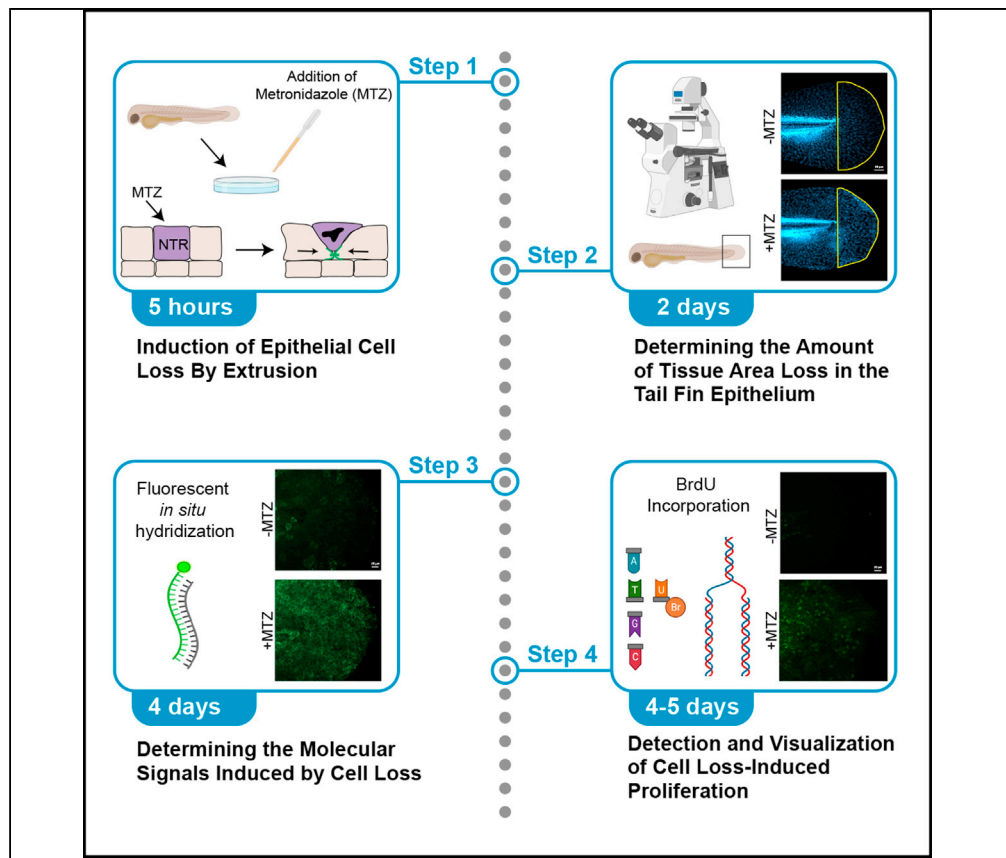


Protocol

A protocol to evaluate epithelial regeneration after inducing cell loss in zebrafish larvae



Epithelial tissues sustain barrier function by removing and replacing aberrant or unfit cells. Here, we describe approaches to evaluate epithelial restorative capacity after inducing cell loss in zebrafish larvae. We provide details to quantify morphological changes to the tail fin epithelium after cell loss, and instructions to interrogate changes in gene expression and proliferation associated with replacement of the lost cells. Together, this approach establishes an *in vivo* vertebrate model for the rapid assessment of molecular pathways controlling epithelial regeneration.

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Highlights

Evaluation of epithelial restorative capacity after cell loss in zebrafish larvae

Quantification of morphological changes related to cell loss in the tail fin epithelium

Instructions to interrogate changes in gene expression after induced cell loss

Steps to assess proliferation associated with replacement of the lost cells

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Protocol

A protocol to evaluate epithelial regeneration after inducing cell loss in zebrafish larvae

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SUMMARY

Epithelial tissues sustain barrier function by removing and replacing aberrant or unfit cells. Here, we describe approaches to evaluate epithelial restorative capacity after inducing cell loss in zebrafish larvae. We provide details to quantify morphological changes to the tail fin epithelium after cell loss, and instructions to interrogate changes in gene expression and proliferation associated with replacement of the lost cells. Together, this approach establishes an *in vivo* vertebrate model for the rapid assessment of molecular pathways controlling epithelial regeneration.

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

BEFORE YOU BEGIN

The following protocol describes the steps required for inducing cell loss by extrusion of the surface epithelial cells in larval zebrafish, and provides the methodology for measuring both the extent of reduced tissue area and the amount of compensatory proliferation to replace the lost cells. To achieve this, the GAL4 enhancer trap line *zc1044a* is used to drive expression of a fusion of the bacterial *nfsB* gene to mCherry (Davison et al., 2007) under the control of upstream activating sequence, or UAS (together, referred to as UAS-Nitroreductase or NTR) in the surface epithelial cells (Eisenhoffer et al., 2017). Under homeostatic conditions the GAL4/UAS-NTR system is inert and labels surface epithelial cells with mCherry in a mosaic fashion. Upon addition of exogenously added metronidazole (MTZ), NTR+ cells convert MTZ into a cytotoxic byproduct that results in extensive DNA damage (Curado et al., 2008) and the subsequent removal of damaged cells through extrusion (Atieh et al., 2021). We describe methods for assessing the amount of cell loss induced after a 5hr MTZ treatment and the extent of compensatory proliferation observed during tissue regeneration and wound healing. These methods allow for the rapid screening of chemical compounds that may be aimed at: 1.) Suppression of extrusion-mediated cell loss and protection from disrupted homeostasis; and 2.) Enhancing the epithelial restorative response after cell loss and injury.

Obtaining zebrafish embryos with nitroreductase (NTR) expression in the surface epithelial cells

© Timing: 1 week



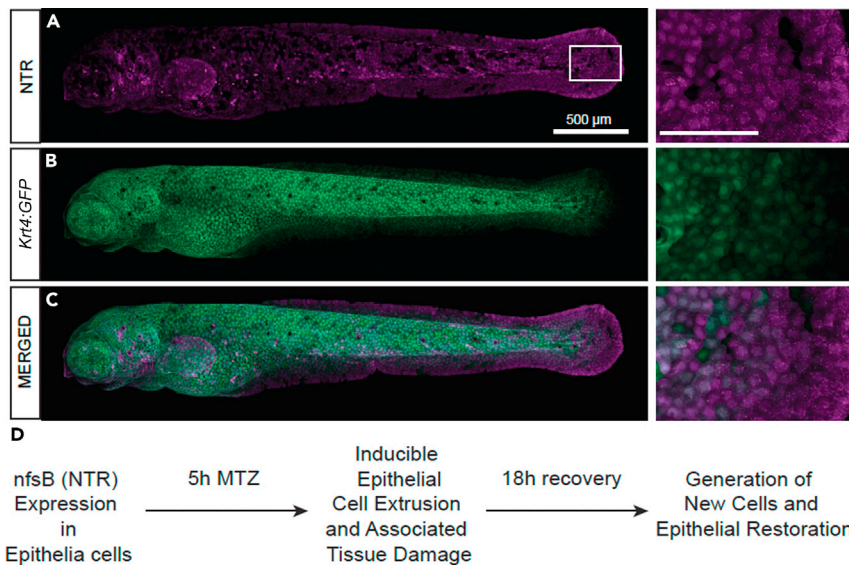


Figure 1. Tiled confocal images of larval zebrafish lines used to label and manipulate the surface epithelia
 (A) Maximum intensity projection of the *Et(Gal4-VP16)^{zc1044a};Tg(UAS-E1b:nfsB-mCherry)^{c264}* (MAGENTA) line showing expression in the surface epithelial cells of 4 day post fertilization (dpf) larval zebrafish. Scale bar for magnified region = 250 μ m.
 (B) The *Tg(krt4:GFP)* (GREEN) transgenic line labels the differentiated surface keratinocytes in the larval zebrafish.
 (C) Merged image of both *Et(Gal4-VP16)^{zc1044a};Tg(UAS-E1b:nfsB-mCherry)^{c264}* (MAGENTA) and *Tg(krt4:GFP)* (GREEN) shows mosaic expression pattern in the surface epithelial cells.
 (D) Schematic of the timeline for the inducible cell loss and epithelial restoration assay.

1. Prepare an incross mating of adult zebrafish from the *Et(Gal4-VP16)^{zc1044a};Tg(UAS-E1b:nfsB-mCherry)^{c264}* transgenic line in the afternoon five days before the intended date of the experiment (Figure 1).
 - a. On the day of the mating (4 days before experiment), pull dividers and allow fish to mate. Collect eggs with E3 medium, discard dead or unfertilized eggs and place in an incubator at 28.5°C.
 - b. On day 1 post fertilization (3 days before experiment) remove any dead embryos and replenish with fresh E3 medium.
- Note:** Although this is not required, embryos may be treated with 0.003% 1-phenyl 2-thiourea (PTU) in E3 throughout their development to block pigmentation and increase the optical clarity of the larvae for subsequent imaging analyses.
2. Sorting fluorescent embryos at day 3 post fertilization (1 day before experiment)
 - a. Anesthetize embryos with 500 μ L of 4 mg/mL Tricaine.
 - b. Sort embryos exhibiting mCherry fluorescence by using a fluorescent dissecting microscope (Figure 1). Collect all mCherry positive embryos into a new dish with E3.
 - c. Collect all embryos lacking mCherry fluorescence into a separate dish with E3. These will be used for enzyme-negative controls.

Note: Transgenic lines expressing green fluorescent proteins (such as *Tg(UAS-1b:Lifeact-EGFP)^{uttm1}* or *Tg(-8.0clnbn:lynEGFP)^{z106b}*) can be used in conjunction with the NTR system to visualize cell-cell junctions, and facilitates capturing extrusion events and subsequent tissue damage during MTZ treatment.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|-----------------------------|---|
| Antibodies | | |
| Rat Anti-BrdU | Abcam | Ab6326 |
| Goat Anti-Rat Alexa Fluor 647 | Thermo Fisher Scientific | A-21247 |
| Chemicals, peptides, and recombinant proteins | | |
| Metronidazole | Sigma-Aldrich | M-3761 |
| DAPI | Thermo Fisher Scientific | D1306 |
| 5-Bromodeoxyuridine | Sigma-Aldrich | B9285 |
| Tricaine-S | Western Chemical | TRICMGR0100 |
| Tween-20 | Sigma-Aldrich | P9416 |
| SSC Buffer 20× Concentrate | Sigma-Aldrich | S6639 |
| Recombinant Human EGF | Prospec Bio | CYT-217-b |
| Recombinant Human Epigen | Peptides International | CYT-601 |
| Triton X-100 | Sigma-Aldrich | T8787 |
| Goat Serum | Sigma-Aldrich | G9023 |
| Bovine Serum Albumin | Sigma-Aldrich | A6003 |
| Paraformaldehyde | Sigma-Aldrich | F8775 |
| Low Melt Agarose | Invitrogen | 16520 |
| Proteinase K | Thermo Fisher Scientific | EO0491 |
| Critical commercial assays | | |
| HCR v3.0 Custom Probe Set epigen | Molecular Instruments | Accession: XM_001344355.8 |
| HCR Amplifier B1 | Molecular Instruments | HCR Amplifier |
| HCR whole-mount probe hybridization buffer | Molecular Instruments | HCR whole-mount probe hybridization buffer |
| HCR whole-mount probe wash buffer | Molecular Instruments | HCR whole-mount probe wash buffer |
| HCR whole-mount amplification buffer | Molecular Instruments | HCR whole-mount amplification buffer |
| MatTek Glass Bottom Dish | MatTek | P35G-1.0-20-C |
| Nunc Glass Bottom Dish | Thermo Fisher Scientific | 150680 |
| Deposited data | | |
| RNAseq Raw Data | NIH Gene Expression Omnibus | GSE140839 |
| Experimental models: Organisms/strains | | |
| Zebrafish: <i>Et(Gal4-VP16)^{zc1044a}, Tg(UAS-1b:nfsB-mCherry)^{c264}</i> | (Eisenhoffer et al., 2017) | zc1044a |
| Zebrafish: <i>Et(Gal4-VP16)^{zc1036a}, Tg(UAS-1b:nfsB-mCherry)^{c264}</i> | (Eisenhoffer et al., 2017) | zc1036a |
| Zebrafish: <i>Et(Gal4-VP16)^{zc1044a}, Tg(UAS-1b:nfsB-mCherry)^{c264}, Tg(UAS-1b:Lifeact-EGFP)^{utm1}</i> | (Eisenhoffer et al., 2017) | utm1 |
| Zebrafish: <i>Tg(krt4:GFP)</i> | (Gong et al., 2002) | N/A |
| Zebrafish: <i>Tg(-8.0cldnb:lynEGFP)^{zf106}</i> | (Haas and Gilmour, 2006) | zf106 |
| Software and algorithms | | |
| GraphPad Prism (version 7.03) | GraphPad | https://www.graphpad.com/scientific-software/prism/ |
| Zeiss Zen Blue 2.6 | Carl Zeiss | https://www.zeiss.com/microscopy/us/products/microscope-software/zen.html |
| Fiji | Image J | https://imagej.net/Fiji |

MATERIALS AND EQUIPMENT

Equipment: Tissue culture 12 well plates (Olympus Catalog #25–101), Glass Pasteur pipettes (VWR Catalog #14672-608) and roller pipette pump (SP Bel-Art Catalog #378980000).

50× E3 Embryo Medium

| Reagent | Final concentration | Amount |
|--------------------------------------|---------------------|----------|
| NaCl | 1.46% w/v | 14.6 g/L |
| KCl | 0.063% w/v | 0.63 g/L |
| CaCl ₂ •2H ₂ O | 0.243% w/v | 2.43 g/L |

(Continued on next page)

Continued

| Reagent | Final concentration | Amount |
|--------------------------------------|---------------------|------------|
| MgSO ₄ •7H ₂ O | 0.407% w/v | 4.07 g/L |
| ddH ₂ O | n/a | Add to 1L |
| Total | n/a | 1 L |

Adjust pH to 7.2 with 0.1 M NaOH solution, autoclave, and store at 4°C for up to 1 year.

Metronidazole

| Reagent | Final concentration | Amount |
|---------------|---------------------|---------------|
| Metronidazole | 1 M | 214 mg |
| DMSO | n/a | 1.25 mL |
| Total | n/a | 1.5 mL |

Incubate in a 50°C water bath for 20 min. Vortex until powder goes into solution.

△ **CRITICAL:** To ensure reproducibility, MTZ is prepared fresh daily and must be kept in the dark.

Tricaine

| Reagent | Final concentration | Amount |
|--------------------|---------------------|------------|
| Tricaine | 4 mg/mL | 4 g |
| ddH ₂ O | n/a | 1 L |
| Total | n/a | 1 L |

Adjust pH to 7.2 with 1 M Tris pH 9.0

BrdU

| Reagent | Final concentration | Amount |
|-----------------|---------------------|-------------|
| BrdU | 10 mM | 15.35 mg |
| DMSO | 5% | 250 µL |
| E3 Embryo Media | n/a | 4.75 mL |
| Total | n/a | 5 mL |

Vortex vigorously and mix on a three-dimensional gentle mixer for 20 min

Block Buffer

| Reagent | Final concentration | Amount |
|----------------------|---------------------|---------------|
| DMSO | 1% | 5 mL |
| Bovine Serum Albumin | 2 mg/mL | 1 g |
| Triton-X100 | 0.5% | 2.5 mL |
| PBS | n/a | 492 mL |
| Total | n/a | 500 mL |

Note: Can be stored at 4°C for up to one month. To ensure reproducibility, MTZ is prepared fresh daily and must be kept in the dark.

| Block Solution | | |
|-----------------------------|---------------------|--------------|
| Reagent | Final concentration | Amount |
| Block Buffer | 90% | 9 mL |
| Heat inactivated-Goat Serum | 10% | 1 mL |
| Total | n/a | 10 mL |

Note: Goat serum is inactivated by heating to 56°C for 30 min and can be stored indefinitely at −20°C

△ **CRITICAL:** Block solution is made fresh on the day used.

STEP-BY-STEP METHOD DETAILS

Induction of epithelial cell loss by extrusion

⌚ Timing: 5 h

1. Prepare zebrafish larvae for metronidazole treatment
 - a. Pipet 15–20 day 4 post fertilization larvae in each well of a 12 well tissue culture plate.
 - b. Prepare treatment groups as follows
 - i. No NTR-mCherry(−), Metronidazole(+)
 - ii. No NTR-mCherry(−), No Metronidazole (−)
 - iii. NTR-mCherry(+), Metronidazole(+)
 - iv. NTR-mCherry(+), Metronidazole(−)

△ **CRITICAL:** The metronidazole (MTZ) induced cell loss assay is always done for 5 h (Figure 2). The key difference between early time point (5 h) and recovery time point (18 h)

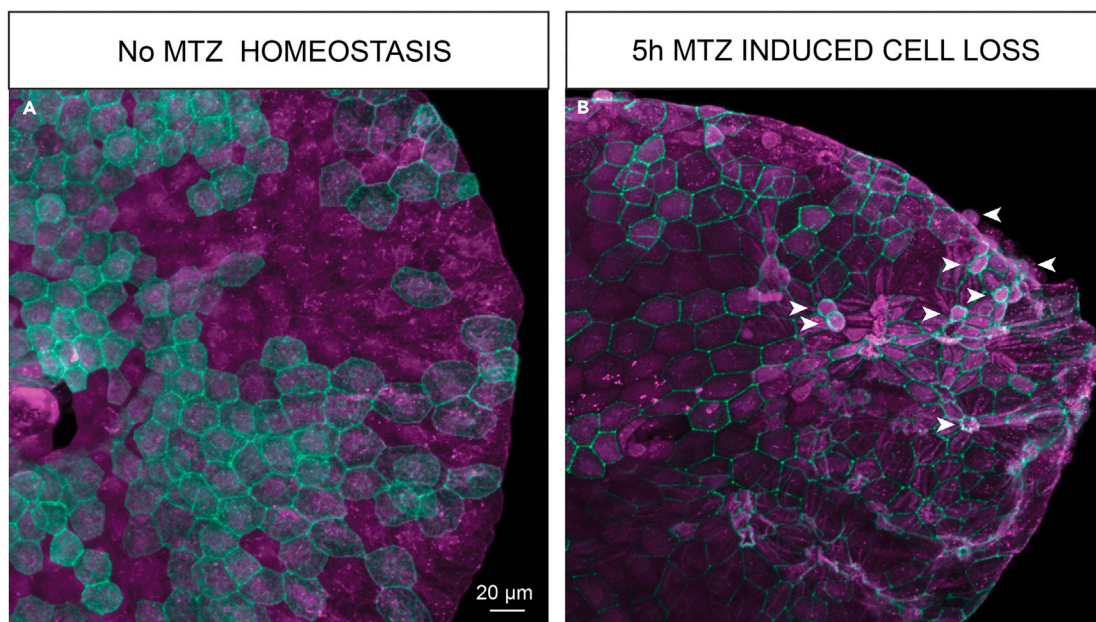


Figure 2. Confocal images of cell loss by extrusion in the larval zebrafish tail fin after MTZ treatment

(A) Confocal image of a wild-type larval zebrafish tail fin. *Et(Gal4-VP16)^{zc1044a};Tg(UAS-E1b:nfsB-mCherry)^{c264}* (MAGENTA), *Tg(UAS-1b:Lifeact-EGFP)^{uttm1}* (GREEN).

(B) Confocal image of cells being extruded (arrowheads) from a larval zebrafish tail fin after 5 h MTZ treatment. *Et(Gal4-VP16)^{zc1044a};Tg(UAS-E1b:nfsB-mCherry)^{c264}* (MAGENTA), *Tg(UAS-1b:Lifeact-EGFP)^{uttm1}* (GREEN).

is that in the latter, MTZ is removed and larvae are allowed to recuperate for 18 h after the initial 5 h induction of epithelial cell loss.

△ **CRITICAL:** If experimental design aims to assay both the initial timepoint after cell loss and the compensatory proliferation observed after damage, all treatment groups during the 5 h damage assay should be doubled.

Note: If screening compounds for enhancement of epithelial restoration additional treatment groups (with/without chemical and with/without induced damage) must be included. In (Wurster et al., 2021), we used human recombinant EGF and EPGN at 250 nM during the induction of damage.

2. Addition of metronidazole.
 - a. Remove as much media as possible and replace with 2 mL of E3 media.
 - b. Add 20 µL of 1 M metronidazole for a final concentration of 10 µM.
 - c. Place all samples at 28.5°C for 5 h.
3. Screening compounds for enhancement of epithelial restoration and function
 - a. Prepare additional treatment groups to include chemical compounds to be screened and their corresponding controls.

Note: When screening chemical compounds, a working concentration should be established, usually within the 1–10 µM range and should be adjusted according to observed tolerance/toxicity.

4. Removing MTZ and chemical compounds.
 - a. Remove E3-MTZ and replace it with 2 mL fresh E3 with no MTZ.
 - b. Replace E3 with fresh E3 two more times with no incubation.
5. Proceed to either Determining the amount of extrusion-induced tissue area loss in the tail fin epithelium (steps 6–8) or [detection and visualization of cell loss-induced proliferation](#) sections of the protocol (steps 12–16).

Determining the amount of extrusion-induced tissue area loss in the tail fin epithelium

⌚ **Timing:** 2 days

The extent of epithelia area loss caused from MTZ treatment and induced extrusion will vary between individual *Et(Gal4-VP16)^{zc1044a};Tg(UAS-E1b:nfsB-mCherry)^{c264}* larvae, therefore methods for determining the extent of cell loss and tissue area reduction between different treatment groups are required. This section of the protocol provides the methodology necessary for measuring the total area of the tail fin in treated larvae, and provides a quick and efficient method for determining the overall extent of tissue area loss (or protection from cell loss and epithelial damage) between a control and experimental group ([Figure 3](#)).

6. Larvae fixation
 - a. Fix larvae for the early damage assay by placing in a 1.7 mL Eppendorf tube containing 1 mL of 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) solution containing 0.05% Triton X-100 and rock on a three dimensional gentle mixer (referred to as a nutator) overnight at 4°C.
7. Sample preparation for imaging.
 - a. After overnight fixation, remove the PFA solution and rinse samples twice with 750 µL PBS-Triton X-100 (0.1%) with no incubation.
 - b. Wash samples with 750 µL PBS-Triton X-100 (0.5%) and rock on mixer for 5 min at room temperature.

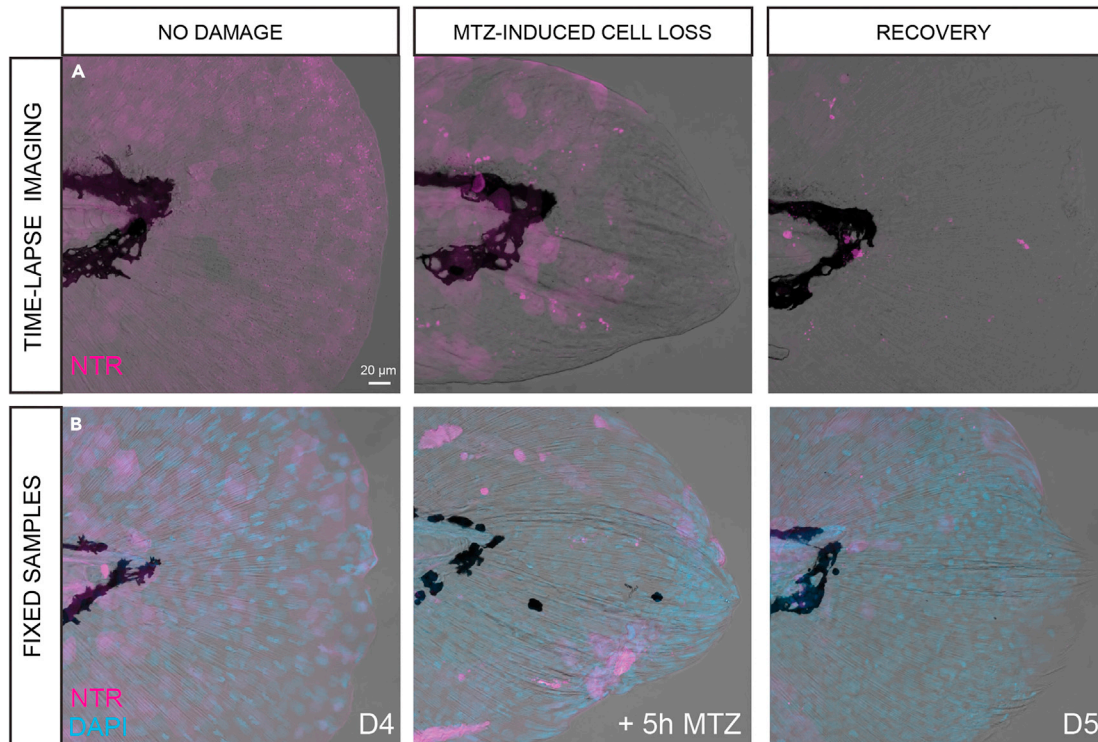


Figure 3. Confocal images of the larval zebrafish tail fin after MTZ-induced cell loss and a 18 h recovery to facilitate tissue restoration
(A) Time-lapse imaging of a larval zebrafish tail fin after 5 h exposure to MTZ and during the 18 h recovery period. *Et(Gal4-VP16)^{zc1044a};Tg(UAS-E1b:nfsB-mCherry)^{c264}* (MAGENTA).
(B) Brightfield images of an untreated larval zebrafish tail fin, after 5 h exposure to MTZ, and after a 18 h recovery period from MTZ-induced cell loss and tissue area reduction. *Et(Gal4-VP16)^{zc1044a};Tg(UAS-E1b:nfsB-mCherry)^{c264}* (MAGENTA) stained with DAPI (BLUE).

- c. Repeat the wash step two additional times.
- d. Prepare a DAPI solution by adding DAPI to PBS-Triton X-100 (0.5%) at 1:1000.
- e. Add 750 µL of PBS-Triton X-100 with 1:1000 DAPI to samples and rock on mixer for 30 min at room temperature.
- f. Wash samples three times with PBS-Triton X-100 (0.5%) and rock on mixer for five min each at room temperature.
- g. Wash samples one time with 750 µL PBS-Triton X-100 (0.1%) and rock on mixer for 5 min at room temperature
- h. Rinse samples with PBS and mix by inverting tube by hand two to three times
- i. Mount larvae microscope slides and seal with clear nail polish

Alternatives: A coverslip sealant such as CoverGrip can also be used to seal the microscope slides.

8. Imaging and quantification of damage to zebrafish epithelia (Figure 4).
 - a. Obtain images of the zebrafish caudal fin.
 - b. Use the polygon tool in Fiji to first draw a vertical line that runs from the dorsal edge of the tail to the ventral edge of the fin. To ensure uniform measurements between samples, always draw the vertical line distal of the notochord.
 - c. Complete the polygon by tracing the distal edge of the caudal fin from the basal end of the notochord until reaching the dorsal edge where the line was started.
 - d. Use the measuring tool in Fiji to calculate the area included in the polygon.
 - e. Divide resulting data into categories based on area thresholds.

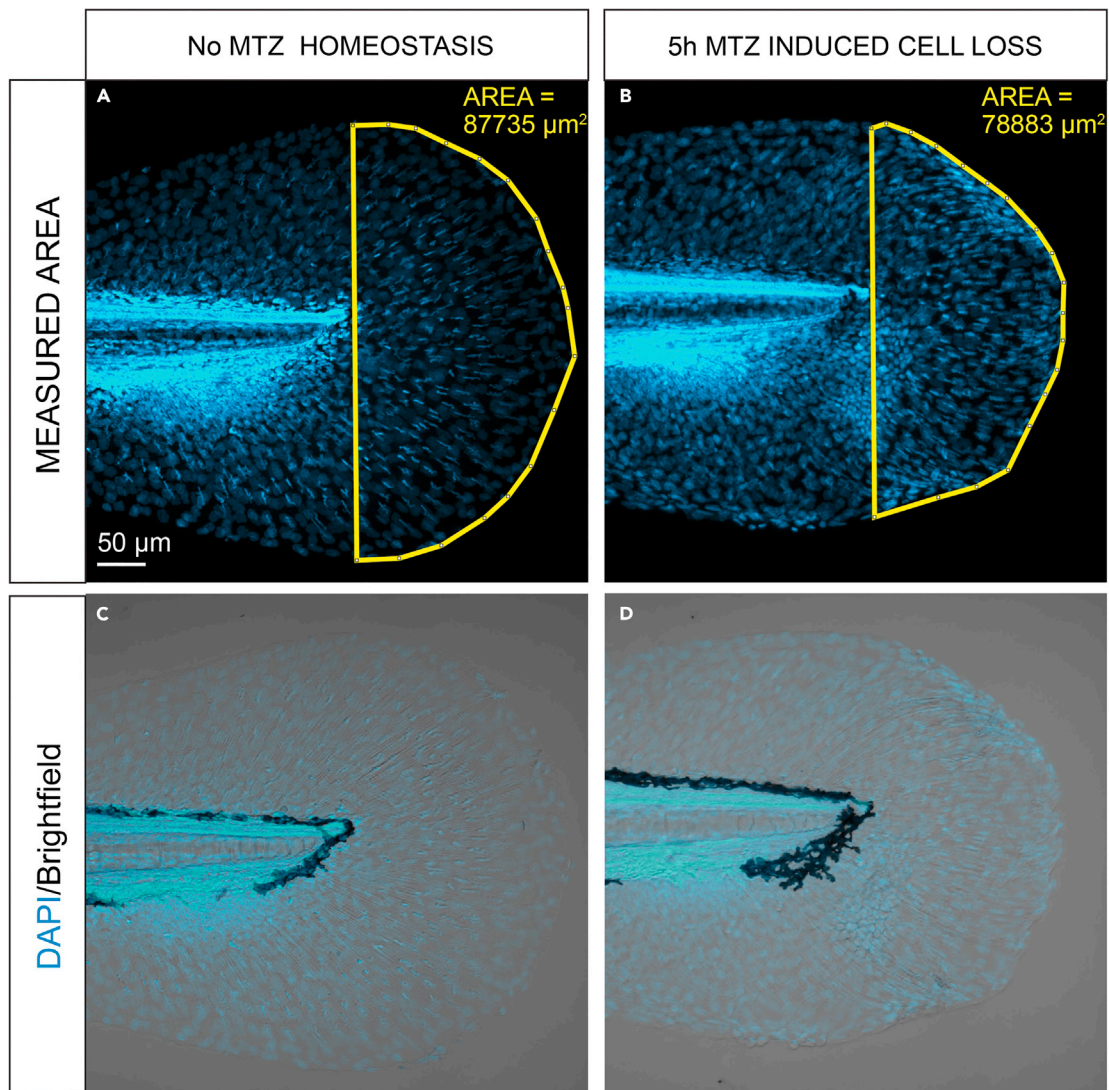


Figure 4. Confocal images of the larval zebrafish tail fin after MTZ-induced cell loss by extrusion

(A) Quantification of the area in the tail fin of an undamaged (Homeostasis) day 4 zebrafish larvae stained with DAPI (BLUE) and measured with the FIJI polygon tool (YELLOW).

(B) Quantification of the area in the tail fin of a day 4 zebrafish larvae after 5 h MTZ treatment (Induced Cell Loss) stained with DAPI (BLUE) and measured with the FIJI polygon tool (YELLOW).

(C) Merge of brightfield image with (3A) stained with DAPI (BLUE).

(D) Merge of brightfield image with (3B) stained with DAPI (BLUE).

△ CRITICAL: In order to accurately measure the fin area, both dorsal and ventral edges of the fin must be captured in the imaging of the tail fin. We find that a 10× image is sufficient for quantification but a 10× with some amount of digital zoom (0.6×) provides a more accurate measurement.

Optional: This approach can also be expanded to include larger regions of the fin, as long as a standardized methodology is uniformly applied.

Note: The semi-automated analysis described here can be scaled up and applied to automated image acquisition and screening platforms, such as the Vertebrate Automated Screening Technology (VAST) (Chang et al., 2012; Jarque et al., 2018).

Determining the molecular signals induced by cell loss in the tail fin epithelium

⌚ Timing: 4 days

MTZ treatment of the *Et(Gal4-VP16)^{zc1044a};Tg(UAS-E1b:nfsB-mCherry)^{c264}* transgenic line results in extensive gene expression changes to promote epithelial tissue repair and regeneration. This section of the protocol describes a modified fixation protocol for downstream use with custom *in situ* probes from Molecular Instruments to detect changes in mRNA of desired target genes using hybridization chain reaction (HCR) (Choi et al., 2018).

9. Larvae fixation

- a. Fix larvae after 5 h MTZ exposure by placing in a 1.7 mL Eppendorf tube containing 1 mL of 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) solution containing 0.1% Triton X-100 and rock on a nutator overnight at 4°C.
- b. Wash larvae twice (5 min each) with 750 μ L PBS.
- c. Wash larvae twice (5 min each) with 750 μ L PBS with 0.1% Tween-20 (PBS-Tween).
- d. Postfix for 20 min with freshly made 4% paraformaldehyde solution and rock at room temp.
- e. Briefly wash larvae twice with 750 μ L PBS-Tween.
- f. Wash larvae twice (5 min each) with 750 μ L PBS-Tween.
- g. Transfer 10–12 larvae into new Eppendorf tube
- h. All subsequent steps can be done in 1.7 mL Eppendorf tubes.

⚠ **CRITICAL:** Our method omits all steps using methanol (dehydration/rehydration) and the proteinase K digestion step from the manufacturer's fixation protocol. This reduces the amount of time between the fixation and the actual *in situ* detection steps. We find that omitting these steps produces reliable results while preserving the tissue morphology of the tail epithelium.

10. mRNA detection by *in situ* hybridization

- a. Follow protocol Multiplexed HCR v3.0 Protocol from Molecular Instruments.

Note: This protocol was used with a HCR V3 custom probe set for *epigen* in zebrafish larvae, XM_001344355.8.

11. Visualization of changes in gene expression within the epithelial cells (Figure 5)

- a. To ensure that differences in gene expression are reproducible, all samples should be mounted as flat as possible for imaging. To achieve this and to ensure that tissue morphology is maintained, samples can be mounted on MatTek dishes (Nunc Brand dishes can be used as an alternative to MatTek) using low melt agarose and imaged using any confocal microscope. For our studies, we used a Zeiss LSM-800 confocal microscope and imaged at both 10 \times and 20 \times .

Note: This fixation and *in situ* detection method can also be applied to identify changes in mRNA expression during the 18 hours after the MTZ recovery step.

Alternatives: This protocol can be modified to accommodate large-scale *in situ* screens by using an automated liquid handler such as the Intavis InsituPro VSi (Bioanalytical instruments).

Detection and visualization of cell loss-induced proliferation

⌚ Timing: 4–5 days

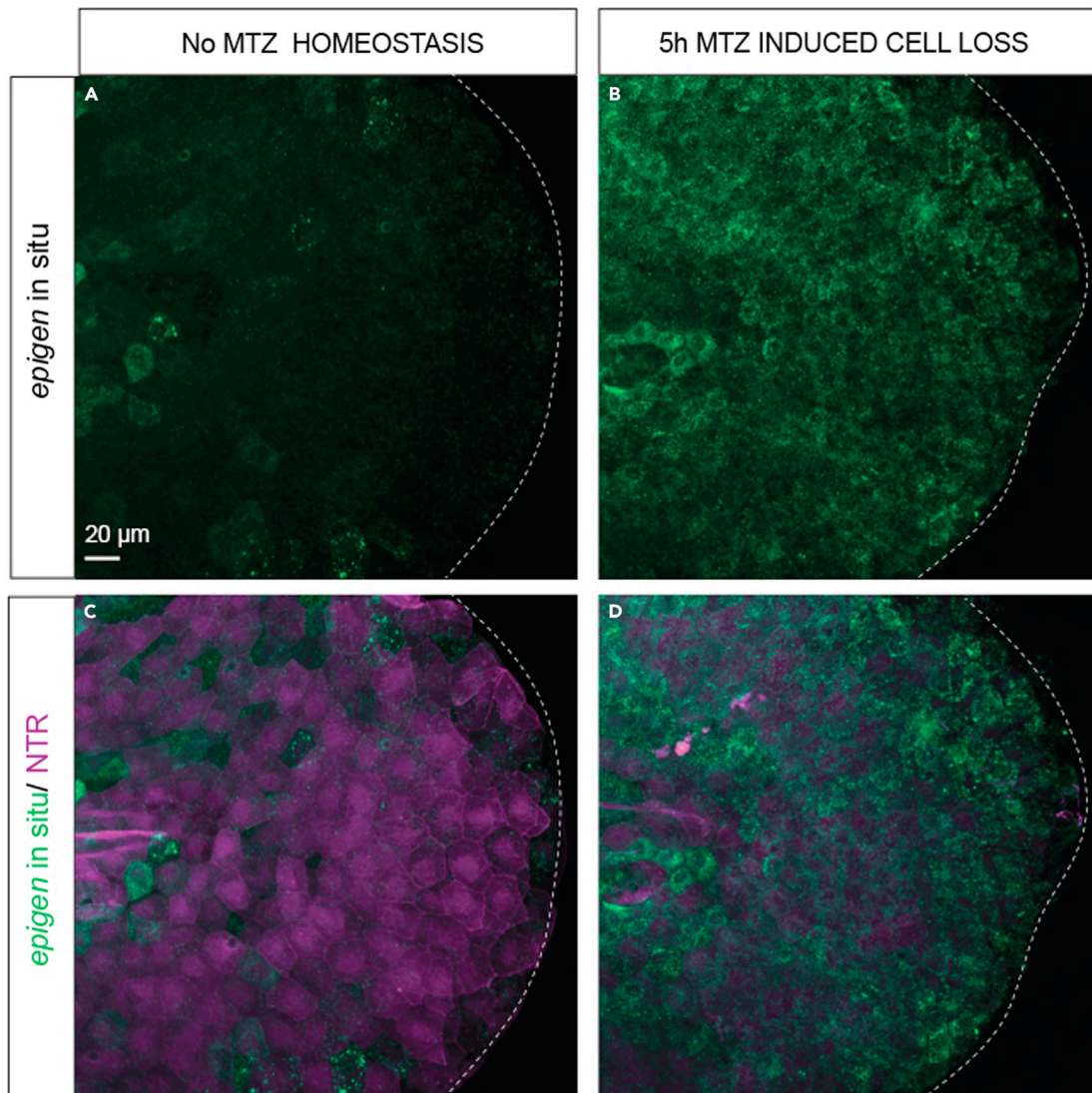


Figure 5. Changes in gene expression in the epithelium after induced cell loss

(A) Confocal images of *epigen* expression (GREEN) in 4dpf tail fin epithelium under homeostatic conditions.
 (B) Confocal images of *epigen* expression (GREEN) after induced cell loss by extrusion.
 (C and D) Merge of *epigen* (GREEN) *in situ* signal with NTR-mCherry (MAGENTA).

Acute treatment of *Et(Gal4-VP16)^{zc1044a};Tg(UAS-E1b:nfsB-mCherry)^{c264}* larvae with MTZ results in extensive cell loss and epithelial tissue damage. For this assay, we remove the MTZ and provide an 18 h recovery period to allow for the generation of new cells to replace the lost cells and restore tissue form and function. One of the hallmarks of tissue repair after damage is the induction of cell division aimed at restoring overall cell numbers (Figure 6). This section of the protocol describes the framework for detecting dividing cells during tissue recovery and provides a method for quickly quantifying cell division in different treatment groups.

Note: Steps 12–16 can be performed after Step 4 in the protocol.

12. Larval recovery from damage.
 - a. Wash each well three times with 2 mL of E3 media.

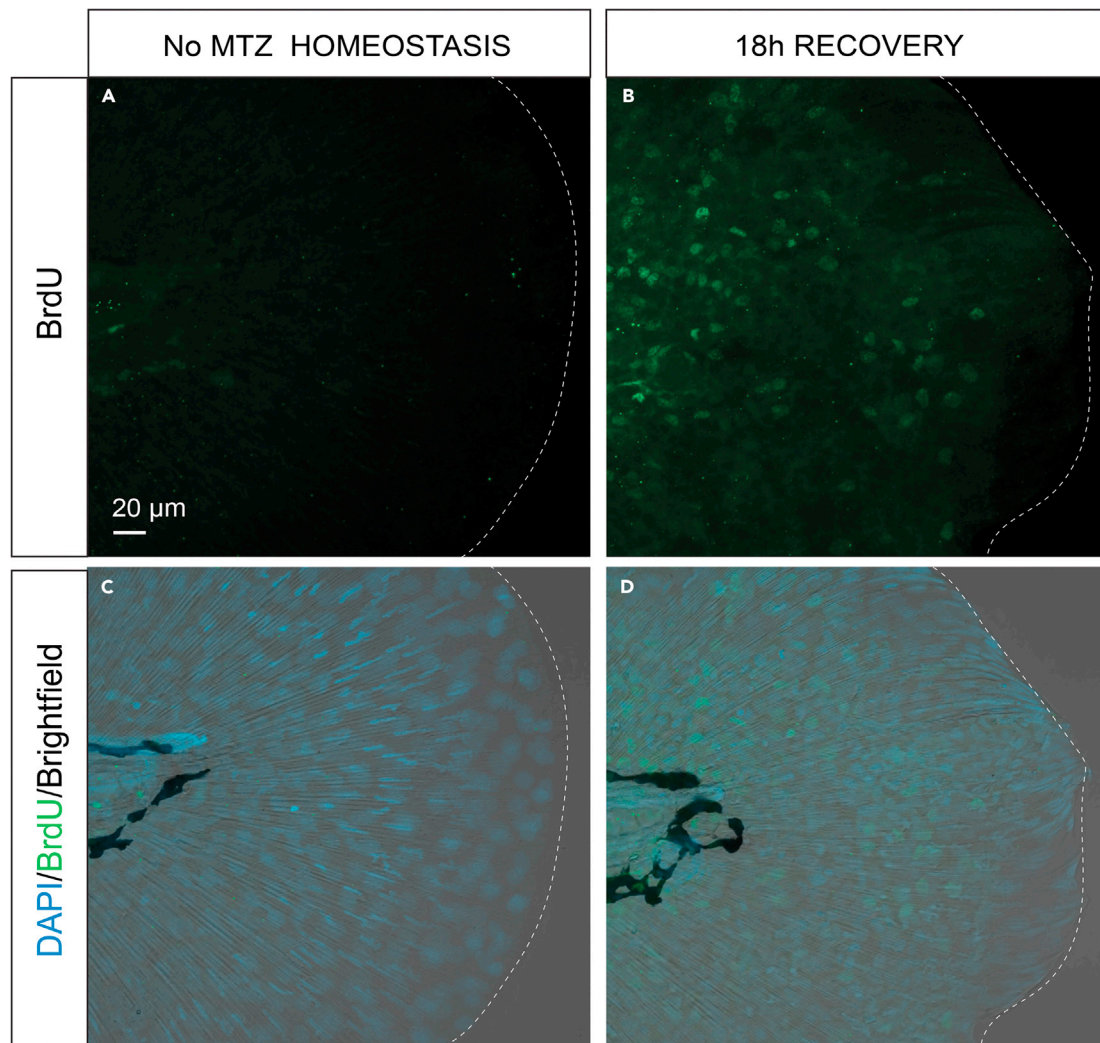


Figure 6. Recovering larval zebrafish tail fin 18 h after MTZ-induced epithelial cell loss

(A) Confocal images of BrdU staining (GREEN) in the tail fin of an undamaged day 4 zebrafish larvae.
 (B) Confocal images BrdU staining (GREEN) of a recovering zebrafish larvae 18 h after MTZ-induced cell loss and damage.
 (C) Merge of brightfield image with (5A) showing BrdU (GREEN) and DAPI (BLUE) to mark all nuclei.
 (D) Merge of brightfield image with (5B) showing BrdU (GREEN) and DAPI (BLUE).

- b. Replace all epithelial restoration compounds in treatment groups (and their corresponding controls) that are meant for the compensatory proliferation portion of the experiment and place at 28.5°C overnight.
13. BrdU incorporation
 - a. Prepare a fresh 10 mM BrdU+ 5% DMSO solution in E3.
 - b. Replace E3 in recovery wells with 2 mL BrdU solution and place at 28.5°C for 30 min.
 - c. Wash each well 2× with 2 mL of E3 and place at 28.5°C for 30 min.
14. Larvae fixation
 - a. Fix larvae for the early damage assay by placing in 1 mL of 4% paraformaldehyde (PFA) in a phosphate buffered saline (PBS) solution containing 0.05% Triton X-100 and rock on a nutator overnight at 4°C.
15. BrdU detection

- a. After overnight fixation, remove PFA solution and wash samples three times with 750 μ L PBS-Triton X-100 (0.5%) and rock on mixer for 5 min at room temperature.
 - b. Wash samples three times with ddH₂O and rock on mixer for 10 min.
 - c. Replace ddH₂O with 2 N HCL solution in ddH₂O with 0.5% Triton-X100 and incubate at room temperature on a nutator for 45 min
 - d. Wash samples three times with 750 μ L PBS-Triton X-100 (0.5%).
16. Antibody staining
- a. Block for 1 h with 1 mL freshly made Block Solution at room temperature on a nutator
 - b. Dilute primary (Rat-anti BrdU) antibody (1:200) in freshly made Block Solution
 - c. Incubate samples in primary antibody overnight on gentle rocking mixer at 4°C
 - d. Rinse samples three times with 750 μ L PBS-Triton X-100 (0.5%) and mix by inverting the tube by hand two to three times.
 - e. Wash samples three times with 750 μ L PBS-Triton X-100 (0.5%) and rock on a mixer for 20 min each at room temperature.
 - f. Block for 1 h with 1 mL freshly made Block Solution at room temperature on a nutator
 - g. Dilute secondary (Goat-anti Rat) antibody (1:200) in freshly made Block Solution
 - h. Incubate samples in a secondary antibody overnight on a nutator at 4°C (keep samples covered or in dark)
 - i. Rinse samples three times with 750 μ L PBS-Triton X-100 (0.5%) and mix by inverting the tube by hand two to three times.
 - j. Wash samples three times with 750 μ L PBS-Triton X-100 (0.5%) and rock on mixer for 5 min at room temperature.
 - k. Prepare a DAPI solution by adding DAPI to PBS-Triton X-100 (0.5%) at 1:1000.
 - l. Add 750 μ L of PBS-Triton X-100 (0.5%) with 1:1000 DAPI to samples and rock on mixer for 30 min at room temperature.
 - m. Rinse samples three times with 750 μ L PBS-Triton X-100 (0.5%) and mix by inverting the tube by hand two to three times.
 - n. Wash samples one time with 750 μ L PBS-Triton X-100 (0.1%) and rock on a mixer for 5 min at room temperature.
 - o. Rinse samples with PBS and mix by inverting the tube by hand two to three times.
 - p. Samples are ready for mounting and imaging.

Note: Additional nucleotide analogs such as EdU, CldU or IdU can be used as an alternative to BrdU or in conjunction with BrdU in studies requiring multiple rounds of labeling of dividing cells (Bayramli et al., 2017; Tuttle et al., 2010). Briefly, BrdU can be administered at the 18 hour recovery time point to label dividing cells. At this point, the larvae can be allowed to recover and be treated with a different nucleotide analog such as CldU or IdU 24 hours later. Subsequent detection of CldU or IdU with BrdU can distinguish cells that divided at one of the two time points (either BrdU positive or CldU/IdU positive) or cells that divided during both time points (cells that are both BrdU and CldU/IdU positive).

Note: Cell division can also be determined by using antibodies to detect the phosphorylation states of key proteins such as Histone H3. One drawback of this approach is that because H3 phosphorylation is transient the result is that many fewer cells are labeled when compared to nucleotide analogs that are incorporated during S phase such as BrdU.

EXPECTED OUTCOMES

MTZ treatment of *Et(Gal4-VP16)^{zc1044a};Tg(UAS-E1b:nfsB-mCherry)^{c264}* drives accelerated cell extrusion that results in epithelial tissue loss. These methods were used (Wurster et al., 2021) to quantify the extent of MTZ-induced cell loss by measuring the area of the tail fin of the larval zebrafish. This approach also demonstrated that addition of the EGF-receptor ligand EPGN is capable of preventing cell loss by extrusion and abrogated the increase in proliferation to compensate for the lost cells.

QUANTIFICATION AND STATISTICAL ANALYSIS

The large amount of cell extrusion observed after MTZ treatment results in a characteristic phenotype due to tissue contraction after the loss of many cells. Although this is observed in a large percentage of treated animals, the severity of damage may vary greatly from individual to individual. In order to uniformly measure the extent of tissue damage, we use the tip of the notochord as a standard landmark common to all samples (treated vs non-treated). To ensure that tissue changes are solely due to MTZ treatment, we leverage the fact that very little cell proliferation or tissue growth is observed under homeostatic conditions at this particular time in development. In addition to following the experimental guidelines, the experimental setup should consist of at least ~50 larvae per treatment group to accurately capture the entire variation in any given experiment. Certain experimental setups, such as treatment with pharmacological inhibitors, may result in a larger variation in the measured area of MTZ treated samples. In these cases, it becomes useful to implement damage categories where samples whose measured areas fall within established ranges can be binned and treated as entire groups. Using this methodology, we are able to conclude that approximately 51% of larvae exposed to 5 h MTZ treatment display mild loss in tissue area, while 34% of larvae exhibit significant reduction of the tail fin epithelium and the remaining 15% of larvae experience severe tissue contraction and damage (Wurster et al., 2021). Pharmacological treatments can shift these percentages and indicate whether an increase or decrease in cell loss and overall tissue area is occurring in the samples.

One hallmark of tissue repair after damage is the induction of cell division. This assay leverages the fact that very little proliferation occurs with the epithelium during the 4th and 5th day of development. A population of sonic hedgehog expressing cells resides near the edge of the notochord and normally contributes to development and formation of the caudal fin (Hadzhiev et al., 2007). However, we observe proliferation in the basal epithelial cells that express the conserved epithelial stem cell marker TP63 (Bakkers et al., 2002; Lee and Kimelman, 2002; Pellegrini et al., 2001; Senoo et al., 2007). Our analysis revealed that the TP63-positive cells are induced to proliferate and are located outside of the area near the notochord where the *shh*-positive cells reside (Wurster et al., 2021). To accurately quantify the number of cells dividing in the epithelium to replace the lost cells, we generate a Region of Interest (ROI) from just outside the notochord (excluding the *shh*-positive cells at the base of the notochord) to the distal end of the caudal fin from the apical edge to the basal edge of the tail. We next quantify all BrdU-positive cells observed throughout the tail fin epithelium from the most distal edge of the tail fin and anteriorly to the urogenital opening. This strategy provides the most accurate representation of the epithelial response to cell loss without introducing region-specific artifacts. Raw counts of the total measured area or number of proliferating cells can then easily be inserted into GraphPad Prism (or other statistical software) for subsequent statistical analysis. Statistical tests utilized will be determined by the number of treatment groups in any given experimental design.

LIMITATIONS

Animals recover rapidly from the induced epithelial cell loss and show very little mortality, providing a robust platform for quickly screening multiple compounds. A limitation to conducting large high-throughput screens is the need for fast exchange of media in many wells at defined timepoints and may require additional equipment or additional lab personnel.

The *Et(Gal4-VP16)^{zc1044a};Tg(UAS-E1b:nfsB-mCherry)^{c264}* transgene is not expressed in adult zebrafish, and therefore limits experiments associated with this protocol to larval stages. Utilization of the *Et(Gal4-VP16)^{zc1044a}* transgene to drive the expression of the effector line *Tg(UAS-E1b:nfsB-mCherry)^{c264}* results in the inability to use in combination with other Gal4 driven transgenes and may require the construction of direct-driven transgenic constructs.

The current protocol induces MTZ-mediated damage in mCherry positive cells throughout the entire epithelia surface and is not limited to a specific region of the larvae. The high level of NTR expression may pose limitations to certain applications, such as whole tissue ablation.

TROUBLESHOOTING

Problem 1

Fish treated with MTZ do not display a noticeable loss of epithelial cells (Figure 2) or decrease in fin area (Figure 3). Lack of this phenotype indicates improper MTZ concentration and/or delivery (steps 1–5 in the [induction of epithelial cell loss by extrusion](#) section).

Potential solution

MTZ should be made fresh on the day of the experiment. MTZ may fail to go into solution if not placed in a water bath and vortexed thoroughly. Additionally, once opened, the MTZ stock container should be sealed with parafilm and stored at 4°C to minimize uptake of humidity from the environment.

Problem 2

Toxicity of pharmacological agents being used for assays or tested for enhancing epithelial restoration may exhibit toxicity (steps 3 and 4 in the [induction of epithelial cell loss by extrusion](#) section).

Potential solution

Dose response curve experiments should be performed and concentration optimized for all agents used.

Problem 3

Increased mortality during 18 h recovery from MTZ-induced damage incubation (step 12 in the [detection and visualization of cell loss-induced proliferation](#) section).

Potential solution

The wash steps intended to remove MTZ are critical for ensuring that fish survival is optimized. A lack of proper washes results in MTZ retention in the media and can be responsible for increased mortality.

Problem 4

Failed detection of BrdU during compensatory proliferation assay (steps 13–16 in the [detection and visualization of cell loss-induced proliferation](#) section).

Potential solution

A common reason for unsuccessful BrdU detection is caused by a failed DNA denaturation step with HCl. It is critical that HCl solution is prepared fresh with ddH₂O and not a buffered solution such as PBS. An alternative approach to HCl is treatment with DNaseI if necessary.

RESOURCE AVAILABILITY

Lead contact

Further information and request for resources and reagents should be directed to and will be fulfilled by the lead contact, George Eisenhoffer (gteisenhoffer@mdanderson.org).

Materials availability

Transgenic zebrafish lines used in this study are available upon request.

Data and code availability

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

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AUTHOR CONTRIBUTIONS

O.E.R. and G.T.E. conceived the method and protocol. K.M.S. and O.E.R. performed all the experiments and subsequent analysis. O.E.R. and G.T.E. wrote the manuscript.

DECLARATION OF INTERESTS

All authors declare no competing interests.

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