Chapter 17

Quantitative Imaging of Endogenous and Exogenous H_2O_2 Gradients in Live Zebrafish Larvae

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Abstract

Quantitative aspects of extracellular H_2O_2 signaling in animals, such as its spatiotemporal dynamics within tissues, remain little understood. Here we detail an optimized, experimental setup for measuring the dynamics and physiological consequences of extracellular H_2O_2 application to live tissues by intravital biosensor imaging in zebrafish larvae.

Key words Hydrogen peroxide, Diffusion, Gradient, Zebrafish, Biosensor, Intravital, Quantitative

1 Introduction

1.1 An Assay to Address Quantitative Questions of H_2O_2 Tissue Signaling

Increasing evidence suggests that extracellular hydrogen peroxide (H_2O_2) acts as a tissue signal, for instance, by regulating inflammation, healing, and regeneration [1–11]. Underlying molecular mechanisms are beginning to be discovered, including redox signaling through redox-sensitive cysteines in signaling proteins [12, 13]. Yet, several important quantitative questions remain unresolved. For example, how far and fast does extracellular H_2O_2 diffuse in intact tissues? What determines its diffusion length, that is, the range on which it can mediate bystander and paracrine effects? Are these length scales consistent with the physiological effects attributed to H_2O_2? Are there distinct zones in tissues, where extracellular H_2O_2 causes signaling without oxidative damage? Quantitatively addressing such questions requires an animal model (1) in which defined concentrations of H_2O_2 can be locally applied, (2) H_2O_2 diffusion through the tissue can be precisely monitored, and (3) physiological responses, for example, morphological changes, or changes in gene expression, can be measured as a function of distance from the local source of H_2O_2.

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1.2 The Zebrafish Model

Over the last decade, transparent zebrafish larvae have become a popular animal model for investigating wound healing and regeneration and for testing the regulatory role of extracellular H_2O_2 in these processes [10, 14]. The main advantages of the zebrafish model include its amenability for direct intravital imaging and pharmacological approaches and the availability of zebrafish larvae at high numbers. The generation time of zebrafish is ~2–3 months, which allows for rapid implementation of genetic manipulations, for example, through gene editing or fluorescent transgene expression, in a tissue-specific manner if desired. The wound responses of zebrafish are comparable to mammals. Zebrafish and mammals possess largely similar repertoires of immune cells [15, 16]. Unlike the mammalian skin, larval zebrafish epidermis is covered by freshwater. Thus, injury of zebrafish epidermis resembles mucosal injury of mammals, namely, injury of the epithelial linings of the upper digestive tract (esophagus, mouth), which are likewise immersed in a freshwater-like solution, that is, saliva.

1.3 A Modified Zebrafish Tail Fin Injury Assay for Local H_2O_2 Application

In zebrafish larvae, H_2O_2 generated by an epithelial NADPH oxidase at the wound site attracts immune cells and mediates tail fin regeneration through oxidation of Src kinases, probably through generating chemotactic oxidized lipid mediators [1, 3, 17]. Wound-associated H_2O_2 production has been visualized using chemical and genetically encoded fluorescent reporters in live larvae [1]. Studying these endogenous H_2O_2 tissue gradients has provided valuable insights into the physiological functions of reactive oxygen species signaling in live animals.

In typical experiments, the tail fin tips of ~3–4 days old zebrafish larvae are amputated, and wound margin H_2O_2 is measured with appropriate fluorescent sensors as previously described [8]. Endogenous H_2O_2 wound patterns dissipate over approximately 60 min after tail fin tip amputation and have been studied by time-lapse microscopy and genetic/pharmacologic manipulation. Below, we detail a modified experimental strategy that allows for the generation of artificial H_2O_2 gradients at zebrafish tail fin wound margins through H_2O_2 bath application. In our setup, the amplitude and duration of the wound H_2O_2 signal can be experimentally controlled to reveal, for example, sufficiency of H_2O_2 for molecular (e.g., transcriptional activation) or morphological (e.g., wound healing, regeneration, inflammation) tissue responses, as well as dose dependency. In addition, tissue mechanisms of extracellular H_2O_2 uptake, diffusion, and metabolism can be studied by live imaging and reaction-diffusion modeling as previously demonstrated [18].

Hydrophilic compounds, such as H_2O_2, cannot easily diffuse through intact zebrafish epidermis and must be locally supplied through an epidermal opening, such as a wound [19]. Thus, the physiological activity of these compounds is hampered by the

extraordinarily fast kinetics of wound closure in zebrafish larvae [19]. This must be kept in mind for hydrophilic agonist/antagonist experiments using this model, including the experiments described below. Fortunately, the recent discovery of osmotic cues as key signals for wound closure and leukocyte recruitment in zebrafish opened up ways to experimentally circumvent this complication [17, 19, 20]. Namely, bathing larvae in an isotonic solution during injury strongly delays wound closure, without being toxic for incubation times <8 h. While the wound is open, it can be efficiently perfused with hydrophilic substances, such as H_2O_2, for example, to probe their tissue metabolism, spatiotemporal dynamics [18], or paracrine signaling functions in live tissue.

In this chapter, we provide an experimental outline that can be modified according to individual research requirements. This protocol was originally developed to quantitatively study extracellular H_2O_2 reaction-diffusion and responses to local H_2O_2 application. We note that it may be adapted to study other hydrophilic agonists of interest, for example, chemokines or growth factors.

2 Materials

2.1 Reagents

1. pCS2+-HyPer: HyPer (Evrogen) subcloned into the pCS2+ expression vector (*see* **Note 1**).
2. pCS2FA-transposase (Tol2kit construct 396) [21].
3. QIAquick Gel Extraction Kit (Qiagen).
4. NotI restriction enzyme.
5. mMESSAGE mMACHINE® SP6 Transcription Kit (Life Technologies).
6. MEGAclear™ Transcription Clean-Up Kit (Life Technologies).
7. E3 embryo medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$.
8. *N*-phenylthiourea (PTU): 0.2 mM in E3 embryo medium.
9. pME-HyPer: HyPer subcloned into pME-MCS (Tol2kit construct 237) [21] (*see* **Note 2**).
10. p5E-*bactin2* (Tol2kit construct 299) [21].
11. p3E-polyA (Tol2kit construct 302) [21].
12. pDestTol2CG2 (Tol2kit construct 395) [21].
13. TE buffer, pH 8.0.
14. LR Clonase™ II Plus enzyme (Invitrogen).
15. One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen).

16. Luria Broth (LB).
17. Ampicillin solution (1000×): 100 mg mL^{-1} in H_2O.
18. QIAprep Spin Miniprep Kit (Qiagen).
19. KCl solution: 1 M in H_2O.
20. Tricaine solution (20×): 4 mg mL^{-1} Ethyl 3-aminobenzoate methanesulfonate (Sigma), 10 mg mL^{-1} Na_2HPO_4, pH 7.2 (*see* **Note 3**).
21. Isotonic E3 embryo medium: 145 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$.
22. Low-melting agarose solution: 1%, dissolved in E3 embryo medium (*see* **Note 4**).
23. Isotonic low-melting agarose solution: 1%, dissolved in isotonic E3 embryo medium (*see* **Note 4**).
24. H_2O_2 solution (Sigma).

2.2 Equipment

1. Hair loop tool: Squeeze a single hair into a 20–200 μL pipette tip and make a short loop protruding out of the tip.
2. Plastic and glass petri dishes.
3. Sterile plastic transfer pipettes.
4. P-2000 Laser-based micropipette puller (Sutter Instrument Co., or a comparable apparatus capable of pulling microinjection capillaries).
5. Nanoject II Auto-Nanoliter Injector (Drummond, or a comparable microinjection apparatus capable of nanoliter volume injections).
6. 3.5 in. long borosilicate glass microinjection capillaries (Drummond).
7. Dissecting microscope.
8. Olympus MVX10 Macroview microscope (or a comparable stereomicroscope equipped for fluorescence imaging).
9. Micro Needle Knife (Fine Science Tools).
10. Nikon Eclipse Ti microscope equipped with a ×20 plan-apochromat NA 0.75 air objective lens, an Andor Clara CCD (charge-coupled device) camera, a light-emitting diode light source (Lumencor), and a motorized stage (or a comparable inverted microscope).
11. LED light source equipped with 438/57 and 475/28 band-pass filters (Lumencor) (or a comparable light source equipped for excitation at 420 and 500 nm).
12. 535/30 emission filter (Chroma Technology) (or a comparable emission filter equipped for acquisition at 520 nm).

13. 35 mm glass bottom microwell dishes (MatTek Corporation).
14. Four-chamber 35 mm glass bottom dish with a 20 mm microwell (Cellvis).

3 Methods

Inconsistencies in tissue thickness and structure necessitate the use of genetically encoded ratiometric H_2O_2 sensors with high dynamic range to obtain consistent and comparable H_2O_2 gradient measurements in live animals. The fluorescent protein-based H_2O_2 sensor HyPer [22] meets these criteria and has been successfully used by multiple groups to measure H_2O_2 gradients in live zebrafish larvae [1, 4, 17–19, 23, 24]. HyPer-expressing zebrafish larvae can be obtained through the injection of in vitro transcribed mRNA into one-cell stage embryos or by the generation of stable transgenic lines that use tissue-specific promoters to drive HyPer expression. The following sections describe detailed methods utilizing HyPer to measure H_2O_2 gradients in live, larval zebrafish tail fins.

3.1 In Vitro Preparation and Zebrafish Embryo Injection of Capped HyPer mRNA

1. Linearize ~3 μg of the pCS2+ HyPer construct using the NotI restriction enzyme for ~2 h at 37 °C.
2. Separate the linearized plasmid from any undigested DNA using agarose gel electrophoresis. Excise and purify the linearized DNA from the gel using the QIAquick Gel Extraction Kit according to the manufacturer's instructions. Elute the linearized fragment in 30 μL of ddH_2O.
3. Prepare capped mRNA with the mMESSAGE mMACHINE® SP6 Transcription Kit using ~1 μg of the linearized plasmid as the template in a 20 μL reaction according to the manufacturer's instructions. Incubate the in vitro transcription reaction for a minimum of 2 h at 37 °C.
4. Add 1 μL of TURBO DNase (provided with mMESSAGE mMACHINE® SP6 Transcription Kit), mix, and incubate for 15 min at 37 °C.
5. Purify the in vitro transcribed mRNA with the MEGAclear™ Transcription Clean-Up Kit according to the manufacturer's instructions (*see* **Note 5**).
6. Dilute the capped HyPer mRNA to 0.5 μg μL^{-1} in ddH_2O, and inject ~2.3 nL into one-cell stage embryos of wild-type AB or *casper* fish.
7. Grow larvae to 3–4 days post fertilization (dpf), changing to fresh E3 medium each day prior to imaging. Use larvae that have ubiquitous expression of HyPer for imaging experiments (*see* **Note 6**).

3.2 Preparation of Transgenic HyPer-Expressing Zebrafish

1. Generate a *bactin2*:HyPer construct by carrying out a multisite Gateway® LR reaction with the p5E-*bactin2*, pME-HyPer, p3E-polyA entry clones, and the pDestTol2CG2 destination vector utilizing the LR Clonase™ II Plus enzyme mix according to the manufacturer's instructions.
2. Transform 3 μL of the reaction into 50 μL of One Shot® TOP10 cells (*see* **Note 7**).
3. Pick single colonies and grow up each in 5 mL LB/ampicillin broth overnight.
4. Purify the construct using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions. Elute in 50 μL of TE buffer or ddH_2O (*see* **Note 8**).
5. Prepare capped Tol2 transposase mRNA in vitro using the pCS2FA-transposase construct as the template (*see* **Note 9**).
6. Prepare a 0.1 M KCl solution containing 10.85 ng μL^{-1} of the *bactin2*:Hyper construct and 10.85 ng μL^{-1} of the Tol2 transposase mRNA. Inject ~2.3 nL of this solution into the cytosol of one-cell stage AB or *casper* embryos.
7. At 2–3 dpf, sort out injected larvae that have mosaic HyPer and cardiac EGFP expression. Raise these animals to sexual maturity.
8. Identify germline transgenic founder (F0) fish by crossing the injected adults with wild-type fish and then screening the 2–3 dpf embryos for HyPer and cardiac EGFP expression. Grow the HyPer-expressing F1 embryos to sexual maturity (*see* **Note 10**).
9. Perform all imaging experiments on the progeny of F1 adults mated to wild-type fish.

3.3 Animal Preparation and Tail Fin Injury to Image Endogenous, Wound-Induced HyPer Signal

Damage to larval zebrafish epidermis triggers H_2O_2 production that is generated on a timescale of minutes and can be accurately captured by wide-field microscopy at low magnification (20×) [1]. We typically mount and capture signals from multiple samples (up to four larvae) during one experiment using a motorized stage (*see* Fig. 1).

1. Anesthetize HyPer-expressing 2–4 dpf zebrafish larvae in anesthetizing E3 medium (E3 containing 0.2 mg mL^{-1} Tricaine) (*see* **Note 11**).
2. Apply ~200 μL of anesthetizing E3 medium to the center of a 35 mm glass bottom microwell dish. Transfer larvae to this droplet with a plastic transfer pipette (*see* **Note 12**).
3. With a hair loop tool, move the larvae to the center of the glass bottom dish. If you are imaging multiple larvae, orient them in order that the anterior and posterior ends of all larvae are

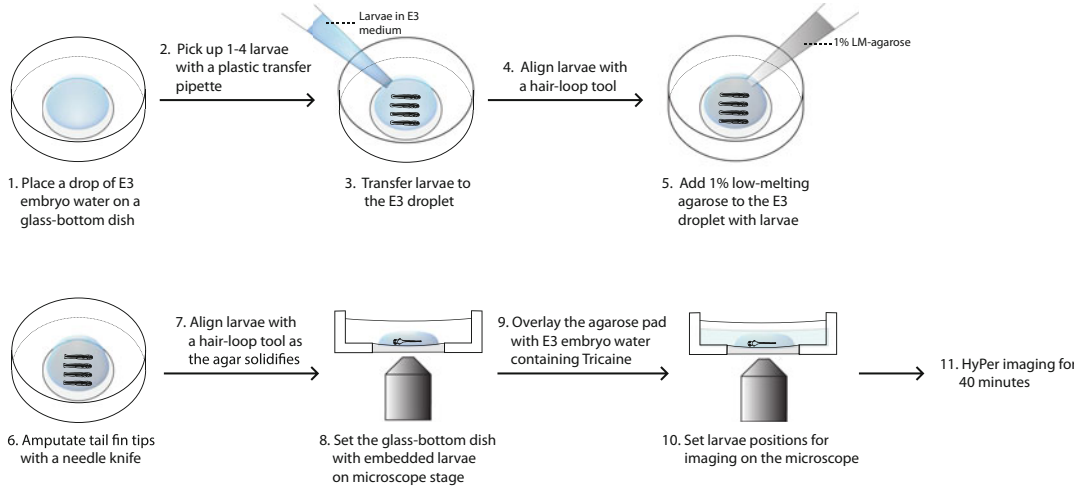

Fig. 1 Experimental schematic for imaging the endogenous, wound-induced HyPer signal in 2–4 dpf zebrafish larvae. 1–4 larvae are embedded in an agarose pad on a glass bottom dish and wounded by tail fin tip amputation (**steps 1–7**). After the agarose solidifies, the dish is set on the microscope stage, and the agarose pad is overlaid with E3 medium (**steps 8** and **9**). Finally, the coordinates of each sample are set, and HyPer imaging is initiated at ~3 min after wounding and proceeds for 40 min (**steps 10** and **11**)

aligned. Next, stack the larvae in column along the dorsal-ventral axes of the animals in the center of the glass bottom dish. Flatten out the tail fins very gently with a hair loop tool.

4. To mount the larvae, gradually mix in ~200 μL of 1% low-melting agarose (dissolved in E3 medium) to the droplet with a plastic transfer pipette. Slowly pipette up and down to create a ~1:1 mixture. After mixing, leave a final volume of ~200 μL (*see* **Note 13**).
5. Using a needle knife, amputate the tail fin tips. Then, quickly realign the larvae, making sure that they are lying with their tail fins flat on the glass bottom dish. We begin imaging the embryos at 3 min after wounding, so note the time of injury (*see* **Note 14**).
6. Fasten the glass bottom dish to the inverted microscope stage. Gently add at least 200 μL of anesthetizing E3 medium to the center of the agarose pad to prevent the sample from drying out during the experiment.

3.4 Imaging the Endogenous, Wound-Induced HyPer Signal

1. Prior to any experiments, mount an unwounded HyPer-expressing larva on a glass bottom microwell dish, and position the sample on the inverted microscope so that the unwounded tail fin is in the field of view.
2. Have the camera set to 8× binning. Using the 20× objective lens, set the illuminating light power and exposure times of the 500 (YFP_{500}) and 420 nm (YFP_{420}) excitation wavelengths so

that the baseline HyPer ratio (YFP_{500}/YFP_{420}) of unwounded tail fin epithelium is ~1. Use unwounded tail fin epithelium at about half the distance from the tip of the tail fin to the tip of the notochord as the reference point (*see* **Note 15**).

3. Set the coordinates of each experimental sample, and choose a focal plane in which the amputated tail fin is clearly in focus under bright-field illumination with a 20× objective lens (*see* **Note 16**).
4. The positions of the experimental samples on the microscope should be set by ~3 min after wounding. At this time, begin imaging. Acquire an image every 1–2 min for at least 40 min (imaging will be completed 43 min after wounding). All images should be acquired at a temperature of 26–28 °C.

3.5 Preparation of Zebrafish Larvae for Imaging Exogenous H_2O_2 Gradients

This method has been optimized for multi-sample imaging in a four-chamber glass bottom dish. This setup permits imaging of multiple samples exposed to four separate conditions (such as four different H_2O_2 concentrations) in a single experiment. We typically mount 1–4 larvae in a single chamber, allowing us to image up to 16 different samples in a single experiment (*see* Fig. 2).

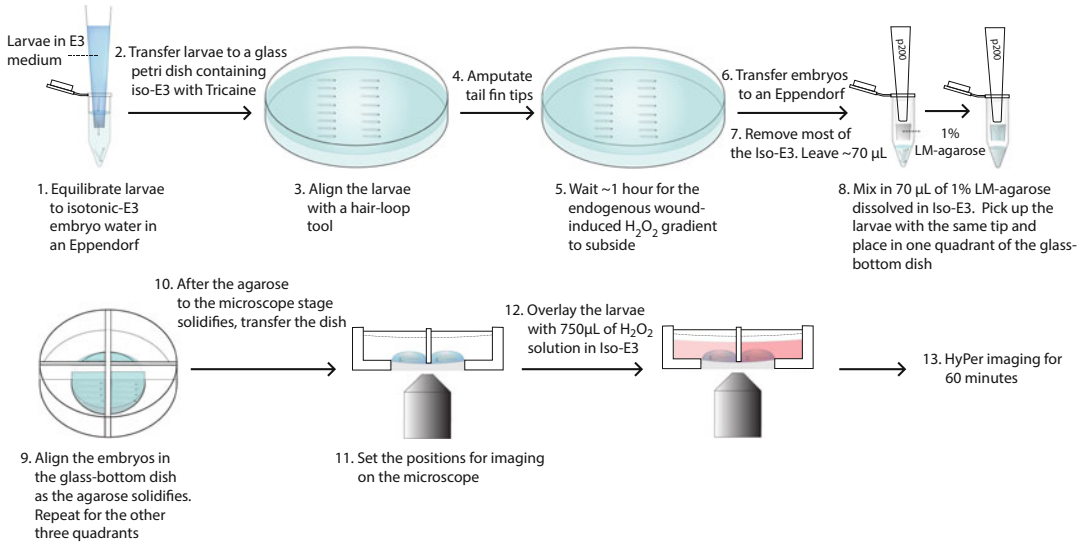

Fig. 2 Experimental schematic for imaging exogenous, H_2O_2-mediated HyPer signals in the larval zebrafish tail fin. Anesthetized 3.5–4 dpf zebrafish larvae are equilibrated to isotonic E3 medium and aligned for tail fin tip amputation in a glass petri dish (**steps 1–3**). Tail fin tips are amputated with a sharpened needle knife, and larvae are incubated in the isotonic E3 bath until the endogenous, wound-induced H_2O_2 gradient has subsided (**steps 4** and **5**). Next, the larvae are transferred to an Eppendorf tube, submerged in a low-melting agarose solution, and transferred to a glass bottom dish (**steps 6–9**). After all the samples are mounted, the glass bottom dish is transferred to the motorized microscope stage, and the coordinates for each sample are set. Finally, the samples are overlaid with H_2O_2 solution, and HyPer imaging is initiated at 60 min after wounding and proceeds for 60 min (**steps 10–13**)

1. Anesthetize HyPer-expressing 3.5–4 dpf zebrafish larvae in anesthetizing E3 medium (*see* **Note 17**).
2. Using a plastic transfer pipette, add 1–4 larvae to an Eppendorf tube containing 1 mL of isotonic E3 medium (iso-E3) supplemented with 0.2 mg mL^{-1} Tricaine. Allow the larvae to settle to the bottom of the tube.
3. Remove the majority of the medium with a transfer pipette, leaving only enough for the larvae to remain completely submerged. Add 1 mL of fresh anesthetizing iso-E3 to the tube. Pipette up and down very slowly with a transfer pipette to suspend the larvae in the medium. Allow them to settle to the bottom of the tube. Repeat this one more time to fully equilibrate the larvae to iso-E3 (*see* **Note 18**).
4. Move the larvae to a glass petri dish filled with anesthetizing iso-E3. Orient the larvae in an optimal position for tail fin tip amputation with a hair loop tool (*see* **Note 19**).
5. Amputate tail fin tips using a freshly sharpened needle knife (*see* **Note 20**).
6. At ~40–45 min after amputation, begin mounting the larvae in the four-chamber glass bottom dish. Transfer larvae that are to be exposed to the same condition in a single chamber to an Eppendorf tube containing 1 mL of anesthetizing iso-E3, and allow them to settle to the bottom of the tube (*see* **Note 21**).
7. Remove the majority of the medium with a transfer pipette, leaving ~70 μL which is enough to completely submerge 4 larvae.
8. Cut off approximately half of a 200 μL plastic pipette tip with a fresh razor blade. With the cut pipette tip and a P200 micropipette, dispense 70 μL of 1% isotonic low-melting agarose to the Eppendorf tube containing the larvae.
9. Gently pipette up the larvae in the same cut pipette tip, and transfer them to a single chamber of the four-chamber glass bottom dish. Quickly align the larvae along their dorsal-ventral axes, and flatten out the tail fins with a hair loop tool before the agarose solidifies.
10. Repeat **steps 6–9** three more times, to mount larvae in the remaining chambers of the glass bottom dish (*see* **Note 22**).
11. Once the agarose solidifies in all the chambers, fasten the glass bottom dish to the inverted microscope stage.

3.6 Imaging Exogenous H_2O_2 Gradients in the Larval Zebrafish Tail Fin

1. Prior to any experiments, mount an unwounded HyPer-expressing larva on a glass bottom microwell dish, and position the sample on the inverted microscope so that the unwounded tail fin is in the field of view.

2. Have the camera set to 8× binning. Using the 20× objective lens, set the illuminating light power and exposure times of the 500 (YFP_{500}) and 420 nm (YFP_{420}) excitation wavelengths in order that the baseline HyPer ratio (YFP_{500}/YFP_{420}) of unwounded tail fin epithelium is ~1. Use unwounded tail fin epithelium at about half the distance from the tip of the tail fin to the tip of the notochord as the reference point (*see* **Note 15**).
3. Freshly prepare H_2O_2 dilutions in iso-E3 supplemented with 0.2 mg mL^{-1} Tricaine (*see* **Note 23**).
4. After fastening the glass bottom dish to the microscope stage, set the coordinates of each experimental sample, and choose a focal plane in which the amputated tail fin is clearly in focus under bright-field illumination with a 20× objective lens (*see* **Note 16**).
5. At 1 h after amputation, add 750 μL of H_2O_2 in anesthetizing iso-E3 to each chamber.
6. Immediately begin image acquisition. Acquire an image every 1–2 min for at least 60 min. All images should be acquired at a temperature of 26–28 °C.

3.7 Image Processing

To calculate HyPer ratio images and spatiotemporal heatmaps of the time-lapse ratio data, we routinely use open-source programs such as Fiji or CellProfiler combined with custom Python scripts based on the Pandas, SciPy, NumPy, and scikit-image libraries. The following steps should be performed to process the data (*see* Fig. 3):

1. Subtract background frame by frame in Fiji by globally subtracting the average intensity of a region outside the larvae from the raw YFP_{500} and YFP_{420} images (*see* **Note 24**).
2. Create a background mask from the YFP_{500} image by applying automatic thresholding on the stack (Image → Adjust → Auto Threshold; *see* **Note 25**) after median filtering (Process → Filters → Filters (2 pixels)). Divide the 8-bit binary image with a fix value of 255 to set the mask values to "1" and the background values to "0." Finally convert the image to 32 bits, and change the background "0" values to NaNs (following thresholding: Process → Math → NaN Background).
3. Calculate the ratio image by dividing the background corrected YFP_{500} and YFP_{420} images (Process → Image calculator → divide (32 bits)), and apply the 16_colors LUT. To remove the background noise, multiply the ratio image with the Image mask.
4. Manually mask out the region of the notochord (following manual selection: Edit → Clear). This is required to exclude ratio data from regions outside the tail fin tissue. Finally, crop a rectangular region of the image series, in which the

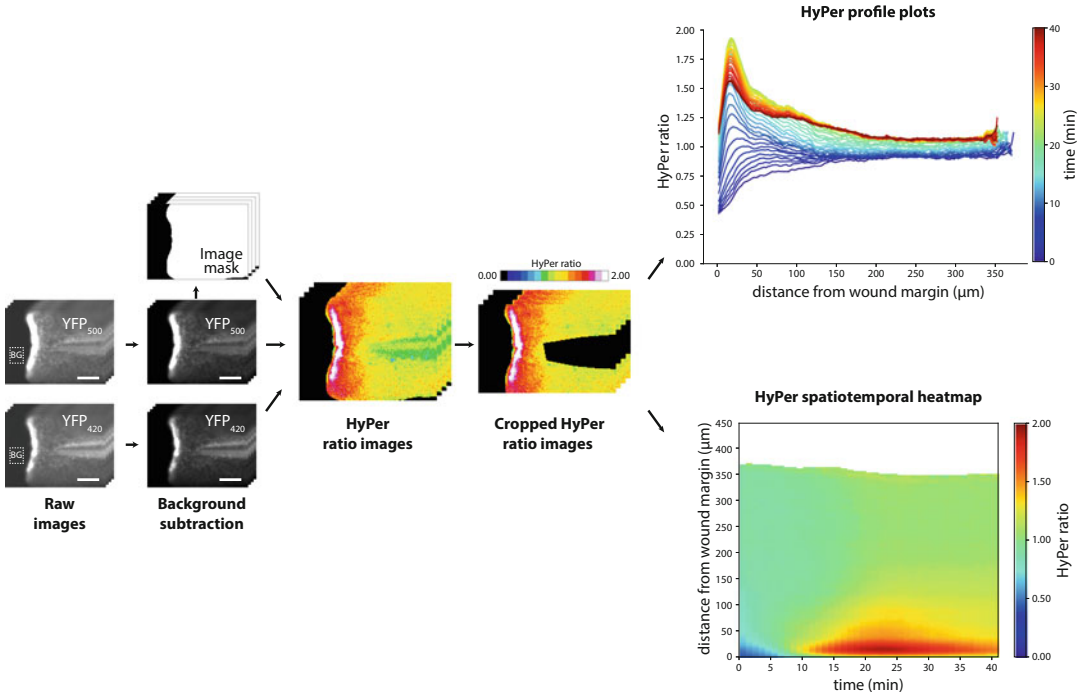

Fig. 3 Workflow of HyPer image processing. HyPer ratio images of wounded TG (bactin2: HyPer) zebrafish are calculated from YFP_{500} and YFP_{420} images after performing background (BG) subtraction and multiplication of the ratio images with a mask derived from the thresholded YFP_{500} image. The notochord region is then manually masked out, and the wounded area of the tail fin is cropped along the vertical axis of the image. The ratio value of every pixel in the time series is then remapped and expressed as a function of binned distance from the wound margin. The data is shown as an average profile plot or spatiotemporal heatmap. Scale bars, 100 μm

contracting/relaxing wound margin fills the vertical axis of the images along the whole time-lapse (*see* **Note 26**).

5. The final step of image processing is to transform the time-lapse image series into averaged profile plots and heatmaps, in which HyPer ratio values of the tail fin are expressed as a function of binned distance from the wound margin. This can be performed in programs such as Python or MATLAB by first applying a distance transform on the cropped HyPer ratio images. The distance transform is an operator which assigns points inside a foreground region (tail fin) the distance closest to the boundary. In our case, the boundary is the wound margin, which is delineated by the background NaN values of the cropped ratio images. HyPer ratio values of each image pixel are thus assigned an exact distance measure from the wound margin. This data is then binned for plotting as individual profile plots for every image timepoint or as heatmaps of the HyPer ratio values as a function of time and distance from the wound margin.

3.8 Possible Downstream Applications and Variations

The amputated larval zebrafish tail fin can be pictured (at least we do it) as a transparent, quasi-1D tissue reaction chamber, a type of "biological microfluidics chamber," in which the spatiotemporal behavior of extracellular H_2O_2 can be microscopically measured with clearly defined temporal (e.g., time of H_2O_2 addition to bath or injury) and spatial (wound margin) reference points. These reference points later allow normalization and integration of quantitative imaging data from different animal experiments into aggregated data matrices that are amenable to downstream analysis by quantitative modeling approaches. For example, by biosensor calibration and implementation of a simplified 1D reaction-diffusion model, we recently inferred extracellular H_2O_2 reaction rates from in vivo data sets generated by the above protocols [18]. Although applied here for studying extracellular H_2O_2, the outlined approach can be, in principle, extended to other hydrophilic substances, such as peptide growth factors or chemokines, given that appropriate imaging reporters are available to monitor their biochemical activity on the cells in the tissue (e.g., Ca^{2+} biosensors for GPCR ligands).

The biosensor imaging assays can be also combined with assays that probe for possible downstream roles of extracellular H_2O_2 gradients, such as leukocyte recruitment, wound closure, regeneration, or spatial regulation of transcriptional regulators (e.g., through in situ hybridization, fluorescence promoter assays). For instance, it could be tested how diffusion length and tissue concentration of extracellular H_2O_2 correlate with functional outputs in vivo. Close spatiotemporal correlations would hereby indicate direct regulatory relationships, and vice versa.

4 Notes

1. Other vectors containing an SP6 RNA polymerase promoter site can be used in place of the pCS2+ vector.
2. Other middle entry vectors, such as the Gateway® pDONR™221 Vector (Life Technologies), can also be used in place of the pME-MCS vector.
3. Prepare anesthetizing E3 medium for larvae imaging fresh each time by diluting Tricaine solution to 1× in E3 embryo medium. Store the 20× Tricaine solution at 4 °C.
4. We find that it is best to prepare these solutions fresh each time. Store the solutions at 42 °C to keep from solidifying. When using pharmacological compounds, add compound to the 1% low-melting agarose immediately before use and mix well.
5. Determine the mRNA concentration by reading the absorbance with a spectrophotometer at 260 nM. Perform agarose

gel electrophoresis on an aliquot of the in vitro transcribed mRNA to test for possible degradation. The 20 μL in vitro transcription reaction generally yields ~20 μg of mRNA. 0.5 μg μL^{-1} is the ideal concentration for mRNA injection into zebrafish embryos. Elute the mRNA from the MEGAclear™ Kit filter cartridge in 25 μL to increase the final concentration of the mRNA.

6. Pigment formation in AB larvae should be inhibited by maintaining the larvae in E3 medium containing 0.2 mM *N*-phenylthiourea (PTU) starting at 24 h post fertilization (hpf). Change this medium daily up to the day of imaging. *Casper* fish, or other pigmentation mutant lines, can be used as alternative.
7. After heat shock, grow up the cells in 250 μL of SOC for at least 1 h at 37 °C shaking. Plate the entire reaction on a LB/ampicillin plate. A successful reaction usually results in a plate with hundreds of colonies. The plate typically consists of clear and opaque colonies. When selecting colonies for overnight growth, select only the clear colonies. These colonies almost always express the construct of interest while opaque colonies never do.
8. Check for positive clones using restriction enzyme digest. We commonly use the PvuII enzyme to test our LR reaction clones.
9. Prepare the Tol2 transposase mRNA using the exact same method described earlier for preparing capped HyPer mRNA with the change of template DNA being the sole variation.
10. We find that screening ~10 injected adults usually results in the discovery of at least one founder fish. When screening F1 embryos for HyPer expression, be sure to obtain clutches of at least 50 embryos each because the transgene will not be transmitted to all progeny.
11. All E3 media should contain 0.2 mg mL^{-1} Tricaine throughout the entire experiment to keep the larvae anesthetized at all times. In experiments with pharmacological compounds, directly add the compound to the anesthetizing E3 medium, and incubate the larvae for 45–60 min prior to wounding. The pharmacological compounds should also be added to the 1% low-melting agarose used for mounting the samples and to the medium overlaying the agarose that prevents it from drying out during the experiment. The effective drug bathing concentrations for zebrafish larvae are typically higher than those used in cell culture or cell-free experiments. Common working concentrations range between ~1 and 100 μM.
12. Multiple larvae can be imaged during the course of a single experiment if a motorized stage is available. We find that

imaging up to four larvae per experiment provides a sufficient amount of time to set up the samples on the microscope for imaging after tail fin tip amputation. Attempting to image more than four samples is not recommended because this usually requires more than the desired 3 min after wounding to set up the samples on the microscope.

13. When mixing in the 1% low-melting agarose, do your best not to move the larvae out of position. This will save you time from having to realign them while the agarose is solidifying.
14. We find that amputating the tail fins up to the tip of the notochord provides us with consistent and comparable results.
15. Ratiometric imaging of HyPer is optimal with excitation wavelengths of 500 and 420 nm and with emission acquired at 516 nm. Because of potential photooxidation of HyPer at strong illuminations, we recommend using neutral density filters and high-sensitivity CCD cameras to reduce the need for strong illumination. Exposure times of up to 300 ms per wavelength can be used. We recommend using high binning (4–8×) because subcellular resolution is not essential.
16. We only use one focal plane for HyPer imaging; consequently, it is important that the larvae tail fins are mounted flat on the glass bottom dish. A tail fin that is not flat will be impossible to focus in a single plane with bright-field illumination. Imaging of these samples will provide inconsistent and incomparable results. We recommend that you do not image these samples.
17. We recommend using 3.5–4 dpf larvae for this experiment. Larvae of this age had the most consistent penetrance and perfusion of exogenous H_2O_2 into the gaping wound.

    In experiments with pharmacological compounds, directly add the compound to the anesthetizing E3 medium, and incubate the larvae for 45–60 min prior to wounding. The pharmacological compounds should be added to the iso-E3 used for equilibration and wounding, the 1% isotonic low-melting agarose used for mounting the samples, and to the H_2O_2 solution that the samples are immersed in during the experiment.
18. Exogenous H_2O_2 can enter the zebrafish larvae through a breach in the epidermis. Amputating the tail fin tips of larvae in their natural hypotonic environment results in rapid wound closure and epithelial barrier reconstitution. Amputation in an isotonic environment blocks wound closure, leaving the wound agape and permitting consistent perfusion of H_2O_2 into the larvae. Prior to amputation, we wash the larvae three times in 1 mL of anesthetizing iso-E3 to equilibrate the larvae to an isotonic environment.

Larvae can stick to the sides of the plastic Eppendorf tube and transfer pipette. Sticking to these surfaces can cause damage to the tail fin epithelium and therefore should be avoided. Always pipette very gently and transfer fish slowly from one medium to another in order to avoid sticking. If larvae get stuck to the Eppendorf tube or transfer pipette, gently pipette the medium up and down until the larvae are knocked off the surface. It is also very important to avoid exposing the fish to air. Any exposure to air will damage the tail fin epithelium of the larvae and render them useless for experiments.

19. Do not amputate the tail fin tips on a plastic petri dish. We find that H_2O_2 perfusion into the wounded tissue is commonly delayed and inconsistent across samples when amputations are done on a plastic surface. We find that amputating tail fins on a glass surface with a sharpened needle knife typically relieves us of these perfusion delays and inconsistencies.

    We recommend aligning the larvae in a pattern that will allow you to amputate a tail fin from one sample and then quickly move on to amputate the next sample without having to realign with a hair loop tool. Minimizing the time difference between tail fin tip amputations synchronizes the endogenous, wound-induced HyPer signal of each sample which is required to subside prior to imaging. Minimal time difference between amputations also results in more consistent and comparable H_2O_2 perfusion into the tissue of the wounded larvae.

20. Always use a sharpened needle knife for tail fin tip amputations. Amputations with a blunt needle knife produce inconsistent and jagged wounds. These wounds are not ideal and result in delayed and inconsistent H_2O_2 perfusion into the wounded tissue across samples.

21. Samples are imaged at ~1 h after tail fin tip amputation. This waiting time is required for the endogenous, wound-induced HyPer signal to subside. We find that it generally takes ~15–20 min to mount larvae in all four chambers of the glass bottom dish. In order to begin imaging at 1 h after amputation, we begin mounting at 40–45 min after amputation. Use this 40–45 min waiting period to prepare the fresh H_2O_2 dilutions that you plan to use for the experiment.

22. To simplify imaging and image processing, mount the larvae in all chambers so that they are all facing the same direction (anterior and posterior ends are aligned).

23. It is best to freshly prepare the H_2O_2 dilutions of interest before each experiment. We recommend preparing the dilutions during the 40–45 min waiting period that is required prior to mounting the larvae. If pharmacological compounds

are being used in an experiment, the compound should be added to the H_2O_2 dilution.

24. Make sure to select a region for background subtraction based on the whole time-lapse, not just a single image, in order to prevent false background subtractions from potential fluorescent objects moving into the field of view during the time-lapse.
25. The adequate thresholding method has to be determined for every imaging setup; we thus recommend to test various thresholding algorithms before making a choice.
26. Depending on the experimental setup, significant wound margin movements can be observed such as closure and contraction during the initial phase of hypotonic wounding and relaxation 30–60 min post isotonic wounding.

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