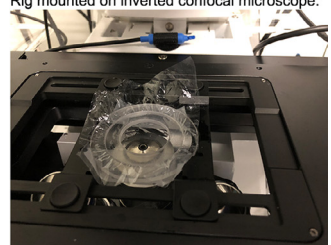


Fig. 1. Live imaging rig design and setup. 1.1. We used a custom fabricated rig for embryo live imaging (adapted from [16]). This consisted of two concentric rings of plexiglass cut to nestle snugly within each other. The outer diameter of the largest ring was set at 10cm, so as to fit in a standard tissue culture plate insert on an inverted microscope stage. A piece of optically clear Teflon film is snapped between the rings so that it is stretched tautly. Dechorionated *Drosophila* embryos are then deposited on the film in a drop of halocarbon oil and covered with a bridged coverslip. Side view of the assembly is provided, with the bridging exaggerated for emphasis. Photographs of (1.2) assembled rig, (1.3) the completed rig on the stage insert, and (1.4) the rig/stage insert mounted on the inverted microscope for imaging are also provided.

our application, we used Chemours Teflon FEP Fluoropolymer, Type A, 100 gauge film (0.001" thickness), available from American Durafilm Co., Inc.

- The film is held taut in a custom rig (Fig. 1) (adapted from [16]), which consists of two concentric plexiglass rings (outer diameter 10cm, 5mm thick) nestled together to hold the film much like an embroidery loop. The diameter of the plastic rings was chosen to fit into a standard culture plate stage insert on an inverted Zeiss LSM700 microscope. This rig is either easily machined from plexiglass, or can be easily 3D printed using common plastic materials. A high degree of mechanical tension on the film is not required, only that which is sufficient to maintain the Teflon film in a taut configuration.
- A bridge was created directly on the film with two layers of clear (Scotch) tape for the coverslip to rest upon without crushing the embryos, and the embryos were covered 22 × 50mm coverslip (1.5 micron thickness). Ensure that sufficient halocarbon oil is present on the Teflon film to allow for contact with the coverslip (see Fig. 1).
- The imaging rig with mounted embryos was then placed on a Zeiss LSM700 inverted confocal microscope equipped with a Plan-Apochromat 20×/0.8 M27 objective. At this point, the imaging rig is now inverted, with the coverslip on the bottom (facing the objective), the Teflon membrane is on the top, and the embryos in the drop of halocarbon oil are sandwiched between them. This particular microscope is equipped with temperature incubation. Temperature was set at 25 degrees C for the duration of imaging, mostly to stabilize the ambient temperature for the duration of the imaging period. If time permits, for the best

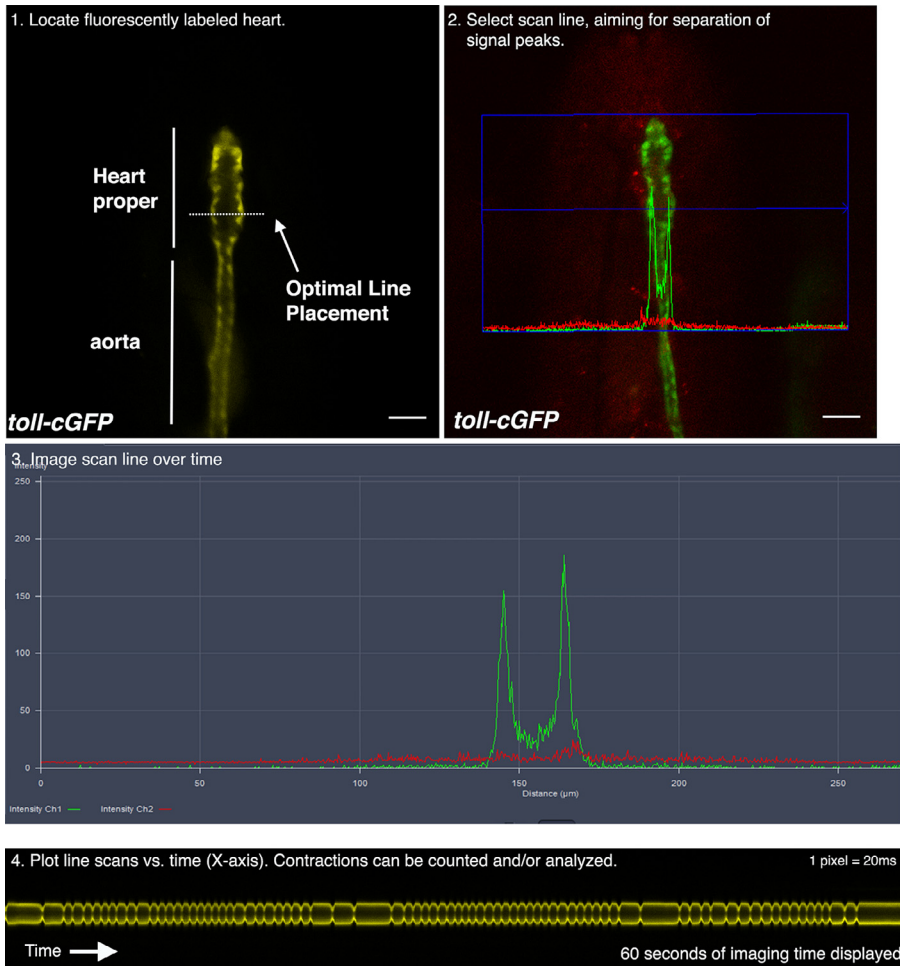


Fig. 2. Assay method. After locating the GFP-expressing embryos on a confocal microscope, the “line select feature available in the Zeiss ZEN software is used to plot the fluorescence intensity of the GFP channel across a line one pixel wide (Fig. 2.1 and 2.2). Lines were chosen so as to obtain good separation of peaks across the heart proper, with each peak corresponding to a wall of the heart proper (Fig. 2.2). The selected line was then imaged using the Line scanning feature, with one image obtained every 20 milliseconds for a total of 100,000 cycles (corresponding to 33 minutes of total imaging time, Fig. 2.3). The line scan time series was then plotted as scan vs time (X-axis) which enables visualization of the movement (contraction) of the walls of the heart proper (Fig. 2.4). Scale bar is 25 microns.

results the embryos in the rig should be left in this configuration for at least 30 minutes prior to imaging, to allow for the embryos to settle in the halocarbon oil droplet, and for the rig to equilibrate to the ambient temperature.

6. Mutant and wild-type sibling embryos in the same clutch were age matched based on the presence of the ring gland, which is visible using the *toll-cGFP* marker [15] and is fully formed by Stage 17.
7. In Line Scan mode and using the GFP fluorescence of the walls of the heart proper as a guide, a line region of interest was drawn that overlapped with both heart proper walls and selected so as to obtain good separation between the peaks of intensity in the GFP channel (see Fig. 2.1). This alignment was checked using the “Line select” feature in the ZEN Black software. The line

region used is one pixel wide. The line of interest may need to be re-drawn several times to obtain good separation of peaks in the GFP channel (Fig. 2.2).

8. For line scanning, a scan speed of 1.57 microseconds/pixel (pixel dwell) was used. Line scans were acquired every 20 milliseconds for over 100,000 cycles (corresponding to approximately 33 minutes). A sample capture of a single timepoint is presented in Fig. 2.3.
9. It is advisable to save the data file at this point, or to use the auto-save feature available in your particular imaging software. For our analyses, an acquisition of 100,000 cycles corresponded to a file size of approximately 250-300 megabytes in the ZEN Black software. On an older machine such as our (2013) setup, this file size can sometimes overwhelm the system memory, and the odds of a system crash and much resulting anguish are high. Newer, more current machines with better hard disk space and scratch volumes will likely ameliorate this issue.
10. Acquired time sequences were then plotted as line scan vs. time (x-axis), each line acquisition corresponding to a single pixel width of the plot (see Fig. 2.4). This was accomplished in the ZEN (Black) software by choosing the "XT" view of the line scan image stack.
11. Line scans were then exported as full resolution images into .tiff files using Zeiss ZEN (Black) software.
12. Exported images were then opened in Adobe Photoshop (CC) and manually examined for analysis.

Method Validation

Using this method, we recorded contractions from over twenty embryos of a variety of genotypes, in this case examining both *akirin* and wild-type sibling embryos. Our analysis, which was first reported in [17], was to examine the number of coordinated vs. uncoordinated contractions in embryos. Periodicity of contractions can clearly be obtained from the X vs time plot, and their numbers recorded and analyzed (see Fig. 2.4). One notable caveat observed from our analysis is that *Drosophila* embryonic hearts do not contract continuously, but rather in trains of contractions that can persist for some time during the imaging period. Despite this issue, our use of this method was focused on recording quality kymographs that show the movement and contraction of the dorsal vessel walls in *Drosophila* embryos (Fig. 2.4). We are currently developing a software-based method for analysis of our output tracks, as well as determining whether our recordings can be integrated into existing workflows, such as the Semi-automated Optical Heartbeat Analysis software [7]

It should be noted that in the accompanying study that produced this method, wild-type embryos bearing the *toll-cGFP* transgene did not display any obvious defects in cardiac patterning when compared with wild-type embryos that did not carry the *toll-cGFP* transgene [15,17]. Specifically, we did not observe a decrease in total number of Mef2-positive cardiomyoblasts or loss of cardiomyoblast sub-population markers (e.g., Seven-up-positive or Dorsocross-positive cells) in *toll-cGFP*-labeled wild-type hearts [17]. Further, among wild-type, *toll-cGFP* embryos examined in our study, we consistently and reproducibly observed coordinated cardiac contractions in wild-type embryos (n=15) as compared with embryos bearing different mutant alleles of *akirin* [17].

Acknowledgements

The authors wish to thank Madison Hupp, Austin Howard, Courtney Willett, and Kristina Palermino for technical advice during the refinement of this protocol. The authors wish to thank Kristine Nowak and members of the Nowak Laboratory for unvarnished, unflinching criticism of the techniques, workflow, and composition of this manuscript. We wish to thank Robert Schulz for generously sharing insect lines. This work was supported by NIH grant R15 GM102826 and American Heart Association AIREA Award 33960109.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

- [1] B. Rotstein, A. Paululat, On the morphology of the *Drosophila* heart, *J. Cardiovasc. Dev. Dis.* 3 (2016) 15–14, doi:[10.3390/jcdd3020015](https://doi.org/10.3390/jcdd3020015).
- [2] A.L. Bryantsev, R.M. Cripps, Cardiac gene regulatory networks in *Drosophila*, *Biochim. Biophys. Acta* 1789 (2009) 343–353, doi:[10.1016/j.bbagr.2008.09.002](https://doi.org/10.1016/j.bbagr.2008.09.002).
- [3] G. Vogler, R. Bodmer, Cellular mechanisms of *Drosophila* heart morphogenesis, *J. Cardiovasc. Dev. Dis.* 2 (2015) 2–16, doi:[10.3390/jcdd2010002](https://doi.org/10.3390/jcdd2010002).
- [4] Y. Tao, R.A. Schulz, Heart development in *Drosophila*, *Semin. Cell Dev. Biol.* 18 (2007) 3–15, doi:[10.1016/j.semcdb.2006.12.001](https://doi.org/10.1016/j.semcdb.2006.12.001).
- [5] L. Qian, R. Bodmer, Probing the polygenic basis of cardiomyopathies in *Drosophila*, *J. Cell. Mol. Med.* 16 (2012) 972–977, doi:[10.1111/j.1582-4934.2012.01529.x](https://doi.org/10.1111/j.1582-4934.2012.01529.x).
- [6] K. Ocorr, G. Vogler, R. Bodmer, Methods to assess *Drosophila* heart development, function and aging, *Methods* 68 (2014) 265–272, doi:[10.1016/j.ymeth.2014.03.031](https://doi.org/10.1016/j.ymeth.2014.03.031).
- [7] K. Ocorr, M. Fink, A. Cammarato, S. Bernstein, R. Bodmer, Semi-automated optical heartbeat analysis of small hearts, *JoVE* (2009) 1–4, doi:[10.3791/1435](https://doi.org/10.3791/1435).
- [8] C. Medioni, M. Astier, M. Zmojdian, K. Jagla, M. Sémériva, Genetic control of cell morphogenesis during *Drosophila melanogaster* cardiac tube formation, *J. Cell Biol.* 182 (2008) 249–261, doi:[10.1083/jcb.200801100](https://doi.org/10.1083/jcb.200801100).
- [9] D.J. Macqueen, I.A. Johnston, Evolution of the multifaceted eukaryotic *akirin* gene family, *BMC Evol. Biol.* 9 (2009) 34, doi:[10.1186/1471-2148-9-34](https://doi.org/10.1186/1471-2148-9-34).
- [10] S.J. Nowak, H. Aihara, K. Gonzalez, Y. Nibu, M.K. Baylies, Akirin links twist-regulated transcription with the Brahma chromatin remodeling complex during embryogenesis, *PLoS Genet.* 8 (2012) e1002547, doi:[10.1371/journal.pgen.1002547](https://doi.org/10.1371/journal.pgen.1002547).
- [11] S.J. Nowak, M.K. Baylies, Akirin: a context-dependent link between transcription and chromatin remodeling, *Bioarchitecture* 2 (2012) 209–213, doi:[10.4161/bioa.22907](https://doi.org/10.4161/bioa.22907).
- [12] F. Bonnay, X.-H. Nguyen, E. Cohen-Berros, L. Troxler, E. Batsche, J. Camonis, et al., Akirin specifies NF- κ B selectivity of *Drosophila* innate immune response via chromatin remodeling, *EMBO J.* 33 (2014) 2349–2362, doi:[10.15252/embj.201488456](https://doi.org/10.15252/embj.201488456).
- [13] N. Liu, X.-W. Wang, J.-J. Sun, L. Wang, H.-W. Zhang, X.-F. Zhao, et al., Akirin interacts with Bap60 and 14-3-3 proteins to regulate the expression of antimicrobial peptides in the kuruma shrimp (*Marsupenaeus japonicus*), *Dev. Comparative Immunol.* 55 (2016) 80–89, doi:[10.1016/j.dci.2015.10.015](https://doi.org/10.1016/j.dci.2015.10.015).
- [14] M.J. Wolf, H. Amrein, J.A. Izatt, M.A. Choma, M.C. Reedy, H.A. Rockman, *Drosophila* as a model for the identification of genes causing adult human heart disease, *Proc. Natl Acad. Sci. USA* 103 (2006) 1394–1399, doi:[10.1073/pnas.0507359103](https://doi.org/10.1073/pnas.0507359103).
- [15] J. Wang, Y. Tao, I. Reim, K. Gajewski, M. Frasch, R.A. Schulz, Expression, regulation, and requirement of the toll transmembrane protein during dorsal vessel formation in *Drosophila melanogaster*, *Mol. Cell. Biol.* 25 (2005) 4200–4210, doi:[10.1128/MCB.25.10.4200-4210.2005](https://doi.org/10.1128/MCB.25.10.4200-4210.2005).
- [16] B.E. Richardson, K. Beckett, S.J. Nowak, M.K. Baylies, SCAR/WAVE and Arp2/3 are crucial for cytoskeletal remodeling at the site of myoblast fusion, *Development* 134 (2007) 4357–4367, doi:[10.1242/dev.010678](https://doi.org/10.1242/dev.010678).
- [17] A.M. Howard, H. Milner, M. Hupp, C. Willett, K. Palermino, S.J. Nowak, Akirin is critical for early tinman induction and subsequent formation of the heart in *Drosophila melanogaster*, *Developmental Biology* 469 (2021) 1–11, doi:[10.1016/j.ydbio.2020.09.001](https://doi.org/10.1016/j.ydbio.2020.09.001).