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A puromycin-dependent activity-based sensing probe for histochemical staining of hydrogen peroxide in cells and animal tissues

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Abstract

Hydrogen peroxide (H_2O_2) is a key member of the reactive oxygen species family of transient small molecules that has broad contributions to oxidative stress and redox signaling. The development of selective and sensitive chemical probes can enable the study of H_2O_2 biology in cell, tissue and animal models. Peroxymycin-1 is a histochemical activity–based sensing probe that responds to H_2O_2 via chemoselective boronate oxidation to release puromycin, which is then covalently incorporated into nascent proteins by the ribosome and can be detected by antibody staining. Here, we describe an optimized two-step, one-pot protocol for synthesizing Peroxymycin-1 with improved yields over our originally reported procedure. We also present detailed procedures for applying Peroxymycin-1 to a broad range of biological samples spanning cells to animal tissues for profiling H_2O_2 levels through histochemical detection by using commercially available anti-puromycin antibodies. The preparation of Peroxymycin-1 takes 9 h, the confocal imaging experiments of endogenous H_2O_2 levels across different cancer cell lines

take 1 d, the dot blot analysis of mouse liver tissues takes 1 d and the confocal imaging of mouse liver tissues takes 3–4 d.

Introduction

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) are a family of transient and redox-active small molecules that have broad functions in biology as signaling agents and/or markers for oxidative stress and damage[1–14]. Indeed, H_2O_2 is a major non-radical ROS that can regulate various downstream targets, including phosphatases, kinases, transcription factors and ion channels, through reversible redox post-translational modifications[15–19]. Aside from mitochondrial activity, an important source of endogenous production of H_2O_2 stems from NADPH oxidase proteins, which are stimulated by growth factors, cytokines, hormones and neurotransmitters[20–29]. NADPH oxidase enzymes play key roles in triggering signaling events that lead to physiological processes such as neural activity and long-term potentiation and depression[30–33], stem cell growth and proliferation[29,34,35], circadian rhythms[36–38] and wound healing[39,40].

Deciphering the complex interplay between sources and targets of H_2O_2 in signaling and stress pathways motivates the development of new methods for probing H_2O_2 across various biological models. In this context, activity-based sensing with small molecule–based fluorescent and bioluminescent probes provides a powerful method to monitor H_2O_2 levels in living systems[41–44]. Our laboratory has advanced this area through the chemoselective H_2O_2-mediated oxidation of boronate protecting groups[41,43,45,46]. Many variations, such as multicolor[47], ratiometric[48,49], two-photon[50], organelle-targeting[51,52], esterase-sensitive[29,53], quinone methide–generating[54] and bioluminescent indicators have been developed[55,56]. These versatile reagents have enabled studies of H_2O_2 biology, offering advantages that include high selectivity and sensitivity, good spatial and temporal resolution and the ability to track H_2O_2 in live cell, tissue and animal models[29,46,53,57,58]. However, cell retention issues minimize the effective use of these reagents in fixed samples.

To address these limitations and expand activity-based sensing of H_2O_2 to fixed specimens, we reported Peroxymycin-1, a novel histochemical probe for H_2O_2 detection that is compatible with fixed-cell and mouse tissue samples (Fig. 1)[59]. Peroxymycin-1 consists of an aryl boronate ester scaffold attached to an alpha-amino group on puromycin, an aminonucleoside antibiotic that can be coupled to nascent proteins synthesized at the ribosome after uncaging the free amine group through H_2O_2-dependent boronate oxidation (Fig. 2a). In our original report, we demonstrated that Peroxymycin-1 can detect H_2O_2 with high selectivity and sensitivity across cells and mouse tissues that are compatible with standard fixation protocols. Specifically, Peroxymycin-1 enables profiling of endogenous levels of H_2O_2 across several types of breast cancer cell lines, showing a correlation between malignancy and higher H_2O_2 levels. Moreover, application to a diet-induced model of nonalcoholic fatty liver disease shows an increase in H_2O_2 levels over healthy controls that is consistent with increased expression of cytochrome P450 2E1 and activity of NADPH oxidases in the liver[60].

Here, we present a detailed protocol for an improved synthesis of Peroxymycin-1. We also detail staining procedures for Peroxymycin-1–treated cell and tissue samples with immunodetection using a commercially available anti-puromycin antibody. The aim of the present protocol is to facilitate the broader use of Peroxymycin-1 by providing a step-by-step procedure with detailed notes for critical steps as well as troubleshooting of potential technical issues. Notably, we have optimized the synthesis of Peroxymycin-1, along with a negative control compound that reacts with H_2O_2 but does not generate puromycin (Ctrl-Peroxymycin-1), where the two-step reaction sequence described in the original report is now performed in a one-pot reaction procedure with improved yield by omitting aqueous work-up and column chromatography purification steps (Fig. 2). This improved synthetic procedure can yield >60 mg of the final product within 1 d. Peroxymycin-1 synthesized by following the optimized protocol also showed less hydrolysis of the boronate pinacol ester to the boronic acid compared to Peroxymycin-1 synthesized by following the original procedure, confirmed by liquid chromatography-mass spectrometry (LC-MS) analysis (Fig. 2c).

Overview of the procedure

In this protocol, we report an optimized synthesis of Peroxymycin-1 and Ctrl-Peroxymycin-1 and outline detailed procedures for applying the histochemical probe to cell and animal experiments. The H_2O_2 levels stained by Peroxymycin-1 can be analyzed in cell and animal tissue samples by confocal imaging or dot blot analysis. The first section of this protocol outlines the optimized two-step, one-pot reaction for synthesizing Peroxymycin-1 and Ctrl-Peroxymycin-1 (Steps 1–17). The second section describes the application of Peroxymycin-1 to profile endogenous H_2O_2 levels in live cells, demonstrating detection of H_2O_2 by Peroxymycin-1 in live cells and histochemical visualization of the incorporated puromycin with primary and secondary antibodies (Steps 18–31). The use of Peroxymycin-1 in mouse samples is described in the third section, demonstrating the utility of Peroxymycin-1 for profiling H_2O_2 levels in mouse liver tissue by both dot blot and confocal imaging (Steps 32–90).

Design strategy

The design concept of Peroxymycin-1 relies on functionalizing the primary amine moiety of puromycin with an activity-based sensing group that induces a H_2O_2-dependent deprotection event that triggers cascade reactions to generate free puromycin for downstream detection. Peroxymycin-1 consists of a puromycin attached to a carbamate-linked aryl boronate pinacol ester (Fig. 2). Oxidative deprotection of the boronate pinacol ester upon treatment with H_2O_2 leads to linker self-immolation and subsequent release of a 1,4-quinone methide species, CO_2 and free puromycin. Puromycin can then incorporate into nascent proteins at the ribosome. The extent of covalent puromycin incorporation thus serves as a proxy for the H_2O_2 level of the sample and can be visualized by immunofluorescence techniques using a standard anti-puromycin antibody. As a negative control, Ctrl-Peroxymycin-1 bears an extra methylene unit in the self-immolative linker, separating the carbamate-linked puromycin from the aryl boronate pinacol ester. The negative control probe is still vulnerable to oxidative deprotection in the presence of H_2O_2, but the electron cascade process to release free puromycin cannot occur.

Limitations

Because Peroxymycin-1 requires the activity-based sensing reaction to occur along with puromycin incorporation into nascent proteins, Peroxymycin-1 requires a relatively long probe incubation time for signal processing (up to 4 h) compared to traditional fluorescent probes (5–30 min). As such, Peroxymycin-1 is not designed for real-time detection of dynamic changes of H_2O_2 in a narrow time window, but rather as a probe for profiling relative H_2O_2 levels across various samples and/or before and after an external stimulus. Because of the incorporation process of uncaged puromycin at the ribosome, Peroxymycin-1 does not provide localized, subcellular information of H_2O_2 levels. This attribute is in contrast to genetically encoded fluorescence proteins (e.g., HyPer[61] and peroxiredoxin-based probes[62]) and traditional small-molecule fluorescence probes with an organelle-targeting localization handle (e.g., MitoPY1)[51,52,63,64].

Comparison of Peroxymycin-1 to other imaging-based H_2O_2 probes

In addition to the high selectivity exhibited by Peroxymycin-1 toward H_2O_2, which is analogous to previously reported boronate-based molecular imaging probes, a key advantage of Peroxymycin-1 is its compatibility with standard immunofluorescence experimental platforms without relying on any genetic manipulation. This feature enables H_2O_2 detection in live cell and animal samples, including mouse tissue, through signal amplification by a desired secondary antibody–fluorophore conjugate. Unlike traditional fluorescent probes for which dye leakage can decrease the signal-to-noise ratio, puromycin is covalently incorporated into nascent proteins after reaction with H_2O_2 so that no probe leakage occurs. Comparison of Peroxymycin-1 with other fluorescence-based sensors is summarized in Table 1. For example, Peroxyfluor-2 is a turn-on fluorescent probe for H_2O_2 with the same boronate trigger as Peroxymycin-1, and its utility has been shown in various live-cell samples[47]. However, this fluorogenic probe is limited to short-term imaging because of limited probe retention in cells, which also makes it difficult to use in tissue and animal samples. Genetically encoded fluorescence proteins offer a complementary set of tools to monitor H_2O_2 level reversibly and ratiometrically in biological samples[61]. Genetic encoding enables expression in specific regions of a cell for organelle-specific H_2O_2 detection in cells and animal models spanning nematodes, zebrafish and plants. Some limitations of genetically encoded probes are their sensitivity to changes in pH in the physiological range; the need for transfection, which can increase incubation time; and complications related to probe concentration compared to that of small-molecule probes.

Finally, immunohistochemical staining of 4-hydroxy-nonenal (4-HNE) is widely used as a marker for oxidative stress and damage in animal tissue samples[65]. This aldehyde product is produced when any one of a number of ROS, such as O_2^-, H_2O_2, hydroxyl radicals and peroxynitrite, acts as a general oxidant. Because of the commercial availability of the anti-4-HNE antibody, this method has seen widespread use. However, the technique is not able to detect ROS directly, much less afford specificity to a particular ROS. 4-HNE staining is also not compatible with live-cell imaging applications.

Applications

Peroxymycin-1 has been successfully used to profile H_2O_2 levels in human cervical epithelial carcinoma cells (HeLa), human epidermoid carcinoma cells (A-431), human breast carcinoma cells (MDA-MB-231 and MDA-MB-468), non-tumorigenic breast epithelial cells (MCF-10A), invasive breast cancer cells (HS 578T) and normal breast cells (HS 578 Bst)[59]. Given these results and because puromycin labeling has found widespread use as a staining platform, we anticipate that cell lines and biological specimens compatible with the anti-puromycin antibody should be compatible with Peroxymycin-1 in a similar experimental setup with not only dot blot and confocal imaging analysis as described in this protocol, but also techniques such as western blot[66], FACS[67] and ELISA[68]. Blot analyses may be appealing because the technique is inexpensive and does not require use of operationally difficult equipment, such as the confocal microscope. In addition to mouse liver, some examples of cell lines and animal samples that have been studied by using puromycin labeling and detection with the anti-puromycin antibody include neurons[69], *Drosophila* larvae[70], and zebrafish[71].

Experimental design

Synthesis of Peroxymycin-1 and Ctrl-Peroxymycin-1—We reported the synthesis of Peroxymycin-1 and Ctrl-Peroxymycin-1 in two steps with an aqueous extraction and column chromatography purification process after the first step[59]. The optimized synthetic procedure reported here takes advantage of a two-step, one-pot reaction setup with only one column chromatography step. Using anhydrous solvent is a key point within this synthesis. Although the reaction is conducted under air, having a large amount of water present in the solution may cause undesired reactivity or decomposition of the starting materials or products. The synthetic procedure is described on the hundred milligrams scale with an overall yield of ~56% for both Peroxymycin-1 and Ctrl-Peroxymycin-1. To conduct the synthesis on a larger scale, users may need to optimize the reaction conditions and purification processes, although in theory, the method outlined in this protocol should be applicable. HPLC may be a viable method for purification, although hydrolysis of the boronate pinacol ester may be observed under aqueous purification conditions.

Applying Peroxymycin-1 and controls for cell experiments—In this protocol, we profiled endogenous H_2O_2 levels in MDA-MB-231, MDA-MB-468, MCF-10A, HS 578T and HS 578Bst cells, but Peroxymycin-1 has also been successfully used to label A-431 and HeLa cells[59]. We anticipate that cell lines and conditions compatible with the anti-puromycin antibody will be compatible with Peroxymycin-1 in a similar experimental setup. However, when using different cell lines or conditions than those described in this protocol, exact parameters for the immunostaining processes such as permeabilization, blocking and antibody incubation time/concentration should be optimized experimentally or based on literature precedents.

Depending on the cell line and conditions, the generation of free puromycin within a cell sample may affect its protein synthesis rate, and Peroxymycin-1 should be normalized by a control puromycin signal to account for possible differences in the protein synthesis rate between samples. A control sample incubated with puromycin should be set up alongside

the Peroxymycin-1 and Ctrl-Peroxymycin-1 samples, and the fluorescence intensity of the control should be used to normalize the Peroxymycin-1 and Ctrl-Peroxymycin-1 signals (experiment described in Anticipated results, and normalization method described in Supplementary information, Data analysis). When using Peroxymycin-1 for the first time with cell lines not reported within this protocol, the puromycin control experiment should always be carried out to ensure that the possible differences in protein synthesis rate between samples does not cause bias in results.

Materials

Biological materials

- Cells: MDA-MB-231, MDA-MB-468, MCF-10A, HS 578T and HS 578Bst cells (UC Berkeley Tissue Culture Facility, RRIDs CVCL_0062, CVCL_0419, CVCL_0598, CVCL_0332 and CVCL_0807, respectively) **!CAUTION** Cells should be regularly checked to ensure that they are authentic and are not infected with mycoplasma ▲ **CRITICAL** The cells used in this protocol were cultured in the UC Berkeley Tissue Culture Facility, but cells from other companies are a suitable alternative. We recommend cells from American Type Culture Collection (e.g., HeLa cells, cat. no. ATCC CCL-2).

- Mice: 8-week-old male C57BL/6 mice (Jackson Laboratory) **!CAUTION** All animal studies were approved by and performed according to the guidelines of the Animal Care and Use Committee of the University of California, Berkeley. Any experiments involving live mice must conform to relevant institutional and national regulations.

Reagents

Synthesis of Peroxymycin-1 and Ctrl-Peroxymycin-1—!CAUTION All reagents in this section are harmful. Handle with gloves and avoid contact with eyes and skin. Volatile materials should be used inside a fume hood.

- 4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzene ethanol (Combi Blocks, cat. no. 651030–55-6) **!CAUTION** 4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzene ethanol is harmful.

- 4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl alcohol (TCI, cat. no. T3496)

- 4-Dimethylaminopyridine (DMAP; Oakwood Chemical, cat. no. 001704) **!CAUTION** DMAP is harmful.

- 4-Nitrophenyl chloroformate, 97% (Gas Chromatography) (AK Scientific, cat. no. A614) **!CAUTION** 4-Nitrophenyl chloroformate is corrosive.

- Methanol for chromatography (Fisher Scientific, cat. no. A452–4) **!CAUTION** Methanol is harmful and volatile.

- Methylene chloride for chromatography (CH_2Cl_2; anhydrous; Fisher Scientific, cat. no. D143–4) **!CAUTION** Methylene chloride is harmful and volatile.

- *N,N*-Diisopropylethylamine (DIPEA; Sigma Aldrich, cat. no. 387649) **!**
  CAUTION DIPEA is corrosive and volatile.

- *N,N*-Dimethylformamide (DMF; anhydrous; Fisher Scientific, cat. no. D143–4) **!**
  CAUTION DMF is harmful.

- Puromycin dihydrogen chloride (Adipogen, cat. no. AG-CN2–0078) **!**
  CAUTION Puromycin dihydrogen chloride is harmful.

Cell staining

- Donkey anti-mouse antibody–Alexa Fluor 488 conjugate (Invitrogen, cat. no. A21202; RRID: AB_141607)

- DMEM (Gibco, cat. no. 10566–016)

- FBS (VWR, cat. no. 97068–085)

- Hoechst 33342 (Invitrogen, cat. no. H21492)

- Mouse anti-puromycin antibody (Kerafast, cat. no. EQ0001; RRID: AB_2620162)

- PBS (Corning, cat. no. 21–040-CM)

Mouse breeding

- High-fat diet (HFD; 60% calories from fat; Research Diets, cat. no. D12492)

- Regular chow (18% calories from protein; Teklad, cat. no. 2018)

Mouse dot blot experiments

- Bicinchoninic acid (BCA) protein assay reagent A (Thermo Scientific, cat. no. 23223)

BCA protein assay reagent B (Thermo Scientific, cat. no. 23224); URL for BCA protein assay protocol: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0011430_Pierce_BCA_Protein_Asy_UG.pdf&title=VXNlciBHdWlkZTogUGllcmNlIEJDQSBQcm90ZWluIEFzc2F5IEtpddA==

- Donkey anti-mouse antibody–Alexa Fluor 647 conjugate (Invitrogen, cat. no. A-31571; RRID: AB_162542)

- Mouse anti-lamin B antibody (Abcam, cat. no. ab16048; RRID: AB_10107828)

- Nonfat dry milk (Bio-Rad, cat. no. 1706404)

- RIPA buffer (1×) (Boston BioProducts, cat. no. BP-116X) !CAUTION RIPA buffer is harmful. Handle with gloves to avoid contact with eyes.

- Tris buffered saline with Tween 20 (20×) (TBST; Santa Cruz Biotechnology, cat. no. sc-362311)

Mouse tissue immunofluorescence

- 16% formaldehyde aqueous solution (Electron Microscopy Sciences, cat. no.15710-S) !CAUTION Formaldehyde is harmful. Handle with gloves to avoid contact with eyes and skin.

- Goat anti-mouse IgG Fab fragment (Jackson ImmunoResearch, cat. no. 115–007-003; RRID: AB_2338476)

- BSA (Fisher Scientific, cat. no. BP1600)

- Donkey anti-mouse Alexa Fluor 647 (LifeTech, cat. no. A31571; RRID: AB_162542)

- Normal donkey serum (Jackson Immunoresearch, cat. no. 017–000-121; RRID: AB_2337258)

- Sucrose (Fisher Scientific, cat. no. 57–50-1)

- Tissue-freezing medium (TFM; General Data, cat. no. TFM-5)

- Triton X-100 (Fisher Scientific, cat. no. BP151–100) **!CAUTION** Triton X-100 is harmful. Handle with gloves to avoid contact with eyes and skin.

Equipment

Synthesis of Peroxymycin-1 and Ctrl-Peroxymycin-1

- 4-ml Wheaton clear glass vials with caps (VWR, cat. no. 66011–548)

- Analytical balance (Denver Instrument Co., A-200DS)

- Analytical thin-layer chromatography plates (250-μm thickness) (Silicycle, cat. no. NC0898660)

- Biotage automated column (Isolera One Accelerated Chromatographic Isolation)

- Biotage SNAP KP-Sil cartridge, silica, 10 g

- Clamps

- Crystallization dish

- Disposable glass Pasteur pipettes (VWR, cat. no.14673–043)

- Disposable scintillation vials, 20 ml (Wheaton, cat. no. 66011–548)

- Dry solvent purification system

- Gravity convection oven

- LC-MS (Agilent 1220 LC System, Advion expression compact mass spectrometer)

- Magnetic stir plate

- Magnetic stir bars (<1 cm)

- Micropipette, 20–200 μl (Sartorius mechanical pipette)

- Needles (Air-Tite veterinary needles 22 gauge (G) × 4 inch thin, cat. no. 89219–270)

- Nitrile gloves (VWR, cat. no. 32934–080)

- Nitrogen gas (N_2)

- Pie block (Chemglass, cat. no. CG-1991-P-13)

- Pipette bulbs

- Rotary evaporator

- Rubber septa

- Sartorius Biohit Optifit tips (Fisher Scientific, cat. no. 14559469)

- Single-neck, round-bottomed flasks (100–500 ml)

- Spatula

- Syringes (HSW Norm-Ject 1-ml Luer-slip syringes, cat. no. 53548–001)

- VWR disposable culture tubes, 16 × 100 mm (VWR, cat. no. 47729–572)

- Whatman Mini-UniPrep transparent syringeless filters (Fisher Scientific, cat. no. 09–923-102)

- Zorbax SB-C18 column (Agilent, 5.0-μm pore size, 4.6 mm × 250 mm)

- XBridge C18 column (Waters, 3.5-μm pore size, 4.6 × 100 mm)

Cell staining

- Eight-well borosilicate chambered cover glass slide (Lab Tek, cat. no. 155409) or 96-well plate (sterile, clear, flat-bottom polystyrene Tissue Culture (TC)-treated microplate; Corning, cat. no. 3598)

- CO_2 incubator (Thermo Scientific, 3586 Napco series 8000 WJ)

- Confocal microscope (Zeiss LSM 710 or Perkin Elmer Opera Phenix high-content screening system)

Mouse dot blot experiments

- Homogenizer (Fisher Scientific, Bel-Art F650000000)

- Imaging system (Licor Odyssey)

- Nitrocellulose membrane (Bio-Rad, cat. no. 162–0146)

- Orbital shaker (Benchmark Scientific Orbi-blotter low-speed shaker)

- Plastic container for dot blot (dimensions: 3.5 × 5 × 3 cm)

Mouse tissue immunofluorescence

- Confocal microscope (Zeiss LSM 710)

- Cover glass (Fisher Scientific, cat. no. 12–544-18)

- Cryostat microtome (Thermo Scientific Microm HM550)

- Dry ice

- Glass staining jar

- Hydrophobic pen (Vector, cat. no. H-4000)

- Intermediate cryomold (Sakura Finetek, cat. no. 25608–924)

- Kimwipes (VWR, cat. no. 89218–057)

- Microscope slide (Fisher Scientific, cat. no. 12–550-343)

- Nail polish (Fisher Scientific, cat. no. 50–949-071)

- Superfrost microscope slides (Fisher Scientific, cat. no. 12–550-15)

- Surgical scissors

- Tweezers

Reagent setup

Peroxymycin-1, Ctrl-Peroxymycin-1, puromycin stock solution—Make a 1 mM stock (1,000×) of Peroxymycin-1 (molecular weight: 731.5 g/mol), Ctrl-Peroxymycin-1 (molecular weight: 745.4 g/mol) or puromycin dihydrochloride (molecular weight: 544.43) in dimethyl sulfoxide (DMSO) in an Eppendorf tube. No degradation was observed when this solution was stored at −20 °C for 1 year.

Peroxymycin-1 (or Ctrl-Peroxymycin-1 or puromycin) incubation medium—Dilute the 1,000× DMSO stock solution of Peroxymycin-1, Ctrl-Peroxymycin-1 or puromycin in DMEM (no FBS) to obtain a concentration of 1 μM for a total volume of 1 ml. The solution should be freshly prepared before each experiment.

PBS with 4% (wt/vol) formaldehyde—Make a 4% (wt/vol) formaldehyde solution by mixing 3 ml of PBS and 1 ml of 16% (wt/vol) formaldehyde solution. The solution should be freshly prepared before each experiment.

PBS with 0.1% (vol/vol) Triton X-100—Mix 0.1 ml of Triton X-100 and 99.9 ml of PBS. Shake the solution on a rotator for 20 min at room temperature (RT; 19–22 °C). The solution can be stored at RT for >1 week unless obvious precipitation is observed. However, we recommend freshly preparing the solution for each experiment.

Permeabilization buffer—Add 0.1 ml of Triton X-100 to 99.9 ml of PBS. Mix the solution in a rotator for 20 min at RT. The solution can be stored at RT for >1 week unless obvious precipitation is observed. However, we recommend freshly preparing the solution for each experiment.

Anti-puromycin antibody incubation solution for cell experiments—Make an anti-puromycin antibody solution (0.3 μl, 1:500 (vol/vol) dilution) in FBS (15 μl, 10% (vol/

vol)) and PBS with 0.1% (vol/vol) Triton X-100 (134 μl). The solution should be freshly prepared before each experiment.

Anti-mouse antibody incubation solution for cell experiments—Make an anti-mouse antibody–Alexa Fluor 488 conjugate incubation solution (1.5 μl, 1:100 (vol/vol) dilution) in FBS (15 μl, 10% (vol/vol)), 1 μM Hoechst 33342 and PBS with 0.1% (vol/vol) Triton X-100 (134 μl). The solution should be freshly prepared before each experiment.

Mice preparation—Feed 8-week-old male C57BL/6 mice (Jackson Laboratory) regular chow or an HFD for 20 weeks. Measure body weight and food intake weekly. **!CAUTION** All the experiments using mice should be performed in agreement with institutional and governmental guidelines. The procedures in this protocol were approved by and performed according to the guidelines of the Animal Care and Use Committee of the University of California, Berkeley. Any experiments involving live mice must conform to relevant institutional and national regulations.

Peroxymycin-1 stock solution for injection into mice—For dosing mice at 10 mg/kg, prepare a stock solution at 2 mg/ml in PBS/PET (PET: 60% (vol/vol) poly(ethylene glycol) 400, 30% (vol/vol) ethanol and 10% (vol/vol) Tween-80) solution mixture (1:1 (vol/vol)). It is important to first dissolve the Peroxymycin-1 solid in PET completely (sonication and vortex can be used) and then add an equal volume of PBS. The solution should be transparent and must be freshly prepared before each experiment.

TBST solution—Dilute 50 ml of the 20× TBST solution in 950 ml of MilliQ water. The solution can be stored at RT for >1 year unless obvious precipitation is observed.

Anti-puromycin antibody incubation solution for dot blot—Dilute 2 μl of the anti-puromycin antibody in 2 ml of the tissue permeabilization buffer (1:1,000 (vol/vol) dilution). The antibody solution can be reused for future experiments (usually a total of two to three blot experiments). After the experiment, transfer the antibody solution to an Eppendorf tube and add sodium azide solution (0.02–0.05% (wt/vol) final). Store at 4 °C until obvious precipitation is observed.

Anti-lamin B1 antibody incubation solution for dot blot—Dilute 2 μl of the anti-lamin B1 antibody in 2 ml of the tissue permeabilization buffer (1:1,000 (vol/vol) dilution). The antibody solution can be reused for future experiments (usually a total of two to three blot experiments). After the experiment, transfer the antibody solution to an Eppendorf tube and add sodium azide solution (0.02–0.05% (wt/vol) final). Store at 4 °C until obvious precipitation is observed.

Anti-mouse antibody incubation solution for dot blot—Dilute 1.0 μl of the anti-mouse antibody–Alexa Fluor 647 conjugate in 10 ml of the tissue permeabilization buffer (1:10,000 (vol/vol) dilution). The antibody solution can be reused for future experiments (usually a total of two to three blot experiments). After the experiment, transfer the antibody solution to an Eppendorf tube and add sodium azide solution (0.02–0.05% (wt/vol) final). Store at 4 °C until obvious precipitation is observed.

30% (wt/vol) sucrose solution—Dissolve 300 mg of sucrose in 1 ml of MilliQ water. The solution should be freshly prepared before each experiment.

Tissue permeabilization buffer—Dissolve 50 mg of BSA in 1 ml of PBS solution containing 0.5% (vol/vol) Triton X-100. The solution should ideally be freshly prepared before each experiment but can also be stored for 1 month at 4 °C.

Mouse tissue blocking buffer—Dilute 20 μl of the anti-mouse IgG Fab fragment solution in 200 μl of the tissue permeabilization buffer (1:10 (vol/vol) dilution). The solution should be freshly prepared before each experiment.

Tissue blocking buffer—Dilute 200 μl of the donkey serum in 2,000 μl of the tissue permeabilization buffer (1:10 (vol/vol) dilution). The solution should be freshly prepared before each experiment.

Anti-puromycin antibody incubation solution for tissue imaging—Dilute 2 μl of the anti-puromycin antibody in 200 μl of the tissue permeabilization buffer (1:100 (vol/vol) dilution). The solution should be freshly prepared before each experiment.

Anti-mouse antibody incubation solution for tissue imaging—Dilute 0.8 μl of the anti-mouse antibody–Alexa Fluor 647 conjugate in 200 μl of the tissue permeabilization buffer (1:250 (vol/vol) dilution). The solution should be freshly prepared before each experiment.

Procedure

Synthesis of Peroxymycin-1 and Ctrl-Peroxymycin-1 ● Timing 7–9 h

1. Transfer 4-nitrophenyl chloroformate (33.7 mg, 0.167 mmol, 1 equiv.) and DMAP (20.4 mg, 0.167 mmol, 1 equiv.) into a 4-ml glass vial by using a spatula. Charge the vial with a small, oven-dried stir bar. (4-Nitrophenyl chloroformate should be stored in a refrigerator and warmed to RT before weighing.)

2. Transfer 4-(hydroxymethyl) phenylboronic acid pinacol ester (39.1 mg, 0.167 mmol, 1 equiv.) for Peroxymycin-1 or 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl) benzene ethanol (41.4 mg, 0.167 mmol, 1 equiv.) for Ctrl-Peroxymycin-1 into a different 4-ml vial by using a spatula.

3. Add anhydrous CH_2Cl_2 (0.54 ml, obtained from a dry solvent purification system) to both vials by using a syringe and needle. White precipitate will form in the solution of 4-nitrophenyl chloroformate and DMAP.

▲ **CRITICAL STEP** Anhydrous solvent is used, but the reaction is carried out in air, because the reaction is not extremely sensitive to air and moisture.

4. Cap both vials and place in a pie block. Cool to 0 °C for 5 min in an ice-water bath while stirring.

▲ **CRITICAL STEP** Both solutions should be completely cooled to 0 °C before they are mixed with each other.

5. Transfer the 4-nitrophenyl chloroformate and DMAP mixture quickly to the stirring 4-(hydroxymethyl) phenylboronic acid pinacol ester solution on the ice-water bath by using a Pasteur pipette. The white precipitate should solubilize when the two solutions are combined.

6. While stirring, warm the reaction to RT over 2 h.

7. Evaporate volatiles by using a gentle flow of N_2 gas at RT (<30 min).

8. Re-dissolve the off-white chunky powder in anhydrous DMF (0.54 ml, obtained from a dry solvent purification system).

9. Weigh puromycin dihydrogen chloride (100 mg, 0.184 mmol, 1.1 equiv.) into a separate 4-ml vial.

10. Dissolve puromycin dihydrochloride in anhydrous DMF (0.54 ml, obtained from a dry solvent purification system).

11. Add DIPEA (0.19 ml, 1.1 mmol, 6.5 equiv.) to the puromycin solution by using a micropipette.

12. Add the puromycin solution by using a Pasteur pipette into a stirred solution of the carbonate intermediate from Step 8 at RT.

13. Stir the reaction solution at RT for 1 h.

? TROUBLESHOOTING

14. Confirm completion of the reaction by LC-MS analysis. A major peak at ~11 min in the UV chromatogram at 275.0 nm (when running a gradient of 5–95% (vol/vol) acetonitrile in water with constant 0.05% (vol/vol) formic acid over 20 min at 1 ml/min by using the XBridge C18 column denoted in Equipment) corresponds to hydrolyzed Peroxymycin-1 (650 mass-to-charge ratio (*m/z*)) (Fig. 2c).

15. Remove volatiles under a gentle flow of N_2 gas (~2 h) or reduced pressure to obtain a clear yellow solid.

■ PAUSE POINT No noticeable decomposition was observed after storing the crude product in the dark at −20 °C for 3 d. However, it is recommended that the product be purified as soon as possible to minimize decomposition.

16. Purify the product by silica gel column chromatography (column diameter: 2 cm, silica gel height: 8–10 cm) by using CH_2Cl_2 as an eluent by increasing methanol concentration (0–5% (vol/vol)). Load crude material onto the column by dissolving in regular CH_2Cl_2 (0.2–0.4 ml). A Biotage flash purification system was used to run an automated silica chromatography column, but the purification process and solvent gradient yielded the same result when performed on a manual silica column. The retention factor on silica thin-layer chromatography plates was 0.3 in 95:5 CH_2Cl_2/methanol.

17. Confirm the identity and purity of the product by NMR spectroscopy (^{1}H NMR and ^{13}C NMR) and LC-MS. This procedure yielded 70 mg of Peroxymycin-1

and 70 mg of Ctrl-Peroxymycin-1 as off-white solids (overall yields of 57% and 56% for Peroxymycin-1 and Ctrl-Peroxymycin-1, respectively)[59].

■ PAUSE POINT The solid form of the product can be stored in the dark at −20 °C for several years. Stock solution of product in DMSO (10 mM) can also be stored at −20 °C (>1-year shelf life).

? TROUBLESHOOTING

Live-cell H₂O₂ detection with Peroxymycin-1 and cell fixation ● Timing 5–6 h

18. Culture adherent cells in a 96-well plate (70–80% confluency). Remove cell culture medium from each well in a laminar hood or biosafety cabinet. Wash cells two times with 100 μl of DMEM (no FBS).

▲ CRITICAL STEP Steps 18 and 19 should be performed by using sterile reagents and equipment.

19. Add Peroxymycin-1 or puromycin incubation medium (a total volume of 100 μl in each well) and then place the plate in a 5% CO_2 humidified incubator at 37 °C for 4 h.

▲ CRITICAL STEP Additional control experiments with puromycin are highly recommended because the incorporation rate of free puromycin to nascent proteins (i.e., protein synthesis rate) may differ from one sample to another. The normalization process discussed in Supplementary information, Data analysis should be performed accordingly.

20. Remove the medium and rinse cells with 150 μl of PBS per well. Aspirate the PBS subsequently. PBS can be replaced by Hank's buffered saline solution with calcium and magnesium. This wash process can be performed without the sterile setup if cells of interest can tolerate short-term, non-sterile conditions.

21. Rinse one additional time with 150 μl of PBS.

22. To fix cells, add PBS with 4% (wt/vol) formaldehyde solution (150 μl per well) and place the plate on a bench at RT for 10 min. Remove solution from the wells.

23. Rinse three times with 150 μl of PBS.

24. Add 150 μl of PBS.

■ PAUSEPOINT The fixed cells can be stored at 4 °C for several weeks if a sufficient amount of PBS is maintained in each well to cover the cells.

Immunodetection of the incorporated puromycin ● Timing 3–6 h

25. Remove the PBS. To permeabilize cells, add PBS with 0.1% (vol/vol) Triton X-100 solution (150 μl) and place cells on a bench at RT for 5 min.

▲ CRITICAL STEP The permeabilization conditions and the following antibody incubation should be optimized for different cell lines, and an additional blocking step after the permeabilization step may be required for certain cell lines.

26. Remove the PBS with 0.1% (vol/vol) Triton X-100. Add anti-puromycin antibody incubation solution for cell experiments (150 μl) and place the plate in a 37 °C incubator for 30 min.

27. Aspirate the solution and add 150 μl of PBS in each well. Repeat this process three times in total.

28. Add secondary anti-mouse antibody incubation solution in each well for cell experiments (150 μl) and place the plate in a 37 °C incubator for 30 min.

29. Aspirate the solution and add 150 μl of PBS in each well. Repeat this process three times in total.

30. Add in PBS and image cells by using an inverted confocal microscope according to the instrument's protocol. Alexa Fluor 488 was excited at 488 nm, and emission was collected at 500–550 nm. Hoechst 33342 was excited at 375 nm, and emission was collected at 435–480 nm.

? TROUBLESHOOTING

31. Analyze the images by using ImageJ or Harmony high-content imaging and analysis software by measuring fluorescence intensity, following Supplementary information, Data analysis.

H_2O_2 detection in mice with Peroxymycin-1 and harvesting of liver ● Timing 6–7 h

32. Inject 10 mg/kg Peroxymycin-1 stock solution in PBS/PET intraperitoneally into regular- or highfat-diet mice.

33. Place mice in cages for 4 h.

34. Fill the chamber with CO_2 until mice are completely euthanized.

35. Cut the mouse belly and harvest liver tissues.

■ **PAUSE POINT** Harvested liver tissues can be stored at −80 °C, although mounting the tissues in TFM is necessary for the imaging.

Dot blot–based visualization of H_2O_2 level in Peroxymycin-1–treated mouse liver ● Timing 20 h

Tissue lysis

36. Cool down RIPA buffer (1 ml) with ice and add the chilled solution to ~10 mg of the harvested liver sample from Step 35.

37. Homogenize the liver sample on ice by using a homogenizer.

38. Separate the supernatant (the lysate solution) and insoluble materials by centrifugation (16,000g, 15 min, 4 °C).

39. Transfer the supernatant to another centrifuge tube.

▲ **CRITICAL STEP** Steps 37 and 38 may be repeated multiple times because insoluble materials tend to heterogeneously spread in the lysate solution and may be difficult to completely separate in a single centrifugation process.

? TROUBLESHOOTING

40. Quantify the total protein amount (mg/ml) in the lysate by BCA assay, following the manufacturer's protocol (URL available in Materials).

41. Dilute the lysate solution to 1 mg/ml with RIPA buffer.

Dot blot preparation and blocking

42. Spot 2 μl of the lysate solution onto a nitrocellulose membrane, pre-cut to 2×4 cm for 15 spots.

43. Place the membrane on a bench at RT for 20 min, until the spotted droplet dries out.

44. Transfer the membrane into a western blot incubation container.

45. Add milk TBST solution (1 ml, 5% (wt/vol)) to the container.

46. Place the container on an orbital shaker at RT for 30 min.

47. Remove the solution from the container.

48. Add TBST solution (1 ml) and place the container on the orbital shaker at RT for 3 min.

49. Repeat Steps 47 and 48 three times and remove TBST solution from the container.

Antibody staining of dot blot

50. Add anti-puromycin or anti-lamin B1 antibody incubation solution for dot blot to the container with the membrane.

51. Place the container on the orbital shaker at 4 °C overnight.

52. Repeat Steps 47 and 48 five times (for a total of five washes) and remove TBST solution from the container.

53. Add anti-mouse antibody incubation solution for dot blot.

54. Place the container at RT for 1 h.

55. Repeat Steps 47 and 48 five times (for a total of five washes) and remove TBST solution from the container.

56. Image the membrane with a blot-imaging system.

Confocal microscopy visualization of H$_2$O$_2$ level in Peroxymycin-1–treated mouse liver ● Timing 3–4 d

Tissue fixation, preservation and cryosectioning

57. Add PBS with 4% (wt/vol) formaldehyde solution to the liver tissue sample (Step 36) in a 15-ml Falcon tube.

58. Place the tube in a 4 °C refrigerator overnight.

59. Remove the PBS with 4% (wt/vol) formaldehyde solution and add 30% (wt/vol) sucrose solution (10 ml).

60. Place the tube in a 4 °C refrigerator overnight.

61. Trim liver tissue with surgical scissors to isolate the desired region of interest.

62. Transfer the liver tissue to the cryomold by using tweezers.

63. Fill the cryomold with TFM so that the entire liver tissue is fully covered.

64. Place the cryomold on a block of dry ice to freeze the TFM and embedded liver tissue.

■ **PAUSE POINT** The cryomold was stored at −80 °C for 1 d before moving to the next step. Although longer storage may be possible, we generally recommend moving on to the next step as soon as possible.

65. Set the cryostat to −20 °C and thaw the TFM/liver tissue block in the cryostat chamber.

66. Section the liver tissue to 30 μm on a slide by using a cryostat. Collect the tissue slice directly onto a superfrost slide.

67. Allow all slides to dry at RT for   30 min.

■ **PAUSE POINT** The slides were stored at −80 °C for 1 d before moving to the next step. Although longer storage may be possible, we generally recommend moving on to the next step as soon as possible. After removing from −80 °C, rehydrate slides by adding enough PBS to fully cover the tissue on the slide.

68. Blot the area around the tissue with a Kimwipe to remove excess moisture.

69. With a hydrophobic pen, delineate a boundary around the tissue sample, leaving a small space around the liver tissue. Dry the boundary at RT for 5 min.

▲ **CRITICAL STEP** Even when the boundary is being dried, the tissue sample should not be allowed to completely dry out, and a minimal amount of buffer should be applied directly onto the tissue sample with a pipette.

70. Place the slide in a staining jar containing PBS and incubate at RT for 3 min.

71. Transfer the slide into another staining jar containing fresh PBS and incubate at RT for 3 min.

72. Repeat Step 71 two times.

Tissue permeabilization and blocking

73. Remove the slide from the jar and add tissue permeabilization buffer (200 μl) directly on the slide. Place a coverslip on the slide to prevent the sample from drying out. Place the slide on a bench at RT for 20 min.

74. Remove the tissue permeabilization buffer.

75. Add mouse tissue blocking buffer (200 μl), place a coverslip on the slide to prevent the sample from drying out, and place the slide on a rocker at RT for 2 h.

▲ **CRITICAL STEP** The incubation with the mouse tissue blocking buffer is critical when the sample of interest is derived from mouse or when anti-mouse secondary antibody–fluorophore conjugate will be used. If a sample is from different species or the anti-puromycin–fluorophore conjugate is used, Step 75 should be omitted.

76. Remove the mouse tissue blocking buffer.

77. Place the slide in a staining jar containing enough tissue permeabilization buffer to completely cover the sample and incubate at RT for 3 min; then, remove the slide from the jar.

78. Repeat Step 77 twice.

79. Add tissue blocking buffer (200 μl) and place the slide on the bench at RT for 2 h.

80. Remove the tissue blocking buffer.

Antibody staining of the tissue sample

81. Add anti-puromycin antibody incubation solution for tissue imaging (200 μl) and place the slide covered with a coverslip on a bench at RT for 1 h.

82. Remove the anti-puromycin antibody incubation solution.

83. Repeat Step 77 twice.

84. Add anti-mouse antibody incubation solution for tissue imaging (200 μl) and place the slide covered with a coverslip on a bench at RT for 1 h.

85. Remove the anti-mouse antibody incubation solution.

86. Repeat Step 77 twice. Additional staining processes such as DAPI staining may be performed during or after Step 84. A staining/wash protocol for the staining of interest should be followed.

87. Add mounting medium (25 μl).

88. Place a coverslip onto the slide. Remove excess mounting medium coming out of the coverslip by using a Kimwipe.

89. Seal the coverslip with nail polish and dry at RT for 5 min.

90. Image the sample by using a confocal microscope. Alexa Fluor 647 was excited with a 633-nm HeNe laser, and emission was collected on a META detector between 638 and 759 nm.

Troubleshooting

Troubleshooting advice can be found in Table 2.

Timing

With commercially available precursors in hand, the synthesis and purification of Peroxymycin-1 is expected to take 7–9 h. The H_2O_2 detection or visualization is expected to take 7 h for cell samples, 25 h for mouse tissue samples in the dot blot platform and >4 d for mouse tissue samples in the confocal imaging platform.

Steps 1–17, synthesis of Peroxymycin-1 and Ctrl-Peroxymycin-1: 7–9 h including purification

Steps 18–24, live-cell endogenous H_2O_2 detection with Peroxymycin-1 and cell fixation: 5–6 h

Steps 25–31, immunodetection of incorporated puromycin: 3–6 h

Steps 32–35, H_2O_2 detection in mice with Peroxymycin-1 and harvesting of liver: 6–7 h

Steps 36–56, dot blot–based visualization of H_2O_2 level in Peroxymycin-1–treated mouse liver: 20 h Steps 57–90, visualization of H_2O_2 level in Peroxymycin-1–treated mouse liver by confocal microscopy: 3–4 d

Anticipated results

Reactivity and selectivity of Peroxymycin-1 toward H_2O_2 in a test tube

The reaction between Peroxymycin-1 (0.3 mM) and H_2O_2 (0.1 mM) can be carried out in PBS (20 mM, pH 7.4), methanol and DMSO solution mixture (64% PBS, 33% methanol and 3% DMSO (vol/ vol/vol) and analyzed by LC-MS at different time intervals (Fig. 3). For reactivity experiments, LC-MS analysis was performed on an XBridge C18 column by using a gradient elution from 5% (vol/vol) to 95% (vol/vol) acetonitrile in water (constant 0.05% (vol/vol) formic acid) over 20 min at 1 ml/min (see Materials for column specifications) (Figs. 2c and 3a). After 25 min of reaction, the ratio of Peroxymycin-1 to puromycin was 91% to 9%. After 6 h of reaction, the ratio of Peroxymycin-1 to puromycin was 56% to 44% (Fig. 3a). The peak corresponding to the hydrolyzed free boronic acid Peroxymycin-1 product was observed at 11.0 min (650 m/z), and puromycin was observed at 8.9 min (472 m/z) (Fig. 2c). For selectivity experiments, LC-MS analysis was performed on an SB-C18 column by using a gradient elution from 5% (vol/vol) to 95% (vol/vol) methanol in water (constant 0.05% (vol/vol) formic acid) over 12 min and an isocratic elution with 95% (vol/ vol) methanol from 12 to 15 min at 0.5 ml/min (see Materials for column specifications) (Fig. 3b). For ROS selectivity studies, the peak corresponding to the hydrolyzed free boronic

acid Peroxymycin-1 product was observed at 14.2 min, and puromycin was observed at 11.5 min.

Analytical data

Peroxymycin-1—Peroxymycin-1 was obtained in 57% yield (70 mg) as an off-white solid.

^{1}H NMR (500 MHz, CDCl$_3$): δ 8.09 (s, 1H), 8.06 (s, 1H), 7.75 (d, J = 7.6 Hz, 2H), 7.24 (d, J = 7.9 Hz, 2H), 7.10 (d, J = 8.0 Hz, 2H), 6.84 (s, 1H), 6.81 (d, J = 8.2 Hz, 2H), 5.77 (d, J = 7.5 Hz, 1H), 5.64 (s, 1H), 5.08 (d, J = 12.6 Hz, 1H), 4.99 (d, J = 12.8 Hz, 1H), 4.65 (m, 1H), 4.49 (m, 1H), 4.41 (m, 1H), 3.98 (m, 1H), 3.88 (d, J = 12.4 Hz, 1H), 3.74 (s, 3H), 3.65 (d, J = 12.2 Hz, 1H), 3.60–3.26 (br s, 6H), 3.01 (m, 1H), 2.96 (m, 1H), 1.32 (s, 12H).

^{13}C NMR (126 MHz, CDCl$_3$): δ 172.3, 158.7, 156.1, 154.4, 150.0, 146.8, 139.1, 137.8, 134.9, 130.2, 128.1, 126.9, 120.8, 114.1, 91.0, 84.7, 83.9, 73.3, 66.9, 61.9, 56.5, 55.3, 50.8, 38.1, 24.8.

Ctrl-Peroxymycin-1—Ctrl-Peroxymycin-1 was obtained in 56% yield (70 mg) as an off-white solid.

^{1}H NMR (500 MHz, CDCl$_3$): δ 8.11 (s, 1H), 8.06 (s, 1H), 7.71 (d, J = 8.0 Hz, 2H), 7.16 (d, J = 7.6 Hz, 2H), 7.10 (d, J = 8.1 Hz, 2H), 6.83 (d, J = 8.6 Hz, 2H), 6.76 (s, 1H), 5.64 (s, 1H), 5.57 (d, J = 8.0 Hz, 1H), 4.66 (s, 1H), 4.44 (m, 1H), 4.40 (m, 1H), 4.22 (m, 2H), 4.01 (m, 1H), 3.89 (d, J = 12.7 Hz, 1H), 3.74 (s, 3H), 3.67 (d, J = 12.8 Hz, 1H), 3.58–3.32 (br s, 6H), 3.01 (m, 1H), 2.92 (m, 1H), 2.88 (m, 2H), 1.31 (s, 12H).

^{13}C NMR (126 MHz, CDCl$_3$): δ 172.4, 158.8, 156.3, 154.7, 150.1, 141.2, 137.9, 135.1, 130.4, 128.4, 128.4, 120.9, 114.3, 91.2, 84.9, 83.9, 73.4, 65.8, 62.1, 56.5, 55.4, 53.6, 50.9, 38.2, 35.7, 24.9.

Detection of endogenous H$_2$O$_2$ in live cells with Peroxymycin-1 and immunostaining—Prepare a 96-well plate with six wells of each of the following cell types: MDA-MB-231, MDA-MB-468, MCF7, MCF10A, HS 578T and HS 578Bst. Follow Steps 18–31 of the above protocol. At Step 19, treat three wells of each cell type with puromycin incubation medium and three wells with Peroxymycin-1 incubation medium. When imaging the cells, stronger fluorescence should be observed for puromycin-treated cells, and the mean fluorescence intensity measurements of the puromycin-treated cells should be used to normalize the fluorescence signal of Peroxymycin-1–treated samples (Fig. 4).

Detection of H$_2$O$_2$ in mouse tissue with Peroxymycin-1 and immunostaining—Feed 8-week-old male C57BL/6 mice with normal chow (NC) or anHFD for 20 weeks. Following Steps 32–35, inject the mice with Peroxymycin-1 and harvest the liver tissue. For analysis by dot blot, follow Steps 36–56. A higher fluorescence ratio should be observed for anti-puromycin to anti-lamin B1 staining in the HFD mice than in the NC control mice (Fig. 5b,d). For immunofluorescence imaging using confocal microscopy, complete Steps 57–90.

Again, a higher fluorescence signal should be observed for HFD mice than for NC control mice (Fig. 5a,c).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Data availability

Data are available through figshare: Fig. 2c, https://doi.org/10.6084/m9.figshare.16639975; Fig. 3a, https://doi.org/10.6084/m9.figshare.16639570; Fig. 4, https://doi.org/10.6084/m9.figshare.16640434; Fig. 5, https://doi.org/10.6084/m9.figshare.16639537; Supplementary Fig. 1, https://doi.org/10.6084/m9.figshare.18480879; Supplementary Fig. 2, https://doi.org/10.6084/m9.figshare.18480885; Supplementary Fig. 3, https://doi.org/10.6084/m9.figshare.18480888; Supplementary Fig. 4, https://doi.org/10.6084/m9.figshare.18480891; Supplementary Fig. 5, https://doi.org/10.6084/m9.figshare.18480894; Supplementary Fig. 6, https://doi.org/10.6084/m9.figshare.18480897; Supplementary Fig. 7, https://doi.org/10.6084/m9.figshare.18480900; Supplementary Fig. 8, https://doi.org/10.6084/m9.figshare.18480903. Source data are provided with this paper.

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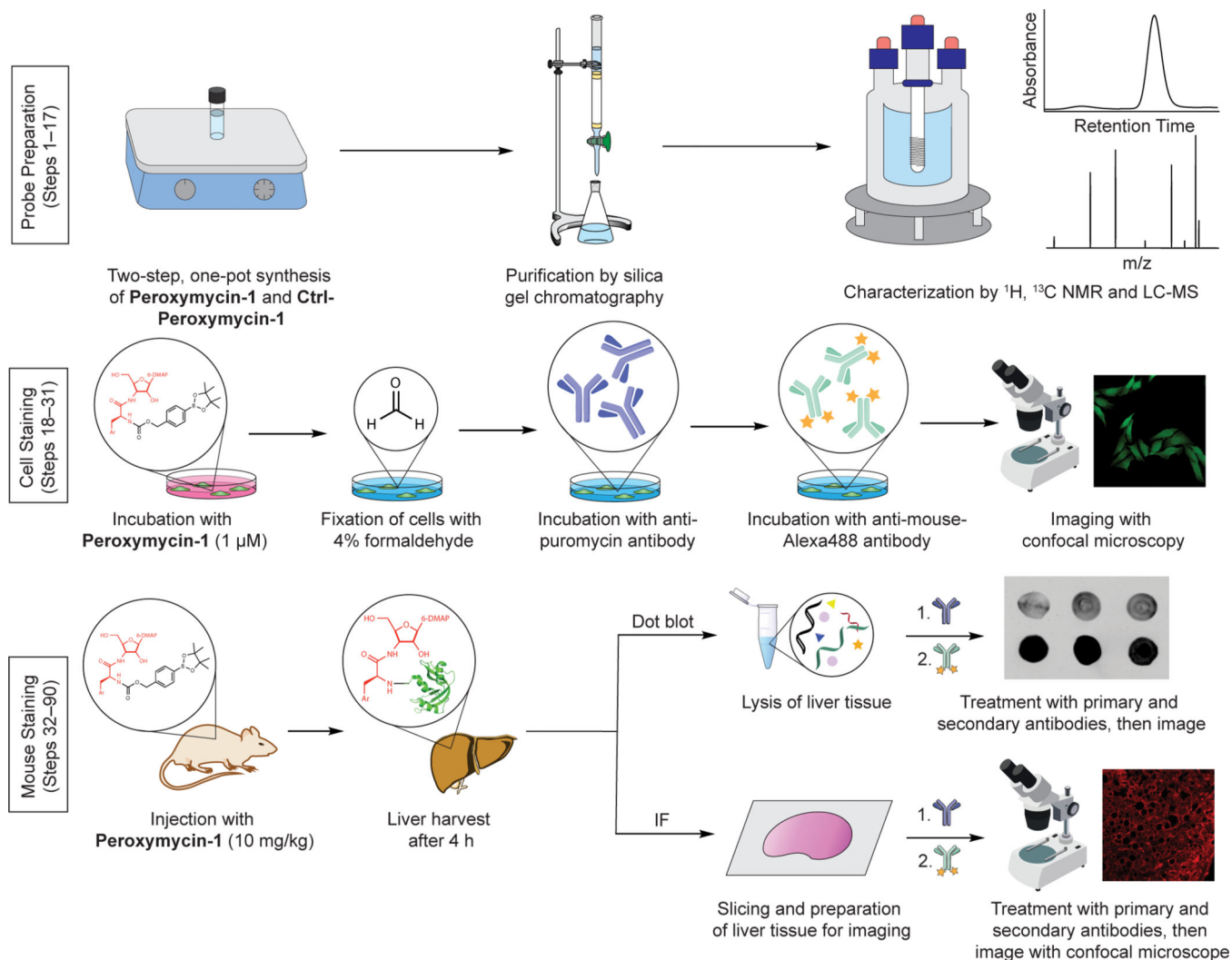
Fig. 1 |. Overview of hydrogen peroxide detection with Peroxymycin-1 in cells and mice.
Ar, 4-methoxyphenyl; IF, immunofluorescence; LC-MS, liquid chromatography-mass spectrometry; 6-DMAP, N,N-dimethyl purin-6-amine. Confocal images reprinted with permission from ref. [59], American Chemical Society.
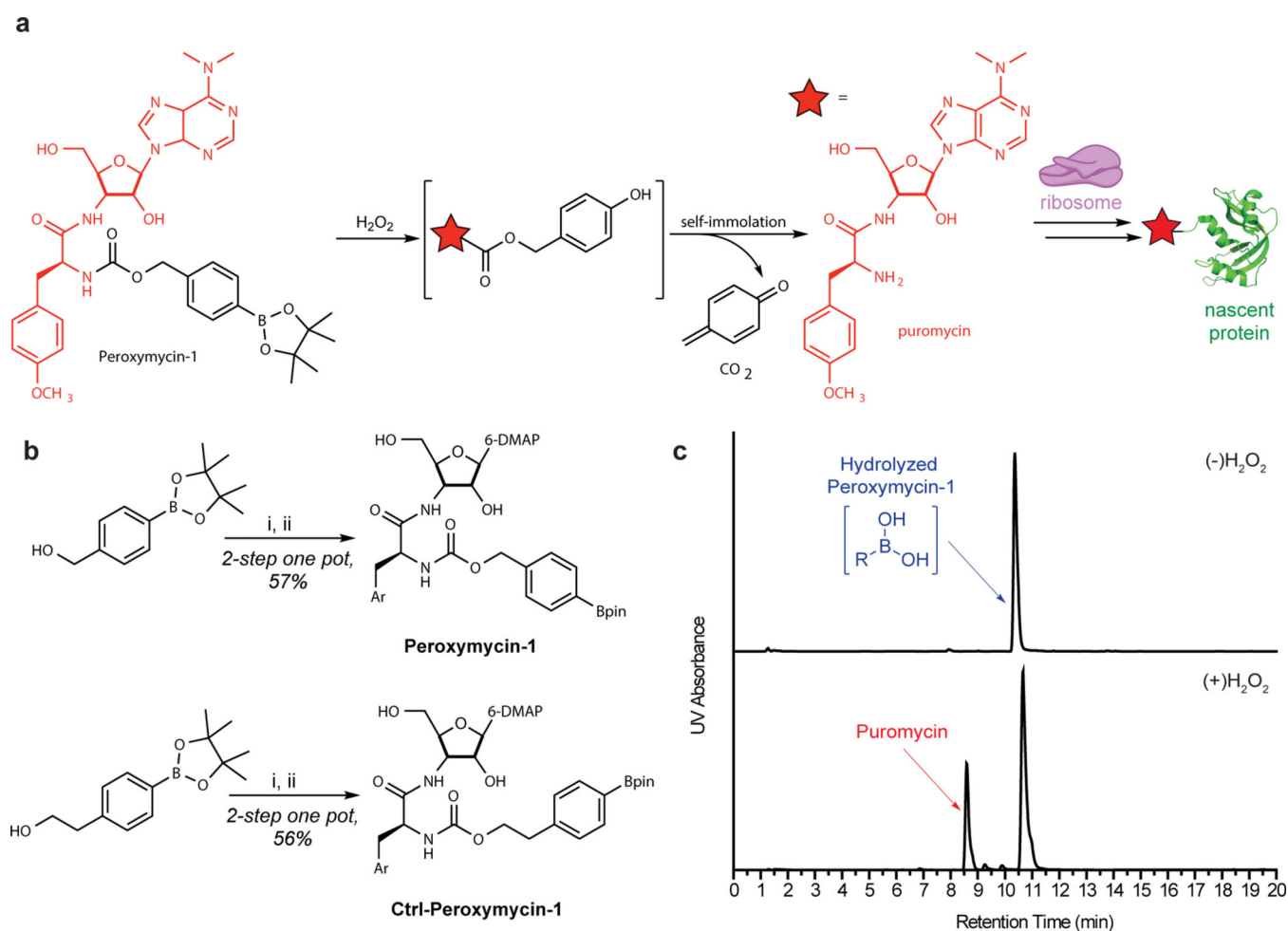
a

b

c

Fig. 2 |. Synthetic and mechanistic scheme of Peroxymycin-1.
a, Mechanism of H_2O_2 detection by Peroxymycin-1. Oxidative deprotection of the boronate pinacol ester occurs in the presence of H_2O_2 and releases puromycin, which is incorporated into nascent proteins by the ribosome. Puromycin shown in red. **b**, Synthetic scheme of Peroxymycin-1 and Ctrl-Peroxymycin-1. Reagents and conditions: (i) 4-nitrophenyl chloroformate, 4-(dimethylamino)pyridine, CH_2Cl_2, 0–23 °C, 2 h; (ii) puromycin • 2HCl, N,N-diisopropylethylamine, dimethyl formamide, 23 °C, 1 h. c, UV chromatogram (275 nm) of purified Peroxymycin-1 (top) and uncaging in the presence of H_2O_2 (0.1 mM) to form free puromycin (bottom). Bpin, boronate pinacol ester.
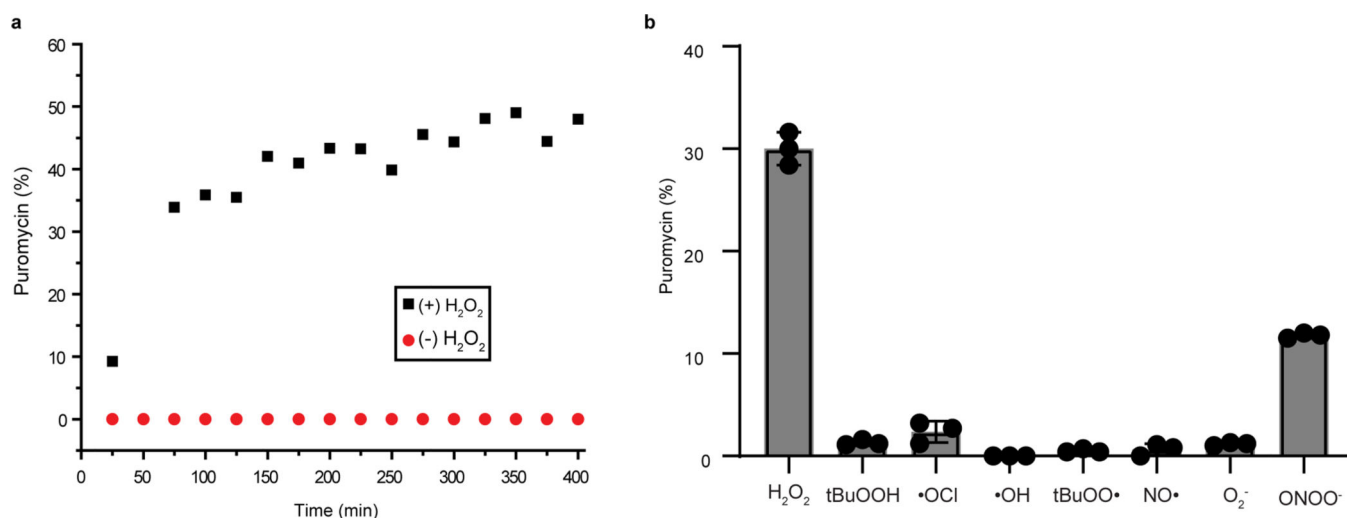
Fig. 3 |. In vitro analysis of Peroxymycin-1 reactivity and selectivity.
a, Reaction kinetics of Peroxymycin-1 (0.3 mM) treated with H_2O_2 (0.1 mM) in PBS analyzed by LC-MS. The percentage of puromycin liberated by the reaction was obtained from its UV absorbance and plotted over 6 h. **b**, Selectivity of Peroxymycin-1 toward various reactive oxygen and nitrogen species. Selectivity of Peroxymycin-1 (0.3 mM) for H_2O_2 (0.1 mM) over other reactive oxygen and nitrogen species was analyzed by LC-MS after treatment with species at 0.1 mM (except $ONOO^-$ at 0.05 mM) for 1 h at room temperature. Error bars denote s.d. ($n = 5$). tBuOOH, tert-butyl hydroperoxide.
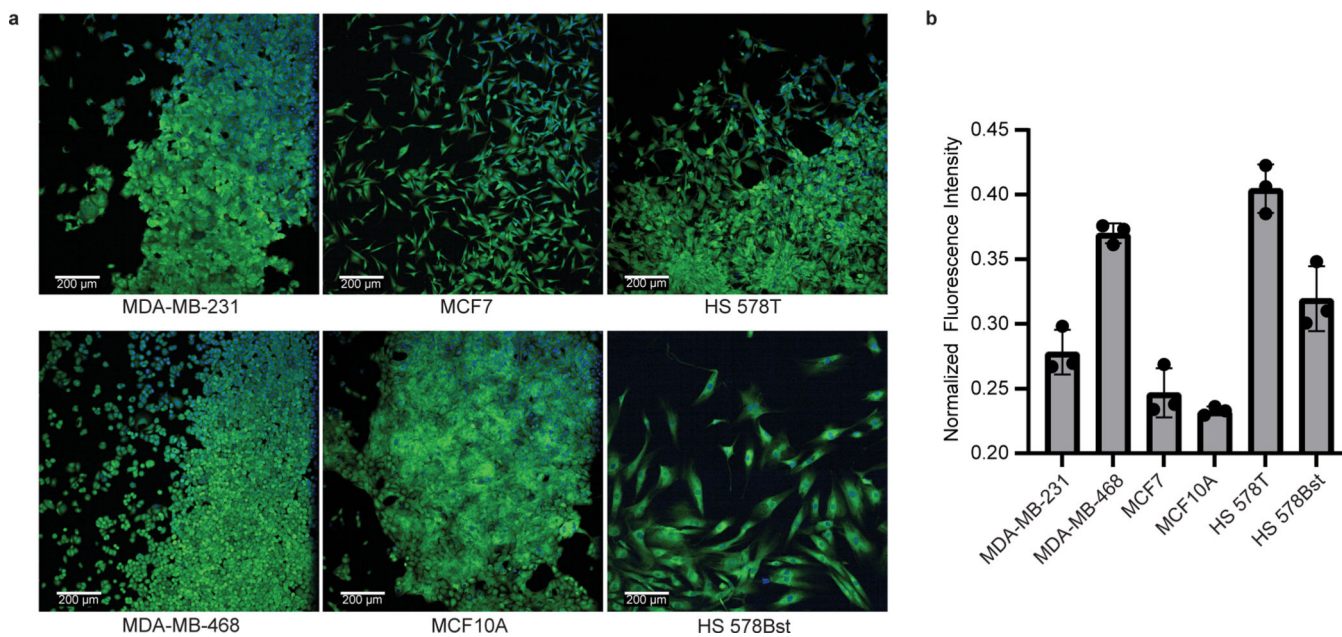
Fig. 4 |. Confocal microscopy images of endogenous H$_2$O$_2$ detection by using Peroxymycin-1.
a, Confocal images of Peroxymycin-1–treated human cell lines (1 μM). Hoechst: nuclear staining (blue channel). Anti-puromycin: staining with mouse anti-puromycin antibody, visualized by donkey anti-mouse antibody–Alexa Fluor 488 conjugate (green channel). Scale bar: 200 μm for all images. **b**, Quantification of fluorescence intensity of the images of Peroxymycin-1–treated cell lines. Values are plotted as Peroxymycin-1 fluorescence intensity ± s.d. ($n = 3$ technical replicates, with 2 fields examined per replicate), normalized to puromycin-treated control cells (for details of the normalization process, see Supplementary information, data analysis).
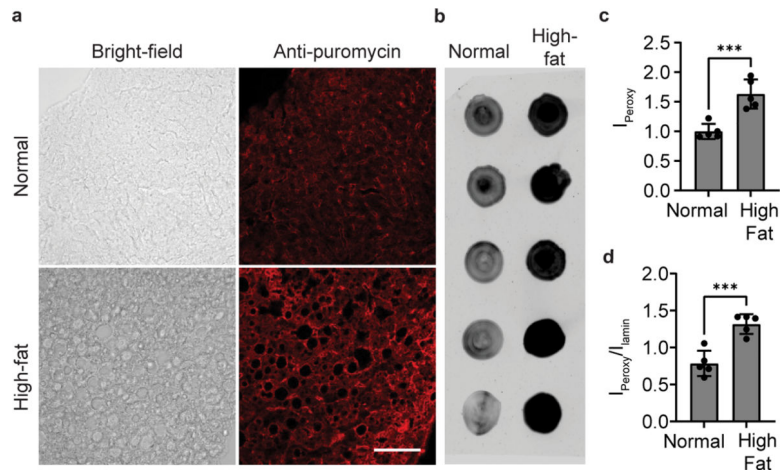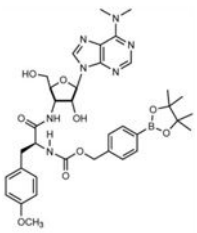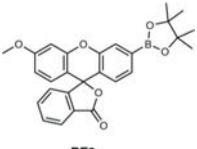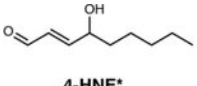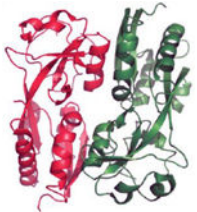
Fig. 5 |. Profiling H$_2$O$_2$ levels with Peroxymycin-1 in liver tissue of mice fed a normal diet and a high-fat diet.

10 mg/kg of Peroxymycin-1 were injected intraperitoneally into mice, and the liver was harvested after 4 h. I$_{Peroxy}$ was divided by I$_{lamin}$ for normalization of the total protein amount in the lysate sample. **a**, Confocal microscopy images of liver tissues of Peroxymycin-1–treated mice, stained with mouse anti-puromycin and anti-mouse–Alexa Fluor 647. Scale bar: 100 μm. **b**, Dot blots of liver tissue lysates probed by using mouse anti-puromycin antibody and then anti-mouse antibody–Alexa Fluor 488. **c** and **d**, Quantification of intensities of **a** and **b**, respectively. Error bars denote s.d. ($n = 5$). ***, $P < 0.001$. Institutional approval for the experiments shown in this figure was obtained from the University of California, Berkeley.High fat, mice raised on high-fat chow; I$_{lamin}$, fluorescence intensity of puromycin–treated sample stained with mouse anti-lamin antibody and anti-mouse antibody–Alexa Fluor 647 conjugates; I$_{Peroxy}$, fluorescence intensity of puromycin-treated sample stained with mouse anti-puromycin antibody and anti-mouse antibody–Alexa Fluor 647 conjugates; Normal, mice raised on normal chow. Confocal and dot blot images reprinted with permission from ref. [59], American Chemical Society.

Table 1.

Comparison of Peroxymycin-1 with other selected imaging-based H_2O_2 probes.

Tool	Application	Pros	Cons	Ref
Peroxymycin-1	Detection of H_2O_2 in fixed cells and mouse tissue (liver).	Peroxymycin-1 is specific to H_2O_2, can be used with multiple dyes and in tissue samples, and does not require genetic engineering.	Samples must be [59] fixed to use with Peroxymycin-1, and the probe has relatively long incubation time. Peroxymycin-1 may also have a potential influence on protein synthesis if used in excess doses.	[59]
PF2	Detection of H_2O_2 in live cells.	PF2 is selective for H_2O_2 over other ROS species, compatible with live cell imaging, and does not require transfection.	PF2 suffers poor retention inside cells, and unsuitable for tissue and live animal imaging.	[47]
4-HNE*	Detection of one of the products of ROS in fixed cells and animal tissue (mice, zebrafish, nematodes, etc.).	4-HNE staining can be used in parallel with other immunostaining techniques and does not require genetic engineering.	4-HNE staining is not specific to a particular ROS, does not give a direct readout of ROS, is not compatible with live cell imaging.	[65]
HyPer family**	Detection of H_2O_2 in live cells and organisms (mice, nematodes, zebrafish, bacteria, etc.).	This prototypical family of H_2O_2-specific genetically encoded sensors is suitable for live cells/animals with organelle- or region- specific expression capability.	The protein sensor can be pH sensitive, difficult to control expression, and requires transfection or other genetic methods to introduce.	[61]

*
4-HNE is the product formed after reaction with ROS. Other structures (Peroxymycin-1 and PF2) in the table are before the detection of H_2O_2.

**
Crystal structure of OxyR (PDB ID: 1I69) is shown.

Table 2

Troubleshooting

Step	Problem	Possible reason	Solution
13	Low reaction yields	Hydrolysis of 4-nitrophenyl chloroformate	In-situ formation of the 4-nitrophenyl carbonate intermediate is key for coupling of the aryl-boronate trigger to puromycin. A deep yellow color change upon addition of the puromycin dihydrochloride solution to the generated 4-nitrophenyl carbonate solution indicates the liberation of nitrophenol upon coupling. Absence of this color change indicates that coupling of the aryl-boronate trigger to puromycin has not occurred and can be fixed by using anhydrous solvents.
17	Multiple peaks were observed in LC analysis after purification	Formation of hydrolyzed boronate (i.e. Pinacol ester was hydrolyzed)	Hydrolyzed Peroxymycin-1 should have 651 m/z. If the hydrolyzed Peroxymycin-1 is the only additional species found, then the mixture is sufficiently pure for use in cell or mouse staining, as the hydrolyzed compound is also active in staining experiments, and is formed when aqueous solution is added to Peroxymycin-1 in the biological experiments and LC-MS characterization experiments. However, if significant concentrations of other compounds such as DMAP (123 m/z), p-nitrophenol (140 m/z), or puromycin (472 m/z), or other unknown comopounds are present then the mixture should be repurified by column chromatography. The extra impurities may be due to the decomposition of 4-nitrophenyl chloroformate, although this was not observed by the authors. Using anhydrous solvent is recommended to decrease the probability of side reactions occurring.
30	Strong background signal was observed for mouse-derived cell lines	Anti-mouse secondary antibody binds to mouse-specific epitopes.	Block the sample with anti-mouse Fab fragment at step 25. Alternatively, use anti-puromycin antibody– fluorophore conjugates, so that use of the secondary antibody can be omitted.
30	Variation in fluorescence intensity between replicate samples observed.	Puromycin incorporation rate may differ between samples.	Perform the cell staining experiment outlined in the Anticipated results section and normalize the Peroxymycin-1 signal by control puromycin signal (see Data Analysis section for details).
39	Tissue lysate solution is still opaque.	The centrifugation process is insufficient.	Repeat the centrifugation multiple times until the solution becomes clearer.