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Imaging Intranuclear Actin Rods in Live Heat Stressed Drosophila Embryos

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TITLE:

Imaging Intranuclear Actin Rods in Live Heat Stressed *Drosophila* Embryos

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SUMMARY:

The goal of this protocol is to inject Rhodamine-conjugated globular actin into *Drosophila* embryos and image intranuclear actin rod assembly following heat stress.

ABSTRACT:

The purpose of this protocol is to visualize intranuclear actin rods that assemble in live *Drosophila melanogaster* embryos following heat stress. Actin rods are a hallmark of a conserved, inducible Actin Stress Response (ASR) that accompanies human pathologies, including neurodegenerative disease. Previously, we showed that the ASR contributes to morphogenesis failures and reduced viability of developing embryos. This protocol allows the continued study of mechanisms underlying actin rod assembly and the ASR in a model system that is highly amenable to imaging, genetics and biochemistry. Embryos are collected and mounted on a coverslip to prepare them for injection. Rhodamine-conjugated globular actin (G-actin^{Red}) is diluted and loaded into a microneedle. A single injection is made into the center of each embryo. After injection, embryos are incubated at elevated temperature and intranuclear actin rods are then visualized by confocal microscopy. Fluorescence recovery after photobleaching (FRAP) experiments may be performed on the actin rods; and other actin-rich structures in the cytoplasm can also be imaged. We find that G-actin^{Red} polymerizes like endogenous G-actin and does not, on its own, interfere with normal embryo development. One limitation of this protocol is that care must be taken during

injection to avoid serious injury to the embryo. However, with practice, injecting G-actin^{Red} into *Drosophila* embryos is a fast and reliable way to visualize actin rods and can easily be used with flies of any genotype or with the introduction of other cellular stresses, including hypoxia and oxidative stress.

INTRODUCTION:

This protocol describes how to inject G-actin^{Red} to visualize the assembly of intranuclear actin rods in heat-stressed embryos that are undergoing an inducible Actin Stress Response (ASR)[1]. We developed this protocol to aid studies of the ASR, which in embryos leads to disrupted morphogenesis and reduced viability, and in adult human cell types is associated with pathologies including renal failure[2], muscle myopathies[3], and Alzheimer's and Huntington's Disease[4–8]. This ASR is induced by numerous cellular stresses, including heat shock[9–11], oxidative stress[4,6], reduced ATP synthesis[12], and abnormal Huntingtin or β-amyloid oligomerization[4–7,9,13–16]. A hallmark of the ASR is the assembly of aberrant actin rods in either the cytoplasm or nucleus of affected cells, which is driven by stress-induced hyperactivation of an actin interacting protein, Cofilin[1,5,6,10]. Unfortunately, key knowledge gaps remain regarding the ASR. For example, the function of the actin rods is not known. We do not understand why rods form in the cytoplasm of some cell types, but the nucleus of others nor is it clear whether the ASR is protective or maladaptive for cells or embryos undergoing stress. Finally, we still do not know the detailed mechanisms underlying Cofilin hyperactivation or actin rod assembly. Thus, this protocol provides a rapid and versatile assay to probe the ASR by visualizing actin rod formation and dynamics in the highly tractable experimental system of the living fruit fly embryo.

The protocol to microinject G-actin^{Red} into living *Drosophila* embryos was initially developed to study the dynamics of normal cytoplasmic actin structures[17] during tissue building events. In those studies, we found that G-actin^{Red} injection did not adversely affect early developmental processes in the embryo, including cytokinesis or gastrulation[17,18]. We then modified the protocol, adapting the embryo handling and G-actin^{Red} injection to allow imaging of actin rods in heat stressed embryos undergoing the ASR[1]. Other methods besides G-actin^{Red} injection can be used to visualize actin in embryos. These methods rely on expressing fluorescent proteins (FPs) tagged to actin or to domains of actin binding proteins, such as Utrophin-mCherry, Lifeact, F-tractin-GFP, and Moesin-GFP (reviewed in[19]). However, using these FP probes requires caution because they can stabilize or disrupt some actin structures, do not equally label all actin structures[20], and in the case of actin-GFP, are highly overexpressed – problematic for the analysis of rod assembly which is not only stress dependent but also actin concentration dependent[1]. Thus, G-actin^{Red} is the preferred probe for rod studies in fly embryos, and the large size of the embryo allows its easy injection.

The workflow of this protocol is similar to other well-established microinjection techniques that have been used for injecting proteins, nucleic acids, drugs, and fluorescent indicators into *Drosophila* embryos[21–27]. However, following the microinjection of G-actin^{Red} here, embryos are exposed to mild heat stress to induce the ASR and intranuclear actin rod assembly. For labs with access to flies and an injection rig, this method should be readily implementable and adaptable for specific lines of study in regard to the ASR, including its induction by different stresses or

modulation in distinct genetic backgrounds.

PROTOCOL:

1. Prepare embryo collection cups and apple juice agar plates

1.1. Five days prior to the injection experiment, construct[28] or procure at least two small, embryo collection cups. Make fresh 60 mm apple juice agar plates to be used with small collection cups[28]. Store plates in plastic boxes covered with damp paper towels at 4 °C.

NOTE: Small embryo collection cups, populated with fly numbers as described in step 1.3, will provide sufficient embryo numbers per experiment, while also ensuring that embryo handling and injection can be done in a short enough time to allow imaging of early developmental stages.

1.2. Warm apple juice plates to 18 °C and add a dab of yeast paste to the center of the plate. Yeast paste is a simple paste of active yeast and distilled water.

1.3. To promote the most generous egg laying, set up collection cups with flies 2 days prior to the experiment. Add at least 100 females and 50 male flies to the collection cups, and top with a prepared apple juice plate (**Figure 1, step 1**). On the days leading up to the injection experiment change the apple juice plates at least twice each day, once in the morning and once in the evening.

NOTE: The best injection and imaging results are obtained when embryo collection cups are kept at 18 °C with a 12 h light on/light off cycle.

2. Prepare a working stock solution of G-actin^{Red} for microinjection

NOTE: This preparation only makes 2 µL of a 5 mg/mL working stock of G-actin^{Red}, so if users are unaccustomed to the microinjection technique, it is advantageous to skip to step 3 and practice the microinjections with a neutral pH buffer to conserve precious working stock. The 10 µg stock of G-actin^{Red} from the vendor can be stored in its original packaging in a 16 oz screw top jar with ~500 g of desiccant at 4 °C for up to 6 months.

2.1. Prepare a G-buffer stock solution in advance: 5 mM Tris-HCl, 0.2 mM $CaCl_2$, pH 8.0. Filter and store at room temperature.

2.2. On the day of injections, prepare 1 mL of a G-buffer working solution in a fresh snap cap microcentrifuge tube on ice using the G-buffer stock from step 2.1, supplemented with the following at the indicated final concentrations: 1 mM dithiothreitol (DTT) and 0.2 mM ATP, pH 8.0.

NOTE: The 1 mL volume of G-buffer working solution is more than what is needed for an experiment but simplifies the preparation. Excess can be discarded after the experiment or users

can scale down according to their preference.

2.3.1. Keeping the 10 µg G-actin^{Red} stock on ice, first add 1 µL of filtered, distilled water to the top of the pink droplet of G-actin^{Red} inside the tube.

2.3.2. Next, add 1 µL of the cold, freshly prepared G-buffer working solution from step 2.2. Pipet up and down ~20 times to mix well with the pipette volume set to 1 µL. The G-actin^{Red} stock will now be at the final dilution of 5 mg/mL.

2.4. Incubate the prepared G-actin^{Red} for 30 min on ice undisturbed.

2.5. Centrifuge the prepared G-actin^{Red} at 16,000 *x g* for 20 min at 4 °C in a microcentrifuge to remove any precipitate.

2.6. Carefully pipet 1.5 µL of the supernatant into a fresh snap cap microcentrifuge tube on ice, avoiding the dark pink pellet.

2.7. Store the prepared G-actin^{Red} supernatant on ice for up to 6 h until ready to load into a microneedle.

NOTE: Microneedles can be pulled from capillary tubes in advance on a micropipette puller, then stored at room temperature on a strip of modeling clay in a 100 x 20 mm Petri dish. Suggested parameters for microneedle pulling can be found in the Pipette Cookbook[29].

3. Collect embryos and mount for injection

3.1. Allow flies to lay embryos for 30 min on apple juice plates with yeast paste at 18 °C.

3.2. While flies are laying, pre-warm an apple juice agar plate **without yeast paste** to room temperature, cut out a 4 cm x 1 cm rectangular wedge of apple juice agar with a razor blade, and place on a 25 mm x 75 mm glass slide.

3.3.1. Harvest plate from the 30 minute collection and dechorionate embryos by pouring fresh bleach, diluted 1:1 with distilled water, onto the plate and swirling the plate for 1 min, as described in[28] (**Figure 1, step 2**).

NOTE: Different brands of bleach are sold at different concentrations. The bleach used here is 6% sodium hypochlorite from the bottle and is diluted to a final concentration of 3% sodium hypochlorite. Other bleach brands at slightly lower concentrations will work equally well.

3.3.2. Pour the bleach and dechorionated embryos into a collection basket (a 70 µm cell strainer) and rinse the plate twice with distilled water from a squirt bottle, adding these washes to the collection basket.

3.3.3. Vigorously rinse the dechorionated embryos in the collection basket with distilled water until no yeast clumps are visible and the basket leaves no pink marks from excess bleach when blotted on a paper towel.

3.4. Using a paintbrush with bristles dampened with distilled water, transfer dechorionated and washed embryos from the collection basket onto the prepared apple juice agar wedge on the glass slide.

3.5. Use a pair of fine tip tweezers or a dissecting needle to arrange ten embryos in a straight line along the long axis of the rectangular agar wedge (**Figure 1, step 3**). Arrange the embryos head-to-tail, such that their anterior pole is facing to the right and dorsal side is facing the researcher (**Figure 1, step 3, magnified**).

3.6. Cut off 0.5 cm of the end of a P200 pipette tip with a razor blade and dip into the "embryo glue" (described in[28]). Generously coat a region 5 mm in width (**Figure 1, step 4**) along the long edge of a 24 mm x 50 mm rectangular coverslip, and let dry, glue side up. Drying will take ~30 s and is complete once the entire glue-coated region appears matte rather than wet or shiny.

NOTE: Prepare "embryo glue" at least 48 h in advance. Add n-Heptane to strips of double-sided tape in a scintillation vial as described in[28].

3.7. Once the "embryo glue" has dried, gently place the coverslip glue side down on top of the row of aligned embryos on the agar, leaving 2-3 mm of space between the edge of the coverslip and the row of embryos.

NOTE: Sticking the embryos too close to the edge of the coverslip may lead to the embryos drying out too much during the course of the experiment.

3.8. Flip the coverslip over so that the embryos are now facing up. They should be stuck in a line along one long edge of the coverslip, and their ventral region facing the closest edge of the coverslip (**Figure 1, step 4, magnified**).

3.9. Desiccate the embryos by placing the coverslip with embryos gently on top of 150 g of fresh blue desiccant stored in a 16 oz screw top jar. Tightly screw on the lid and incubate for 8-10 min (**Figure 1, step 5**).

3.10. After desiccation, remove the coverslip from the desiccant jar and tape each short side of the coverslip to a microscope slide, **embryo side up**, with two 4 cm^2 pieces of double-sided tape so that the embryo coverslip will fit onto the injection stage (**Figure 1, step 6**).

3.11. Add 2-3 drops of Halocarbon 27 oil with a Pasteur pipette to cover the aligned embryos and protect them from further dehydration (**Figure 1, step 6**).

4. Inject and heat stress embryos to promote actin rod formation

NOTE: All injections are done in a temperature-controlled room at 18 °C.

4.1. Prepare humid incubation chambers from a glass Petri dish at least 100 mm x 20 mm in size and line the chamber with twists of lab tissue wipers dampened with distilled water (**Figure 1, step 8**). Pre-warm the incubation chambers at 32 °C or the desired incubation temperature prior to injecting embryos.

4.2. Open the airflow valve for the microinjector and turn on the microinjector (compressed air or house air with a pressure of at least 90 psi is suitable).

4.3. While embryos are desiccating, backload the previously prepared G-actin^{Red} supernatant into the microneedle using a micro loader tip. Set the pipette to draw up 1-1.5 µL.

NOTE: Because of the viscosity of the actin, loading volumes may not be accurate and there may be enough actin left to load at least one to two more microneedles. Up to 60 embryos can be injected per loaded microneedle if the microneedle is calibrated properly and does not become clogged during the course of the experiment.

4.4. Attach the microneedle to the needle holder and tighten the screw. Connect the air tube to the microinjector and ensure that the backflow pressure on the microneedle equilibrates to 30 hPa.

4.5. Calibrate the microinjector settings to expel a 100 µm diameter bubble of G-actin^{Red} (~500 pL) on a slide micrometer. Rotate the pressure knob (500-1500 hPa) and injection pulse time knob (0.1-0.5 s) on the microinjector to get the right bubble size. Adjust these settings each time a new microneedle is loaded to account for variability in actin viscosity and microneedle tip size.

NOTE: The prepared G-actin^{Red} is viscous and there may be air in the tip of the microneedle that should be expelled before injecting embryos. If the G-actin^{Red} does not readily expel from the tip of the microneedle, gently break the microneedle tip against the edge of the slide micrometer.

4.6. Place the slide with mounted embryos onto the microscope stage.

NOTE: Every injection set up will be different, so researchers will have to adjust their injection method accordingly. Here the embryos are moved with respect to a stationary microneedle, injecting each embryo by running the embryo into the microneedle.

4.7. Adjust the micromanipulator stage and focus of the 10x objective on the light microscope so that the embryos are visible. The embryos are in the correct focal plane when the outlines of the vitelline membrane are sharpest and the embryo appears largest. Choose embryos to inject that are in the correct developmental stage, so that by the time the post-injection incubation is complete, most of the clutch reaches the desired developmental stage (e.g., inject embryos at Bownes' stage 2-3[30] in order to observe rods at cellularization after heat stress at 32 °C).

4.8. Use the microneedle controls to bring the needle into the same focal plane as the embryos.

NOTE: If the microneedle catches on the coverslip while moving the stage or microneedle, then the microneedle is too close to the slide and is not in the correct focal plane. The microneedle should be parallel to the coverslip, and not at a significant angle (**Figure 1, step 7**).

4.9. Insert the microneedle into the embryo so that it hits the embryo in the middle of its ventral region, at the embryo "equator". Trigger injection with the foot pedal or "inject" button when the microneedle tip is visible inside the middle of the embryo (**Figure 1, step 7**).

4.10. Inject the G-actin^{Red} once and slowly remove the microneedle. Move the stage and repeat for each embryo of the appropriate developmental stage.

NOTE: Expansion of the embryo is normal as the G-actin^{Red} is injected and a bit of cytoplasm may leak out of the embryo.

4.11. After all the embryos have been injected, place the slide with the embryos in the prepared humid incubation chamber and close the lid (**Figure 1, step 8**). If trying to obtain embryos that reach cellularization, heat stress the embryos at 32 °C for 60-75 min in the humid incubation chamber.

NOTE: Incubation times are noted that allow visualization of rods in cellularizing heat stressed embryos. The incubation time will be longer for non heat stress control embryos because development will be slower at a lower temperature[31]. These control embryos can be incubated in humid chambers at temperatures such as 18 °C or 25 °C, depending on design of the specific experiment and the question to be asked. The minimum incubation time necessary for the G-actin^{Red} to diffuse throughout the embryo is 30 min.

5. Image actin rods in heat stressed embryos by confocal microscopy

5.1. While the embryos are being heat stressed, turn on the confocal microscope and select the 561 nm laser channel.

5.2. Move the objective lens (25x, 40x or 63x recommended) to the working position.

5.3. If imaging heat stressed embryos, then set the heated stage incubator to achieve an internal temperature of 32 °C. A point-and-shoot or infrared thermometer can be used to check the temperature at or near the objective.

5.4. Remove the slide with injected embryos from the humid incubation chamber after incubation is complete (**Figure 1, step 9**).

5.5. Working quickly, gently pry off the double-sided tape pieces that were used to adhere the

coverslip with mounted embryos to the slide (**Figure 1, step 9**).

5.6. Stick two 2.5 cm long pieces of double-sided tape together and cut the tape in half lengthwise to make two strips, 2.5 x 0.5 cm long (**Figure 1, step 10**).

CAUTION: Be gentle during these steps as coverslips can easily shatter if too much force is applied.

5.7. Stick two-thirds of the length of each tape strip onto the first coverslip, flanking each side of the embryos in Halocarbon 27 oil (**Figure 1, step 10, orange**), leaving one-third of the tape strips hanging off the edge of the first coverslip where the embryos are stuck. Use gloved hands and be careful not to touch the embryos during this step.

5.8. Gently place a second rectangular coverslip on top of the tape strips to sandwich the embryos between the coverslips (**Figure 1, step 10, blue**). Align the 25 mm edges but keep the 50 mm edges offset from one another by 1 cm in width.

NOTE: This second coverslip's full surface will become the new imaging surface that will face the objective lens, **so take care not to get fingerprints or Halocarbon 27 oil on this second coverslip surface**. The offset is necessary so that extra Halocarbon 27 oil can be added to evenly immerse the embryos. If needed, add Halocarbon 27 oil at the seam where the two coverslips meet at the top of the sandwich and it will coat the embryos by capillary action (see dashed lines in **Figure 1, step 10**).

5.9. Gently tap down on the areas of the coverslip that are directly on top of the tape strips with the blunt side of a razorblade to get the coverslip to adhere to the tape.

5.10. Flip the coverslip sandwich over and place on a lab tissue wiper to keep the imaging surface clean and carry to the confocal microscope (**Figure 1, step 11**). Ensure that the tape is completely stuck to both coverslips before imaging.

5.11. Confirm that the heated stage is at temperature and if using an inverted microscope, add immersion liquid onto the selected objective lens.

5.12. Place the coverslip sandwich onto the stage carefully to ensure that the new imaging surface (2nd coverslip) is the one touching the immersion liquid (**Figure 1, step 11**).

NOTE: If needed, adhere the coverslip sandwich to the stage with two small pieces of double-sided tape to prevent unnecessary movement during imaging if the coverslips do not fit well in the heated stage.

5.13. Focus on an embryo that is in cellularization (Bownes stage 4a[30]) or desired developmental stage using either transmitted light or fluorescence.

5.14. Once an embryo has been brought into focus, switch to the laser acquisition mode on the confocal microscope and adjust laser power and gain, frame size, tiling, and projection settings as desired.

5.15. Take surface-view images through the focal planes of the embryo's nuclei to find intranuclear actin rods. Rods should appear in multiple orientations as bright streaks or dots inside the comparatively dark nuclei (**Figure 2A, 2C**).

6. Alternative imaging experiments

6.1. Perform FRAP to investigate actin turnover along the length of intranuclear actin rods or in cytoplasmic actin structures, such as the tips of the plasma membrane furrows during cellularization.

6.2. In the imaging software, choose a rectangular region around furrow tips or actin rods in which to acquire the experiment.

6.3. Choose a small square region of the center of an actin rod within the rectangular acquisition region to bleach. Ensure that the tips of the actin rod are visible and do not get bleached during the course of the experiment to allow for accurate tracking of the rod inside the nucleus.

6.4. Set the bleach laser to iterate 50x and set the bleach laser power to maximum.

6.5. Choose a time course to acquire images every second for a total up to 120 s at maximum pixel dwell speed and set the laser to bleach after the first two seconds of image acquisition.

6.6. Quantify the data to determine the half-time of fluorescence recovery[1].

REPRESENTATIVE RESULTS:

A schematic workflow of embryo handling is depicted in **Figure 1,** and a timetable for a typical experiment is presented in **Table 1**. An estimate for a good experimental outcome is that for every 10 embryos injected, at least half of the embryos viewed will be at the correct developmental stage, undamaged, and exhibit a robust ASR with heat stress at 32 °C. This ASR will be evidenced by the assembly of intranuclear actin rods as shown in the representative surface view image of an embryo in **Figure 2A** (right panel). Actin rods will appear in several orientations (parallel or perpendicular to the imaging plane) inside the nuclei and can be imaged through several focal planes. In comparison, control embryos incubated at 18 °C will not display actin rods (**Figure 2A**, left panel). The percent nuclei containing rods can be quantified, as demonstrated in **Figure 2B**. In addition, FRAP experiments may be performed on rods (**Figure 2C**). A suggested quantification method for FRAP data is referenced in[1] and an example of a fluorescence recovery plot for a bleached versus unbleached region of an actin rod is shown in **Figure 2D**.

If an embryo is severely damaged by injection or becomes too dry during the experiment, mitotic

asynchrony might be observed and cellularization will be disrupted. Sometimes, rods may not be visible because of a failure to get enough actin injected into the embryo. If this happens, ensure that the amount of G-actin^{Red} injected is 500 pL (measured with a micrometer in step 4.5) and confirm that this amount remains consistent between embryos by doing a test injection into the surrounding oil to check the size of the G-actin^{Red} bubble in between each embryo microinjection. Additionally, to ensure rod visualization, work quickly to add the coverslip and move the embryos to the heated microscope stage once they are taken from the humid chamber in step 5.4, as rod assembly is reversible[1] and rods can disassemble if the embryos are kept at a temperature less than 32°C for more than 30 min.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic overview of embryo handling during the experiment.

(**1**) Adult flies in embryo collection cups lay embryos on apple juice agar plates. (**2**) Embryos are dechorionated with 1:1 bleach:distilled water, poured into a collection basket, and thoroughly washed with distilled water to remove bleach and debris. (**3**) Embryos are transferred with a paintbrush to a rectangular apple juice agar wedge on a slide and arranged on their sides, head-to-tail, with dorsal region facing the edge of the agar. (**4**) A 5 x 50 mm region of a glass coverslip (orange) is coated with "embryo glue" and pressed down gently onto the row of embryos arranged on the agar to adhere them to the coverslip. (**5**) The coverslip with embryos is inverted so that embryos face up. The embryos are desiccated in a screw top jar. (**6**) Immediately after desiccation, the coverslip is taped to a slide, embryos facing up, and embryos are covered with Halocarbon 27 oil. (**7**) A microneedle previously loaded with prepared G-actin^{Red} is used to make a single injection into the center of the ventral region of each embryo, with needle positioned parallel to the coverslip. (**8**) After injection, embryos are incubated inside a Petri dish humidified with damp lab tissue wipers at the control temperature (18 °C) or with heat stress (32 °C). (**9**) After incubation, the coverslip with the embryos on it is removed from the slide. (**10**) Two pieces of double-sided tape are layered on top of each other, sliced in half lengthwise, and placed on either side of the oil surrounding the embryos on the first coverslip. A second coverslip (blue) is placed on top of the first to create a new imaging surface, offset so that it leaves a gap for more oil to be added to cover the embryos as necessary. (**11**) If imaging on an inverted confocal microscope, the coverslip sandwich is inverted so that the second coverslip faces the objective. Imaging is done in an incubated chamber, and actin rods are visualized over several focal planes of each embryos' nuclei.

Figure 2: Representative results of actin rods in heat-stressed embryos.

(**A**) Actin rods are not seen in an embryo that was incubated at the control temperature of 18 °C (left panel), but are seen in the nuclei of an embryo that was heat-stressed at 32 °C (right panel). (**B**) Quantification of the percentage of nuclei with actin rods from a representative experiment. Each dot represents one embryo where rods and nuclei were counted in the entire imaged region (n = 22 embryos at 18 °C; n = 23 embryos at 32 °C; error bars show standard deviation). A Student's t-test, with unequal variance assumed, was used to calculate the p-value. (**C**) A representative time series shows FRAP on an actin rod. The portion of the rod that was bleached is indicated by a white arrowhead. Pre-bleach is 2 s prior to the bleach step. Time = 0 s is the bleach step, and fluorescence recovery was tracked until 60 s post bleach. (**D**) A plot shows

recovery dynamics for actin fluorescence in a bleached region of a rod, compared to an unbleached region in the same rod. Rods are remarkably stable and actin within them does not turnover. Thus, no recovery is seen.

Table 1: Experimental workflow with suggested timetable.
This timetable summarizes the expected time it will take to complete each step of the protocol.

Table 2: Troubleshooting suggestions.
This table provides suggestions for troubleshooting to aid the successful completion of the protocol.

DISCUSSION:

The significance of this method is that it utilizes the well-established protocol of microinjection in *Drosophila* embryos[21–27] to enable new research regarding the ASR and accompanying actin rod assembly. A major advantage of injecting G-actin^{Red} into live embryos is that the ASR can be studied under a variety of contexts. For future studies, these contexts may include injecting embryos of other genotypes as part of a mutant screen or exposing injected embryos to different stress conditions, such as oxidative stress[4,32]. Although not described in detail here, this injection technique can also be modified to inject nucleic acids, other proteins, drugs and indicator dyes (for examples, see[21–24,32]) to study the ASR. Thus, this method presents a number of approaches for identifying the range of stresses that induce ASR, further characterizing cellular responses during ASR (e.g. changes in mitochondrial activity), and uncovering new molecules and mechanisms underlying intranuclear actin rod assembly.

Some critical steps of the protocol include the following: In step 3.9, embryos must be properly desiccated to ensure successful injection and best embryo health. Desiccation time will depend on the ambient temperature and humidity of the laboratory, so it is recommended to practice the mounting, desiccation, and injections with a neutral pH buffer first to establish this parameter for handling the embryos. In step 4.5, the microneedle and injection settings must be fine-tuned to allow injection of enough G-actin^{Red} into embryos. If too little G-actin^{Red} is injected into the embryo, actin rods may not be easily visualized, since the formation of actin rods is dependent on the concentration of free actin[1]. Additionally, it will be difficult to get consistent results from FRAP experiments if there is not enough G-actin^{Red} injected, since the fluorescence intensity will not be high enough to overcome background fluorescence. Therefore, it is important to calibrate the bubble size each time a new needle is loaded and used. G-actin^{Red} is viscous and tends to clog inside the microneedle. Sometimes, this can lead to injecting variable amounts of G-actin into the embryos. If the microneedle is clogged and clearing the microneedle with high pressure fails, it may be necessary to attempt breaking the tip of the microneedle further or even loading a new microneedle and injecting a fresh set of embryos. Finally, in step 4.11, embryos must be incubated at elevated temperature and for sufficient time for the ASR to be induced and rods to form[1]. The temperatures of all incubators should be constantly monitored, time to transfer embryos from incubator to incubator must be limited, and a timer should be used for all incubations. Other possible problems are listed in **Table 2** with accompanying troubleshooting tips.

One major limitation of this protocol is that exceptional care must be taken to preserve the health of the embryos during injections, incubations and imaging. The protocol has been designed to maximize embryo health, and with significant practice, a researcher can complete all steps of the protocol with embryo development progressing at the rates expected per temperature[31]. A second limitation of the protocol is the necessity for a microinjection rig, which can be fairly expensive and is not common equipment for every fly lab. However, if an adjacent lab is equipped to inject other embryos (e.g., *Xenopus*, Zebrafish, and *Caenorhabditis elegans*) or adherent cells, the injection rig used is likely suitable for *Drosophila* injections. In that case, only the shape of the needle need be adapted for *Drosophila* embryos according to the guidelines of the Pipette Cookbook[29]. Alternatively, there are some less expensive micoinjector options on the market (e.g., analog microinjectors), which can significantly reduce the cost of assembling an injection rig.

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DISCLOSURES:

No conflicts of interests declared.

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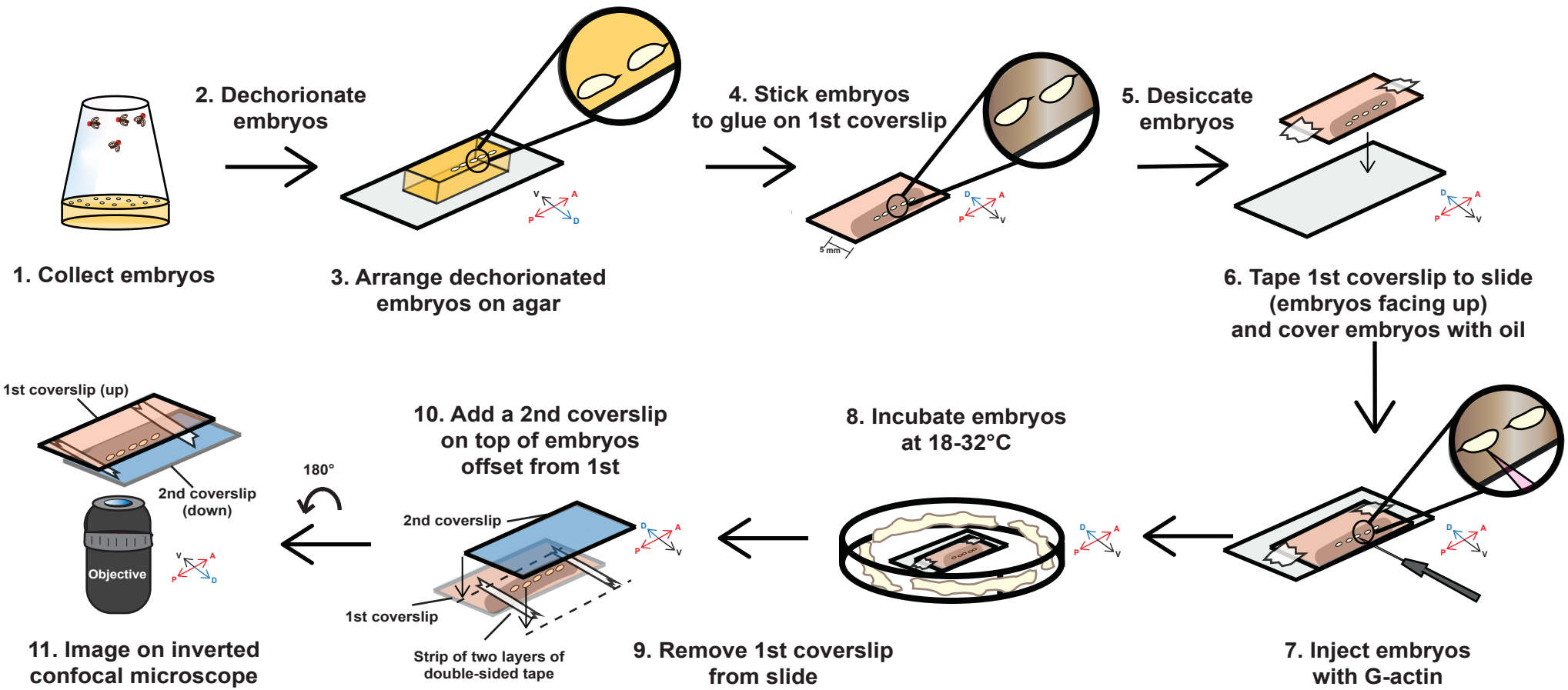
Figure 1



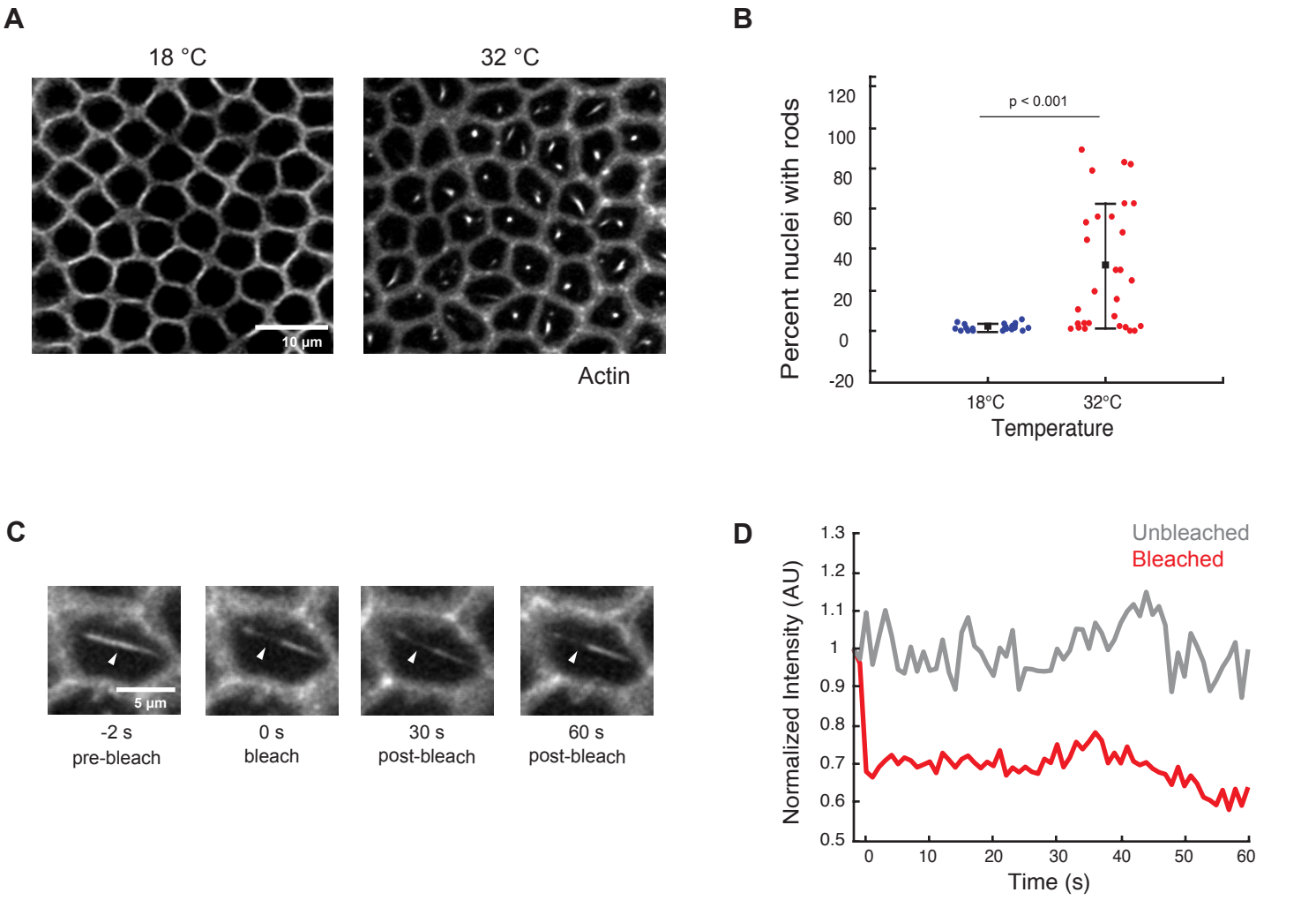


Table 1. Experimental workflow and suggested timetable

Order	Step	Required time for each step
1	1.1	5 days in advance
2	1.2-1.3	2 days in advance
3	2.7 Note	1 day in advance
4	2.1-2.7	1 h
5	3.1	30 min
6	3.2-3.11	15-30 min
7	4.1	30 min in advance
8	4.2-4.4	1 min
9	4.5-4.10	10-20 min
10	4.11	30 min-1+ h
11	5.1-5.3	1 h in advance
12	5.4-5.10	5 min
13	5.11-6.6	15 min-1+ h

Description
Make embryo collection cages. Pour apple juice agar plates.
Set up collection cages with adult male and female flies.
Pull capillary tubes to make microneedles.
Prepare G-actin.
Allow flies to lay eggs.
Collect, mount, desiccate embryos. Cover embryos with oil.
Prepare humid incubation chambers.
Load microneedle.
Calibrate G-actin bubble size and inject embryos.
Incubate/heat stress embryos.
Turn on microscope and incubation stage.
Sandwich embryos between coverslips for imaging.
Image intranuclear actin rods in embryos.

Table 2. Troubleshooting suggestions.

Potential problem
Flies do not lay enough embryos.
No G-actin is expelled from the microneedle.
Difficult to calibrate a small enough bubble size.
Embryos release from the glue on the coverslip during injecting.
Embryos dry out during temperature incubation.
Oil does not completely cover the embryos in between the first and second cov
Intranuclear actin rods are not visible in heat-stressed embryos.
Large bubbles of G-actin are visible around the injection site of embryos.

Suggestions

Set up the cup at least 5 days in advance (refer to steps 1.1-1.3). Change plates 3x per day leading up to the experiment to encourage egg layi

Increase pressure and time settings on microinjector. Break the microneedle tip further (refer to step 4.5 Note). Since major clogs may not cle

Adjust the pressure and time settings (refer to step 4.5). Since the microneedle tip opening might be too large, load a new needle.

Adjust “embryo glue” consistency for future coverslips by adding more double-sided tape to the heptane solution (refer to step 3.6 Note).

Make sure that the slide is level in the incubation chamber (refer to step 4.11) and that the oil is not touching anything that might wick it awa

Add extra oil via capillary action to the small gap between the coverslips (refer to step 5.8 Note).

Inject a larger volume of G-actin (refer to step 4.5). Confirm that the temperature of both the post-injection incubation (refer to step 4.11) ar

Inject a smaller volume of G-actin (refer to step 4.5). Increase the embryo desiccation time to promote better retention of the injected actin i

Table of Materials

Click here to access/download;Table of Materials;Biel_ActinRods_JoVEMaterials_Table_Revised.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Adenosine triphosphate (ATP)	Millipore-Sigma	A23835G	Component of G buffer
Apple juice, Mott's, 64 fl oz	Mott's	014800000344	Component of apple juice plates
Bacto Agar	BD	214010	Component of apple juice plates
Bleach, PureBright Germicidal, 6.0% sodium hypochlorite	KIK International	059647210020	For dechorionating embryos
Calcium chloride	Millipore-Sigma	C1016500G	Component of G buffer
Cell strainer, 70 µm	Falcon	352350	For collecting dechorionated embryos
Confocal microscope, LSM 880 34-channel with Airyscan	Zeiss	0000001994956	For imaging intranuclear actin rods
Desiccant	Drierite	24001	For desiccating embryos
Dissecting microscope, Stemi 508 Stereoscope with 8:1 zoom	Zeiss	4350649000000	For arranging embryos on agar wedge
Dissecting needle, 5 in	Fisher Scientific	08965A	For arranging embryos on agar wedge
Dithiothreitol (DTT)	Fisher Scientific	BP1725	Component of G buffer
Double-sided Tape, Scotch Permanent, 0.5 in x 250 in	3M	021200010323	For making embryo glue
Embryo collection cage	Genessee Scientific	59100	For housing adult flies and collecting embryos
Fine tip tweezers, Dumont Tweezer, Style 5	Electron Microscopy Sciences	72701D	For arranging embryos on agar wedge
Glass capillaries, Borosillicate glass, thin 1 mm x 0.75 mm	World Precision Instruments, Inc.	TW1004	For microneedles
Halocarbon oil 27	Millipore-Sigma	H8773100ML	For hydration of embryos
Heated stage incubator	Zeiss	4118579020000, 4118609020000, 4118609010000	For confocal imaging
Lab Tissue Wipers, KimWipes	Kimberly-Clark	34155	Lab tissue wipers

Light microscope, Invertoskop 40C Inverted Phase contrast microscope, refurbished	Zeiss	Discontinued	Injection microscope
Methyl-4-hydroxybenzoate	Millipore-Sigma	H36471KG	Component of apple juice plates
Microinjector, FemtoJet4x	Eppendorf	5253000025	Microinjector
Micro loader tips, epT.I.P.S. 20 µL	Eppendorf	5242956003	For loading microneedles
Micromanipulator and injection stage with x,y,z dials for needle adjustment	Bernard Instruments, Inc (Houston, TX)	Custom	For performing microinjections
Micropipette puller, Model P-97, Flaming/Brown	Sutter Instruments	P97	For pulling capillary tubes to make microneed
Microscope cover glass 24x50-1.5	Fisher Scientific	12544E	For mounting embryos
Microscope slides, Lilac Colorfrost, Precleaned, 25 x 75 x 1mm	Fisher Scientific	22037081	For mounting embryos for injection
n-Heptane	Fisher Scientific	H3601	Component of embryo glue
Objective, 10x	Zeiss	Discontinued	10x objective for injection microscope
Objective, C-Apochromat 40x/1,2 W Korr. FCS	Zeiss	4217679971711	40x water objective for confocal
Objective, LD LCI Plan-Apochromat 25x/0.8 Imm Cor	Zeiss	4208529871000	25x mixed immersion objective for confocal
Objective, Plan-Apocrhomat 63x/1.40 Oil DIC f/ELYRA	Zeiss	4207829900799	63x oil objective for confocal
Paintbrush, Robert Simmons Expression E85 Pointed Round size 2	Daler-Rowney	038372016954	For transferring embryos
Paper towels, Kleenex C-fold paper towels, white	Kimberly-Clark	884266344845	For blotting cell strainer
Pasteur pipette, 5 3/4 in	Fisher Scientific	1367820A	For covering embryos with oil
Petri dish, glass, 100 x 20 mm	Corning	3160102	For humid incubation chamber
Petri dish, plastic, 60 x 15 mm	VWR	25384092	For apple juice plates

Pipette, Eppendorf Reference 0.5-10 µL	Eppendorf	2231000604	For loading the microneedle
Pipette tip, xTIP4 250 µL	Biotix	63300006	For adding embryo glue to coverslip
Razor blade	VWR	55411050	For cutting agar wedge, tape, pipette tips
Rhodamine-conjugated globular actin, human platelet (non-muscle; 4x10 µg)	Cytoskeleton, Inc.	APHR-A	G-actin^Red
Scintillation vial, 20 mL Glass borosillicate with polyethylene liner and urea caps	Fisher Scientific	033377	For making embryo glue
Screw top jar, 16 oz	Nalgene	000194414195	For storing desiccating embryos
Stage micrometer	Electron Microscopy Sciences	602104PG	For calibrating volume of G-actin injection
Sucrose	Millipore-Sigma	840971KG	Component of apple juice plates
Trizma base	Millipore-Sigma	T15031KG	Component of G buffer
Yeast, Lesaffre Yeast Corporation Yeast, Red Star Active Dry, 32 oz	Lesaffre Yeast Corporation	117929157002	Component of yeast paste

Point-by-point of changes made to Biel et al.,

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
   - *Done.*

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
   - *Done.*

3. Please define all abbreviations during the first-time use.
   - *Done.*

4. Please ensure that the Abstract is between 150-300 words.
   - *Done.*

5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Genesee Scientific, (Cytoskeleton, Inc.), Nalgene, Drierite, Sigma, Fisher Scientific, VWR, Kimberly-Clark, Electron Microscopy Sciences, Eppendorf, Kim Wipe, etc.
   - *Done.*

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.”
   - *Done to the best of our ability*.

7. The Protocol should contain only action items that direct the reader to do something.
   - *Done to the best of our ability*.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

- *Done*.

9. Please ensure you answer the "how" question, i.e., how is the step performed? Please include all the button clicks, Knob turns etc. wherever applicable.

- *Done*.

10. 4.5: How is this done?

- *We have edited 4.5 to include this information (lines 248-250). The text now reads:*

> "Rotate the pressure knob (500-1500 hPa) and injection pulse time knob (0.1-0.5 s) on the microinjector to get the right bubble size. Adjust these settings each time a new microneedle is loaded to account for variability in actin viscosity and microneedle tip size"

11. 5.3: How do you identify this visually?

- *We have edited 5.3 to include this information (lines 305-306). The text now reads:*

> "A point-and-shoot or infrared thermometer can be used to check the temperature at or near the objective."

12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. 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.

- *Done*.

13. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. 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.

- *We believe that this is a repeat of Editorial Comment 12.*

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

- *We have edited the Discussion to explicitly include the requested information regarding critical steps (lines 468-487); modifications (lines 462-463); troubleshooting (lines 486-487); limitations (lines 489-493 and 493-494); significance (lines 456-458); and future applications (lines 459-461).*

15. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the table in alphabetical order.

- *Done*.

Reviewers' comments:

Reviewer 1:

Major Concerns:

1. It is difficult to follow the introduction as Drosophila embryo and neural background are intermixed, it goes from one to the other and back again to the first.

- *We have edited the Introduction so that it is more focused and the logic is more linear (lines 56-94).*

2. The description in 4.5 of how the embryos are arranged is confusing. The head to tail terminology in the figure legend makes more sense.

- *We have edited 3.5 where we described the arrangement of the embryos on the agar wedge (lines 188-191). The text now reads:*

> "3.5. Use a pair of fine tip tweezers or a dissecting needle to arrange ten embryos in a straight line along the long axis of the rectangular agar wedge (**Figure 1, step 3**). Arrange the embryos head-to-tail, such that their anterior pole is facing to the right and dorsal side is facing the researcher (**Figure 1, step 3, magnified**)."

3. In the note on 4.11 - it is unclear what the control embryos are, the conditions they will be kept in, and how they differ from the experimental. This is a critical area of importance.

- *We have edited the Note for 4.11 to clarify that the controls are embryos that are not heat-stressed (lines 290-295). The text now reads:*

> "NOTE: Incubation times are noted that allow visualization of rods in cellularizing heat stressed embryos. The incubation time will be longer for non heat stress control embryos because development will be slower at a lower temperature[31]. These control embryos can be incubated in humid chambers at temperatures such as 18 °C or 25 °C, depending on design of the specific experiment and the question to be asked."

4. In relation to 5.10 and Fig. 1 step 11 - the images are confusing. It is hard to follow where the tape and the two coverslips are. Perhaps one coverslip can be colored to make it easier to follow? Also, the reason for the offset is not clear from the protocol.

- *We have edited Figure 1 by highlighting the 1st coverslip in orange and the 2nd coverslip in blue throughout the figure, and changed the opacity of the coverslips in Figure 1, steps 10 -11 to more clearly identify which coverslip is which. We have also added a Note to 5.8 to explain why the offset is needed (lines 325-334). The text now reads:*

> "5.8. Gently place a second rectangular coverslip on top of the tape strips to sandwich the embryos between the coverslips (**Figure 1, step 10, blue**). Align the 25 mm edges but keep the 50 mm edges offset from one another by 1 cm in width.
>
> NOTE: This second coverslip's full surface will become the new imaging surface that will face the objective lens, **so take care not to get fingerprints or oil on this second coverslip surface**. The offset is necessary so that extra oil can be added to evenly immerse the embryos. If needed, add oil at the seam where the two coverslips meet at the top of the sandwich and it will coat the embryos by capillary action (see dashed lines in **Figure 1, step 10**)."

Minor Concerns:

1. In 1.1, please indicate the size of collection cups needed and the rationale.

- *We have edited 1.1 to include the cup size and rationale for embryo collection (lines 99-105). The text now reads:*

> "1.1. Five days prior to the injection experiment, construct[28] or procure at least two small, embryo collection cups. Make fresh 60 mm apple juice agar plates to be used with small collection cups[28]. Store plates in plastic boxes covered with damp paper towels at 4 °C.
>
> NOTE: Small embryo collection cups, populated with fly numbers as described in 1.3, will provide sufficient embryo numbers per experiment, while also ensuring that embryo handling and injection can be done in a short enough time to allow imaging of early developmental stages."

2. In 1.2, please indicate why it is important to set up the embryo collections days before the experiment, and why the light/dark cycle and temperature matter. Also, not every reader will know what yeast paste is.

- *We have edited 1.2 to include a description of yeast paste, moved the rest of the text from 1.2 into an additional step 1.3, and included a rationale for the temperature and light/dark cycle (lines 107-117). The text now reads:*

"1.2. Warm apple juice plates to 18 °C and add a dab of yeast paste to the center of the plate. Yeast paste is a simple paste of active yeast and distilled water"

1.3. To promote the most generous egg laying, set up collection cups with flies two days prior to the experiment. Add at least 100 females and 50 male flies to the collection cups, and top with a prepared apple juice plate (**Figure 1, step 1**). On the days leading up to the injection experiment change the apple juice plates at least twice each day, once in the morning and once in the evening.

NOTE: The best injection and imaging results are obtained when embryo collection cups are kept at 18 °C with a 12 hr light on/light off cycle."

3. It is unclear why so much G-actin buffer is made, and in 2.3.2 it is confusing to call in 1ml of G-buffer, when you are using 1 ul.

- *We have edited 2.2 to clarify the excess buffer preparation and 2.3.2 to address the confusing language (lines 134-136 and 141, respectively). The text now reads:*

"2.2 NOTE: The 1 mL volume of G-buffer working solution is more than what is needed for an experiment but simplifies the preparation. Excess can be discarded after the experiment or users can scale down according to their preference."

"2.3.2. Next, add 1 µL of the cold, freshly prepared G-buffer working solution from 2.2."

4. 2.6 - why use a 1.5 ml microcentrifuge tube for the 1.5ul?

- *Since it does not matter what volume the tube is, we edited 2.6 to remove the 1.5mL designation (line 150). The text now reads:*

"2.6. Carefully pipet 1.5 µL of the supernatant into a fresh snap cap microcentrifuge tube on ice, avoiding the dark pink pellet."

5. In 3.3.1, it would be useful to reference the bleach concentration.

- *We added a Note to 3.3.1 to clarify the bleach concentration (lines 172-174). The text now reads:*

"NOTE: Different brands of bleach are sold at different concentrations. The bleach used here is 6% sodium hypochlorite from the bottle and is diluted to a final concentration of 3% sodium hypochlorite. Other bleach brands at slightly lower concentrations will work equally well."

6. In 3.3.3 it is unclear what the embryos are being rinsed with.
   - *We edited the text to clarify that the embryos are being rinsed with water (lines 180-181). The text now reads:*

> "3.3.3. Vigorously rinse the dechorionated embryos in the collection basket with distilled water until no yeast clumps are visible…"

7. In 3.4 it is unclear what the paint brush has been damped with.
   - *We edited the text to clarify that the brush is dampened with water (line 184). The text now reads:*

> "3.4. Using a paintbrush with bristles dampened with distilled water…"

8. In 3.7 - how long does the glue take to dry and how is dry assessed.
   - *We edited 3.6 to include this information (lines 195-196). The text now reads:*

> "3.6. …Drying will take ~30 sec and is complete once the entire glue-coated region appears matte rather than wet or shiny."

9. In 5.15, it seems worth referencing the figure with the images.
   - *We edited 5.15 to add a reference to Figure 2 (line 362). The text now reads:*

> "5.15. …Rods should appear in multiple orientations as bright streaks or dots inside the comparatively dark nuclei (**Figure 2A, 2C**)."

10. It is unclear why the imaging needs to be done on an inverted microscope.
    - *Imaging need not be done on an inverted microscope. We edited the legend of Figure 1 to clarify (lines 429-432). The text now reads:*

> "(**11**) If imaging on an inverted confocal microscope, the coverslip sandwich is inverted so that the second coverslip faces the objective."

Reviewer #2:

Major Concern:
[The] authors have already published this protocol in detail in the articles in Figard et al., 2019 and Figard et al., 2011. I don't see any reasoning why to publish twice the same protocols. Could the authors justify why to publish it again? I do not see additional information on the proposed protocol that could help the readers to understand the methodology better.

- *We have submitted this method because we were invited to do so by JoVE editor, Dr. Stephanie Weldon, following the publication of our Cell Reports paper*

in 2019 (Figard et al., 2019). We have provided far more detail in this method than what was included in the Cell Reports paper; and this level of detail is expected to allow a novice to do these experiments rather than someone who already has injection experience. This JoVE method will also be accompanied by a video protocol, which the Cell Reports publication lacked.

Our earlier JoVE publication (Figard & Sokac 2011) did not include methods for embryo injection. Where there was overlap (e.g. making embryo collection cups), we strived to refer to the 2011 paper and not repeat information here.

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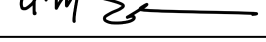
Signature:	
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