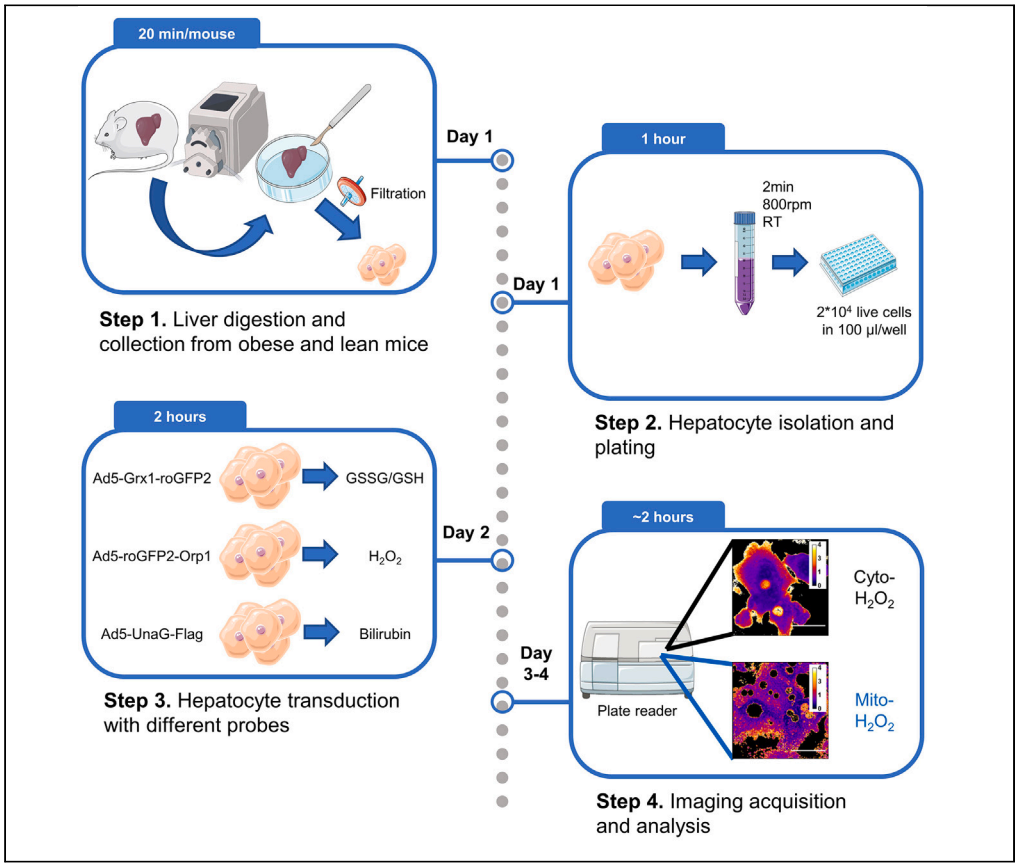
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Quantifying mitochondrial redox and bilirubin content in intact primary hepatocytes of obese mice using fluorescent reporters



Assessing the physiological role of H_2O_2 requires sensitive techniques to quantify H_2O_2 and antioxidants in live cells. Here, we present a protocol to assess the mitochondrial redox state and unconjugated bilirubin levels in intact live primary hepatocytes from obese mice. We described steps to quantify H_2O_2, GSSG/GSH, and bilirubin content in the mitochondrial matrix and the cytosol using the fluorescent reporters roGFP2-ORP1, GRX1-roGFP2, and UnaG, respectively. We detail hepatocyte isolation, plating, and transduction and live-cell imaging using a high-content imaging reader.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Thomas Belmas, Marc Liesa, Michaël Shum

michael.shum@crchudequebec.ulaval.ca

Highlights

Isolation of primary hepatocytes from high-fat-fed mice

Use of fluorescent reporters to measure GSSG/GSH, H_2O_2, and bilirubin in hepatocytes

Imaging protocol in live cells using a high-content imaging system

Images analysis procedure to evaluate redox balance in live hepatocytes from obese mice

Belmas et al., STAR Protocols 4, 102408
September 15, 2023
https://doi.org/10.1016/j.xpro.2023.102408

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Quantifying mitochondrial redox and bilirubin content in intact primary hepatocytes of obese mice using fluorescent reporters

Thomas Belmas,[1,2] Marc Liesa,[3,4] and Michaël Shum[1,2,5,6,*]

[1]Endocrinology – Nephrology Research Axis, CHU de Québec-Université Laval Research Center, Québec, QC, Canada

[2]Department of Molecular Medicine, Faculty of Medicine, Université Laval, Québec, QC, Canada

[3]Institut de Biología Molecular de Barcelona, IBMB, CSIC, Barcelona, Catalonia, Spain

[4]Department of Medicine, Endocrinology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

[5]Technical contact

[6]Lead contact

*Correspondence: michael.shum@crchudequebec.ulaval.ca
https://doi.org/10.1016/j.xpro.2023.102408

SUMMARY

Assessing the physiological role of H_2O_2 requires sensitive techniques to quantify H_2O_2 and antioxidants in live cells. Here, we present a protocol to assess the mitochondrial redox state and unconjugated bilirubin levels in intact live primary hepatocytes from obese mice. We described steps to quantify H_2O_2, GSSG/GSH, and bilirubin content in the mitochondrial matrix and the cytosol using the fluorescent reporters roGFP2-ORP1, GRX1-roGFP2, and UnaG, respectively. We detail hepatocyte isolation, plating, and transduction and live-cell imaging using a high-content imaging reader.

For complete details on the use and execution of this protocol, please refer to Shum et al.[1]

BEFORE YOU BEGIN

Reactive oxygen species (ROS) are powerful oxidants formed by the partial reduction of oxygen, which include the superoxide anion, O_2^{-}; hydrogen peroxide, H_2O_2 and hydroxyl radical $HO^{\cdot}$.[2] Mitochondria are a major source of ROS with H_2O_2 released by mitochondria being a key signaling molecule, communicating the mitochondrial redox state to the rest of the cell.[3] There are several tools available to study ROS actions, such as electron paramagnetic (spin trapping) resonance (EPR/ESR), which directly measures the content of different ROS, as well as indirect measurements using chemical reporters or detecting the oxidative modifications on proteins and lipids.[4] Most of these methods have important limitations including the requirement of stringent controls, instability of ROS measured or being unspecific.[5] Furthermore, these approaches cannot be properly used to measure ROS *in vivo* (live cells).

To overcome these issues, redox-sensitive fluorescent proteins were developed[2,6,7] to detect H_2O_2 and redox pairs in living organisms with unmatched spatiotemporal resolution. These include, H_2O_2 probes such as Hyper,[8–12] ro-green fluorescent protein (GFP)-based H_2O_2 and GSSG/GSH sensors[13–16]; and Förster resonance energy transfer (FRET)-based sensors.[17] The ratiometric properties of roGFP2 are valuable because it enables roGFP2 fluorescence to report on H_2O_2 or GSSG/GSH content without being confounded by changes in roGFP2 expression, sensitivity or photobleaching between cells or organelles. An important advantage of using roGFP-based sensors is their reversible oxidation and being insensitive to changes in pH.

In this protocol, we used roGFP-based H_2O_2 and GSSG sensors to measure the redox state in live hepatocytes. Fluorescent properties of GFP were engineered to develop a redox sensitive GFP

(roGFP2), achieved by introducing two cysteine of GFP.[18] This pair of cysteines can form a disulfide bond in an oxidizing environment, which causes changes in GFP fluorescence properties. Oxidized roGFP2 will shift its excitation peak from 490 nm to 400 nm, while preserving its emission peak at 511 nm roGFP2 can be fused to protein to specifically detect H_2O_2 using ORP1 or oxidized glutathione (GSSG) using GRX1. roGFP2-ORP1 (oxidant receptor peroxidase 1) and GRX1-roGFP2 (glutaredoxin 1) acts as a primary oxidant acceptor and then passes on the oxidation to a target protein, roGFP2. Additionally, adding a suitable targeting sequences, allowed to locate roGFP2 in various subcellular compartments and thus measure the redox state selectively in mitochondria, endoplasmic reticulum, nucleus, and cytoplasm.[8,16,19,20] Contrary to other ROS detection methods, genetically encoded proteins are more specific and sense H_2O_2 in a reversible manner in the upper nanomolar range, meaning that they enable dynamic observation of redox balance by real-time live-cell imaging.

Whereas different tools have been developed to measure oxidants, few methods can directly detect antioxidants. Unconjugated bilirubin is the final product of heme catabolism and is a powerful antioxidant.[21] While multiple methods exist to measure conjugated and unconjugated bilirubin in blood and cell lysates, the discovery of the UnaG protein from eel, which emits green fluorescence when it binds bilirubin allows us to measure intracellular bilirubin in live cell. UnaG binding to bilirubin is reversible allowing the detection of bilirubin dynamics in real-time. Additionally, fusing UnaG to different targeting sequences enabled the detection of unconjugated bilirubin in different subcellular compartments.[21] Using these probes, our group showed that the mitochondrial biliverdin exporter ABCB10 (ATP-binding cassette B10) increases the content of unconjugated bilirubin in the mitochondrial matrix and the cytosol to determine the redox state in hepatocytes from obese mice.[1] Here, we provide a protocol to measure in the mitochondria and the cytosol GSSG/GSH level by using the Grx1-roGFP2, H_2O_2 level by using roGFP2-Orp1 and Bilirubin using UnaG probes.

Institutional permissions

All experiments were approved by Institutional Animal Care and Use Committee at the Animal Research Committee at the University of California, Los Angeles (UCLA).

Mice

Mice were housed in ventilated cages (two to five mice per cage) and water/food was provided *ad libitum*, under 12-h light: dark cycle, and at a room temperature of +22°C to 24°C. Obesity and insulin resistance were induced by high fat diet (HFD) feeding staring at 3–4 weeks of age (D12451, Research Diets; 45 kcal % fat) for 26 weeks. Lean controls were mice fed a chow diet for the same period.

Material

Timing: [20 min] Preparation of 96 well-plates. Must be done the day before.

Note: Steps 1–2 should be done in sterile conditions, in a biological hood (i.e., biosafety cabinet)

1. Coat the bottom of the 96 well-plates with 30 µL/well of 0,01% rat-tail collagen solution and incubate for 2 h at +21°C in a biosafety cabinet.
2. Wash twice with PBS and let dry overnight (with lid on in a biosafety cabinet protected from light).

Collagen Solution

Reagent	Final concentration	Stock concentration
Collagen	0.01%; 0.1 µg/mL	100%; 1 mg/mL in water

Coat the 96 well plate with 30 µL of collagen 0.01%.
Store at +4°C.

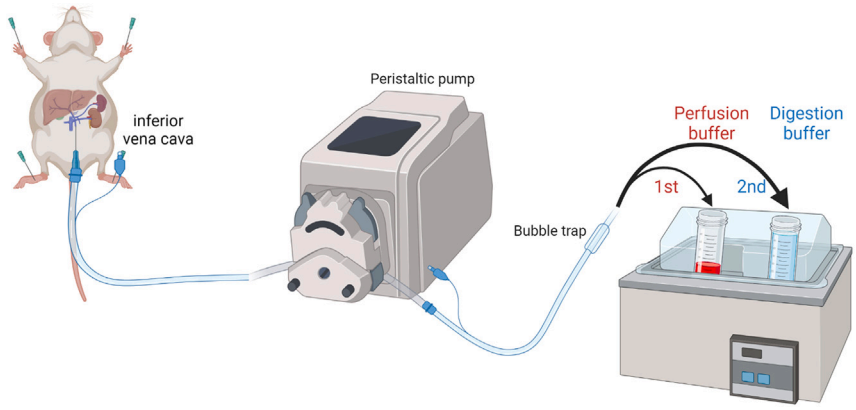


Figure 1. Schematic representation of tubing set-up for perfusion/digestion

Tubing set-up

3. Connect the tube to the peristaltic pump, with one end in the perfusion buffer (see Figure 1).
4. Connect a drip chamber to the outlet end of the tubing to avoid any bubbles during the perfusion.
5. Connect the 23-gauge needle to the end.
6. Rinse the perfusion apparatus with 70% ethanol throughout the tubing.
7. Run air through the tubing.
8. Run 20 mL of sterile water to wash residual ethanol.
9. Fill the tubing with Perfusion buffer.

Note: Avoid having long tubes, as the flow of the perfusion and digestion buffer will decrease their temperatures before reaching the liver. The temperature of the buffer when reaching the liver should should be 37°C.

Note: You might need to cut and connect the tubing of the pump to the smaller tubing of the drip chamber. Seal the connection with parafilm.

⚠ CRITICAL: Avoid bubbles in the system as bubbles can block liver vessels and therefore perfusion.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Ad5-Grx1-roGFP2 (GSSG/GSH probe)	Welgen	www.welgen.com upon request
Ad5-roGFP2-Orp1 (H_2O_2 probe)	Welgen	www.welgen.com upon request
Ad5-UnaG-Flag (bilirubin probe)	Welgen	www.welgen.com upon request
Chemicals, peptides, and recombinant proteins		
EDTA 0.5 M, Ph 8.0	Thermo Fisher	15575020
DMEM low glucose	Fisher Bioreagents	BP2482-500
Phosphate-buffered saline (PBS) 10×	Invitrogen	02-023-1A
L-Glutamine solution	Hylabs	BP507/500D
Penicillin-Streptomycin solution	Invitrogen	03-020-1B
Fetal bovine serum (FBS)	Invitrogen	03-031-1B
Collagen	Sigma-Aldrich	04-007-1A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Trypan blue solution	Sigma-Aldrich	C3867-1VL
M199	Gibco	11150067
Glutamax	Gibco	35050061
Insulin	Gibco	12585014
Triiodothyronine	Sigma	709719-1MG
Dexamethasone	Sigma	D4902-100MG
Experimental models: Organisms/strains		
Mouse strain: C57BL/6J (Wild type, male, 34 weeks old)	The Jackson laboratory	RRID: IMSR_JAX:000664
Software and algorithms		
Excel		
Harmony High-Content Software		https://www.perkinelmer.com/fr/product/harmony-4-8-office-hh17000001
GraphPad		https://www.graphpad.com/features
ImageJ		https://imagej.nih.gov/ij
Other		
HEPES (1 M)	Sigma-Aldrich	H0887-100ML
NaCl	Sigma	S6546-1L
KCl	Sigma	58221-500ML-F
$Na_2HPO_4 \cdot 12H_2O$	Sigma	71663-1KG
Collagenase type IV	Sigma	C4-28-100MG
$CaCl_2$	Sigma	C4901-500G
Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium	Invitrogen	01-050-1A
Penicillin-Streptomycin	Gibco	15140148
3 Bulldog Clamps Surgery	Fine Science Tools	18051-35
Cell strainer, 70 µm	Corning	CLS431751-50EA
Hemacytometer		
Peristaltic pump	Thermo Scientific	72-330-100
Mouse dissection tray	N/A	N/A
Sterile 50 mL centrifuge tubes	Corning	430829
Sterile 25 mL serological pipettes	Bio-SORFA	315100
23-gauge winged needle	Terumo	SV23BLK
Tubing material	Thermo Scientific	6416-14
Water bath	N/A	N/A
70% ethanol	N/A	N/A
Drip chamber	Qosina	23240
ViewPlate-96 Black, TC treated	PerkinElmer	6005182
Operetta high-content confocal microscope		

MATERIALS AND EQUIPMENT

Prepare buffer solutions

Timing: [30 min] Preparation of the different buffers required for hepatocytes isolation.

- Prepare Perfusion Media, Plating Media, and Maintenance Media, 50 mL per mouse per buffer as described below.
- One hour before the experiment, prepare fresh Digestion Media.
- Warm Perfusion and Digestion media at +42°C (as described in materials and equipment).

Perfusion Media		
Reagent	Final concentration	Amount
NaCl	8 µg/mL	8 g
KCl	0,2 µg/mL	0,2 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
$Na_2HPO^{4-}12H_2O$	0,1 µg/mL	0,1 g
Hepes	2,38 µg/mL	2,38 g
Total		**1 L**

Adjust pH between 7,6 and 7,7 with NaOH 1 N.
Filtered on 0.22 µm filter.

⚠ CRITICAL: Just before perfusion, add 50 µL EDTA 0.5 M pH 8 in 50 mL of Perfusion Media.

Digestion Media			
Reagent	Final concentration	Stock concentration	Amount
Perfusion Media **without EDTA**	X	X	50 mL
Collagenase Type IV (Sigma-C4138)	0.4 mg/mL		20 mg
$CaCl_2$*	0.75 mg/mL	375 mg/mL	100 µL
Total			**50 mL**

*$CaCl_2$ is very important to ensure maximal activity of Collagenase Type IV.

Plating Media			
Reagent	Final concentration	Stock concentration	Amount
M199 + Glutamax (1×)	N/A	N/A	500 mL
Penicillin	1 U/mL	100 U/mL	5 mL
Streptomycin	1 µg/mL	100 µg/mL	5 mL
Serum	10%	N/A	50 mL
Insulin	10 nM	100 U/mL	7,8 µL
Triiodothyronine	100 nM	1 mM	50 µL
Dexamethasone	500 nM	10 mM	25 µL
Total			**500 mL**

Maintenance Media			
Reagent	Final concentration	Stock concentration	Amount
M199 + Glutamax (1×)	N/A	N/A	500 mL
Penicillin	1 U/mL	100 U/mL	5 mL
Streptomycin	1 µg/mL	100 µg/mL	5 mL
Dexamethasone	100 nM	10 mM	5 µL
Total			**500 mL**

High-content fluorescence microscope system

Live cell imaging was performed using the Operetta System Spinning Disk Fluorescent Microscope from Perkin-Elmer, with the hepatocytes cultured on ViewPlate 96-well Black Plates at 37°C, 5% CO_2. A Xenon lamp with the following filters was used to detect roGFP2: the oxidized form of roGFP2 (roGFP2ox) detected using Excitation 460–490 nm and collected at Emission 500–550 nm; a reduced form of roGFP2 (roGFP2red) with Ex 410–480 nm and Em 500–550 nm and for UnaG, Ex 460–490 nm and Em 500–550 nm. To quantify fluorescence, Harmony Perkin Elmer Software was used to select cells and threshold the images to remove background fluorescence. Average fluorescence intensity values were calculated per field, containing 20–30 cells. Results for roGFP2-Orp1 and Grx1-roGFP2 were expressed as oxidized/reduced probe fluorescence ratio.

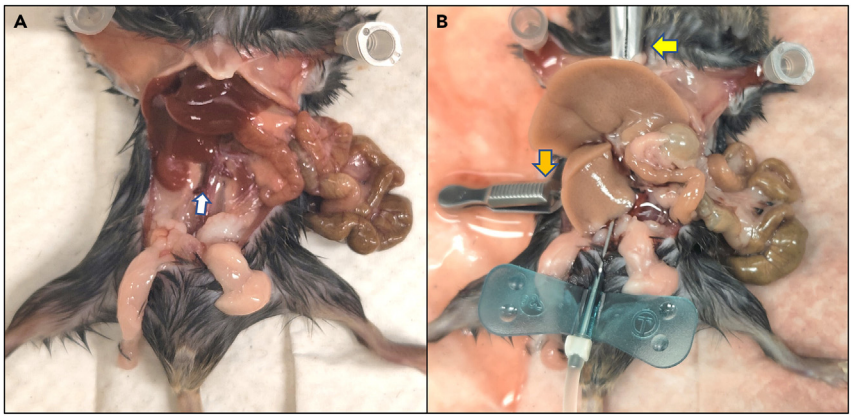


Figure 2. Picture showing the cannulation of the IVC for liver perfusion

(A) The white arrow shows the inferior vena cava (IVC).

(B) Cannulation of the IVC with two clamps used, one clamp holding the needle inserted in the IVC (orange arrow) and another one clamping the IVC behind the liver (yellow arrow) to prevent the perfusion not inflating the liver and just reaching the heart. Perfusion of the IVC starts at 2 mL/min before cutting the portal vein. Image is taken after 4 min perfusion and see how the liver inflated and some of the blood was washed, changing the color of the liver to a whitish color and losing elasticity.

Alternatives: This method can be done with any confocal microscope, but the high-content confocal system offers a rapid automated analysis capable of performing experiments in 6-well up to 384-well plates.

STEP-BY-STEP METHOD DETAILS

Liver digestion and collection

Timing: 15–20 min/mouse

⚠ CRITICAL: Proper perfusion of the liver is essential for maximum yield of hepatocytes.

Note: Two mice can be performed simultaneously using two pumps or a dual-independent channel pump.

1. Anesthetize one mouse using isofluorane and wait until the mouse is fully anesthetized.
   a. Test whether the mouse is fully anesthetized, by checking the withdrawal reflex (pinch the mouse's foot with pliers).
   b. Place the mouse in a prone position and spray the abdomen with 70% ethanol to avoid contaminating samples with hair.
   c. Use a tweezer to lift the skin near the genitals and, with scissors, make an incision and cut the peritoneum in the longitudinal and horizontal axis to expose the abdominal cavity.
   d. First, locate the inferior vena cava (IVC) in the abdominal cavity and the portal vein underneath the liver (Figure 2A).
   e. Insert 1 cm of the needle into IVC and clamp it with a small bulldog-type clamp at the edge of the vessels and the needle (Figure 2B).
   f. Clamp the superior IVC by cutting the diaphragm first, between the liver and the heart.
   g. Start the peristaltic pump at 2 mL/min.
   h. Then cut the portal vein to allow proper perfusion.

Optional: Ketamine/xylazine can be used in place of isofluorane. You can inject ketamine (30 mg/mL) and xylazine (6 mg/mL) IP in 100 µL PBS.

2. Perfuse the IVC with Perfusion Media with EDTA (store at +4°C up to 1 month) using the peristaltic pump at a 5 mL/min rate. For a better reaction, use up to 50 mL. The liver should start to appear pale and beige.
   a. Apply gentle pressure on the portal vein regularly.
3. When 2–3 mL of Perfusion Media are left, switch rapidly the tubing from Perfusion Media to Digestion Media (freshly made 1 h before experiment). Use up to 50 mL.

   Note: The collagenase concentration might need to be changed depending on the batch and digestion efficiency (see troubleshooting).

   Note: Some air bubbles can enter the tubing during the switch of perfusion to Digestion Media. However, they will be trapped in the air bubble trap.

   Note: After the Digestion Media perfusion, the liver is supposed to increase in volume, whitening, and lose elasticity if well digested.

   ⚠ CRITICAL: Set the pump at a low speed before inserting the needle to avoid the introduction of air bubbles inside the liver vasculature.

4. Excise the liver.
   a. Carefully remove the needle from the liver.
   b. Resect the liver from the body cavity and directly remove the gallbladder.
   c. Place the liver in a petri dish with 10 mL of Plating Media (store the media at +4°C up to 1 month).
   d. Remove the liver capsule, hold it with forceps, and shake it in the media. Once the liver is well digested, hepatocytes will be released easily just by shaking.
   e. Transfer the suspension into Falcon 50 mL.
   f. Complete to 30 mL with Plating media.

Hepatocyte isolation

Timing: 1 h

5. Under culture hood.
   a. Do several gentle up and down with a 10 mL graduated pipette.
   b. Pass the liver pieces through a 100 µm nylon cell strainer into a 50 mL tube.
   c. Spin down cells for 5 min at +21°C; 200 g.
   d. Aspirate the supernatant but keep a certain volume above the pellet, and re-suspend cells in 30 mL Plating Media.

   Note: Centrifuge with low acceleration and low brake to avoid stressing the hepatocytes.

6. Cell counting (Mix well the cells before each counting since the cells quickly precipitate).
   a. Do a 1/10 dilution of the cells.
   b. Mix well, add 20 µL of cells suspension with 20 µL Trypan blue, and add 10 µL to the hemacytometer.
   c. Count both live and dead cells in all 4 large quadrants.
   d. Formulas.
      i. Viability: Live cells/Total cells.
      ii. Cells per mL: Average cells per square × Trypan blue Dilution (2) × dilution factor (10) × 10^4.
      iii. Number of cells / volume (mL).

Note: Viability should be above 80%. Lower viability may result in significant cell mortality in the next few hours.

Note: With this method we expect ~40–60 × 10^6 cells from a single mouse liver.

7. Cell plating.
   a. To assess redox status with a high-content microscope, plate cells into a dark 96-well plate.
   b. Seed 2 × 10^4 live cells per well in 100 µL.
   c. Shake gently in straight horizontal and vertical movement (avoid swirling) to make sure that cells are evenly attached and leave cells in the incubator at +37°C for 4 h.
   d. 4 h later, change ~70% of Plating Media to remove unattached or dead hepatocytes with Maintenance Media (store at +4°C up to 1 month). At this time, hepatocytes can be cultured without FBS.

Note: Density separation by centrifugation in Percoll is used to separate viable hepatocytes from dead hepatocytes and cell debris. However, in our hands, it doesn't work for hepatocytes from obese mice. Therefore, good viabilibity is key to obtained appropriate hepatocyte culture.

Note: When changing the media, keep ~30% of the media as hepatocytes full of lipid droplets are sensitive to air.

Hepatocyte transduction and imaging of redox and bilirubin probes

Timing: 2.5 h

8. The same day, after changing the media for maintenance media, incubate primary hepatocytes with adenoviruses for 2 h.
   a. Multiplicity of Infection (MOI) 400 for roGFP2 adenoviruses.
   b. MOI 10 for UnaG adenoviruses.
9. 2 h later, wash the cells twice with maintenance media.

Note: different probes can be use in the same plate and imaged sequentially. Ex. 12 well with cytosolic-roGFP2-ORP1, 12 well with mito-roGFP2-ORP1, 12 well with mito-GRX1-roGFP2, etc…

Image acquisition

Timing: ~2–8 h (see Figure 3)

10. 24 h after transduction, perform live-cell imaging using the High-content Fluorescence Microscope imaging system.
11. Set the temperature at +37°C and the CO_2 at 5% 1 h before imaging.
12. Select the right plate properties.
13. Select the right filter for each channel:
    a. Oxidized form of roGFP2 (roGFP2ox) with Ex 460–490 nm and Em 500–550 nm.
    b. Reduced form of roGFP2 (roGFP2red) with Ex 410–480 nm and Em 500–550 nm.
    c. UnaG with Ex 460–490 nm and Em 500–550 nm.
14. Select the objective (we recommend 20×) and select at least 5 different fields for each well to be imaged.
15. Select one of the wells and one of the fields to adjust the focus, intensity of the light, and exposure.
16. Repeat this last process on multiple wells.
17. Once the image previews are satisfying, acquire the images of all the wells selected.

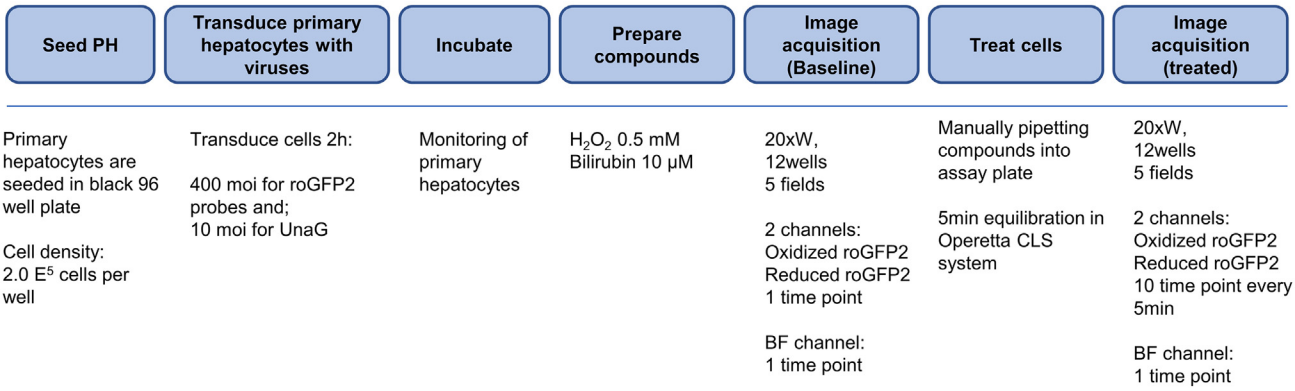


Figure 3. Workflow of imaging redox and bilirubin probes

Cells are plated in black 96-well plate at a density of $2.0e^5$ cells per well. Then 4 h later, Primary hepatocytes are transduced 2 h with the adenoviruses then washed. 24 h–48 h later cells are imaged with the high-content microscope. If cells are treated with different compounds, prepare them before imaging the fluorescence in baseline conditions. Then, treat the cells outside the microscope. Allow 5 min equilibration for the temperature and CO_2 before acquiring images in treated conditions.

Note: depending on the number of fields, intensity of the light and exposure, the minimal time between the images and the condition will vary.

Image analysis

Image analysis can be performed using the Harmony software. The analysis sequence can be saved and applied to multiple images (see Figure 4).

The general procedure is.

18. In your analysis sequence, add the "Find image region" panel with selected output population: image region reduced channel.
19. Select the cells or mitochondria using the common threshold or absolute threshold properties contained in "Find image region" panel.
20. Add another "find image region" and select whole image region using roGFP2 reduced channel.
21. Repeat with the roGFP2 oxidized channel.
22. To perform a Mask of selected object to calculate the background, add another "Select region" window with:

    Output population: whole image oxidized.

    Region: whole image region oxidized.

    Method: Restrict by Mask.

    Population: Image region reduced (selected in step 2).

    Mask region: Image region reduced (selected in step 2).

    Check Use inverted mask.

    Name output region Background Oxidized.

23. Repeat with the whole image reduced and name output region Background Reduced.

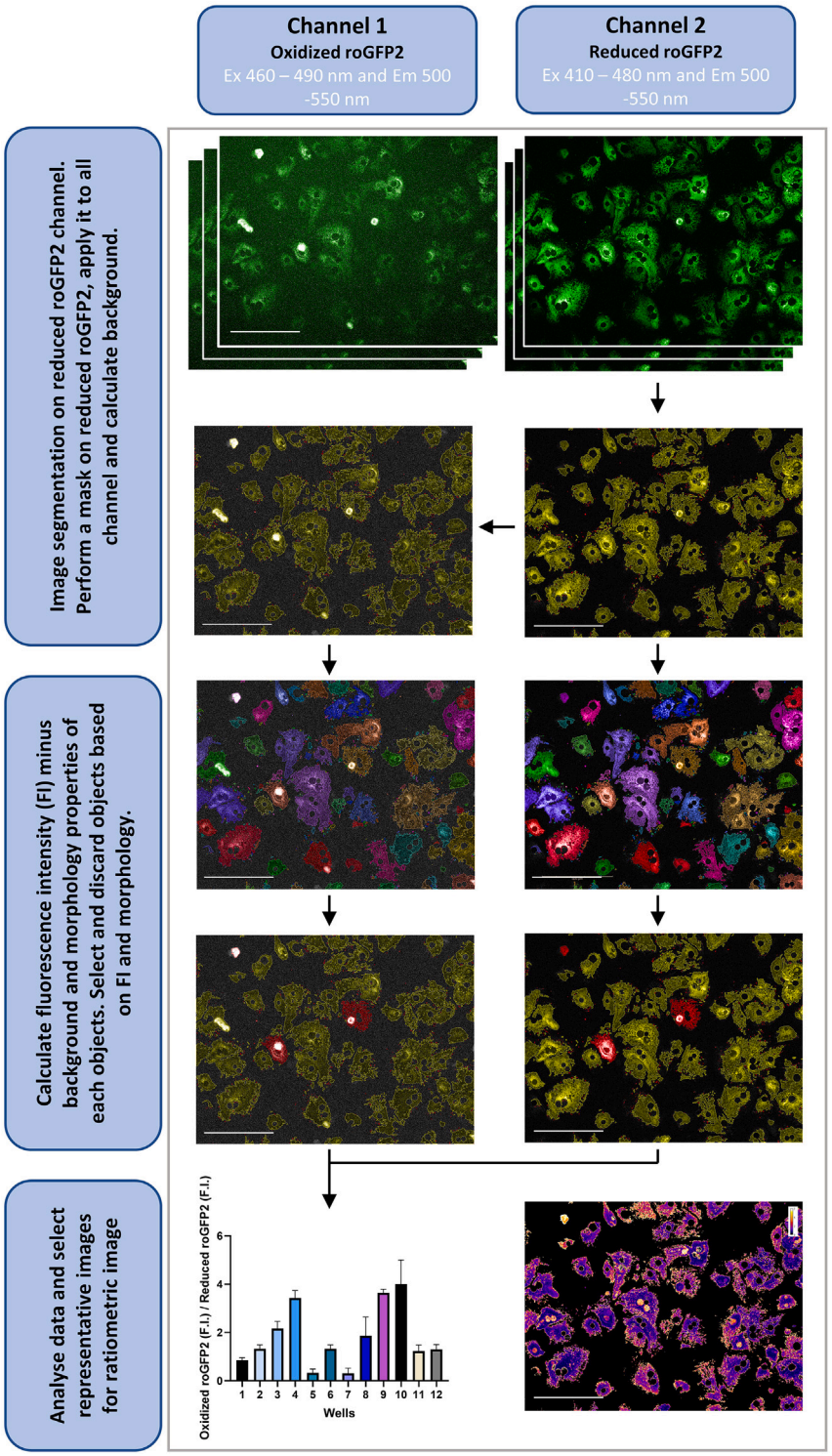


Figure 4. Image analysis process

To analyze images, the user can export images and use its own software analysis or use the Harmony software. The integrated analysis of the software allows for fast and batch analysis of all images. Here, we give an example on how to analyze roGFP2 redox probes. Reduced roGFP2 signals can be segmented to perform a mask using the common or absolute threshold parameters. Then this mask can be selected and applied to all the channels. The obtained images can be reverted on each channel to select the outside of the object threshold which will be the background. The next

Figure 4. *Continued*

step is to identify with the same thresholding parameters all objects and calculate their fluorescence intensity (FI) and morphology properties. Based on these values (FI and morphology), the user can select the objects and filter them based on their size, FI, FI std, roundness, etc... Artifacts or unwanted objects can be discarded. Finally, the FI and background intensity from each channel are calculated and can be subtracted using the formula option in the define results panel. The users will obtain two results oxidized roGFP2 minus background and reduced roGFP2 minus background. The ratio of oxidized roGFP2 over reduce roGFP2 will give an estimate of oxidized levels of the probes that can be compared between conditions. Selected images can be exported as raw images (Tiff) using the right click and transform with image J to obtain a ratiometric image as representative images among different conditions.

24. Add "calculate intensity properties" panel to calculate both reduced and oxidized background fluorescence intensity.
25. Add a "calculate morphology properties" panel to calculate properties of the image region reduced populations.
26. Add also a "calculate the intensity properties" panel.
27. Repeat the same process with the oxidized channel.
28. Then add a "select population" panel based on the reduced channel population.
29. You can then select the cells based on properties: roundness, area, and intensity, stdev, etc... This selection is useful to remove debris and dead cells.
30. In the defined results panel, you can select the results to show in the evaluation and also add a formula to subtract background from FI of selected region.
31. Go the evaluation window and start analysis. Harmony will calculate the mean fluorescence intensity,
32. Export the data.

Note: To adjust the analysis sequence, multiple images from different experiments are required to test whether the analysis sequence is working with all the conditions tested for your specific experiments.

Note: Each individual image for each excitation needs to be exported in tiff manually. Then, we use Image J to overlay the oxidized images on the reduced images.

Note: The imaging parameters such as time of exposure, the number of fields per well and the number of wells will influence the time of image acquisition. Therefore, these parameters will limit the temporal resolution of real time measurements of the redox state. In our experiments, we could image 24 wells, obtaining 4–5 images per well. For example, if one well took 15 s to image, these means that we could measure changes in fluorescence every 6 min (24 wells × 15 s = 360 s / 60 s = 6 min). Thus, to determine whether the acute response to the addition of bilirubin or oxidations, we spaced the treatment between individual wells for 15 s, on a heater plate set at 37°C and then add the plate back in the system to image the cells every 10 min. The time taking out and putting in the plate was recorded as well.

Note: We recommend testing the dynamic range of the redox probes ORP1-roGFP2 and roGFP2-Grx1 using oxidants and reducing agents. This is achieved by measuring roGFP2 ratiometric fluorescence before and 30 min after treating separate groups of cells with H_2O_2 0.5 mM to maximally oxidize ORP1-roGFP2, diamide 0.25 mM to oxidize roGFP2-GRX1 and DTT 0.25 mM to maximally reduce both probes. These treatments will work for both cytosolic and mitochondrial targeted probes.

Note: For the UnaG probes, it is recommended to verify the expression levels of UnaG-Flag among the different conditions to control for the expression of the probe.

Note: This protocol has been optimized for primary hepatocytes but could be used also with other cells. Optimization for adenoviral transduction and fluorescence detection in other cells and different batches of the same viruses are needed.

EXPECTED OUTCOMES

The probes should respond to oxidants such as H_2O_2 (Figures 5A and 5B) and reducing agents, such as dithiothreitol (DTT). These agents can establish the dynamic range of the probes in hepatocytes and other cellular models. In hepatocytes from high-fat fed mice, an increase of 15%–20% were observed in cytosolic and mitochondrial H_2O_2 and oxidized glutathione (GSSG/GSH) (Figures 5C and 5D). Bilirubin supplemented to the media can be used to test the UnaG probe (Figure 6A) and the antioxidant capacity of bilirubin can be tested using the roGFP2-ORP1 to detect H_2O_2 (Figure 6B) and GRX1-roGFP2 to detect oxidized glutathione (GSSG/GSH) (Figure 6C).

QUANTIFICATION AND STATISTICAL ANALYSIS

Graph Pad 11 were used for statistical analyses, which included Student's *t* tests or Mann-Whitney *U* tests when comparing two groups and one-way analysis of variance (ANOVA) with Dunnet post hoc.

LIMITATIONS

This method cannot provide absolute concentration values of H_2O_2, oxidized glutathione and bilirubin. It can only be used to determine fold changes between groups.

Primary hepatocytes in culture de-differentiate after plating. Thus, experiments cannot go beyond 72 h after plating.

TROUBLESHOOTING

Problem 1

Poor liver digestion.

Potential solutions

Make sure that you added the $CaCl_2$ to the Digestion media as calcium is required for the collagenase activity. You can also try different concentrations of collagenase, as the efficiency of the collagenase can vary from one batch to another. You can increase the concentration up to 1 mg/mL.

You can gently apply pressure on the portal vein for 5 s every 30 s to enhance the perfusion of the entire liver.

Problem 2

Poor hepatocyte viability after isolation.

Potential solutions

Poor viability can be caused by incomplete or over-digestion. Adjust the digestion conditions by titrating the collagenase.

Problem 3

Weak fluorescence of the redox probes.

Potential solutions

For the UnaG, bilirubin 10 μM can be used to treat the cells and observed increase fluorescence in both the cytosolic and mitochondrial compartment. Weak fluorescence can also be caused by low expression levels of the probes. UnaG is fused with a 3xFlag tag which enable the detection of UnaG expression between conditions. Additionally, MOI and transduction conditions can be adjusted to ensure proper expression of the fluorescent probes. For the UnaG or the redox probes, bilirubin 10 μM or H_2O_2 0.5 mM can be used, respectively, to treat the cells and observed increase fluorescence in both the cytosolic and mitochondrial compartment.

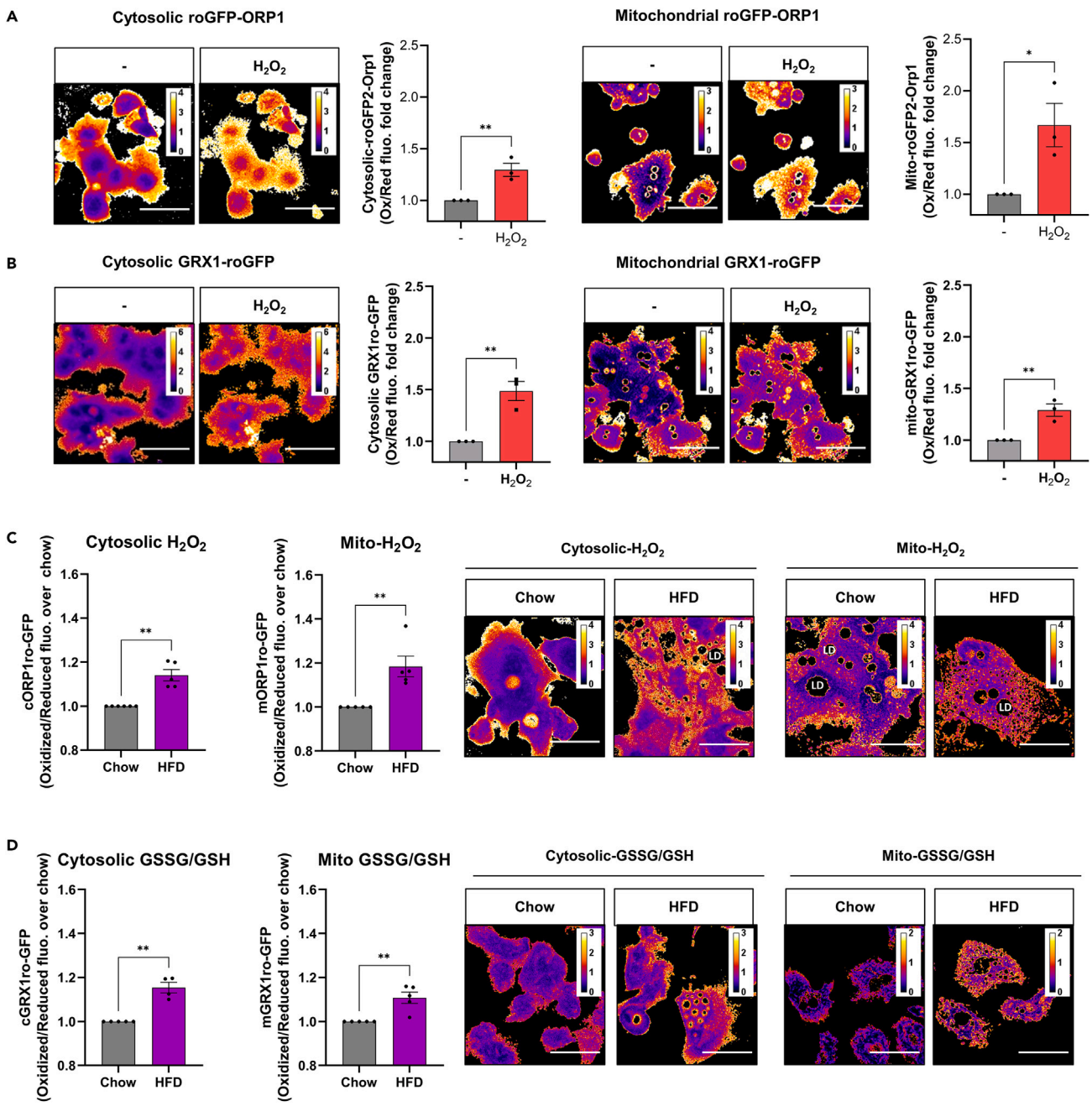


Figure 5. roGFP2 based probes to detect changes in cytosolic and mitochondrial H_2O_2 and oxidation induced by high fat diet feeding in mouse hepatocytes

(A) Primary hepatocytes from chow diet-fed mice were transduced with adenovirus encoding Cyto-roGFP2-Orp1, measuring cytosolic H_2O_2 content, Mito-roGFP2-Orp1, measuring mitochondrial matrix H_2O_2 content and treated with H_2O_2 0.5 mM for 20 min.

(B) Cyto-Grx1-roGFP2 measuring cytosolic GSSG/GSH or Mito-Grx1-roGFP2 measuring mitochondrial GSSG/GSH and treated with H_2O_2 0.5 mM for 20 min.

(C) Primary hepatocytes from lean (chow diet) and HFD-fed mice transduced with adenovirus encoding Cyto-roGFP2-Orp1, Mito-roGFP2-Orp1, (D) Cyto-Grx1-roGFP2 measuring cytosolic GSSG/GSH or Mito-Grx1-roGFP2 measuring mitochondrial GSSG/GSH. Here, we used high-content microscope to image primary hepatocytes. The ratio of green fluorescence emitted by oxidized roGFP2 divided by reduced roGFP2 is proportional to H_2O_2 content (Orp1) and GSSG/GSH (Grx1) respectively. Scale bar, 100 μm. n = 3–5 mice/group and independent isolations; *p < 0.05, **p < 0.01, Mann-Withney T-test. Data are represented as mean ± SEM. Some analysis and images were previously published in.[1] The results show the average changes in fluorescence of 5 images per mouse in fold change compared to chow-fed mice.

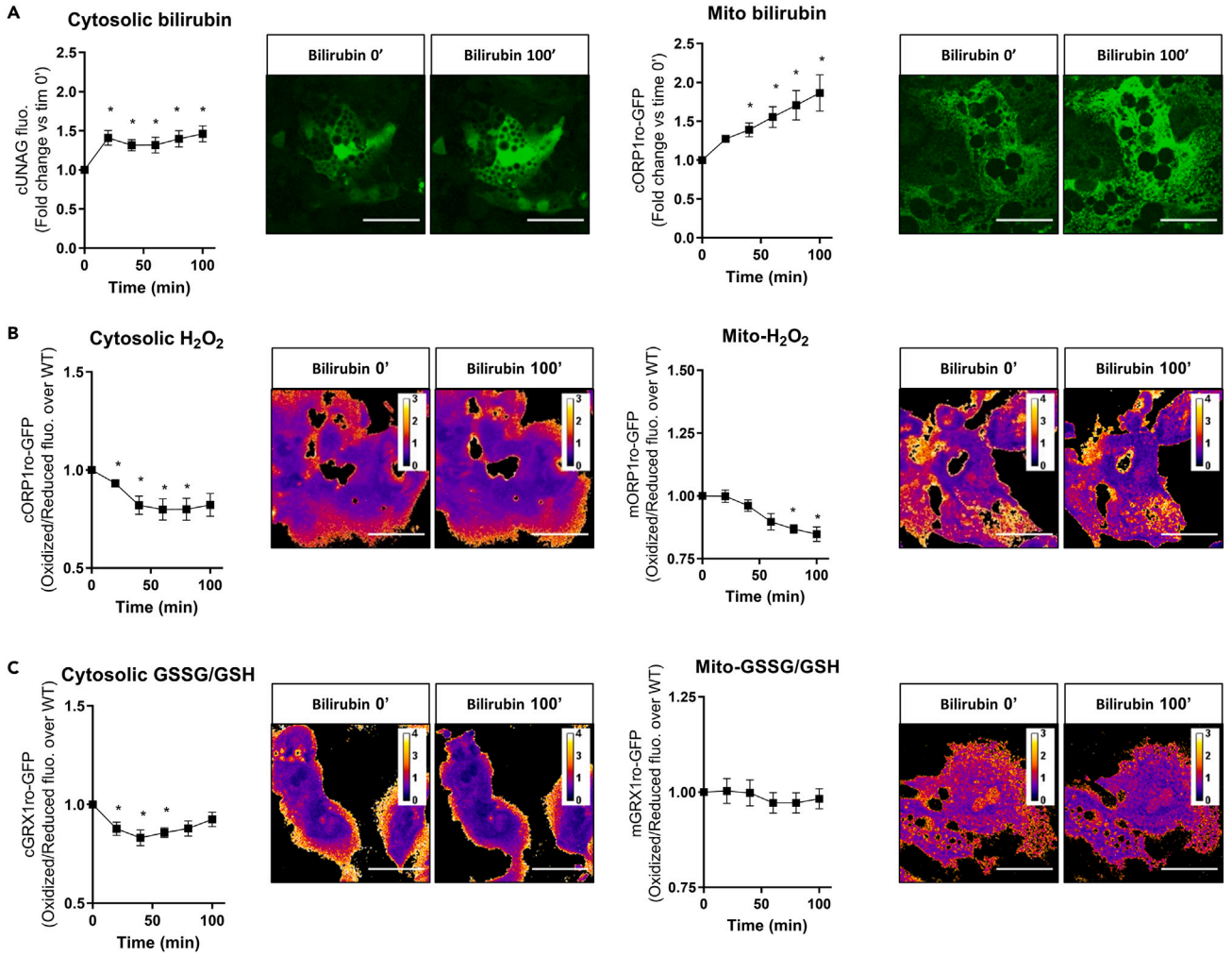


Figure 6. Mitochondrial and cytosolic redox state response to bilirubin supplementation

Primary hepatocytes were transduced with adenovirus encoding cytosolic UnaG (cUnaG), to measure cytosolic bilirubin levels, or with mitochondrial matrix targeted UnaG (mUnaG), to measure matrix bilirubin levels.

(A) Fold increase in cytosolic bilirubin levels and mitochondrial bilirubin levels after adding bilirubin (10 μM) to the hepatocyte culture media.

(B and C) (B) This increase in bilirubin were associated with lower H_2O_2 levels in the cytosol and the mitochondrial matrix whereas (C) exogenous bilirubin was able to reduce oxidized glutathione (GSSG) in the cytosol but not the mitochondrial matrix. Data are represented as mean ± SEM. n = 5 mice/group and independent isolations; one-way ANOVA, *p < 0.05 vs. time 0′ min. Scale bar = 100 μm. The results show the average changes in fluorescence of 5 images per mouse in fold change compared to time 0′.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Michael Shum (Michael.shum@crchudequebec.ulaval.ca).

Materials availability

This study did not generate any unique materials or reagents.

Data and code availability

We did not generate any unique datasets or code.

ACKNOWLEDGMENTS

We thank Nate Miller of the Imaging Core at UCLA for his assistance with image analysis using the Operetta Imaging system. M.S. was funded by the CRCHU de Québec, CHU de Québec foundation, and NSERC RGPIN-2021-03443. M.L. was funded by NIAAA 1R01AA026914 and is funded by MCINN/AEI/FEDER- PID2021-127278NB-I00. All the schematic diagrams in this manuscript were created with www.biorender.com.

AUTHOR CONTRIBUTIONS

T.B. wrote the manuscript. M.L. reviewed the manuscript. M.S. wrote the manuscript, designed experiments, and collected and analyzed data.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Shum, M., Shintre, C.A., Althoff, T., Gutierrez, V., Segawa, M., Saxberg, A.D., Martinez, M., Adamson, R., Young, M.R., Faust, B., et al. (2021). ABCB10 exports mitochondrial biliverdin, driving metabolic maladaptation in obesity. Sci. Transl. Med. *13*, eabd1869. https://doi.org/10.1126/scitranslmed.abd1869.

2. Dikalov, S.I., and Harrison, D.G. (2014). Methods for detection of mitochondrial and cellular reactive oxygen species. Antioxid. Redox Signal. *20*, 372–382. https://doi.org/10.1089/ars.2012.4886.

3. Murphy, M.P. (2009). How mitochondria produce reactive oxygen species. Biochem. J. *417*, 1–13. https://doi.org/10.1042/BJ20081386.

4. Halliwell, B., and Gutteridge, J.M.C. (2015). Free Radicals in Biology and Medicine (Oxford University Press). https://doi.org/10.1093/acprof:oso/9780198717478.001.0001.

5. Brewer, T.F., Garcia, F.J., Onak, C.S., Carroll, K.S., and Chang, C.J. (2015). Chemical approaches to discovery and study of sources and targets of hydrogen peroxide redox signaling through NADPH oxidase proteins. Annu. Rev. Biochem. *84*, 765–790. https://doi.org/10.1146/annurev-biochem-060614-034018.

6. Jiang, X., Wang, L., Carroll, S.L., Chen, J., Wang, M.C., and Wang, J. (2018). Challenges and opportunities for small-molecule fluorescent probes in redox biology applications. Antioxid. Redox Signal. *29*, 518–540. https://doi.org/10.1089/ars.2017.7491.

7. Kostyuk, A.I., Panova, A.S., Kokova, A.D., Kotova, D.A., Maltsev, D.I., Podgorny, O.V., Belousov, V.V., and Bilan, D.S. (2020). In vivo imaging with genetically encoded redox biosensors. Int. J. Mol. Sci. *21*, 8164. https://doi.org/10.3390/ijms21218164.

8. Belousov, V.V., Fradkov, A.F., Lukyanov, K.A., Staroverov, D.B., Shakhbazov, K.S., Terskikh, A.V., and Lukyanov, S. (2006). Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. Nat. Methods *3*, 281–286. https://doi.org/10.1038/nmeth866.

9. Bilan, D.S., Pase, L., Joosen, L., Gorokhovatsky, A.Y., Ermakova, Y.G., Gadella, T.W.J., Grabher, C., Schultz, C., Lukyanov, S., and Belousov, V.V. (2013). HyPer-3: a genetically encoded H_2O_2 probe with improved performance for ratiometric and fluorescence lifetime imaging. ACS Chem. Biol. *8*, 535–542. https://doi.org/10.1021/cb300625g.

10. Ermakova, Y.G., Bilan, D.S., Matlashov, M.E., Mishina, N.M., Markvicheva, K.N., Subach, O.M., Subach, F.V., Bogeski, I., Hoth, M., Enikolopov, G., and Belousov, V.V. (2014). Red fluorescent genetically encoded indicator for intracellular hydrogen peroxide. Nat. Commun. *5*, 5222. https://doi.org/10.1038/ncomms6222.

11. Markvicheva, K.N., Bilan, D.S., Mishina, N.M., Gorokhovatsky, A.Y., Vinokurov, L.M., Lukyanov, S., and Belousov, V.V. (2011). A genetically encoded sensor for H_2O_2 with expanded dynamic range. Bioorg. Med. Chem. *19*, 1079–1084. https://doi.org/10.1016/j.bmc.2010.07.014.

12. Pak, V.V., Ezeriņa, D., Lyublinskaya, O.G., Pedre, B., Tyurin-Kuzmin, P.A., Mishina, N.M., Thauvin, M., Young, D., Wahni, K., Martínez Gache, S.A., et al. (2020). Ultrasensitive genetically encoded indicator for hydrogen peroxide identifies roles for the oxidant in cell migration and mitochondrial function. Cell Metab. *31*, 642–653.e6. https://doi.org/10.1016/j.cmet.2020.02.003.

13. Calabrese, G., Peker, E., Amponsah, P.S., Hoehne, M.N., Riemer, T., Mai, M., Bienert, G.P., Deponte, M., Morgan, B., and Riemer, J. (2019). Hyperoxidation of mitochondrial peroxiredoxin limits H2 O2 -induced cell death in yeast. EMBO J. *38*, e101552. https://doi.org/10.15252/embj.2019101552.

14. Gutscher, M., Sobotta, M.C., Wabnitz, G.H., Ballikaya, S., Meyer, A.J., Samstag, Y., and Dick, T.P. (2009). Proximity-based protein thiol oxidation by H_2O_2-scavenging peroxidases. J. Biol. Chem. *284*, 31532–31540. https://doi.org/10.1074/jbc.M109.059246.

15. Linzner, N., Loi, V.V., Fritsch, V.N., Tung, Q.N., Stenzel, S., Wirtz, M., Hell, R., Hamilton, C.J., Tedin, K., Fulde, M., and Antelmann, H. (2019). *Staphylococcus aureus* uses the bacilliredoxin (BrxAB)/Bacillithiol disulfide reductase (YpdA) redox pathway to defend against oxidative stress under infections. Front. Microbiol. *10*, 1355. https://doi.org/10.3389/fmicb.2019.01355.

16. Morgan, B., Van Laer, K., Owusu, T.N.E., Ezeriņa, D., Pastor-Flores, D., Amponsah, P.S., Tursch, A., and Dick, T.P. (2016). Real-time monitoring of basal H_2O_2 levels with peroxiredoxin-based probes. Nat. Chem. Biol. *12*, 437–443. https://doi.org/10.1038/nchembio.2067.

17. Enyedi, B., Zana, M., Donkó, Á., and Geiszt, M. (2013). Spatial and temporal analysis of NADPH oxidase-generated hydrogen peroxide signals by novel fluorescent reporter proteins. Antioxid. Redox Signal. *19*, 523–534. https://doi.org/10.1089/ars.2012.4594.

18. Hanson, G.T., Aggeler, R., Oglesbee, D., Cannon, M., Capaldi, R.A., Tsien, R.Y., and Remington, S.J. (2004). Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. J. Biol. Chem. *279*, 13044–13053. https://doi.org/10.1074/jbc.M312846200.

19. Panieri, E., Millia, C., and Santoro, M.M. (2017). Real-time quantification of subcellular H_2O_2 and glutathione redox potential in living cardiovascular tissues. Free Radic. Biol. Med. *109*, 189–200. https://doi.org/10.1016/j.freeradbiomed.2017.02.022.

20. Van Lith, M., Tiwari, S., Pediani, J., Milligan, G., and Bulleid, N.J. (2011). Real-time monitoring of redox changes in the mammalian endoplasmic reticulum. J. Cell Sci. *124*, 2349–2356. https://doi.org/10.1242/jcs.085530.

21. Park, J.-S., Nam, E., Lee, H.-K., Lim, M.H., and Rhee, H.-W. (2016). *In cellulo* mapping of subcellular localized bilirubin. ACS Chem. Biol. *11*, 2177–2185. https://doi.org/10.1021/acschembio.6b00017.