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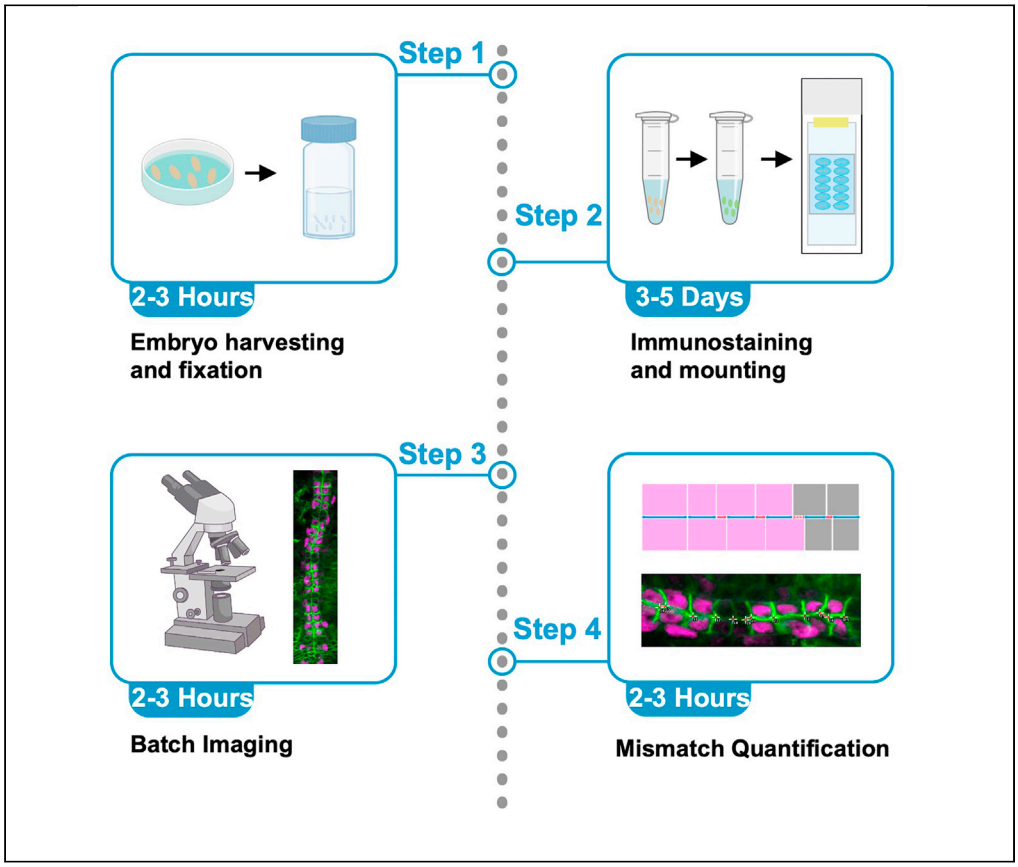
# Protocol for batch imaging and quantification of cellular mismatch during *Drosophila* embryonic heart formation




How individual cells form precise connections with partners in a complicated environment has been a longstanding question. However, most cell matching studies have used qualitative approaches, which may miss subtle but significant morphological changes. Here, we describe the use of embryonic *Drosophila* heart formation as a simplified system to quantitatively study cell matching. We provide a step-by-step protocol for large-scale embryo preparation and immunostaining and imaging details. We also describe steps for quantifying cellular mismatch from the batch images.

Shaobo Zhang, Timothy E. Saunders

shaobo@u.nus.edu (S.Z.)
dbsste@nus.edu.sg (T.E.S.)

## Highlights

Succinct staining protocol for large-scale embryo preparation for imaging

Clear protocol for imaging *Drosophila* embryo heart morphology

Tools for quantifying cellular mismatch from imaging data

Zhang & Saunders, STAR Protocols *2*, 100817 December 17, 2021 
https://doi.org/10.1016/j.xpro.2021.100817

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# Protocol for batch imaging and quantification of cellular mismatch during *Drosophila* embryonic heart formation

Shaobo Zhang[1,5,6,*] and Timothy E. Saunders[1,2,3,4,7,*]

[1]Mechanobiology Institute, National University of Singapore, Singapore 117411, Singapore

[2]Department of Biological Sciences, National University of Singapore, Singapore 117558, Singapore

[3]Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, Proteos, Singapore 138673, Singapore

[4]Division of Biomedical Sciences, Warwick Medical School, University of Warwick, Coventry CV4 7AL, UK

[5]Present address: Department of Ophthalmology, University of California San Francisco, San Francisco, CA, USA

[6]Technical contact

[7]Lead contact

*Correspondence: shaobo@u.nus.edu (S.Z.), dbsste@nus.edu.sg (T.E.S.)
https://doi.org/10.1016/j.xpro.2021.100817

## SUMMARY

**How individual cells form precise connections with partners in a complicated environment has been a longstanding question. However, most cell matching studies have used qualitative approaches, which may miss subtle but significant morphological changes. Here, we describe the use of embryonic *Drosophila* heart formation as a simplified system to quantitatively study cell matching. We provide a step-by-step protocol for large-scale embryo preparation and immunostaining and imaging details. We also describe steps for quantifying cellular mismatch from the batch images. For complete details on the use and execution of this protocol, please refer to Zhang et al. (2018 and 2020).**

## BEFORE YOU BEGIN

### Fly preparation

Timing: 3 days

Preparing flies for imaging is a standard protocol. Here, we outline the particular approach we use but there are many different approaches that enable imaging of embryos.

1. *Drosophila melanogaster* strains were maintained on standard fly food medium at 25°C, 60% humidity, and 14 h/10 h light-dark cycles.
2. 3–5 days old flies are caged in a fly cage (Flystuff, Cat #59–100) with the food plate (with yeast paste added) changed every day. Plates with laid embryos are collected during cage day 3–10 for further steps.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Rabbit anti-Tinman | Laboratory of Manfred Frasch (Azpiazu and Frasch, 1993) | N/A |
| Mouse anti-Seven up 2D3 | Developmental Studies Hybridoma Bank | Cat# SEVEN-UP 2D3; RRID: AB_261807 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Mouse anti-Spectrin | Developmental Studies Hybridoma Bank | Cat#3A9 (323 or M10-2); RRID: AB_528473 |
| Mouse anti-Fas3 | Developmental Studies Hybridoma Bank | Cat#7G10 anti-Fasciclin III; RRID: AB_528238 |
| Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 | Thermo Fisher Scientific | Cat#A21235; RRID:AB_2535804 |
| Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 | Thermo Fisher Scientific | Cat#A-11031; RRID:AB_144696 |
| Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific | Cat#A-11001; RRID:AB_2534069) |
| Chemicals, peptides, and recombinant proteins | | |
| Heptane | Macron Chemicals | Cat#MAL5177-16 |
| 20% Paraformaldehyde | Fisher Scientific | Cat#50980493 |
| Phosphate buffered saline | Sigma-Aldrich | Cat#P0261 |
| Triton | Sigma-Aldrich | Cat#9002-93-1 |
| AquaMount | Polysciences | Cat#18606-20 |
| Bovine Serum Albumin (BSA) | Fisher Scientific | Cat#SH3057402 |
| Experimental models: Organisms/strains | | |
| D. melanogaster: w[1118] | Bloomington Drosophila Stock Center | Cat#3605 |
| Software and algorithms | | |
| R | R Project | N/A |
| RStudio | RStudio | RRID:SCR_000432 |
| MATLAB | MathWorks | RRID:SCR_001622 |
| ImageJ | Fiji | RRID:SCR_002285 |
| Other | | |
| Nikon SpinningDisk_W1_LiveSR | Nikon | N/A |
| MatTek mounting dish | MatTek | Cat#P35G-0-10-C |

## MATERIALS AND EQUIPMENT

Imaging microscope: we have mainly used a Nikon SpinningDisk W1 microscope to achieve higher speed of imaging multiple embryos. A CFI Plan Apochromat 100×/1.45 NA oil immersion objective was used, and the imaging z-stack interval was set to be 0.3 μm. For each embryo ~30 z-slices were taken.

## STEP-BY-STEP METHOD DETAILS

### Embryo harvesting and fixation

Timing: 2 h

To achieve high-quality preparations, we applied the hand devitellinization method to fix embryo samples. This method does not expose samples to methanol, which can destroy cell membrane and other structures (Hobro and Smith, 2017; Hoetelmans et al., 2001) (especially here, the methanol fixation method leads to weak integrin immunostaining signal). In addition, some reagents such as fluorescently labeled phalloidin, will not work if the embryos have been exposed to methanol.

1. Harvest the overnight embryo plate of the fly cage in the morning. Add ~2 mL halocarbon oil into the plate. Rotate the plate to let the oil evenly cover the plate surface.
2. Identify the embryos from early stage 16 using completed closed dorsal closure and three segments of hindgut as an identification mark under a stereomicroscope. Select ~50–100 embryos using a micro dissecting probe through hand-picking to move them to a marked region on the plate.
3. Prepare a small sized filter paper (~1 $cm^2$), put it on the Petri dish lid, and add one or two drops of bleach solution (prepared fresh daily) to infiltrate the filter paper.

4. Transfer the embryos that have been harvested on the plate to the surface of the bleach-infiltrated filter paper.
5. Dechorionate these embryos in the bleach-infiltrated filter paper for ~1–2 min until their chorion (egg shell) detached.
6. Pick up the filter paper (with embryos attached on top) by tweezer and attach this to a paper towel to dry it.
7. Add three drops of water onto the plate, move the dried filter paper (with embryos attached on top) to one of the water- drops to wash it for 10 s, pick it up and dry it with paper towel. Repeat this three times.
8. Use a micro dissecting probe under a stereomicroscope to pick up the dechorionated and washed embryos (during this step, the embryos will simply attach to the probe) and move them into a 5-mL glass vial with ~1 mL of formaldehyde-saturated heptane.
9. Incubate the embryos in this solution on a nutator for 1 h at room temperature.
10. Using a glass pipette, transfer the embryos to a small petri dish lid. Allow the heptane to evaporate (~30 s). Ensure that the embryos stay in the neck of the pipette when transferring them. Embryos entering the body of the pipette tend to stick to the sides and are lost. Also, the embryos tend to sink quickly toward the tip of the pipette. It is best to allow them to sink and to transfer them in one to two drops onto the petri dish lid.
11. Immediately cover the embryos with PBS solution (~2 mL) after the heptane evaporates.
12. Using a stereomicroscope to monitor the procedure, use a 23-gauge needle mounted on a 3-mL syringe to carefully poke a small hole at the surface of the vitelline membrane at the anterior end of the embryo. The vitelline membrane will open up and detach from the embryo.
13. Gently "push" the fixed embryos out from the vitelline membrane through the hole by applying pressure from the opposite end.
14. The membrane will remain stuck to the bottom of the petri dish lid and the devitellinized embryo will move into the PBS solution.
15. Add 100 μL Triton X-100 into the PBS solution in the petri dish lid. Pipette solution for a few times to mix the solution and allow the embryos to float up in the solution.
16. Collect and transfer the devitellinized embryos to a 1.5 mL Eppendorf tube by using 200 ul pipette.

**Pause point:** Store embryos at 4°C or proceed immediately with staining of the embryos.

### Immunostaining, mounting, and batch imaging

**Timing: 3–5 days**

To achieve higher efficiency of imaging multiple embryos, we have improved the immunostaining steps, especially for embryo mounting. Here, we describe the process for imaging Fasciclin III (Fas3)/Spectrin and Tinman (Tin). Our lab is currently developing a Tin::GFP reporter line that will be made available once fully validated.

17. Rinse the fixed embryos with 1% BSA/PBT (PBS with 0.1% Triton added)
18. Wash the fixed embryos twice with 1% BSA/PBT. Each time keep the Eppendorf tube on the nutator for 20 min incubation.
19. Block the embryos were with 10% BSA/PBT for 1 h on the nutator.
20. Remove the blocking medium and add primary antibodies (anti-Fas3 or anti-Spectrin 1:300, and anti-Tin 1:1000) diluted in 5% BSA/PBT. Incubate the primary antibody on a nutator in 4°C for overnight.
21. Wash the sample four times with 1% BSA/PBT for four times. Each time, lay the Eppendorf tube on a gently moving nutator for 10 min.

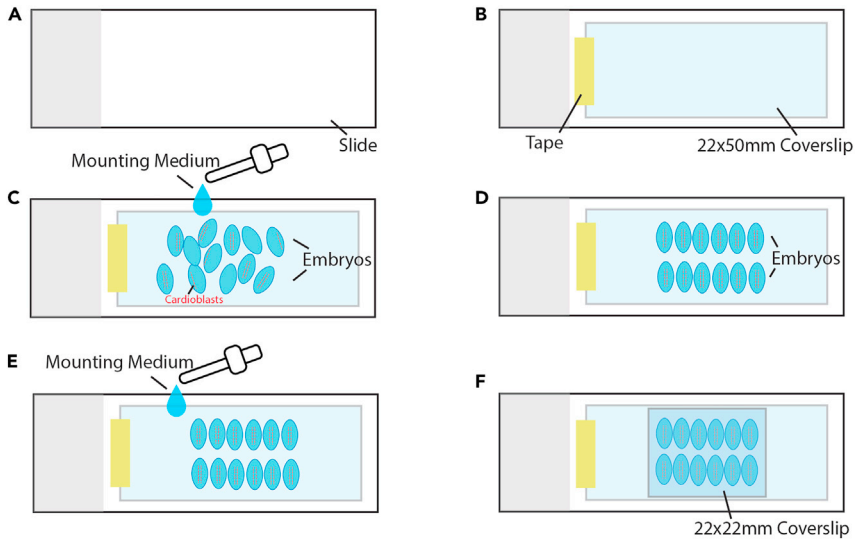


**Figure 1. Embryo mounting for batch imaging**

(A–F) Schematic illustrations of the double-side mounting steps of the *Drosophila* embryos for batch imaging of the heart cell alignment. See description in text for further details.

22. Add secondary antibody (Alexa Fluor 488 for anti-Fas3/anti-Spectrin and Alexa Fluor 568 for anti-Tinman) diluted in 5% BSA/PBT for 2 h at room temperature, lay the Eppendorf tube on a gently moving nutator.
23. Wash procedure: three rinses with PBT. For each time, lay the Eppendorf tube on a gently moving nutator for 10 min.
24. Mounting: to increase the efficiency of imaging batches of embryos at the right orientation, we have implanted the mounting procedures as listed below (Figure 1):
    a. Place a 22 × 50 mm coverslip on top of the microscope slide, and tape one end of the coverslip to the slide (Figures 1A and 1B).
    b. Transfer the embryos (~50–100) to the center of the coverslip and dry the samples by using filter paper (Figure 1C).
    c. Add one drop of Aqua-mount medium on top of the embryos, and gently spread the medium.
    d. Put the slide under stereomicroscope and move the embryos next to each other in a line by using the probe, orientate the embryos to make sure that either dorsal side or ventral side is facing up (Figure 1D).
    e. Add another drop of Aqua-mount medium at the side of the embryos and gently angle the 22 × 22 sized coverslip over the sample with one edge touching the 22*50 coverslip (Figures 1E and 1F).
    f. Gently lower the top coverslip over the sample and seal the top coverslip with nail polish. Keep the slide in dark for overnight to make sure the medium dry before imaging.
25. Imaging: As the embryos are aligned next to each other in a line, it will be easy to locate and image dozens of embryos in a batch. At the same time, as double-sided coverslips have been used, it will allow imaging the heart structure with higher efficiency.

*Note:* during the mounting step, it is difficult to make sure that dorsal/ventral sides of all the embryos are facing up, thus by flipping the double-mounting coverslip, it increases the imaging efficiency.

*Optional:* Sequential staining: In some cases, the two desired primary antibodies (e.g., anti-Fas3 and anti-Spectrin in Zhang et al., 2018) originate from the same host species (e.g.,

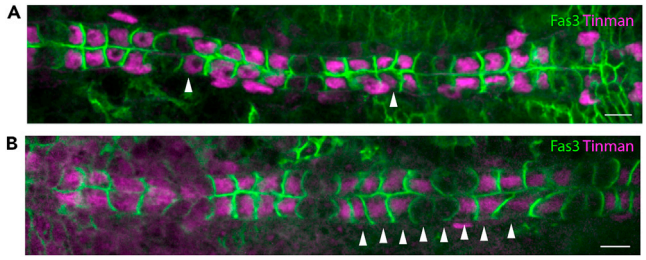


**Figure 2. *Drosophila* heart cell alignment**

(A and B) Examples of immunostaining images of the *Drosophila* embryonic heart cells by using the immunostaining methods. Magenta = Tinman. Green = Fasciclin III. Arrowheads point to the cells with cell contact mismatch. Anterior is to left. Scale bar, 10 μm.

mouse) and need to be stained within the same samples. Sequential staining can be applied. After finishing steps 1–8 for first primary antibody, apply post-fixation for 20 min with 4% formaldehyde/PBT, then, repeat from step 1 to step 8 for second primary antibody. However, please do note that for the sequential staining procedure, as the fluorescence pattern for second primary antibody will have some mixed signals from the first primary antibody, the more important primary antibody should be applied first. For example, in (Zhang et al., 2018), we principally wanted to examine the expression pattern of Fas3 under different genetic perturbations and observe the cardioblast matching morphology at the same time. The anti-Fas3 primary antibody was used first and anti-Spectrin antibody was applied in the sequential staining steps.

*Note:* Most primary antibodies can be reused a few times, i.e., store the primary after the first incubation at 4°C for further usage).

*Note:* when aligning the embryos next to each other in Aqua-mount mounting medium, keep this process short (normally within half an hour), as the Aqua-mount mounting medium will become sticky after some time, which makes it difficult to move embryos. If the mounting medium become sticky, try adding another drop.

## EXPECTED OUTCOMES

From a cage of healthy flies, this procedure can likely be repeated daily for 2–3 days. Around 100 embryos should be collectable for preparation and imaging. The protocol should be repeated at least once with different cages to ensure reproducibility. Successful implementation will provide sufficient image data to enable robust quantification. Clear image stacks of the embryonic *Drosophila* heart are needed for the following cell mismatch quantification (Figure 2).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Mismatch quantification

The *Drosophila* embryonic heart has 52 cells at each side and they arrange in a repeated 4-2 cell arrangement pattern (4 Tinman-positive CBs and 2 Svp-positive CBs) through segments A1 to A7 (Figure 3A). Here, segments A2-A5 of the embryos at stage 16 were selected for cardioblast mismatch quantification. Partner cells were assigned by their cell type marker (Tinman antibody staining) and their relative positions in the above-mentioned repeated 4-2 cell arrangement (Figure 3A). Contact mismatches between cells in the contralaterally opposite sides of the heart was measured based on their membrane contacts (Figure 3B), which were labeled through the above-mentioned procedures. The following steps are applied to perform the quantification analysis:

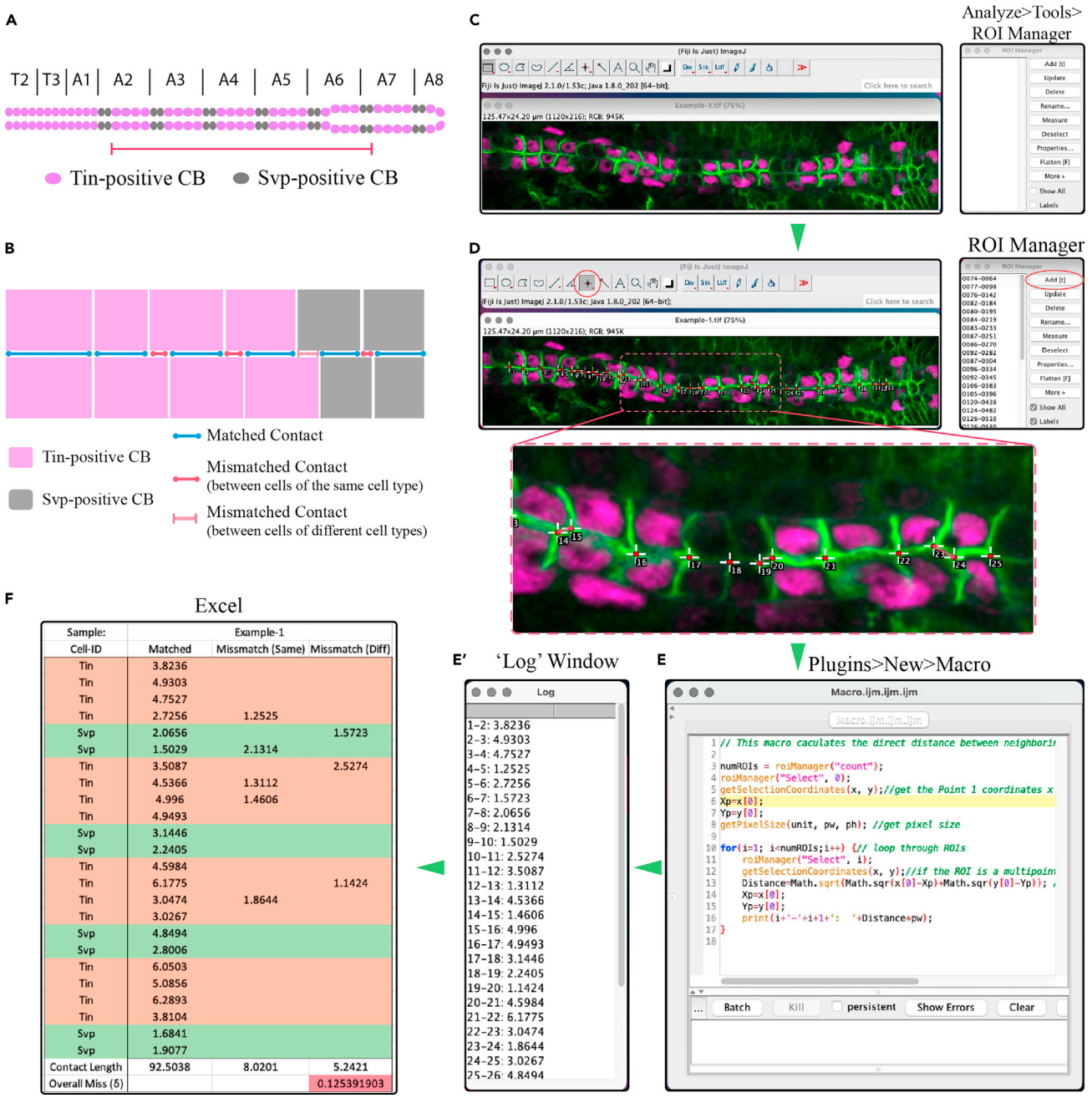


**Figure 3. Quantitative analysis of *Drosophila* heart cell contact mismatch**

(A) Schematic of the *Drosophila* heart cell arrangement pattern at Stage 16.

(B) Schematic of the definition of heart cell contact mismatch.

(C) Open image stack and the ROI manager in Fiji.

(D) Label out the crossed junction points of cell lateral boundaries and the middle contact line between the two contralateral sides and register the points in the ROI manager. Red circles marked the Fiji functions used in this step. Bottom panel shows a zoom-in image of the labeled heart. Lines between Point 14–15 and 23–24 show the mismatched contacts.

(E) Run the macro code to quantify the Euclidean distance on the x-y plane of neighboring junction points.

(E′) Result of the Euclidean distances.

(F) Arrange the Euclidean distances into the corresponding categories and calculate the mismatch in Excel.

1. Open image file (tiff file here) in Fiji (Schindelin et al., 2012) (Figure 3C).
2. Adjust the brightness to get the best contrast of the images and apply a Gaussian Blur filter with Sigma (Radius)=0.5 pixels.
3. Open the ROI manager ('Analyze'> 'Tools'> 'ROI Manager') (Figure 3C).
4. Select the 'Point Tool' on the menu bar and manually label out the crossed junction points of cell lateral boundaries and the middle contact line between the two contralateral sides and registered each point by adding into 'ROI manager' (Figure 3D).
5. The membrane contact length between neighboring junction points was estimated by measuring their Euclidean distance on the x-y plane through running a customized Macro code (Figure 3E) (the Macro code is provided at the end of this section).
6. The distances between labeled points will show up in the Log window (Figure 3E'). Copy and paste the data into a spreadsheet program. Manually assign each contact length to different cells and classified into 'matched contact', 'mismatched contact between same cell types', or 'mismatched contact between different cell types' (Figure 3F), based on the partner cell assignment (Figure 3B).
7. The total Mismatch is calculated by dividing the sum of mismatched contact length by total membrane contact length (Figure 3F). With this method, the mismatch value can range between 0 and 1. In wild-type embryos, we typically found a mismatch around 0.13. We recommend at least $n \geq 20$ embryos to gain suitable statistical power.

*Note:* It is important to have high quality data for this analysis. If the heart displays defects, such as the two sides are not completely joined, then this analysis cannot be performed. The number of defective hearts increased substantially in mutant embryos, such as perturbation of Cdc42 signaling (Zhang et al., 2018).

Macro Code:

```
// This macro calculates the direct distance between neighboring points

numROIs = roiManager("count");

roiManager("Select", 0);

getSelectionCoordinates(x, y);//get the Point 1 coordinates x and y

Xp=x[0];

Yp=y[0];

getPixelSize(unit, pw, ph); //get pixel size

for(i=1; i<numROIs;i++) {// loop through ROIs

roiManager("Select", i);

getSelectionCoordinates(x, y);// get the Point i coordinates x and y     Distance=-
Math.sqrt(Math.sqr(x[0]-Xp)+Math.sqr(y[0]-Yp)); //calculate the distance between two
points

Xp=x[0];

Yp=y[0];

print(i+'-'+i+1+':    '+Distance*pw);

}
```

## LIMITATIONS

The above-described quantification approach is designed to detect alteration in cardioblast mismatch under conditions where the cell expression pattern is not perturbed. When the 4-2 expression pattern of Tinman-Seven-up is significantly altered, for example due to over-proliferation of Tinman positive cardioblasts by over-activating Heartless (Yadav et al., 2021), it can be difficult to define the partner cells. This is especially the case between opposite cells of the same type, as they lose the 4-2 arrangement pattern which is used to define the partner cells of the same cell types. In this scenario, our quantification technique will not give very instructive information. The experimental protocols will still be useful to generate images of heart cell arrangement with high quality and quantity.

Additionally, as we used fixed samples to examine the cell mismatch, this approach will not give dynamic information about how the cell mismatch adjust during the matching stages. But similar quantitative concept can be applied for live imaging data by segmenting the membrane marker signals and applying the similar cell mismatch quantification strategies (see Zhang et al., 2018).

## TROUBLESHOOTING

### Problem 1

Tinman antibody is not commercially available (immunostaining, mounting, and batch imaging, step 20).

### Potential solution

An alternative commercially available antibody that can be used to differentiate cardioblast cell types is the anti-Seven up antibody (DSHB Cat# SEVEN-UP 2D3; RRID: AB_261807) which labels the non-Tin expressing CBs. However, this antibody is mouse origin (same as anti-Fas3 and anti-Integrin), sequential staining will need to be applied to label the cell boundaries as well as the cell type.

### Problem 2

During the mounting steps, when aligning the embryos in the mounting medium, the embryos may move around and make the alignment process difficult (immunostaining, mounting, and batch imaging, step 24d).

### Potential solution

After transferring the embryos onto the coverslip, try to dry up the samples. When applying the mounting medium, 1 or 2 drops will be enough. Also, try to flatten the mounting medium to make it into a thin layer that just covers the embryos.

### Problem 3

When adjusting the embryo orientation to make the dorsal side facing up, it is difficult to differentiate the dorsal/ventral side (immunostaining, mounting, and batch imaging step 24d).

### Potential solution

To solve this issue, we have used double-coverslip mounting. Thus, when performing the imaging, if the dorsal side of some embryos is facing down, just flip the double-coverslip and perform another batch imaging. This mounting strategy maximizes the imaging efficiency.

### Problem 4

During the mounting steps, especially with the aligning of many embryos (>50), the mounting medium may dry up and it becomes hard to align the embryos after several minutes (immunostaining, mounting, and batch imaging step 24d).

### Potential solution

Try to shorten the period of embryo alignment. It may take some practice to become familiar with aligning procedure. Thus, in the beginning, try to limit the number of embryos in the mounting steps. Further, if the mounting medium becomes dry, apply another drop of mounting medium.

### Problem 5

During the mismatch measurement step, due to the curved shape of the heart and some distortion of the embryos caused during the fixation and mounting steps, it is hard to distinguish in the same z-plane when trying to label the crossed junction points Image (Quantification Analysis step 4).

### Potential solution

To overcome this, search the surrounding z-planes (1–2 planes above/below) to look for the most obvious crossed junction points. In the final membrane contact length measurement, the z-direction distance is ignored here but can easily be accounted for by estimating the Euclidean distance between the points (by adding the z-distance into the Euclidean distance calculation step). Here is the code for this quantification step:

```
// This macro calculates the direct distance between neighboring points

numROIs = roiManager("count");

roiManager("Select", 0);

getSelectionCoordinates(x, y);//get the Point 1 coordinates x and y

Roi.getPosition(channel, z, frame); //get the Point 1 coordinates z

Xp=x[0];

Yp=y[0];

Zp=z;

getVoxelSize(px, py, pz, unit);

for(i=1; i<numROIs;i++) {// loop through ROIs

roiManager("Select", i);

getSelectionCoordinates(x, y); //get the Point i coordinates x and y

Roi.getPosition(channel, z, frame); //get the Point i coordinates z

Distance=Math.sqrt(Math.sqr(px*(x[0]-Xp))+Math.sqr(py*(y[0]-Yp))+Math.sqr(pz*(z-
Zp))); //calculate the distance between two points

Xp=x[0];

Yp=y[0];

print(i+'-'+i+1+':    '+Distance);

}
```

### Problem 6

When labeling the crossed junction points of cell lateral boundaries and the middle contact line between the two contralateral sides, as it is a manual process, the labeling process can generate variations (Quantification Analysis step 4).

### Potential solution

When performing the labeling, try to zoom in the image to label the crossed junction and include a larger n for each genetic condition (n>20) for robust statistical analysis.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact upon reasonable requests, Timothy Saunders (timothy.saunders@warwick.ac.uk).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

The published article includes all datasets generated or analyzed during this study. Raw data are available on reasonable request from the corresponding author.

## ACKNOWLEDGMENTS

We thank Manfred Frasch for sharing anti-Tinman reagent. We acknowledge Anqi Huang and Christopher Amourda for helping set up the fixation and immunostaining protocols. This work was supported by the National Research Foundation Singapore under an NRF Fellowship to T.E.S. (NRF2012NRF-NRFF001-094) and a Singapore Ministry of Education Tier 2 grant to T.E.S. (MOE2018-T2-2-138).

## AUTHOR CONTRIBUTIONS

S.Z. and T.E.S. designed the project. S.Z. performed the experiments and data quantification. S.Z. and T.E.S. wrote the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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