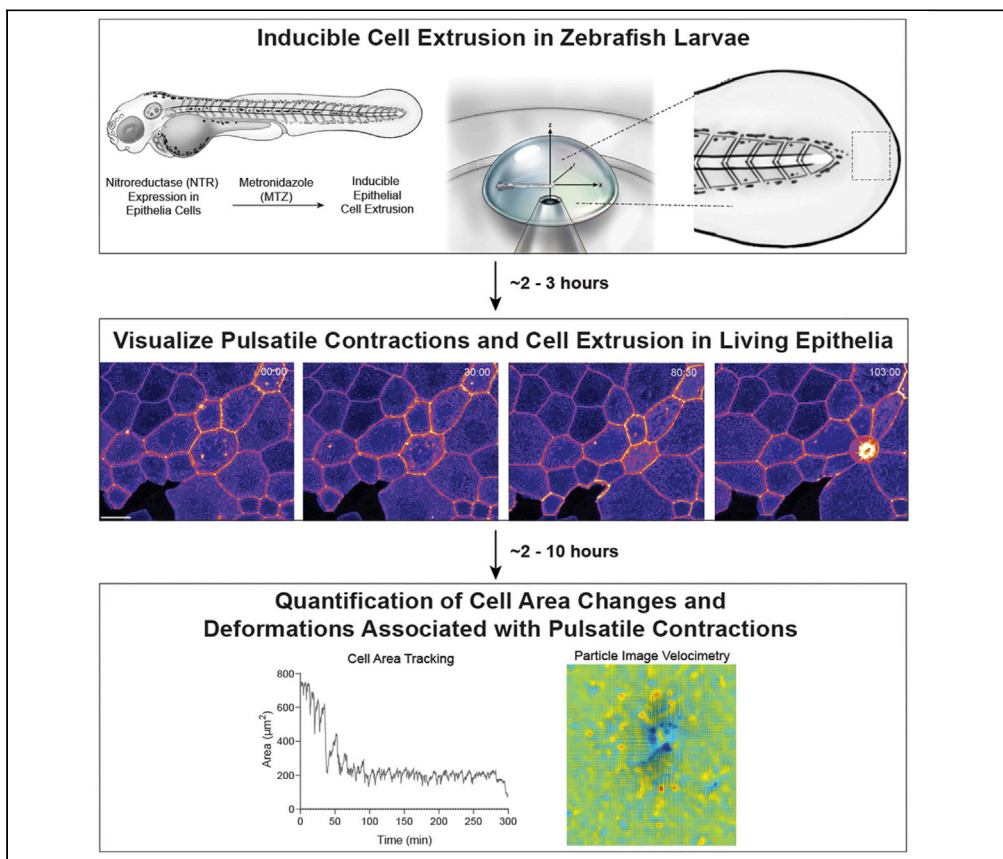


## Protocol

# Protocol for quantitative analysis of pulsatile contractions and cell extrusion in epithelial tissues of larval zebrafish



Cell elimination by extrusion is important for epithelial homeostasis, but knowing when and where cells will extrude has made *in vivo* studies difficult. Here, we describe a step-by-step protocol for inducing cell extrusion from the larval zebrafish epidermis. We detail how to capture the dynamics of extrusion via time-lapse imaging and describe how existing protocols can be implemented for the analysis of cell shape changes preceding extrusion events and derivation of mechanical measurements associated with these shape changes.

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### Highlights

Inducible cell extrusion in the epidermis of larval zebrafish

Use of time-lapse confocal imaging to capture the dynamics of cell extrusion

Description for analysis of cell shape changes preceding extrusion events

Steps for measurements of deformation associated with the cell shape changes

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## Protocol

## Protocol for quantitative analysis of pulsatile contractions and cell extrusion in epithelial tissues of larval zebrafish

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<https://doi.org/10.1016/j.xpro.2021.100600>

## SUMMARY

Cell elimination by extrusion is important for epithelial homeostasis, but knowing when and where cells will extrude has made *in vivo* studies difficult. Here, we describe a step-by-step protocol for inducing cell extrusion from the larval zebrafish epidermis. We detail how to capture the dynamics of extrusion via time-lapse imaging and describe how existing protocols can be implemented for the analysis of cell shape changes preceding extrusion events and derivation of mechanical measurements associated with these shape changes.

For complete details on the use and execution of this protocol, please refer to Atieh et al. (2021).

## BEFORE YOU BEGIN

Here, we describe the procedure of inducing damage in a subset of cells within the outer epidermis of zebrafish larvae and methods for time-lapse imaging to track the dynamic remodeling and mechanical alterations within the tissue preceding epithelial cell extrusion. This is accomplished by expression of the genetically encoded enzyme nfsB (also referred to as nitroreductase, or NTR) fused to mCherry (Davison et al., 2007) in the surface epithelial cells using the GAL4 enhancer trap line *zc1044A* (Eisenhoffer et al., 2017). Upon exogenous addition of metronidazole (MTZ) to the plate or well containing the larvae, mCherry fluorescent epithelial cells expressing NTR will convert the MTZ to a cytotoxic byproduct that promotes DNA damage (Curado et al., 2008). The damaged cells are then eliminated by extrusion (Atieh et al., 2021), a process by which neighboring cells form a contractile actomyosin ring and eject the cell from the tissue while sealing the hole from its exit (Roseblatt et al., 2001). The protocol below describes the specific steps for inducing extrusion and imaging 4 days post-fertilization (4dpf) *Et(Gal4-VP16)<sup>zc1044A</sup>;Tg(UAS: nfsB-mCherry)* zebrafish larvae in combination with the *Tg(UAS:Lifeact-GFP)<sup>utm1</sup>* transgenic line (Eisenhoffer et al., 2017) to fluorescently label for F-actin in the surface epithelial cells. As fluorescent labeling of the epithelial cell boundaries is key for quantitative analysis of these dynamic events, any fluorescently tagged membrane marker expressed in epidermal cells can be used in combination with the *Et(Gal4-VP16)<sup>zc1044A</sup>;Tg(UAS: nfsB-mCherry)* zebrafish line required for inducing epithelial cell extrusion. We do, however, recommend that the fluorescently tagged membrane-bound protein be stable or slow recycling in order to facilitate assessment of cellular shape changes and tissue movements by Particle Image Velocimetry (PIV) analyses (Thielicke and Stamhuis, 2014, Thielicke and Stamhuis, 2018).



**Note:** All procedures described in this protocol have been approved by the University of Texas MD Anderson's Institutional Animal Care and Use Committee (protocol#00001283-RN02). Experiments that are performed following this protocol should be approved by the user's Institutional Animal Care and Use Committee.

**Note:** Plan on breeding adult zebrafish the appropriate amount of days prior to the experiment (i.e. to use 4dpf larvae, adult zebrafish should be bred 5 days prior to the experiment). Embryos/larvae can be between 1 and 7dpf, the developmental time window during which the enzyme nitroreductase (NTR) expression has been characterized (Eisenhoffer et al., 2017). The expression of NTR is key to the protocol as it will catalyze the prodrug metronidazole (MTZ) when added, therefore producing a cytotoxic agent that promotes cell death (Curado et al., 2008).

**Note:** Low-melt agarose should be prepared at least 2 h prior to mounting larvae in order to allow it to adjust to the appropriate temperature in the heating block.

**Note:** Larvae do not need to be treated with an inhibitor of pigmentation as they express fluorescently labelled proteins and are therefore amenable to optical live microscopy.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Zebrafish: <i>Et(Gal4-VP16)<sup>zrc104a</sup>; Tg(UAS-E1b:nsfB-mCherry); Tg(UAS-E1b:Lifeact-EGFP)<sup>utm1</sup></i>	(Davison et al., 2007) (Eisenhoffer et al., 2017)	N/A
Software and algorithms		
MATLAB R2019a	MathWorks	<a href="https://www.mathworks.com/products/matlab.html">https://www.mathworks.com/products/matlab.html</a>
PIVLab	(Thielicke and Stamhuis, 2014)	<a href="https://www.mathworks.com/matlabcentral/fileexchange/27659-pivlab-particle-image-velocimetry-piv-tool-with-gui">https://www.mathworks.com/matlabcentral/fileexchange/27659-pivlab-particle-image-velocimetry-piv-tool-with-gui</a>
Fiji	ImageJ	<a href="https://imagej.net/Fiji">https://imagej.net/Fiji</a>
Tissue Analyzer	(Aigouy et al., 2016)	<a href="https://grr.gred-clermont.fr/labmirouse/software/WebPA/index.html">https://grr.gred-clermont.fr/labmirouse/software/WebPA/index.html</a>
Chemicals, peptides, and recombinant proteins		
Metronidazole (MTZ)	Sigma	M-3761
DMSO	Sigma	276855
Tricaine-S	Western Chemical Product	TRICMGR0100
Low melt agarose	Thermo Fisher Scientific	16520050
Other		
Stereo microscope	Leica Camera AG	KL-300-LED
Laser scanning confocal	Zeiss	LSM-800
Glass Pasteur pipets	VWR	14672-608
Glass Bottom Dish – 1 compartment	MatTek	P35G-1.0-20-C
Glass Bottom Dish – 4 Compartments	Greiner	627870
Dumont #5 Forceps	Fine Science Tools	11251-10

## MATERIALS AND EQUIPMENT

- E3 media
  - 34.8 g NaCl.
  - 1.6 g KCl.
  - 5.8 g CaCl<sub>2</sub>·2H<sub>2</sub>O

- MTZ solution (1M): in 1.5mL tube
  - Weigh 214 mg of MTZ
  - Add 1.2 mL of DMSO
  - Incubate in 42°C–50°C water bath for 50 min
  - Vortex until the rest of the MTZ is dissolved before using

**Note:** MTZ is prepared fresh and can be used at any point during that same day.

△ **CRITICAL:** MTZ solution needs to be kept in the dark

- Tricaine solution (4 mg/mL): in 15 mL tubes
  - Weigh 4 g of tricaine powder
  - Add 1 L of ddH<sub>2</sub>O and mix on stir plate
  - Adjust pH to 7.2 using 1M Tris pH9
  - Aliquot in 15 mL conical vials
  - Store at –20°C
- Low-melt agarose (0.5%): in 1.5 mL tubes
  - Weigh 250 mg of low-melt agarose
  - Add 50 mL of E3 embryo medium
  - Microwave for 5 s intervals quickly mixing the solution in between, until agarose has dissolved and E3 starts bubbling
  - Aliquot in 1.5 mL Eppendorf tubes and incubate in heating block at 42°C

**Note:** Agarose solutions can be kept in heating block for up to a month.

## STEP-BY-STEP METHOD DETAILS

### Treatment with metronidazole (MTZ)

⌚ Timing: 3 h

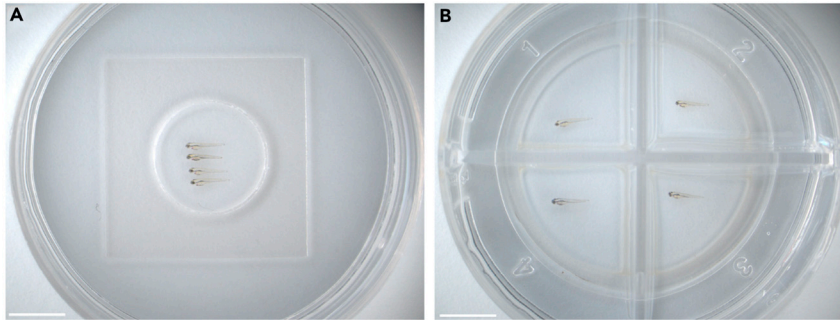
This step is key to inducing cell damage and extrusion in the epidermis. While NTR expression does not alter the physiological functions of epithelial cells, addition of the prodrug metronidazole (MTZ) leads to the production of a cytotoxic DNA intercalating agent that induces damage and cell extrusion.

1. Prepare MTZ solution.
2. Add solution to larvae at a final concentration of 10 mM and pipet well to avoid MTZ crystallization at the bottom of the well.

**Note:** For the study by (Atieh et al., 2021), MTZ was usually added to 4 larvae at a time in a 6-well plate at final volumes of 50  $\mu$ L of MTZ solution to 5 mL of E3 embryo medium. For a higher number of larvae, we found that MTZ was less efficient at inducing extrusion and damage.

3. Incubate at 28°C for 2 h in the dark.

**Note:** MTZ final concentration and incubation time can be adjusted depending on the amount of cellular extrusion desired for the particular experiment. Changing the MTZ concentration or incubation time determines the amount of cell extrusion induced (Figure 1F in (Atieh et al., 2021)).



**Figure 1. Example of glass bottom dishes containing 4dpf zebrafish larvae mounted in low-melt agarose for imaging**  
(A) Zebrafish larvae mounted in a glass bottom MatTek dish with one compartment. Scale bar, 5 mm.  
(B) Zebrafish larvae mounted in a glass bottom Greiner dish with 4 compartments. Scale bar, 5 mm.

### Immobilization and mounting of zebrafish larvae in agarose

⌚ Timing: 5–10 min

This step is necessary to immobilize zebrafish larvae for time-lapse imaging. For additional information and a video tutorial on mounting larval zebrafish for imaging, please see (Eisenhoffer and Rosenblatt, 2011).

4. Add 5–7 drops of tricaine/well to anesthetize larvae.
5. Using a glass pipet, transfer the desired number of larvae in a dry 35 mm glass bottom dish and add a drop of tricaine to the dish for 10–20 s.

**Note:** For simultaneous imaging of larvae in different drug conditions, 4-compartment glass bottom dishes can be used (Figure 1B) (also see step 10).

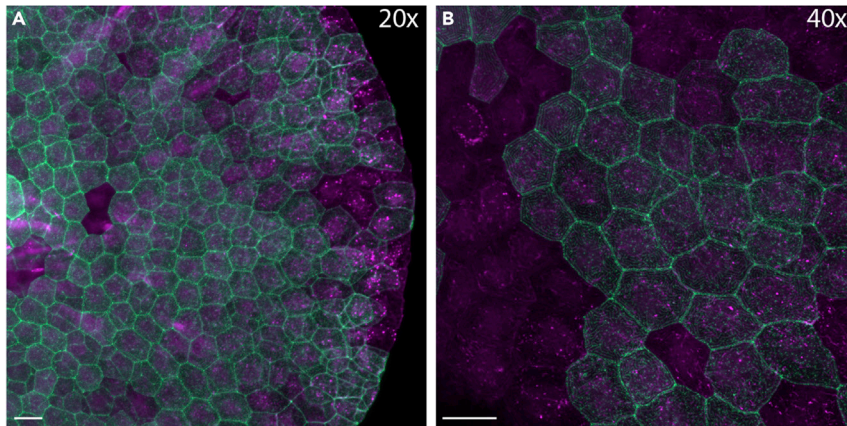
6. Pipet any excess liquid around larvae and add 100  $\mu$ L of 0.5% agarose (see materials and equipment for low melt agarose preparation).
7. Under a routine stereo microscope, center larvae in agarose so that they are all mounted in the same direction. Larvae should be mounted to clearly see both dorsal-ventral and anterior-posterior axes, so that the edge of the tail appears flat (Figure 1A).
8. Keep repositioning larvae so that they remain completely flat until agarose starts solidify (this should take 1–2 min at most).
9. When agarose is completely solidified (should become opaque), add a drop of tricaine and complete with 1 mL of E3.

**⚠ CRITICAL:** Larvae should be perfectly flat when agarose polymerizes especially for high resolution imaging. This will avoid acquiring thick z-stacks and will allow reducing the time interval between image frames acquired to capture both pulsatile contractions and extrusion events.

### Time-lapse confocal imaging

⌚ Timing: varies depending on the desired time of acquisition (up to 10 h)

10. Acquire Z-stack images using a laser-scanning confocal microscope equipped with either a 20 $\times$  air objective (0.8  $\mu$ m/pixel) (Figure 2A) or a 40 $\times$  water objective (0.18  $\mu$ m/pixel) (Figure 2B) depending on the desired x-y resolution (X-Y dimensions 512  $\times$  512 or 1024  $\times$  1024). Depending on the area acquired (portion of the epithelium vs whole tail), reduce or increase z-step from 1 to 3  $\mu$ m.



**Figure 2. Representative examples of epithelial cells at different magnifications time-lapse imaging**

(A) Maximum intensity projection of epithelial cells in the tail fin using a 20× air objective (for multi-positional acquisition). Scale bar, 20 μm.

(B) Maximum intensity projection of epithelial cells using a 40× water objective (for high-resolution acquisition). Scale bar, 20 μm.

Epithelial cells express Nitroreductase (NTR-mCherry, shown in magenta) and Lifact-EGFP (green).

- a. For in-depth analysis of pulsing events, we recommend that the time-step does not exceed 30 s as pulses last on average between 1 and 2 min.
- b. For acquisition of the whole tail fin epithelium using a 20× objective (Figure 2A), top bottom and right margins of approximately 5 μm will facilitate capture of extrusion events and prevent the tail from going out of frame due to pulsing and eventual whole tissue area reduction.

**Note:** If you decide to monitor multiple conditions simultaneously, acquire images at a 5 min interval using a 20× air objective in a 4-compartment glass bottom dish (Figure 1B). This approach facilitates incubation of larvae with different pharmacological agents, before or during MTZ treatment and imaging.

**Alternatives:** While the use of a laser-scanning confocal microscope is described, alternative microscopy platforms, such as spinning disk microscopy, are also compatible with this protocol. Potential limitations of these alternatives are discussed below.

11. Generate maximum intensity projections for all captured z-stack files (Figure 2).
12. Save maximum intensity projections in 8-bit Tiff file format.

### Quantitative and statistical analyses

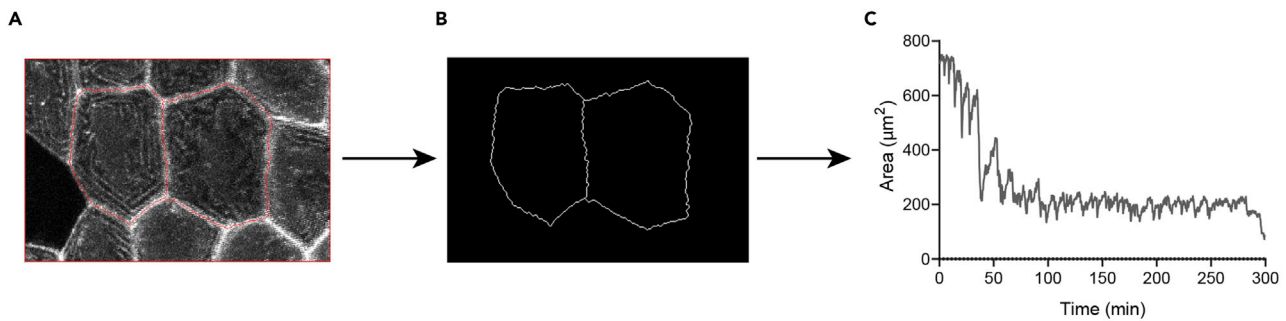
#### Measuring cell area using Tissue Analyzer

For a better familiarization with the Tissue Analyzer software, we suggest referring to (Aigouy et al., 2016)

In this step, we use the ImageJ plugin Tissue Analyzer to track cell area changes that occur following MTZ treatment, prior to extrusion. Tissue Analyzer allows tracking of cell area over time based on segmentation by junctional protein fluorescence.

13. Split the time-lapse Tiff file into individual images that represents each time point.
14. Open the Tissue Analyzer plugin in Fiji and drag image sequences into the “Image List” box.
15. In the “Segmentation” section, click “Detect Bonds” and set parameters.

**Note:** We advise starting with “Detect Bonds Preview Only” as this will quickly process the first image of the sequence and give an idea of what the segmentation will look like. Here, you can adjust parameters such as the minimum size of desired detected cells.



**Figure 3. Cell area analysis using Tissue Analyzer**

(A) Cell-cell Bond detection after correction.

(B) Segmented processed image.

(C) Scatter dot graph representing a single cell example of the area fluctuation overtime of an epithelial cell.

- Once the parameters look correct, you can process all images at once by clicking “Detect Bonds (save watershed)”. This will automatically generate segmented images of epithelial cells (Figures 3A and 3B).

**Note:** For the study by (Atieh et al., 2021), strong and weak blurs were set at 10 and 1 respectively and cell size was set to be detected at a minimum of 15 pixels.

- If mistakes in cell-cell junction detection are still present, you can manually correct them by either removing (right click over the junction) or adding (left click to draw junction) junctions.

**Note:** In the case of manual correction being performed, do not forget to hit the “save” button in the bottom right side of the software before moving on to the next image.

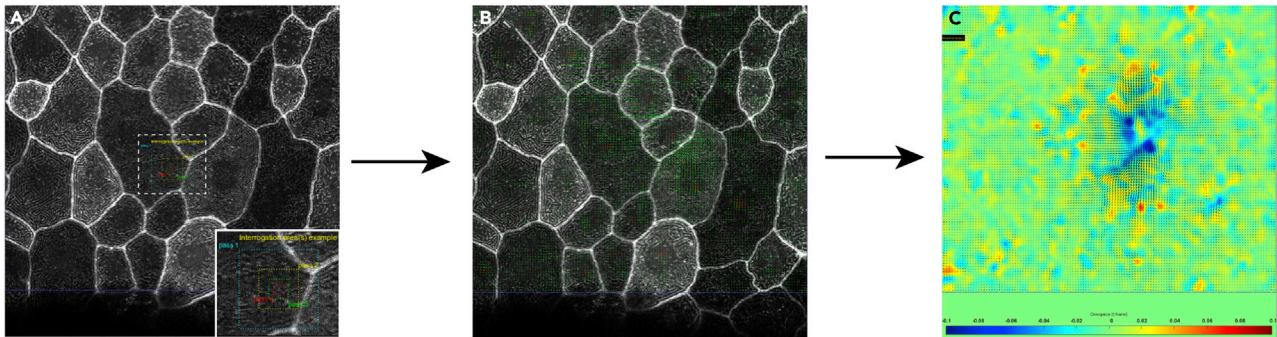
- After all image sequences have been verified, process the segmentation in the “PostProcess” section by clicking “Finish all”.
- Go to the “Plots” section to generate an excel sheet containing quantified parameters (i.e., cell area, cell perimeter,... etc).
- In (Atieh et al., 2021), we tracked single cell areas over time (Figure 3C). Retrospective analysis can facilitate assignment as extruding cells, neighboring cells and non-extruding cells. In order to generate single plots averaging multiple pulses, align pulses by overlapping minimal area values.

**△ CRITICAL:** For images containing many epithelial cells, Tissue Analyzer assigns cell ID numbers for each frame. Cell IDs are however different for every frame so we recommend quantifying a total of 1 to 6 cells at a time to ensure accurate tracking, especially when analyzing a significant amount of time-frames.

#### Measuring cell contraction and expansion using Particle Image Velocimetry (PIV)

For a better familiarization with the PIVlab software, we suggest referring to (Thielicke and Stamhuis, 2014). A detailed video to walk you through the PIV lab software is also available on <https://www.youtube.com/watch?v=Sp3Ounq07Qc>

- Spilt Tiff file into image sequences.
- Load image sequences in PIVlab. Add and import images. For this protocol, the sequencing style used was 1-2,2-3,3-4,...
- In the “Analyses settings” section, start by selecting the region of interest that will be analyzed in Exclusion (ROI, Mask).



**Figure 4. Tissue flow analysis using PIVlab**

(A) Setting passes and interrogation windows.

(B) PIV analysis after selection of velocity limits.

(C) Convergence/Divergence (i.e., cellular contraction/expansion) colormap as generated by the software.

**Note:** PIV does not ignore non-fluorescent parts of the image. Therefore, be sure to exclude any cells that are not fluorescently tagged (which can be the case for the zebrafish epithelial Gal4-UAS lines).

24. Adjust image fluorescence in the “Image pre-processing” section.

**Note:** For this protocol, we applied the CLAHE, highpass and denoise filters .

25. In the PIV settings section, we applied four passes:  $128 \times 128$ ,  $64 \times 64$ ,  $32 \times 32$ , and  $16 \times 16$  pixel-size interrogation window with 50% overlap each (Figure 4A).
26. At this point, images can then be analyzed in the “Analysis” section.
27. Once analysis is completed for all frames (Figure 4B), calibrate your images by inserting the time interval and the scale of your acquisition in the “Calibration” section.
28. If you find some mistakes in the analysis (i.e., out of range arrows), you can correct them in the “Post-processing” section by selecting velocity limits .
29. For plot generation, you can choose the type of measurements performed (velocity magnitude, divergence,...) in “Plots → Derive parameters/modify data”.
30. For plot appearance, heatmaps and scale bars can be adjusted in “Plots → Modify plot appearance” (Figure 4C).
31. For getting numerical values of measurements go to “Extractions → Parameters from area”. The software will prompt you to draw the area you wish to get measurements from.
32. Measurements will automatically be saved as text files and movies can be saved as AVI files.

## EXPECTED OUTCOMES

In (Atieh et al., 2021), we have uncovered the appearance of pulsatile cellular contractions preceding extrusion as a result of MTZ treatment of NTR expressing larvae and subsequent DNA damage. Using this protocol, we were able to quantify the dynamic changes in cell area during pulsing prior to extrusion and assess the impact on the mechanical state of the epithelium.

## LIMITATIONS

As previously mentioned, larvae need to be mounted flat, especially for high resolution imaging (using a  $40\times$  objective) of a small part of the epithelium. This will allow acquiring a smaller z-stack, and therefore decreasing the time interval between frames.

For fast acquisitions (with a time-step  $< 3$  min) and especially when using a water or oil objective, multi-positioning is complicated. Therefore, running an experiment with a control and a drug-



treated or transgenic line simultaneously imaged at high resolution is not an option and can only be achieved using a 20× air objective (see step 11). It is important to note that if pulsing dynamics are compared between conditions, they will not be from larvae imaged exactly in parallel.

For analysis of pulse dynamics, both images of whole tail (20×) or groups of epithelial cells (40×) allow for area tracking and segmentation. However, for analysis of pulsing for extended periods of time, until extrusion occurs, we recommend using imaging the whole tail 20× images to allow a better view of the overall tissue, as catching one or multiple extrusion events in a limited field at high resolution can be challenging.

The tail fin epithelium can contract at the end of the movies after many cells have been extruded from the tissue. While analysis of cell area and pulsatile contractions (steps 13–20) occur prior to extrusion events, the extrusion of many cells from the tissue can cause movement and drift in the movies, precluding analysis at later time points.

## TROUBLESHOOTING

### Problem 1

If the tail fin epithelium appears folded or severely damaged during imaging, this can occur if the low-melt agarose is too concentrated and creates a stiff environment around the larvae. These larvae should not be used for analysis.

### Potential solution

It is recommended to routinely make new aliquots of low melt agarose for mounting and immobilization and test each batch for consistency and rate of solidification before beginning experiments.

### Problem 2

If larvae cannot be mounted flat or if stack size is too big, increasing time interval between frames during acquisition may be the only option. In this case, analysis of the dynamics of pulsatile contractions may not be very precise.

### Potential solution

One possible solution is to acquire images using a spinning disk microscope where acquisition is much faster. It is important to note that in the latter case, if junctional fluorescence is not very bright, samples will be more sensitive to bleaching and image resolution will be compromised, making image analysis more challenging (see problem 1).

### Problem 3

If acquired fluorescence images are low in resolution (i.e. too much noisy background, low fluorescence intensity... etc), cell area tracking and segmentation through Tissue Analyzer can be challenging.

### Potential solution

We advise processing the image in Fiji to reduce noise and increase junctional fluorescence intensity. This can be achieved through increasing brightness threshold, subtracting fluorescence background or applying blurring filters.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, George T. Eisenhoffer ([GTEisenhoffer@mdanderson.org](mailto:GTEisenhoffer@mdanderson.org))

### Material availability

The transgenic zebrafish lines used in this paper are available upon request.

## Data and code availability

The published article includes all data sets and codes generated or analyzed during this study.

## ACKNOWLEDGMENTS

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## AUTHOR CONTRIBUTIONS

Y.A, O.E.R., and G.T.E conceived the method and protocol. Y.A. performed all the experiments and subsequent analysis. Y.A. and G.T.E. wrote the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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