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Live imaging and analysis of muscle contractions in Drosophila embryo

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January 31, 2019

To: Phillip Steindel, Ph.D.
Review Editor
JoVE

Re: Revised manuscript submission

Dear Dr. Steindel,

Thank you for inviting us to revise and resubmit our manuscript entitled "Live imaging and analysis of muscle contractions in *Drosophila* embryo". We thank the editor and the reviewers for overall positive evaluation of our work. We appreciate reviewers' comments and critiques that helped us to revise and improved the quality of our manuscript. We attached our response, point by point, to the reviewers' critiques and comments. We hope that our revised manuscript will be found acceptable for publication.

We look forward to hearing from you.

Sincerely,

Vlad Panin
(on behalf of other co-authors of the manuscript)

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TITLE:

Live Imaging and Analysis of Muscle Contractions in *Drosophila* Embryos

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KEYWORDS:

muscle contractions, embryo, rolling, development, drosophila, live imaging, fluorescent marker

SUMMARY:

Here, we present a method to record embryonic muscle contractions in *Drosophila* embryos in a non-invasive and detail-oriented manner.

ABSTRACT:

Coordinated muscle contractions are a form of rhythmic behavior seen early during development in *Drosophila* embryos. Neuronal sensory feedback circuits are required to control this behavior. Failure to produce the rhythmic pattern of contractions can be indicative of neurological abnormalities. We previously found that defects in protein O-mannosylation, a posttranslational protein modification, affect the axon morphology of sensory neurons and result in abnormal coordinated muscle contractions in embryos. Here, we present a relatively simple method for recording and analyzing the pattern of peristaltic muscle contractions by live imaging of late stage embryos up to the point of hatching, which we used to characterize the muscle contraction phenotype of protein O-mannosyltransferase mutants. Data obtained from these recordings can be used to analyze muscle contraction waves, including frequency, direction of propagation and relative amplitude of muscle contractions at different body segments. We have also examined body posture and taken advantage of a fluorescent marker expressed specifically in muscles to accurately determine the position of the embryo midline. A similar approach can also be utilized to study various other behaviors during development, such as embryo rolling and hatching.

INTRODUCTION:

Peristaltic muscle contraction is a rhythmic motor behavior similar to walking and swimming in humans[1–3]. Embryonic muscle contractions seen in *Drosophila* late stage embryos represent an example of such a behavior. *Drosophila* is an excellent model organism to study various developmental processes because embryonic development in *Drosophila* is well characterized,

relatively short, and easy to monitor. The overall goal of our method is to carefully record and analyze the wavelike pattern of contraction and relaxation of embryonic muscles. We used a simple, non-invasive approach that offers a detailed visualization, recording and analysis of muscle contractions. This method can also be potentially used to study other in vivo processes, such as embryonic rolling seen in late stage embryos just prior to hatching. In previous studies, embryonic muscle contractions were mostly analyzed in terms of frequency and direction[1,2]. In order to estimate the relative extent of contractions as they progress along the body axis in the anterior or posterior direction, we have used embryos expressing GFP specifically in muscles. This analysis provides a more quantitative way to analyze muscle contractions and to reveal how body posture in embryos is maintained during series of peristaltic waves of muscle contractions.

Peristaltic muscle contractions are controlled by central pattern generator (CPG) circuits and communications between neurons of the peripheral nervous system (PNS), the central nervous system (CNS), and muscles[4,5]. Failure to produce normal peristaltic muscle contractions can lead to defects such as failure to hatch[2] and abnormal larval locomotion[6] and can be indicative of neurological abnormalities. Live imaging of peristaltic waves of muscle contraction and detailed analysis of contraction phenotypes can help uncover pathogenic mechanisms associated with genetic defects affecting muscles and neural circuits involved in locomotion. We recently used that approach to investigate mechanisms that result in a body posture torsion phenotype of ***p****rotein* ***O****-****m****annosyl****t****ransferase* (*POMT*) mutants[7].

Protein O-mannosylation (POM) is a special type of posttranslational modification, where a mannose sugar is added to serine or threonine residues of secretory pathway proteins[8,9]. Genetic defects in POM cause congenital muscular dystrophies (CMD) in humans[10–12]. We investigated the causative mechanisms of these diseases using *Drosophila* as a model system. We found that embryos with mutations in *Drosophila protein O-mannosyltransferase* genes *POMT1* and *POMT2* (a.k.a. *rotated abdomen* (*rt*) and *twisted* (*tw*)) show a displacement ("rotation") of body segments, which results in an abnormal body posture[7]. Interestingly, this defect coincided with the developmental stage when peristaltic muscle contractions become prominent[7].

Since abnormal body posture in POM mutant embryos arises when musculature and epidermis are already formed and peristaltic waves of coordinated muscle contractions have started, we hypothesized that abnormal body posture could be a result of abnormal muscle contractions rather than a defect in muscle or/and epidermis morphology[7]. CMDs can be associated with abnormal muscle contractions and posture defects[13], and thus the analysis of the posture phenotype in *Drosophila POMT* mutants may elucidate pathological mechanisms associated with muscular dystrophies. In order to investigate the relationship between the body posture phenotype of *Drosophila POMT* mutants and possible abnormalities in peristaltic waves of muscle contractions, we decided to analyze muscle contractions in detail using a live imaging approach.

Our analysis of peristaltic contraction waves in *Drosophila* embryos revealed two distinct contraction modes, designated as type 1 and type 2 waves. Type 1 waves are simple waves propagating from anterior to posterior or vice versa. Type 2 waves are biphasic waves that initiate

at the anterior end, propagate halfway in the posterior direction, momentarily halt, forming a temporal static contraction, and then, during the second phase, are swept by a peristaltic contraction that propagates forward from the posterior end. Wild-type embryos normally generate a series of contractions that consists of approximately 75% type 1 and 25% type 2 waves. In contrast, *POMT* mutant embryos generate type 1 and type 2 waves at approximately equal relative frequencies.

Our approach can provide detailed information for quantitative analysis of muscle contractions and embryo rolling[7]. This approach could also be adapted for analyses of other behaviors involving muscle contractions, such as hatching and crawling.

PROTOCOL:

1. Preparation

1.1. Prepare a fly cage by making approximately 50 holes in a 100 mL capacity tri-corner plastic beaker using a hot 25 G needle (see **Table of Materials**).

1.2. Prepare 60 mm x 15 mm Petri dishes with apple juice-agar (3% agar and 30% apple juice).

1.3. Prepare fresh yeast paste by mixing dry yeast granules and water. Spread the yeast paste onto the apple agar plates to increase egg laying.

1.4. Anaesthetize about 50–60 flies (use approximately equal numbers of males and females) on CO_2 and put them in the fly cage.

NOTE: Using an increased proportion of females (up to ~2:1 ratio of females:males) may help increase the amount of laid eggs for some genotypes.

1.5. Attach an apple juice-agar Petri dish with yeast paste to the fly cage tightly and seal it with modeling clay. Make sure it is sealed at all corners.

1.6. Wait until flies wake up from anesthesia and then invert the cage such that the Petri dish is now at the bottom. Put the cage into an incubator with controlled temperature (25 °C) and humidity (60%).

1.7. Allow flies to lay eggs for 2–3 hours, replace the apple plate with a fresh one, and let the plate with eggs age for 19–20 hours in an incubator.

NOTE: Prior to the above step, flies must be synchronized to facilitate collection of stage 17e-f (19-21 h AEL) embryos. This can be achieved by transferring flies to a cage with a fresh yeast-apple juice-agar plate 3–4 times for 12 hours (once every 3–4 hours). Keeping flies at controlled circadian light environment (LD cycle) can also help with collecting a synchronized population of embryos, but this was not essential in our experiments.

2. Collection of embryos

2.1. Carefully pick embryos with a wet paintbrush and place them in a collecting glass dish filled with 1x PBS.

2.2. Select the embryos that have their tracheae filled with air. Air-filled tracheae indicate that embryos have reached Stage 17, and their peristaltic muscle contractions should have begun. Tracheae become clearly visible when they are filled with air, which can serve as a marker for Stage 17.

2.3. Place an apple juice agar slab on a glass slide and carefully transfer embryos from PBS to the slab. Line up the embryos with their ventral side up.

NOTE: Dorsal and ventral sides of embryos can be distinguished by the position of dorsal appendages on the eggshell.

2.4. Make a rectangular wax boundary on another glass slide using a wax pen (see **Table of Materials**).

2.5. Place a double-sided sticky tape within that boundary and gently pick up the embryos by lowering this slide onto the agar slab. Apply gentle pressure to ensure that embryos stick to the tape well, with their dorsal side up. If necessary, embryos can be rolled on the tape to correct their orientation. Do all manipulations while monitoring embryos under a dissection microscope.

2.6. Cover embryos with 1x PBS for live imaging of muscle contractions.

NOTE: Some procedures described above are related to basic Drosophila techniques used in many studies. More detailed descriptions of common Drosophila techniques can be found elsewhere[14].

3. Recording of embryos

3.1. Perform live imaging of mounted embryos on an epiflourescence microscope with a time-lapse function and a digital camera with suitable emission filters (see **Table of Materials**) using a 10x water immersion objective lens.

NOTE: Here we used embryos expressing GFP in muscles. Other fluorescent markers with suitable excitation light and emission filter sets can also be used (e.g., for tdTomato detection, one can use a Chroma ET-561 filter set for excitation and emission around optimal 554 nm and 581 nm, respectively).

3.2. Perform live video recording of embryos using suitable software (see **Table of Materials**) for about 1–2 hours with an acquisition rate of 4 frames/s.

NOTE: To analyze rolling of the developing embryo within its shell, embryos without expression of fluorescent markers can be used. To this end, regular transmitted light illumination without spectral filters is applied to visualize embryo motion within the shell (See **Movie 2**).

4. Analysis of the recordings

4.1. Export the recorded video directly into Image J for further analyses (e.g., as AVI files).

4.2. In ImageJ, crop the video recordings to the size of individual embryos by drawing a box around each embryo and then clicking on **Image** > **Crop**. This greatly reduces the size of video files without affecting its resolution and facilitates their analysis.

4.3. Rotate cropped images to achieve vertical position of the embryo midline relative to the screen, by clicking on **Image** > **Transform** > **Rotate**.

NOTE: Selecting **Preview** during this process will provide guidance for rotation, showing gridlines to ensure vertical position of the midline.

4.4. Analyze embryo rolling:

4.4.1. Mark the position of one or both tracheae in the first frame of the video at a point midway between posterior and anterior ends. Click on **Analyze** > **Tools** > **ROI manager** and record this position as **slice number-y coordinate-x coordinate** by drawing a box of approximate width of 30 pixels and height of 10 pixels around it and typing **t** on the keyboard. Ensure that when typing **t**, a region of interest is selected on the video. Alternatively, select the **Add (t)** tab on the ROI manager to record the position of trachea instead of typing the command.

NOTE: The region of interest can vary in shape or size depending on the embryonic region or developmental event being studied.

4.4.2. Mark the position of the same area of the trachea after each peristaltic contraction. Measure the distance from the pre-contraction position to the post-contraction position by drawing a line connecting the centers of each box and typing **m** on the keyboard. Convert the distance to micrometers using a known scale of images. Alternatively, measure the distance in micrometers in a single step by clicking on **Analyze** > **Set Scale** and enter the known pixel-to-micron conversion factor to yield a report in microns.

NOTE: A distance in pixels can be entered together with its corresponding distance in micrometers.

4.4.3. Correlate the distance and direction of each rolling event with the direction of muscle contraction propagation in at least 8 embryos for statistically significant differences.

4.5. Analyze embryonic muscle contractions:

4.6. Use embryos expressing fluorescent markers in muscles (e.g., we used transgenic flies expressing a fusion construct of Myosin Heavy Chain promoter and GFP called MHC-GFP[5]) to analyze muscle contraction parameters such as contraction amplitude.

4.7. Use the recording of fluorescent readout and draw a region of interest (e.g., a box of ~40 pixels x 120 pixels) centered on the muscles (which are clearly visible due to the presence of fluorescent marker) of a particular body segment, and select the **Add (t)** tab on the ROI manager to record the position of the ROI. Click on **ROI manager** > **Measure** to record the average fluorescent intensity of each region of interest selected for each frame of the video.

4.8. Move the box to the centers of other body segments of interest and click on **Add (t)** in the ROI manager to record their positions. This will give regions of interest of identical size in all body segments to be analyzed. Select at least one posterior, one medial, and one anterior segment, e.g., A7, A4, and T2, respectively.

4.9. In the ROI manager, select all regions of interest recorded as **slice number-y coordinate-x coordinate** (e.g., by selecting while holding **Ctrl**) and click on **More** > **Multi measure** to measure the mean fluorescent intensity of each region of interest for all frames of the video, and report each measurement in a table. Each region of interest is a column of the table, and each frame is a row. Transfer the table to a spreadsheet program for further analyses.

4.10. Plot a graph with frame number on the x-axis and mean fluorescent intensity on the y-axis. Frame number can be converted to time using the frame rate (4 frames/s) of the video **(Figure 1A)**.

4.11. Determine muscle contraction amplitude by estimating the increase in GFP fluorescence intensity relative to the baseline. Muscle contractions increase the GFP intensity as they bring more GFP into the vicinity of the focal area as more muscles get pulled in during these contractions (**Movie 1**)[7]. Establish a baseline fluorescence as the average intensity between contraction waves. Normalize GFP intensity to the baseline by dividing every ROI intensity value by the baseline intensity.

NOTE: Each profile has a different baseline fluorescence, as there may be different expression levels in different muscle segments. One potential complication is that the GFP fluorescence may change over time due to photo bleaching. This can be resolved by monitoring changes in fluorescence baseline and using a sufficient sample size for wave analyses (we normally use sets of 10 fluorescent waves and confirm that the baseline is approximately constant by taking an average of only those peak minima as baseline that have decreased in fluorescence by 10% or less relative to the initial minima peak). A pulse-LED illumination may be also applied to mitigate that problem.

4.12. Compare muscle contractions on left and right sides of the embryo by analyzing peak intensities on both sides of the embryo for same segments. Use contraction amplitude and time

of peak intensities to examine differences in extent and timing of peristaltic muscle contraction waves propagating along both sides of the embryo.

4.13. Compare normalized intensity of GFP at different segments (e.g., at anterior, medial and posterior regions) during muscle contraction wave propagation to examine changes in the contraction as the wave propagates. This analysis also determines the direction of the wave (i.e., whether it propagates toward anterior or posterior regions of the embryo).

REPRESENTATIVE RESULTS:

Normal peristaltic muscle contractions are shown in a *WT* (*wild-type*, Canton-S) embryo in **Movie 1**. The average frequency of peristaltic waves of muscle contractions in our analysis was 47 contractions per hour and the average amplitude was 60% above baseline for WT embryos. Embryo rolling is shown for a *WT* embryo in Movie 2, with the white arrow marking the initial position of a trachea and a black arrow depicting the position of a dorsal appendage. The dorsal appendage (exterior) does not move whereas the tracheae (interior) does, indicating that the embryo has rolled within its shell

In our analysis of pattern of muscle contractions, we designated a peristaltic contraction as a type 1 wave if its profile has a peak that arises at the anterior region first, followed by peaks at middle and posterior regions (backward wave) or a profile in which the peak first arises at posterior segments and then propagates toward anterior regions (forward wave) (**Figure 2A** and **Movie 1**). We also observed another type of waves that we designated as Type 2. Type 2 waves start at one end of the embryo (usually anterior), proceed toward the middle regions, and then return to their origin as a sweeping wave re-initiated at the opposite end (**Figure 2A** and **Movie 1**, wave 4). *POMT* mutant embryos show abnormal relative frequency of type 1/type 2 wave generation (**Figure 3**), which results in body posture abnormality, the body torsion (or "rotation") phenotype (**Figure 4**).

Movie 1 shows peristaltic muscle contractions in a *wild-type* embryo.

Movie 2 shows embryo rolling in a *wild-type* embryo.

Figure 1A shows muscle contraction amplitude monitored over time as normalized GFP intensity at different embryo segments (anterior, middle and posterior). Peaks during 165-178 s time period represent a simple forward wave (Type 1). **Figure 1B** shows that there is no difference in the amplitude (depicted as GFP intensity) and time of muscle contractions on right and left sides of the embryo.

Figure 2 shows Type 1 and Type 2 muscle contraction profiles generated using GFP intensity as a measure of contraction amplitude. A type 1 wave is a single wave generated at the anterior or posterior end of the embryo that continues propagation towards the opposite end. Type 2 is a biphasic wave in which the wave propagates to the middle of the embryo during the first phase and then returns to the origin as a peristaltic contraction reinitiated at the opposite end. Each

wave line represents normalized GFP intensity detected in successive body segments of an embryo, and peaks correspond to muscle contraction. Slant appearance of the peaks illustrates that muscle contractions propagate along successive segments, from anterior to posterior, or vice versa, and thus peaks occur in a consecutive manner in successive body segments.

Figure 3 includes graphs of contraction wave series in *WT* and *POMT* mutant embryos. The graphs illustrate that Type 2 contraction waves are generated at increased relative frequency in *POMT* mutants, as compared to *WT* embryo. The series of waves in *WT* embryo (top graph) depicts the contractions shown in **Movie 1**.

Figure 4 shows fixed *WT* and *POMT* mutant embryos with muscles stained with fluorescein-conjugated phalloidin to highlight embryo body posture. The curved dashed line illustrates the body posture phenotype of a POMT mutant.

FIGURE AND MOVIE LEGENDS:

Figure 1: Muscle contraction amplitude. (**A**) GFP intensity is plotted against (Y- axis) time (X-axis) for different body segments of the embryo. (**B**) GFP intensity (Y- axis) plotted against time for left and right sides of the same segment of a contracting embryo. Frame rate is 4 frames/sec for both graphs.

Figure 2: Type 1 and Type 2 peristaltic muscle contraction wave profiles. (**A)** Type 1 wave profile in which individual lines represent normalized GFP intensities of particular body segments over time, while the peaks indicate contraction events. (**B**) Type 2 wave profile that shows an example of a biphasic contraction wave, plotted in the same way as in A.

Figure 3: Series of contraction waves generated by *wild-type* and *POMT* mutant embryos. Blue and red bars depict Type 1 and Type 2 waves, respectively. Note that the *POMT* mutant generates an increased proportion of Type 2 waves, as compared to *WT*. The *WT* graph represents contractions shown in **Movie 1**.

Figure 4: Fixed and stained *POMT* mutant (rt^{-}) and WT embryos. Note the rotation in body segments of the *POMT* mutant embryo, highlighted by a dashed line tracing the position of midline. Muscles are visualized using staining with fluorescein-conjugated phalloidin. Anterior is to the left, dorsal is up. Scale bar is 100 μm.

Movie 1: Example of peristaltic muscle contractions of a *WT* embryo. Muscle contractions are shown in a pseudocolor format to illustrate increase in GFP intensity when contraction occurs (most bright pixels are red). The video was acquired at 4 frames per second (fps) and is shown at 20 fps.

Movie 2: A wild-type embryo rolling within its shell. White arrow indicates the initial position of a trachea, and black arrow indicates the position of a dorsal appendage. Note that as the embryo rolls, tracheal position changes but the dorsal appendage does not move, which illustrates that

the embryo rolls inside its eggshell.

DISCUSSION:

Our method provides a quantitative way to analyze important embryo behaviors during development, such as peristaltic muscle contraction waves, including wave periodicity, amplitude and pattern, as well as wave effect on embryo rolling and posture. This can be useful in analyses of different mutants to study the role of specific genes in regulating these and other behaviors during embryonic development. We have used changes in muscle-specific GFP marker intensity to analyze muscle contraction amplitude, frequency and direction of contraction wave propagation in embryos. These changes in GFP signal reflect the extent of contractions, as contracting body segments bring more GFP into an ROI and the vicinity of the focal area. This approach significantly simplifies analyses and gives a better visual representation of the pattern of peristaltic contraction waves.

In our experiments, we used genotypes with muscle-specific transgenic expression of GFP to visualize and study in detail muscle contractions during embryonic development. Other studies used a similar approach to analyze larval motion such as crawling and bending[5, 15]. A similar technique to study coordinated muscle contractions was previously applied for sandwich preparation of embryos, which is a more invasive approach that may affect embryo behavior and development[3]. In contrast, our method is completely non-invasive and the embryos are unperturbed during assays. Our protocol doesn't require the embryos to be dechorionated or devitellinated, and live embryos of interest can be recovered after assays and propagated for further analyses.

Our method can potentially be developed further for a high content analysis (HCA)-based screening to isolate and analyze mutations that affect embryonic muscle contractions and other behaviors and developmental processes. This strategy, for instance, can be used to simultaneously record muscle contractions of many embryos and for assessing their response to various stimuli, drugs, or environmental changes.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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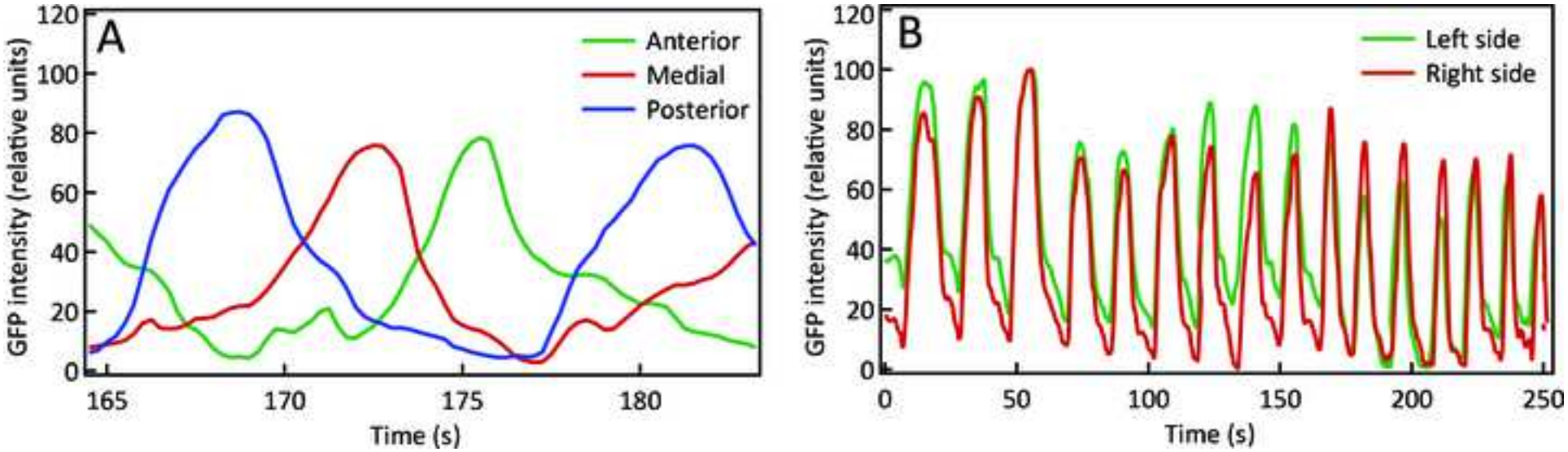

Figure 2

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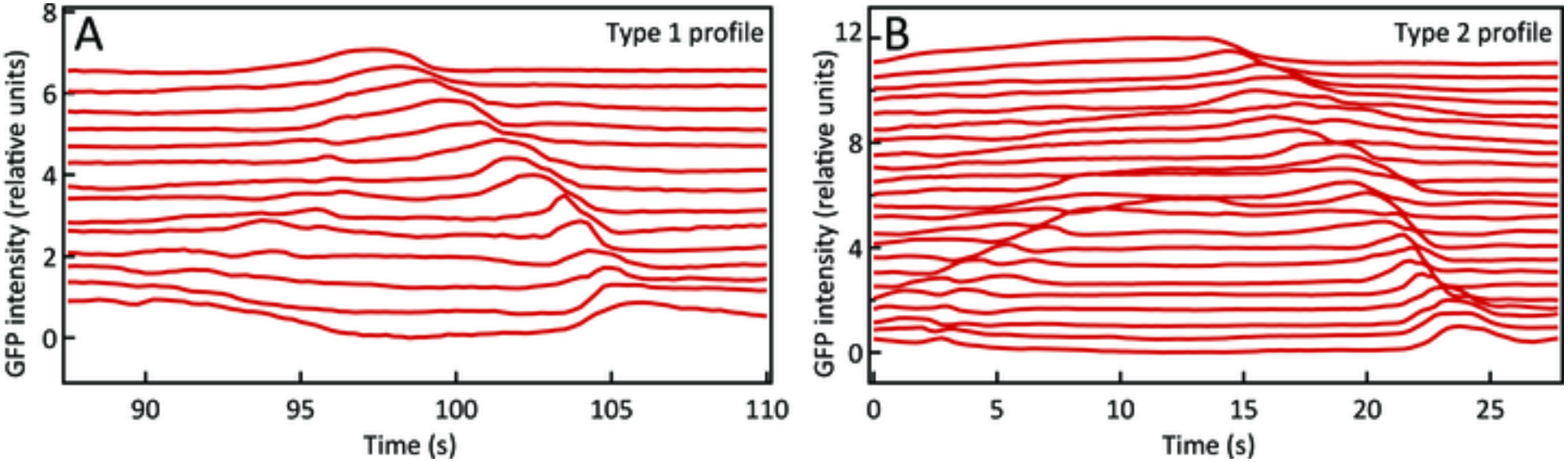

Figure 3

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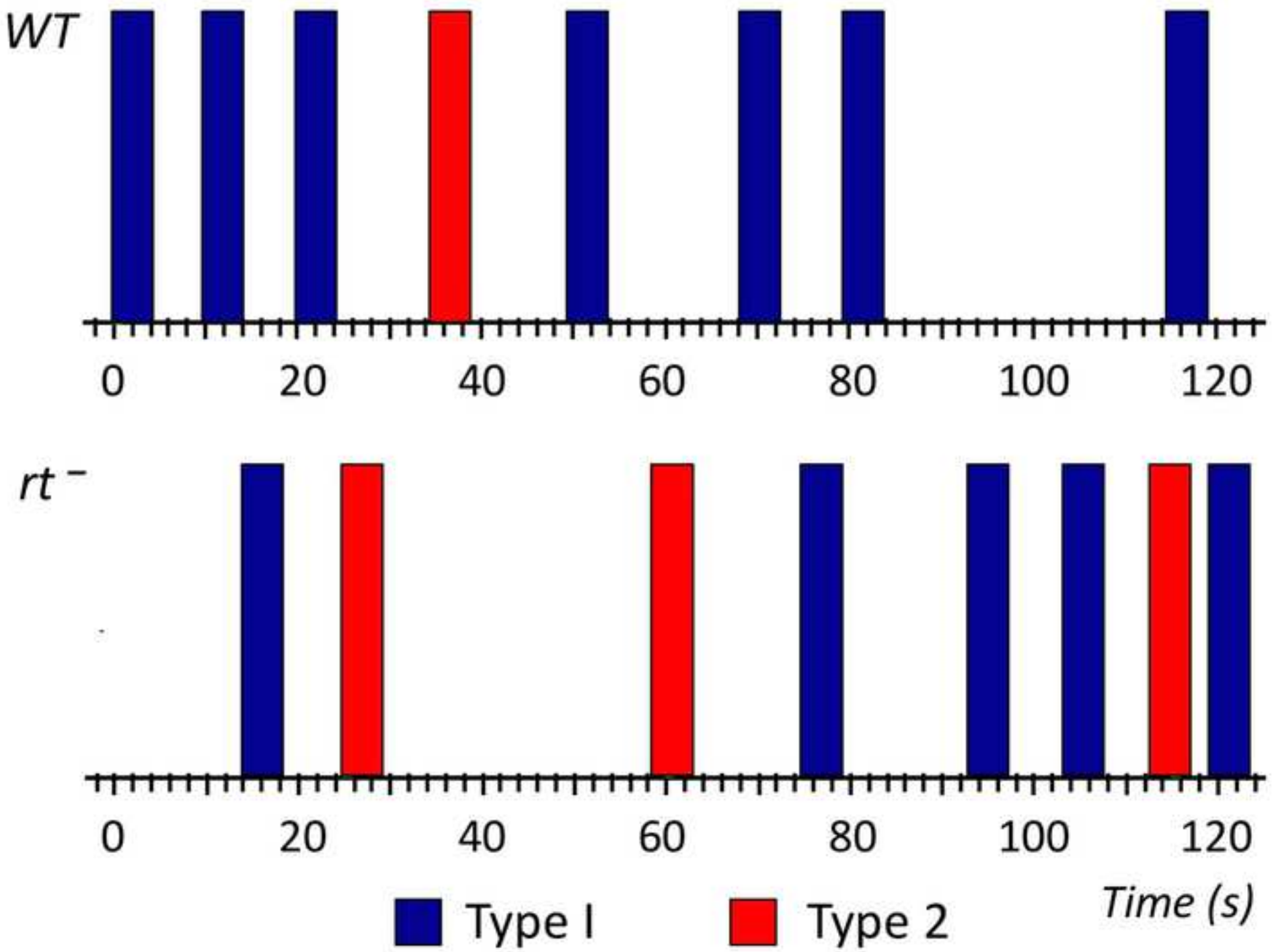

Figure 4

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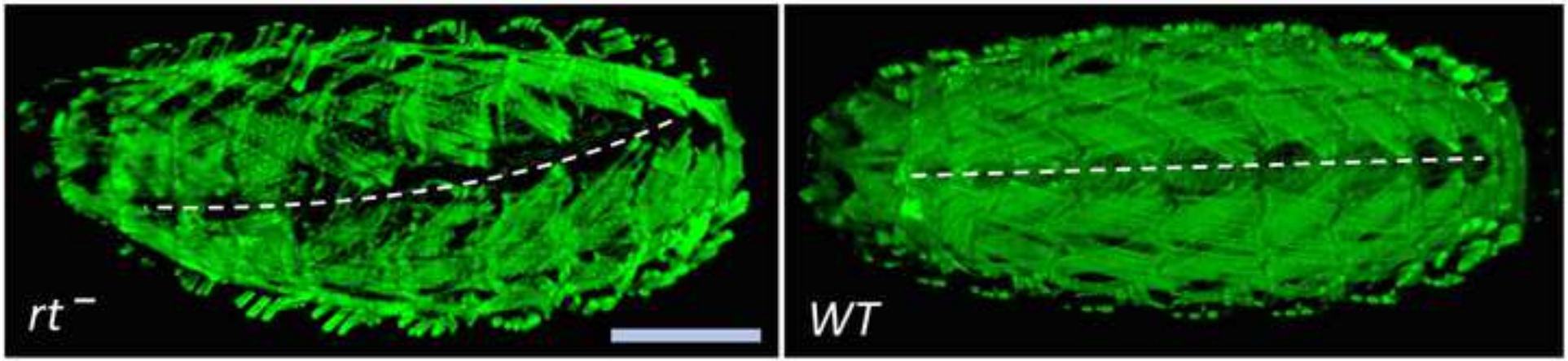

Movie 1

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Video or Animated Figure

WT thermo(1).avi

Movie 2

Click here to access/download

Video or Animated Figure

Rolling video JOVE(1).avi

Click here to access/download;Table of Materials;jove materials Revised.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Digital camera	Hamamatsu CMOS ORCA-Flash 4.0	C13440-20CU	With different emission filters
Forceps	FST Dumont	11254-20	Tip Dimensions 0.05 mm x 0.01 mm
LED	X-cite BDX (Excelitas)	XLED1	
Microscope	Carl Ziess Examiner D1	491405-0005-000	Epiflourescence with time lapse
Needle	BD	305767	25 G x 1-1/2 in
Paintbrush	Contemporary crafts		Any paintbrush will work
Petri dishes	VWR	25384-164	60 mm x 15 mm
Software	HCImage Live		
Thread Zap Wax pen	Thread Zap II (by BeadSmith)(Amazon)	TZ1300	Burner Tool
Tricorner plastic beaker	VWR	25384-152	100 mL

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We thank the editor and the reviewers for overall positive evaluation of our manuscript. We appreciate reviewers' comments and critiques that helped us to revise the manuscript. We believe that this improved the quality of our manuscript. We hope that our revised manuscript will be found acceptable for publication.
Below is our response, point by point, to the reviewers' critiques and editorial comments.

Reviewer #1:

Minor Concerns:
Lines 48-85: consider combining paragraphs to shorten introduction. For methods paper, there is too much focus on previous results. Instead, emphasize the use of the method and explain the author's application for the method as well as different possible applications.
Response: The suggested changes have been made. Please see lines 44-70 and lines 108-111.

Line 107: clarify/elaborate. Should users replace agar plates every 3 hours, and then grow for 20 hours? This explanation was unclear.
Response: This has been clarified. Please see 1.7 and lines 140-142.

Line 116: Explain why take embryos whose trachea are expanded. Describe in detail how such embryos can be distinguished from others.
Response: This detail has been included. Please see 2.2.

Line 151: A few notes on potential problems in mounting or imaging and possible solutions may be helpful.
Response: Our mounting and staging technique is quite standard and notes are provided throughout the protocol to avert possible problems that may occur during these steps.

Line 171: I suggest dividing the analysis section in subsections e.g. 4a-measuring contractions by tracheal position, 4bmeasuring contraction with muscle specific GFP expression.
Response: This suggestion has also been included. Please see 4a (line 216) and 4b (line 248).

Line 285: more comments on general use or other applications of methods here (and/or in introduction).
Response: Suggested changes have been made in the introduction and further applications of the method are mentioned in the discussion (lines 404-408).

Reviewer # 2:

Major Concerns:
1. The most serious concern I have is that the method aims at quantifying contraction frequencies and amplitudes (see abstract), however it does not do that as presented. Not a single number is given. Without a major revision including these numbers I do not believe that this protocol will be very useful. Robustness needs to be shown. What number of animals is needed for a statistically significant difference?

Response: Number of embryos analyzed for both kinds of analysis in the paper was at

least 8. This information is now included in the manuscript (line 244). An example how relative frequencies of Type 1 and 2 waves can be analyzed by our method is shown in new Figure 4 for wild-type and POMT mutants. More extended analyses of series of contraction waves using our protocol can be also found in Baker et al, 2018 (ref. [7]).

Minor Concerns:
2. The intro could mention the difference of type 1 vs type 2 waves.
Response: Type 1 vs. type 2 difference is now included in the introduction. (Lines 96-104).

3. Staging will not be precise if the plate is changed every 12h. This would require a change every hour (1.7).
Response: We do not advise to change the plate every 12 h, rather we suggest to change the plate 3-4 times every 12 hrs (see note on 1.7.). In addition, for more precise staging, we use the event of trachea been filled with air (stage 17e, Pereanu et al 2007).

4. As the embryos are not dechorionated, they will not dry out. Thus, the statement in the protocol is incorrect (2.6).
Response: This statement has been corrected.

5. Type 2 waves seem to split rather than to invert, thus the text is not accurate.
Response: We did not indicate that type 2 waves invert. Rather, we describe a type 2 wave as biphasic, with opposite direction of propagation at the two phases. We think that describing the type 2 wave as a split wave would be less clear and potentially confusing.

6. Figure 3 has no labeling.
Response: Genotypes and dashed lines showing rotation of body segments have been added to Fig 3.

7. Senseless mutants are mentioned in the introduction but not elsewhere. Either delete or mention them in the results section.
Response: We have deleted the mention of *sens* mutants.

Reviewer # 3:

Major Concerns:
1. The precision of using a box to mark trachea positions (section 4.4) is not clear to me. A point may be more precise, but at the very least the authors should list the size of the box.
Response: The idea here is to mark and record the position of the trachea before and after muscle contractions because when the embryo rolls inside the eggshell the position of the trachea changes. This can be achieved by using a box or a point (as pointed out by the reviewer). A relatively small rectangular box, e.g., ~ 30x10 pixels, is sufficient to mark and record the position of the trachea. This information is now included in the manuscript (See 4a).

2. Regarding the muscle contraction analysis, it is not clear what is in the ROI that the authors are measuring. Is there a specific muscle, or set of muscles that is being measured?
Response: The ROI has fluorescent readout from muscles in it and we are measuring its intensity because when muscles contract the GFP intensity increases as compared to

baseline. Ideally, the ROI should include all muscles from one anterior, one medial and one posterior body segment should be taken for analysis of wave patterns. To illustrate this better we have now included a movie that demonstrates muscle contractions and how fluorescence readout increases in response to contractions (See Movie 1).

3. It is unclear to me how the GFP fluorescence increased as a function of muscle contraction (section 4.11). Did additional muscles get pulled into the ROI? Are the authors now measuring a smaller region with the same amount of GFP because the muscle is contracted?
Response: Muscle contractions increase the GFP intensity as they bring more GFP into the vicinity of the focal area[1] (Section 4b, lines 282-284). This is demonstrated in the movie of muscle contractions that we have now included in our manuscript (Movie 1).

4. I do not think that normalized GFP intensity can necessarily be an indication of contraction strength (section 4.12). It might be extent of contraction, but "strength" indicates force production.
Response: We have changed “strength” to “extent” throughout the manuscript.

5. I suppose the eventual video will mitigate some of this, but in many cases it took effort to determine exactly how a protocol would be completed. Cartoons showing how boxes are drawn and ROIs are determined could be of great help to the eventual readers.
Response: The reviewer is correct to point out that the eventual video will mitigate the confusion, but we have made additional changes and added movies and graphs that will help the reader to follow the protocol better. Drawing boxes in Image J is fairly straightforward and determining ROIs depends upon what the reader is interested in analyzing. E.g., we drew ROIs around trachea and muscles for our analysis. The video will also circumvent these concerns.

6. How the data in Figure 2A was generated is not clear to me. Better description of what the figure is, or another cartoon, could be of great help.
Response: Figure 2A shows an example of a peristaltic muscle contraction wave obtained by drawing ROIs and measuring normalized GFP intensity for muscle segments in an embryo over time. Each wavy line represents normalized GFP intensity from different anterior and posterior body segments of an embryo and peaks represent muscle contraction. The peaks appear slant because muscle contractions begin in a particular anterior/posterior segment and then occur in other segments in a consecutive manner (anterior to posterior or posterior to anterior). They do not occur simultaneously in all body segments. Therefore, muscle contraction peaks appear one after other throughout the body segments over time.

Minor Concerns:
1. Section 3.4 what is the acquisition rate?
Response: 4 frames/second, see 3.2.

2. Section 4 might be more accessible to the reader if it were divided into two sections (1) embryo rolling and (2) muscle contractions.
Response: This suggestion has been incorporated as Section 4a and 4b in the manuscript.

3. Line 175: You say, "Alternatively, the distance can be measured in pixels". I think that you mean microns.

Response: Yes, this has been corrected.

Reviewer # 4:

Minor Concerns:
*Please add a concluding sentence at the end of Introduction to summarize the observations and lead to protocol.
Response: Added, see lines 108-111.

*Protocol, section 1.7, "allow flies to lay eggs for 19-20 hrs": This seems to be a lengthy window for egg laying, because eggs collected after this time window will be 020 hours old and therefore they would not be synchronized. Wouldn't it be better to use more adult females, decrease the egg laying time window to 24 hours, and then age the eggs laid in this time window until they are 19-20 hours old?
Response: We agreed with the Reviewer and made the suggested changes (see 1.4 and 1.7).

*Protocol, section 2.3: It will be helpful if the authors show an image or provide some description so that the reader knows how to differentiate ventral from dorsal side in embryos at that stage.
Response: This has now been added, see note on 2.3.

*Protocol, section 4.14, and line 221: … if its profile has a peak, “which” arises at the anterior …
Response: Grammar Corrected, See representative results, line 328.

*Figure and Table legends, line 242: Instead of "Fig 1 A." use "Fig 1. A."
Response: Legends fixed.

Reviewer # 5:

Major Concerns:
1. It was surprising that the authors do not provide actual examples of visualizing wild-type or defective muscle contraction. Only single images are provided, which could be accomplished with fixed tissue. A movie of this type is critical to someone using this method for experimental analysis. We do not see what peristaltic movements, for example, mean or how one determines that these are defective.
Response: To make sure our readers understand what peristaltic muscle contractions are, we have included an example of movie (Movie 1), as well as graphs that depict series of contraction waves in WT and POMT mutants (Fig.4), illustrating that mutants generate a higher proportion of type 2 peristaltic waves, which is a characteristic phenotype of these mutants.

2. Related to the above, even a series of still images from a time-lapse analysis of muscle contraction with time stamps would be helpful and seems appropriate.
Response: To illustrate peristaltic muscle contractions, we included movies that show fluorescent (Movies 1) and regular light (Movie 2) images of contraction waves.

3. Diagrams of the method of mounting embryos and diagrams/overlays of how measurements were taken is also appropriate. These details are unique to this particular

method, and should be fully explained with visual aids/examples.
Response: Our mounting and staging techniques are standard and routinely done (as pointed out by the reviewer) and are mentioned elsewhere[2]. Drawing ROIs for taking measurements in Image J is fairly straightforward and will be shown in the eventual video.

4. In contrast to Parts 1 and 2, much of which is available elsewhere, Part 3 is lacking many details that would ensure success. For example 1) how often is an image collected over the 3-hour period? 2) How is the embryo kept in place and in focus when it begins significant muscle contraction? 3) How is the embryo kept alive and hydrated during this time but does not drown from lack of oxygen for this three hour period (simply placing PBS on a slide is not sufficient if one is imaging for 3 hours, 4) how did they prevent photobleaching of the GFP, 5) what magnification is adequate, and so on. Also, what drivers and fluorophores are adequate for robust muscle expression (stock sources, genotypes of successful combinations and options are appropriate). The "Protocol" simply lists the equipment used. Does this mean that someone who does not have these specific resources or someone not familiar with this particular microscope or software cannot use this protocol in any manner? The method should be translatable to other setups. What type of image is saved, for example (see below).
Response: 1) The video is a continuous video of approximately 1-2 hrs and can be taken continuously up to the point of embryo hatching. 2) The embryo is inside the eggshell, which is sticking to a tape and thus does not move significantly when contracting. 3) The embryo remains alive and hydrated and PBS can be replenished if need be without disturbing the microscope setup or the embryo slide as we use water immersion lenses. 4) Photo bleaching of GFP is a potential complication and we have explained in detail how to circumvent this potential problem (see Note in section 4b. 6) Adequate magnification used is 10X and is mentioned in Section 3.1. We have not used any muscle specific Gal4 driver rather we have used a transgenic fusion construct of myosin heavy chain promoter and GFP (MHC-GFP) to express GFP in muscles (See section 4b). Other fluorophores have also been mentioned in the manuscript (See note in 3.1). The equipment details have been moved to table of materials. We are sure other microscopes and software setups can be suitable for using with this protocol but for analysis we have only used Image J and would recommend using that.

5. Part 4 is good on some details of how data is collected but is lacking in others. For example, how does one recognize that an embryo is rolling in the image?
(What does this look like? A movie or series of time point images of an example again seems appropriate).
Response: The reviewer brings a good point here and to that end we have included a movie (Movie 2) showing a WT embryo rolling.

Minor Concerns:
1. Much of the experimental detail provided to the reader is general and very redundant with many other publications. Parts 1 and 2 describe very routine methods for embryo collection that have been available for more than 2 decades. These do not merit publication but, at the very least, none are cited.
Response: We agree that Parts 1 and 2 include elements of routine methods that were used in many previous studies. We decided to provide the description of these well-known steps, instead of simply including references, for a more complete and convenient protocol that can be easily used by researchers even without significant experience of working with Drosophila. However, per reviewer's suggestion, we added

now a reference for a well-known compendium of Drosophila techniques that describes in greater detail some basic procedures related to our protocol, such as handling flies and collecting embryos (Roberts 1998).

2. It would be useful to include a section on troubleshooting. One example where this might be useful in Part 2 is on line 125 where they state that embryos will stick to the tape with their dorsal side up. This seems surprising unless one positions the embryos, so more information on this aspect would be useful.
Response: This is a routine procedure and embryos can be easily positioned this way. Embryos with the chorion tend to stick well to adhesive tape, but they still can be rolled to achieve necessary orientation. We added this information in section 2.5.

3. Detail in this last section discusses general guidelines for imaging ("crop images to make file smaller") but does not mention image resolution or whether this is modified. Others such as the file type that can be successfully imported into ImageJ, necessary plugins, and so on may be very useful in translating this procedure to other microscopes or dedicated software.
Response: We included details on video resolution and file type in 4.2 and 4.1, respectively.

Reviewer # 6:

Major Concerns: 1. It needs major improvement in grammar and detail for a reader to follow.
Response: We edited the manuscript to add more details, and improve its clearness and grammar.

2. It is also recommended that the authors alter the figures to include, as important examples, wave data from the mutants so that the reader can see "mutant behavior".
Response: As suggested, we included examples of wave movies (Movies 1, 2) and graphs that illustrate mutant behavior in wave generation (Fig. 4)

Minor Concerns:
2. Summary is misleading. The authors are not recording embryonic developmental processes. The authors are recording muscle contraction.
Response: Changes to the summary have been made as suggested

3. In the abstract, coordinated muscle contractions are not seen "early" during development in the Drosophila embryo (early in embryonic development is stage 5 for example). Coordinated contraction occurs at the end of embryonic development (stage 17). To that end, instead of stages, hours after egg laying should be included.
Response: Hours after egg laying (AEL) are now included, see note in 1.7.

4. At the end of the introduction, the authors mention briefly that Senseless mutants also show abnormal contraction patterns. These data should be included so that the readers have a sense of the different types of contraction behaviors that can be identified with this technique. Also it would be wise to include a conclusion at the end of the introduction. As is, the reader is left hanging.
Response: To avoid confusion and make the introduction more straightforward, we removed the mention of senseless mutants. Detailed information on contractions of sens mutants can be found in ref. [7], however, it's beyond the main scope of our manuscript.

5. The manuscript, but particularly the protocol, is poorly written and filled with grammatical mistakes. The authors should rewrite the manuscript using the same tense and in a more formal tone. The manuscript should be edited so that it is not vague and please remove jargon. More detail should be given. As examples, what are the genotypes of the "wildtype" embryos with green muscles? How many different muscle GAL4 drivers have been tested? Do the wave patterns change depending on which driver (with associated background) is used? One could imagine differences in wave patterns with different "wildtype" controls. The authors should also be consistent in terms of using abbreviations and while referring to Image J functions and commands. i.e. how to find the command in ImageJ.
Response: Wildtype is Canton-S. The green muscles are a green fluorophore conjugated to a stain called phalloidin, which stains actin filaments of muscles (See Fig 3 legend). Muscle Gal4 driver has not been used instead we used a fusion construct of myosin heavy chain promoter and GFP (MHC-GFP) to express GFP in muscles (See section 4b). To the best of our knowledge different Gal4 drivers should not significantly change wave patterns but then we can't say for sure. These details are now added to the manuscript.

6. In the protocol and abstract the authors mentioned that this technique could be used to examine other embryo behaviors like "rolling" and "hatching". However, the authors have not explained how to analyze and interpret embryos "rolling" or "hatching" in control or mutant embryos. They have also not provided any graphs or images to demonstrate this process.
Response: We have added a rolling video for a wild type embryo (Movie 2). POMT mutants do not show defects in embryo rolling, but our method can be useful to assess rolling in other mutants Similarly, our live imaging approach can be used for studying hatching or other embryo movements during late embryonic stages.

Responses to editorial comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: We corrected spelling and grammar issues.

2. Please provide an email address for each author.
Response: We have provided the email addresses of all authors (lines 13-15).

3. Please revise the Introduction to include the following: A clear statement of the overall goal of this method; The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application. Please focus on the general types of results acquired.
Response: We have included the information requested above in the introduction (lines 50-60).

4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u. Please abbreviate liters to L to avoid confusion.
Response: These changes have been made throughout the manuscript.

5. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.
Response: These changes have been made.

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Response: We have moved commercial information about the microscope, camera etc., to the table of materials. Our analysis part of the protocol however pertains to specific software "Image J" and is still included in the protocol itself.

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Response: We have removed personal pronouns throughout the protocol except for a few notes within the protocol.

8. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
Response: The tense of the protocol is now imperative wherever possible except for a few "notes". We have discussed the protocol in the discussion.

9. Section 4: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).
Response: Software steps are now explained in more detail and numerical values for certain ROIs have been provided (see edits in Section 4).

10. 1.1: Please specify the capacity of the beaker used. How many holes are made? What is the size of the needle?
Response: Information requested above is now included in 1.1.

11. 1.4: Approximately how many flies are needed?
Response: Information added in 1.4.

12. 3.1-3.5,4.1-4.14:Please write the text in the imperative tense to direct the reader to do something (e.g., "Do this," "Ensure that," etc.).
Response: The tense has been changed to imperative throughout the protocol.

13. Please combine some of the shorter Protocol steps so that individual steps contain

2-3 actions and maximum of 4 sentences per step.
Response: These recommended changes have been made throughout the protocol.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response: Done.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia**.**
Response: Done.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the substeps where the details are provided must be highlighted.
Response: Done.

17. Discussion: Please discuss critical steps within the protocol.
Response: Critical step moved to the protocol (see Note on step 6 of 4b).

18.Figures 1 and 2: Please change the time unit “sec” to “s”. Please combine all panels of one figure into a single image file. Please include a title for each figure in the figure legend.
Response: The recommended changes have been made.

19. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.
Response: Items are now sorted in alphabetical order in the revised table of materials.

20. References: Please do not abbreviate journal titles.
Response: Full journal titles are now provided.