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Corresponding Author:	Giulia Nigro, Ph.D Institut Pasteur Paris, France FRANCE
Corresponding Author's Institution:	Institut Pasteur
Corresponding Author E-Mail:	giulia.nigro@pasteur.fr
Order of Authors:	Aline Stedman
	Antonin Levy
	Philippe Sansonetti
	Giulia Nigro
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TITLE:

Analyzing Oxidative Stress in Murine Intestinal Organoids Using Reactive Oxygen Species-Sensitive Fluorogenic Probe

AUTHORS AND AFFILIATIONS:

Aline Stedman[1,a], Antonin Levy[1,b], Philippe J. Sansonetti[1,2,c], Giulia Nigro[1,d]*

[1]Molecular Microbial Pathogenesis Unit, Institut Pasteur, INSERM U1202, 75015 Paris, France
[2]Chaire de Microbiologie et Maladies Infectieuses, Collège de France, 75231 Paris, France

Present address:
[a]Sorbonne Université, CNRS UMR7622, INSERM U1156, Institut de Biologie Paris Seine (IBPS) - Developmental Biology Unit, 75005 Paris, France.
[b]Molecular Radiotherapy, INSERM U1030, Gustave Roussy, Université Paris-Saclay, F-94805 Villejuif, France
[c]The Center for Microbes, Development and Health, Institut Pasteur Shanghai and Chinese Academy of Sciences, Shanghai, China
[d]Microenvironment and Immunity Unit, Institut Pasteur, INSERM U1224, 75015 Paris, France

Email addresses of the authors:
Aline Stedman (aline.stedman@sorbonne-universite.fr)
Antonin Levy (antonin.levy@gustaveroussy.fr)
Philippe J. Sansonetti (philippe.sansonetti@ips.ac.cn)
Giulia Nigro (giulia.nigro@pasteur.fr)

*Email address of the corresponding author:
Giulia Nigro (giulia.nigro@pasteur.fr)

KEYWORDS:

Murine intestinal organoids, ROS detection, Flow cytometry analysis, Live imaging detection, intestinal stem cells, ROS-sensitive dye, oxidative stress, fluorogenic probes

SUMMARY:

The present protocol describes a method to detect reactive oxygen species (ROS) in the intestinal murine organoids using qualitative imaging and quantitative cytometry assays. This work can be potentially extended to other fluorescent probes to test the effect of selected compounds on ROS.

ABSTRACT:

Reactive oxygen species (ROS) play essential roles in intestinal homeostasis. ROS are natural by-products of cell metabolism. They are produced in response to infection or injury at the mucosal level as they are involved in antimicrobial responses and wound healing. They are also critical secondary messengers, regulating several pathways, including cell growth and differentiation. On the other hand, excessive ROS levels lead to oxidative stress, which can be deleterious for cells

and favor intestinal diseases like chronic inflammation or cancer. This work provides a straightforward method to detect ROS in the intestinal murine organoids by live imaging and flow cytometry, using a commercially available fluorogenic probe. Here the protocol describes assaying the effect of compounds that modulate the redox balance in intestinal organoids and detect ROS levels in specific intestinal cell types, exemplified here by the analysis of the intestinal stem cells genetically labeled with GFP. This protocol may be used with other fluorescent probes.

INTRODUCTION:

Reactive oxygen species (ROS) are natural by-products of cellular metabolism. They can also be actively produced by specialized enzymatic complexes such as the membrane-bound NADPH-Oxidases (NOX) and Dual Oxidases (DUOX), which generate superoxide anion and hydrogen peroxide[1]. By expressing antioxidant enzymes and ROS scavengers, cells can finely tune their redox balance, thereby protecting tissue homeostasis[2]. Although ROS can be highly toxic to the cells and damage DNA, proteins, and lipids, they are crucial signaling molecules[2]. In the intestinal epithelium, moderate ROS levels are required for stem and progenitor cell proliferation[3]; high ROS levels lead to their apoptosis[4]. Chronic oxidative stress is linked to many gastrointestinal diseases, such as inflammatory bowel diseases or cancer. As an example, in a mouse model of Wnt-driven intestinal cancer, elevated ROS production through activation of NADPH-oxidases was found to be required for cancer cells hyperproliferation[5, 6]. Defining how intestinal cells and, in particular, stem cells manage oxidative stress and how the cellular environment can impact this capacity is essential to understand the etiology of this disease better[7].

In a tissue, different cell types present a basal oxidative state that may vary according to their function and metabolism and the expression of varying levels of oxidant and antioxidant molecules[4, 7]. Monitoring ROS *in vivo* is very challenging. Cell permeable dyes that emit fluorescence according to their redox state have been developed to visualize and measure cellular ROS in living cells and animals. However, their efficacy depends on their diffusion inside living tissues and their rapid readout, making them difficult to use in animal models[8].

In the past, the study of the effect of compounds on ROS generation was done using cell lines, but this may not reflect the *in vivo* situation. The intestinal organoid model, developed by the group of Clevers[9], enables the growth of intestinal primary cells *ex vivo*. Culture of intestinal crypts in matrices, in the presence of defined growth factors, leads to three-dimensional structures, called organoids (mini-gut), which reproduce the crypt-villus organization, with cells from the different epithelial lineages lining an internal lumen, and the intestinal stem cells residing in small crypts-like protrusions.

Here, taking advantage of this model, a simple method is described to study oxidative stress in primary intestinal cells at the single-cell resolution by adding a commercially available ROS-sensitive dye into the organoid culture medium.

Plate readers are often used to detect ROS production in a total population. This protocol uses flow cytometry or imaging assay to detect ROS in a particular cell type with genetically modified cells or specific antibody staining. This work involves mouse intestinal organoid culture and ROS

visualization by confocal imaging and quantification by flow cytometry. Using Lgr5-GFP mice-derived small intestinal organoids, it has been shown that it is possible to specifically analyze the level of oxidative stress in intestinal stem cells upon different treatments. This protocol can be adapted to test the influence of exogenous molecules, such as microbiota-derived muramyl-dipeptide (MDP)[10], on the ROS balance, after stimulating organoids with the selected compounds.

PROTOCOL:

All animal experiments were carried out after approval by the Institut Pasteur Use Committee and by the French Ministry of Agriculture no. 2016-0022. All the steps are performed inside a tissue culture hood.

1. Preparation of reagents and materials for culturing intestinal organoids

1.1. To prepare growth culture medium, mix advanced DMEM/F-12 supplemented with 1x glutamine, 1x penicillin/streptomycin (P/S) solution, 10 mM of HEPES, 50 ng/mL of murine EGF, 20 µg/mL of murine Noggin 500 ng/mL of mouse R-Spondin1 (see **Table of Materials**). Leave the medium at room temperature (RT) during the crypt's extraction.

NOTE: Freeze the unused medium in aliquots at -20 °C. Avoid freeze and thaw.

1.2. Fill a 50 mL tube with 40 mL of Advanced DMEM/F-12 and keep it on ice.

NOTE: Keep the unused medium at 4 °C. It will be used for organoids passaging.

1.3. Pre-warm the cell culture plates (µ-Slide 8 well chambers and/or 96-well round bottom) in the incubator at 37 °C.

1.4. Thaw basement membrane matrix (BMM) (see **Table of Materials**) aliquots on ice (before starting the protocol or at least 1 h before plating crypts).

NOTE: The BMM will quickly solidify if not kept cold.

1.5. Prepare washing/flushing solution adding 1% penicillin-streptomycin solution to DPBS (DPBS-P/S).

1.6. Fill a 100 mm petri-dish with 10 mL of cold DPBS-P/S. Fill six 15 mL tubes with 10 mL of DPBS and label them from F1 to F6.

1.7. Prepare 30 mL of 10 mM EDTA solution by dilution from 0.5 M EDTA in DPBS. Fill two 15 mL tubes with 10 mL of 10 mM EDTA, and label them E1 and E2.

1.8. Keep all solutions pre-cooled at 4°C and keep them on the ice during the procedure.

2. Intestinal organoids culture

2.1. Sacrifice a 8-10 weeks-old Lgr5-EGFP-IRES-creERT2 (Lgr5-GFP) mouse according to the national rules and regulations.

2.2. Collect 5-8 cm of the jejunum encompassing the region between the duodenum (5 cm from the stomach) and the ileum (10 cm from the cecum) and keep in cold DPBS-P/S on ice.

2.3. Clean the intestinal content by flushing with 5-10 mL of cold DPBS-P/S.

NOTE: Home-made flushing syringes can be obtained by plugging a 200 µL tip onto a 10 mL syringe nozzle.

2.4. Open the intestine longitudinally using ball tip scissors (see **Table of Materials**) (to prevent damaging the tissue).

2.5. Using forceps, transfer the tissue into a petri dish containing cold DPBS-P/S at room temperature and shake it to rinse.

2.6. With a plastic Pasteur pipette, grab the intestine by aspiration and transfer it into a 15 mL tube labeled **E1** containing 10 mL cold 10 mM EDTA.

2.7. Invert the tube 3 times and incubate on ice for 10 min.

2.8. Using a plastic Pasteur pipette, transfer the tissue in tube **F1** containing 10 mL DPBS. Vortex for 2 min (on normal vortex, holding the tube by hand and ensuring that the intestine swirls nicely).

2.9. Put 10 µL of the fraction in a petri dish and assess the quality of the fraction under a microscope.

NOTE: All vortex steps are performed at maximum speed, and the quality of each fraction should be assessed under the microscope (**Figure 1**).

2.10. With a plastic Pasteur pipette, grab the intestine by aspiration and transfer it in tube **F2** containing 10 mL DPBS and vortex for 2 min.

2.11. Repeat step 2.10, transferring the tissue in tube **F3** containing 10 mL DPBS and vortex for 2 min.

2.12. Repeat EDTA incubation as in step 2.6, transferring the tissue in tube **E2** containing 10 mM EDTA.

2.13. Invert the tube 3 times and incubate on ice for 5 min.

2.14. Repeat step 2.10, transferring the tissue in tube **F4** containing 10 mL DPBS and vortex for 3 min.

2.15. Repeat step 2.14, transferring the tissue in tube **F5** containing 10 mL DPBS and vortex for 3 min.

2.16. Repeat step 2.15, transferring the tissue in tube **F6** containing 10 mL DPBS and vortex for 3 min.

2.17. Combine the best fractions filtering by gravity through a 70 µm cell strainer into a 50 mL tube (on ice) to eliminate villi and significant debris.

NOTE: Usually, F5 and F6 are the fractions containing numerous crypts and less debris.

2.18. Spin the crypts at 150 x *g* at 4 °C for 3 min.

2.19. Empty the tube, disrupt the pellet mechanically, and add 5 mL of cold DMEM/F12.

2.20. Put 10 µL of the suspension in a petri dish and count the number of crypts present in the aliquot manually under a microscope.

NOTE: Do not count single cells or small debris.

2.21. Calculate the volume (V) of crypts suspension required in µL, considering that 300 crypts are plated per well, W is the number of wells, and N is the number of crypts counted out of 10 µL of the suspension.

NOTE: V = 300 x W x 10/N. Then transfer the solution to a new 15 mL tube. If a small volume is used in the planned experiment, a 1.5 mL centrifuge tube can be used.

2.22. Spin the crypts at 200 x *g* at 4 °C for 3 min.

2.23. Carefully remove the supernatant using a pipette.

2.24. Mechanically disrupt the pellet and gently add growth culture medium to obtain a concentration of 90 crypts/µL.

2.25. Add 2 volumes of undiluted BMM to have a final concentration of 30 crypts/µL. Carefully pipette up and down without introducing air bubbles into the mix.

NOTE: Always keep the tube on ice to avoid BMM solidification.

2.26. Plate 10 µL of the crypts/BMM mix into each well. For Flow cytometry analysis, use round-bottom 96-well plates. Distribute 10 µL at the center of each well as a dome. For imaging, use µ-slide 8 well (see **Table of Materials**) and deposit the 10 µL as a thin layer.

NOTE: Plate the organoids as a thin layer for the imaging assay to enable their in-depth imaging.

2.27. Leave the plate for 5 min at RT to allow the BMM to solidify. Place the plate in the incubator at 37 °C and 5% CO_2 for 15 min.

2.28. Add 250 µL of growth medium into each well, taking care not to detach the BMM.

2.29. Place the plates in the incubator at 37 °C and 5% CO_2.

2.30. Perform the ROS analysis between days 4 and 6 of culture. Otherwise, change the medium and split the organoids after the appearance of several and long budding structures and when dead cells accumulate into the organoids lumens.

3. Organoids passaging

3.1. Start passaging small intestinal organoids from the 6th day of culture, when significant budding structures have formed, and the organoids lumens have become dark.

NOTE: The organoid's lumen becomes dark due to the accumulation of dead cells, debris, and mucus. Avoid letting the organoids overgrow before splitting. The splitting ratio depends on the organoids' growth. Passaging the organoids with a ratio of 1:2 at day 6 and 1:3 at day 10 is recommended.

3.2. Fill a 15 mL tube with 4 mL of cold Advanced DMEM/F-12 and keep it on ice.

NOTE: Here, volumes for a 96-well culture plate are provided. If a different format is used, adjust the volume accordingly.

3.3. Carefully aspirate the medium with a pipette or a vacuum pump from the wells without touching the BMM domes, and discard it.

3.4. Add 100 µL of cold Advanced DMEM/F-12 per well. Pipette up and down to break the BMM and transfer the content of the well into the 15 mL tube.

3.5. Wash the well with 200 µL cold Advanced DMEM/F-12 and collect it in the same tube.

NOTE: If passaging multiple wells from the same experimental condition, the contents of the wells can be pooled in the same 15 mL collecting tube.

3.6. Spin the 15 mL collecting tube at 100 x *g* for 5 min at 4°C.

3.7. Discard the supernatant and add 1mL of cold Advanced DMEM/F-12 to the pellet. Using a P1000 tip, take up a P10 tip (without filter) and pipette up and down at least 20 times.

3.8. Add 4 mL of cold Advanced DMEM/F-12 to the tube. Spin at 300 x *g* for 5 min at 4°C.

3.9. Aspirate the supernatant with a pipette or a vacuum pump without disturbing the pellet. Then, disrupt the pellet mechanically.

3.10. Add BMM diluted in growth culture medium (2:1 ratio). Carefully pipette up and down without introducing air bubbles into the mix.

3.11. Plate 10 µL of the crypts/BMM mix into each well.

3.12. Keep the plate for 5 min at RT to allow the BMM to solidify. Place the plate in the incubator at 37 °C and 5% CO_2 for 15 min.

3.13. Add 250 µL of growth medium into each well.

NOTE: Be careful not to detach the BMM.

3.14. Place the plates in the incubator at 37 °C and 5% CO_2.

4. Preparation of reagents and materials to assess oxidative stress in intestinal organoids

4.1. Prepare a 250 mM stock solution of inhibitor N-acetylcysteine (NAC) (see **Table of Materials**), resuspend 10 mg with 245 µL of DPBS. Use at 1 mM final concentration.

4.2. Prepare a 50 mM stock solution of inducer Tert-butyl hydroperoxide (tBHP), 70% in water, dilute 3.22 µL with 496.8 µL of DPBS. Use at 200 µM final concentration.

4.3. For the Flow cytometry study, prepare a 250 µM working solution of a fluorogenic probe (see **Table of Materials**) by diluting the stock solution 1/10 in DMSO. Use at 1 µM final concentration.

NOTE: As indicated in the manufacturer's instructions, the fluorogenic probe is sensitive to light and oxygen. Stocks and aliquots should not be open and close too many times.

4.4. For the imaging study, prepare a 1.25 mM working solution of the fluorogenic probe by diluting the stock solution 1/2 in DMSO. Use at 5 µM final concentration.

4.5. Prepare a final solution of 0.1 µg/mL DAPI in DPBS, to be used for dead cell discrimination in the Flow cytometry assay.

4.6. Dilute Hoechst 33342 to 1.25 mg/mL in DPBS. Use at 5 µg/mL final concentration to be used for nuclear staining in the imaging assay.

4.7. Warm DMEM without phenol red at 37 °C.

NOTE: These steps describe using negative and positive controls that must be included in any assays, using the conditions indicated in **Figure 2A**. The assay can be used to test anti- or pro-oxidant compounds. The steps are the same, and the only difference is when the compounds are added before using the fluorogenic dye.

5. Visualization of oxidative stress in 3D organoids by confocal microscopy

5.1. Take the organoids plated in the µ-Slide 8 well chambers and add 1 µL NAC stock solution in the corresponding wells to obtain a final concentration of 1 mM.

5.2. Incubate for 1 h at 37 °C and 5% CO_2.

5.3. Add 1 µL tBHP stock solution in the corresponding wells to obtain a final concentration of 200 µM.

5.4. Incubate for 30 min at 37 °C and 5% CO_2.

5.5. Add 1 µL per well of the 1.25 mM dilution of the fluorogenic probe to obtain a final concentration of 5 µM.

5.6. Add 1 µL per well of the 1.25 mg/mL dilution of Hoescht to obtain a final concentration of 5 µg/mL.

5.7. Incubate for 30 min at 37 °C and 5% CO_2.

5.8. Remove the medium without disturbing the BMM. Gently, add 250 µL of warm DMEM without phenol red.

NOTE: If a long-term acquisition is planned, add growth factors compounds to DMEM without phenol red.

5.9. Image the organoids using a confocal microscope equipped with a thermic chamber and gas supply that detects the fluorogenic probe (ROS).

NOTE: The excitation/emission (ex/em) for the fluorogenic probe is 644/665, ex/em for Hoechst (nuclei) is 361/486, and ex/em for GFP (intestinal stem cell from the Lgr5-GFP mice) is 488/510. A 63x oil immersion objective is used to detect signals in stem cells. Do not change laser settings between samples. A 20x objective might be used to allow an overview of ROS production.

5.10. Use the positive control to set up laser intensity and time exposure for the ROS signal and check that this signal is lower in the negative control.

5.11. Using eyepiece screen the slide to identify the organoids expressing GFP and adjust laser intensity.

NOTE: This step is manually performed. The eyepiece screens the slide to identify the GFP expressing organoids.

5.12. Define positions to obtain a stitched image of the whole organoid. Setup a z-stack of 25 µm (step size 5 µm) to get a section of the organoids showing one layer of cells.

NOTE: Refer to the microscope user manual to optimize the setup. Using living cells, the acquisition should be done within 1 h after the end of the incubation.

5.13. Open the images in an open-source image processing software (see **Table of Materials**).

5.14. Go through the z-stack and choose the section in which the middle of the organoids is well represented and create a new image with the selected area.

5.15. Quantify the images as per steps 5.15.1 – 5.15.5.

5.15.1. Select the freehand line tool.

5.15.2. Draw a line following the nuclei.

NOTE: Select only regions presenting GFP-positive cells if only stem cells are analyzed.

5.15.3. Increase the line width to cover the cell layer with the line without including the luminal debris.

5.15.4. Select the channel for the ROS signal and measure the fluorescence intensity in the selected region and annotate the values.

5.15.5. Draw a line where there is no signal and measure the fluorescent intensity of the background that will be subtracted to the previous value to get the final intensity.

6. Quantification of the oxidative stress on the dissociated organoids using flow cytometer

6.1. Add 1 µL NAC stock solution in the wells for negative controls to obtain a final concentration of 1 mM.

NOTE: Use the organoids plated in the 96-well round-bottom plates.

6.2. Incubate for 1 h at 37 °C and 5% CO_2.

6.3. Add 1 µL tBHP stock solution in the corresponding wells to obtain a final concentration of 200 µM.

6.4. Incubate for 30 min at 37 °C and 5% CO_2.

6.5. With a multichannel pipette, remove the medium without disturbing the attached BMM and transfer it to another 96-well round bottom plate. Keep this plate aside.

6.6. Add 100 µL of trypsin, and with a multichannel pipette, pipette up and down at least five times to destroy the BMM.

6.7. Incubate for not more than 5 min at 37 °C and 5% CO_2.

6.8. With a multichannel pipette, pipette up and down at least five times to dissociate the organoids.

6.9. Spin at 300 x *g* for 5 min at RT.

6.10. Discard the supernatant by inverting the plate. Add back the medium collected in step 6.3 to the corresponding wells and resuspend the cells by pipetting up and down 5 times.

6.11. Add the fluorogenic probe at the final concentration of 1 µM. Add 1 µL per well from the 250 µM dilution and incubate for 30 min at 37 °C and 5% CO_2.

NOTE: Do not add the fluorogenic probe to the wells needed for the instrument's settings (**Figure 2B**).

6.12. Spin at 300 x *g* for 5 min at RT.

6.13. Resuspend the cells with 250 µL of 0.1 µg/mL DAPI solution. Transfer the samples in the proper Flow cytometry tubes, keep the tubes on ice, and proceed with the analysis.

NOTE: Add PBS instead of DAPI to the wells needed for the instrument's settings (**Figure 2B**).

6.14. Optimize the forward and side scatter voltage settings on unstained control and laser voltages for each fluorophore using mono-stained samples.

6.15. Using an appropriate gating strategy (**Figure 4A**), collect a minimum of 20,000 events.

NOTE: 50,000 events are preferred. Detailed acquisition settings vary according to the instrument used.

REPRESENTATIVE RESULTS:

As a proof of concept of the described protocol, the crypts obtained from the Lgr5-eGFP-IRES-CreERT2 mouse line were used in which intestinal stem cells display mosaic GFP expression, which was established by Barker et al., to characterize intestinal stem cells[10] initially and allow to map these cells based on their GFP expression. A model is thereby provided to compare ROS levels in a specific cell type population upon different treatments. A ROS inhibitor (NAC) was used, and an inducer (tBHP), known to act on cellular ROS to visualize changes in their levels.

Figures 1A and **1B** show representative images of fractions F1 and F4 obtained during the crypts extraction procedure for the intestinal organoid culture. Each fraction must be checked under a microscope or binocular during the extraction procedure to follow crypts detachment and define those fractions enriched in crypts, rather than villi, single cells, or debris. The chosen fractions are then pooled together and passed through a 70 µm cell strainer to remove all the remaining fragments of villi and obtain a preparation with only crypts (**Figure 1C**). The crypts start to close within a few hours of embedding in BMM, and at D1, round organoids were observed (**Figure 1D**). After 3-5 days, the organoids will appear with budding structures representing the "newly formed crypts." The organoids are ready for ROS analysis (**Figures 1E** and **1F**).

In the protocol of imaging oxidative stress by confocal microscopy, the slide containing the organoids, incubated with the probe, was imaged with a confocal fluorescence microscope equipped with lasers and filters to detect the Hoechst (ex/em: 361/486), the GFP (ex/em: 488/510) and the fluorogenic probe (ex/em): 644/665) signals. A confocal microscope equipped with 20x air and 63x oil immersion objective allowed the visualization of ROS. In Lgr5-GFP mice, the GFP-positive cells are Lgr5-expressing intestinal stem cells. **Supplementary Figure 1** shows representative images obtained with the 20x objective providing an overview of the ROS in several organoids. **Figure 3** shows representative images, obtained with the 63x oil objective, of intestinal organoids expressing GFP, non-treated (NT), or pre-incubated or not with the ROS inhibitor NAC, and stimulated or not for 30 min with the ROS inducer tBHP.

In the presence of the inhibitor, the only signal from the dead cells contained in the lumen of the organoid is visible. In the non-treated organoid, the basal ROS levels are shown, proving that stem cells produce higher ROS than differentiated cells (according to the microscope settings, the ROS signal might also be visualized in non-stem cells). GFP-positive cells present a more significant cytoplasmic signal with the inducer in the presence of the fluorogenic probe, demonstrating that ROS levels increase particularly in stem cells after treatment.

Figure 4 shows representative results obtained when analyzing ROS production in intestinal organoids stimulated or not with ROS inhibitor or inducer, using a Flow cytometer equipped with 405 nm, 488 nm, and 630 nm lasers. The gating strategy presented in **Figure 4A** makes it possible to evaluate ROS production at the level of the whole organoids cell population, defining intact and living cells based on physical parameters and DAPI exclusion (SSC-A *vs.* FSC-A and DAPI *vs.* FSC-A) and FSC-H *vs.* FSC-A) or only in the intestinal stem cells, further gated on cells with GFP high signal. **Figure 4B** shows the ROS levels in the total population upon collection of 50,000 events. Basal ROS levels in the non-treated (NT) cells decrease after stimulation with the inhibitor

(NAC), and on the contrary, increase after challenge with the inducer (tBHP). Cells pre-treated with the inhibitor and then stimulated with the inducer present a lower level than those stimulated with the inducer alone. The results were then analyzed using appropriate software, obtaining the median fluorescent intensity (MFI). The obtained values are presented as a ratio over the non-treated cells, as shown in the graph presented on the right of **Figure 4B**. **Figure 4C** shows the same parameters described in **Figure 4B** in the stem cells, gated as GFP positive cells, showing a 3.5-fold decrease in ROS level upon NAC treatment and 4-fold increase upon tBHP treatment over non-stimulated cells. This result demonstrates that following this protocol, it is possible to quantify differences in ROS levels at the level of the whole cell population or in GFP positive stem cells upon their treatment of the organoids with specific compounds.

FIGURE LEGENDS:

Figure 1: Representative images of crypts and organoids. (**A**) Example of fraction F1 obtained after the first incubation with EDTA, enriched in villi (square), with some debris (star) and crypts (circle)**.** (**B**) Example of fraction F4 enriched in crypts. (**C**) Suspension presenting only isolated crypts obtained after the filtration with a 70 µm cell strainer (scale bar, 200 µm). (**D**, **E, and F**). Typical organoids were obtained after 1, 3, and 5 days respectively, after embedding the crypts in BMM (scale bar, 100 µm).

Figure 2: Outline of the experimental plan. (**A**) Conditions used in this protocol included in each experiment: non-treated wells (NT), inducer-treated wells (tert-Butyl hydroperoxide - tBHP), inhibitor-treated wells (N-acetyl cysteine - NAC), and inhibitor- and inducer-treated wells (NAC-tBHP). (**B**) Plate format for the flow cytometry assay. Each condition is plated in triplicate (line A). Lines B, C, and D include wells for flow cytometer setting with only the fluorogenic probe, only DAPI, or non-stained (NS) samples.

Figure 3: Representative confocal images of ROS staining in organoids. Stitched images were obtained with a confocal microscope equipped with a high-speed EMCCD Camera, 63x/1.4 oil objective, and slit 35 µm, using the lasers 405, 488, 640, and filters 460/50, 535/50, 700/75 to acquire Hoechst, GFP, and the fluorogenic probe respectively. Confocal optical sections of organoids non-treated (NT), treated with the ROS-inhibitor (NAC), with the ROS-inducer (tBHP), or pre-treated with the ROS inhibitor and then stimulated with the ROS-inducer (NAC-tBHP). In grey, nuclei stained with Hoechst; in green, Lgr5-GFP cells; in red, the fluorogenic probe (scale bar, 50 µm).

Figure 4: Representative flow cytometry analysis of ROS in cells derived from organoids. (**A**) Schematic representation of the gating strategy used in flow cytometry analysis: gating for cell shape (exclusion of dead cells and debris accumulated in the organoids lumen), gating for living cells (cells not incorporating DAPI-laser 405), gating for single cells (doublet discrimination), and stem cells (GFP positive cells-laser 488) (FSC: forward scatter, SSC: side scatter). The ROS signal has been acquired using the 630 laser. (**B**) On the left, histograms were obtained with an appropriate software showing the intensity ROS signals for the total living population (after gating around 10,000 events per condition) in the different samples NT: non-treated; NAC:

inhibitor-treated; tBHP inducer-treated; NAC-tBHP: inhibitor- and inducer-treated. On the right, a typical example of the calculated ratio for MFI values over the NT samples obtained during an experiment starting from 3 samples per condition (mean ± SD) (*** P = 0.0003). (**C**) Same as in B for the GFP positive population (1,000 events per condition) (* P = 0.02).

Supplementary Figure 1: Representative confocal images of ROS staining in organoids. Stitched **i**mages were obtained with a confocal microscope equipped with a high-speed EMCCD Camera, 20x objective, and slit 35 µm, using the lasers 405, 488, 640, and filters 460/50, 535/50, 700/75 to acquire Hoechst, GFP, and fluorogenic probe respectively. Confocal optical sections of organoids non-treated (NT), treated with the ROS-inhibitor (NAC), with the ROS-inducer (tBHP), or pre-treated with the ROS inhibitor and then stimulated with the ROS-inducer (NAC-tBHP). In grey, nuclei stained with Hoechst; in green, Lgr5-GFP cells; in red, fluorogenic probe (scale bar, 100 µm).

DISCUSSION:

This work provides a step-by-step protocol to isolate murine jejunal crypts, culture them into 3D organoids, and analyze ROS in organoids by combining a ROS-sensitive fluorogenic probe with qualitative microscopy imaging of whole organoids and quantitative ROS measurement using flow cytometry on single cells following organoid dissociation.

The first critical step in this method is the crypts extraction procedure. Indeed, the quality of crypts preparation is the key to successful organoids formation. It is therefore essential to obtain fractions with enriched crypts and few cellular debris or dying cells. The crypts may be found in different fractions to those indicated in the protocol, as dissociation may vary with the age and health status of the mouse. The number of EDTA incubations can be modified accordingly. If crypts do not seem to be detaching after fraction 4, a 3 min EDTA incubation needs to be repeated. Inversely, suppose crypts already detach after the first EDTA incubation. In that case, the second EDTA incubation may not be necessary, and the sequential vortex steps in DPBS should be done until fractions are obtained with enough crypts devoid of debris. If no dissociation occurs, make sure DPBS without Ca^{2+} and Mg^{2+} is used to prepare the collecting tubes, and replace EDTA with a new solution. Crypts are fragile structures, so they should be kept as much as possible on ice and rapidly plated after isolation.

Different plates and drop volumes may be used to cultivate organoids. For instance, crypts can also be plated in 24 or 48 well plates after adjusting the crypts concentration, the volume of the BMM drop, and the medium added in each well. Multiple drops may be plated in the same well in a 12- or 6-well plate. Generally, crypts decrease in size and round up to form small round organoids at day 1 of culture. Formation of new buds should be observed 2-3 days after the plating.

For studying changes in ROS levels in intestinal stem cells, the advantage of the Lgr5-eGFP-IRES-CreERT2 mouse line was taken. A caveat of this model is the selective silencing of the knocked-in allele and the consequent mosaicism of the GFP expression, which can be absent in patches of stem cells or entire crypts. During the imaging protocol, not all the organoids will present stem

cells expressing GFP; therefore, not all the organoids will be considered unless it is possible to rely on the spatial position of the cells. Instead, this must be considered when analyzing the GFP negative cell population in the Flow cytometry protocol. Indeed, as it is impossible to rely on the spatial position, the GFP-negative population will be composed of non-stem cells and GFP-negative stem cells.

Here a protocol is provided for the qualitative evaluation of ROS in intestinal organoids. A critical aspect for this part is linked to the working distance of the objectives that are used. The organoids are grown in BMM; they are not attached to the bottom of the well, introducing a distance from the objective focus plan. For this reason, it is critical to plate the organoids in a thin layer of BMM, to minimize this issue. Even in this optimized setting, not all the organoids will be in the correct position to be adequately imaged.

A quantitative analysis of the images might be done using an appropriate image analysis software, evaluating the mean fluorescent intensity of the images in the ROS signal channel as described in the protocol. For this purpose, it is necessary to acquire a high number of images to get a sufficient number of events to be statistically significant. As mentioned before, using the Lgr5-GFP mice, not all the organoids will express GFP, requiring a considerable number of samples to be imaged.

During the flow cytometry procedure, a critical step is the dissociation of the organoids into single cells. If the dissociation is too harsh, cells may die and release DNA. A rock-inhibitor, Y-27632, to counteract anoikis, and DNAse may be added to the dissociation buffer if they do not interfere with the studied pathway. Trypsin dilution or reduced incubation times may be used.

Finally, it is crucial to define the best time point to analyze ROS production after the different treatments (anti- or pro-oxidant) tested. In the case of drugs that rapidly induce ROS within minutes or hours, the imaging assay can be used to determine when there is the maximal induction by adding the fluorogenic probe before the tested compounds. The fluorescence intensity of the probe after organoids stimulation may vary between experiments performed on different days. Therefore, it is crucial always to calculate the ratio with the non-stimulated samples and add controls (oxidant /antioxidant) to verify the reactivity of the probe. NAC and tBHP were used as negative and positive controls as they gave the most conclusive results. Still, other reagents may be used, such as resveratrol as an antioxidant or paraquat/menadione as oxidants. Incubating cells for too long with the fluorogenic probe may be toxic and even modify the cell redox balance, so incubation times must also be tightly controlled. Cells stained with the probe may be fixed and analyzed a few hours after. In this case, for the flow cytometry analysis, the DAPI cannot discriminate between living and dead cells. Instead, a fixable dye for live/dead discrimination should be used before fixation.

Organoids may also be grown for several days (more than 7), but this will increase the number of living and proliferative cells and the dead cells that accumulate in the organoids lumens, generating high background, particularly in the imaging assay. If an abnormal increase in the fluorogenic probe signal is observed, ensure that the solution used to resuspend stimulating

compounds is not pro-oxidant per se (i.e., ethanol).

One concern to consider when using this protocol is that live imaging and cell dissociation followed by flow cytometry may induce oxidative stress in cells and generate a background signal. Fixation of the organoids may be considered according to the experiment. Another limitation arises from the difficulty in the in-depth imaging of organoids grown in a 3D matrix. As mentioned in the protocol, the BMM should be distributed on the slide as a thin layer to limit this aspect.

Here, the protocol is designed using a commercially available fluorogenic dye. Its primary advantage is its compatibility with multi-color staining of organoids so that specific cell types. For instance, antibody staining for cell surface markers immediately after the fluorogenic probe incubation may be done to detect particular sub-types. However, the probe is not specific to a specific ROS species as it can detect Superoxide, Nitrite peroxide, and hydrogen peroxide[11–13]. For this reason, it is generally used to detect global oxidative stress. Although commercialized as a cytosolic-only probe, the selected fluorogenic probe could be found to reach mitochondria[14]. As its specificity can vary between different cellular contexts, we suggest using other complementary approaches to measure ROS when possible. Alternative dyes such as probes specific to detect mitochondria-generated superoxide anions could be used[10]. A repertoire of chemiluminescent probes was also developed to detect specific ROS species with high sensitivity, such as luciferin-based probes[15, 16]. These have the advantage of being compatible with *in vivo* imaging but can't be used to map ROS production with specific cell types. Finally, this protocol can be applied to other types of organoids, for instance, colonic organoids derived from human biopsies. In this case, the culture growth medium should be adapted accordingly[17]. To further analyze the redox machinery within intestinal cells, the organoids culture and dissociation procedures described in this protocol can be combined with transcriptomic and proteomic approaches on whole organoids or Fluorescence activated cell sorted (FACS) organoids cells.

ACKNOWLEDGMENTS:

This work was supported by French National Research Agency (ANR) grant 17-CE14-0022 (i-Stress).

DISCLOSURES:

The authors have nothing to disclose.

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Figure1

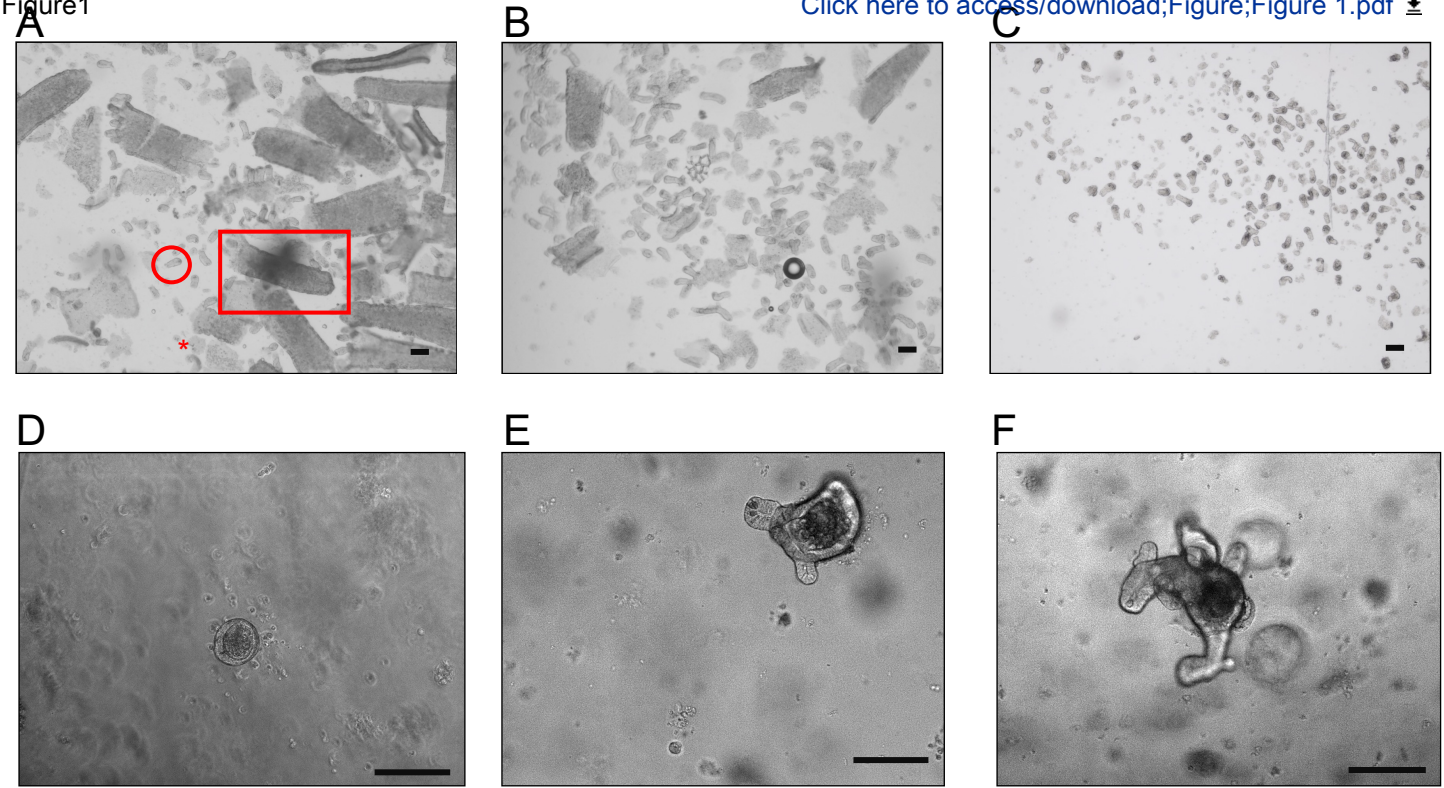


Figure2

A

Control	Non-treated	NT
Positive signal	tert-Butyl hydroperoxide	tBHP
Middle signal	N-acetyl cysteine and tert-Butyl hydroperoxide	NAC-tBHP
Negative signal	N-acetyl cysteine	NAC

B

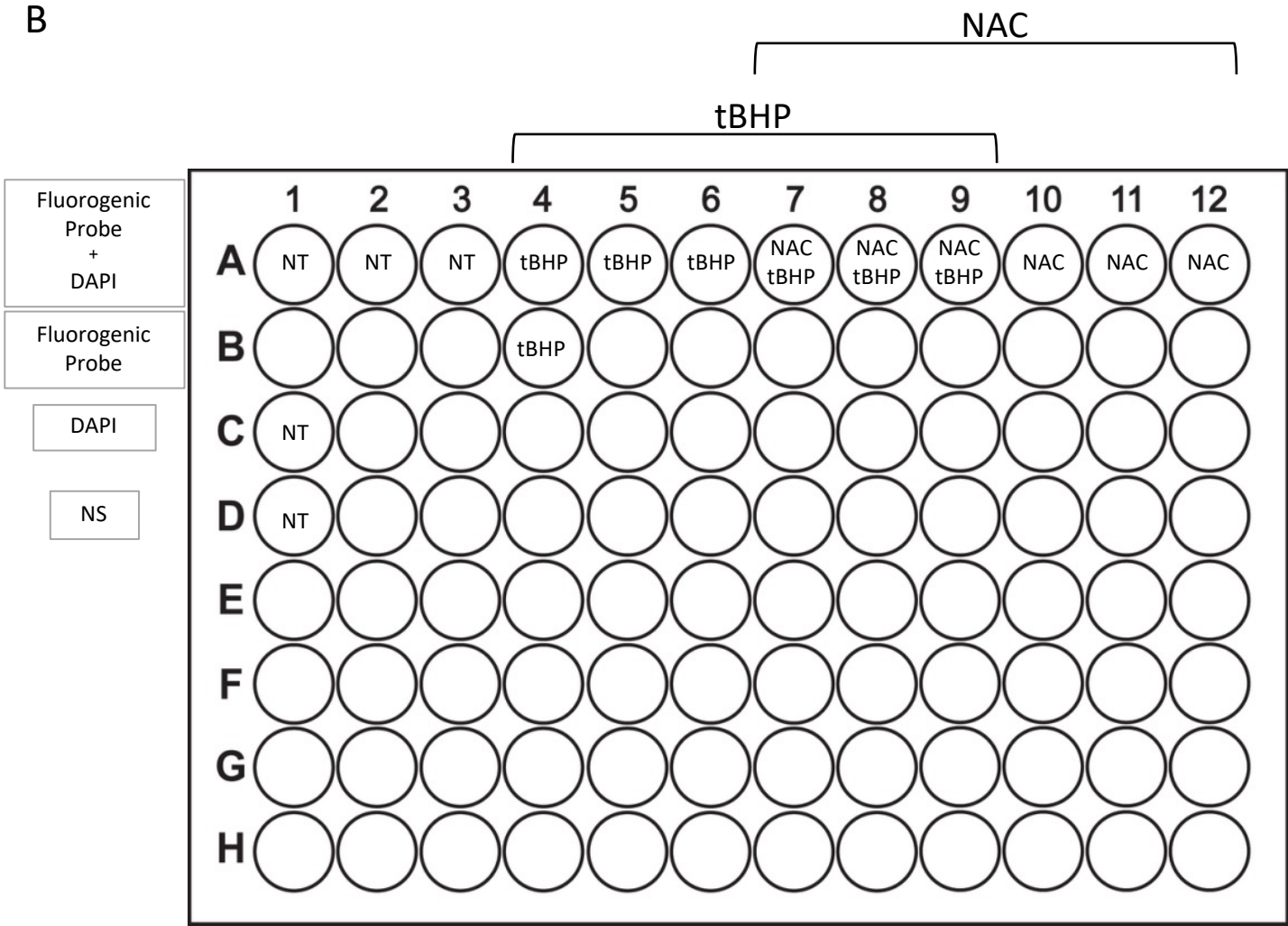


Figure3

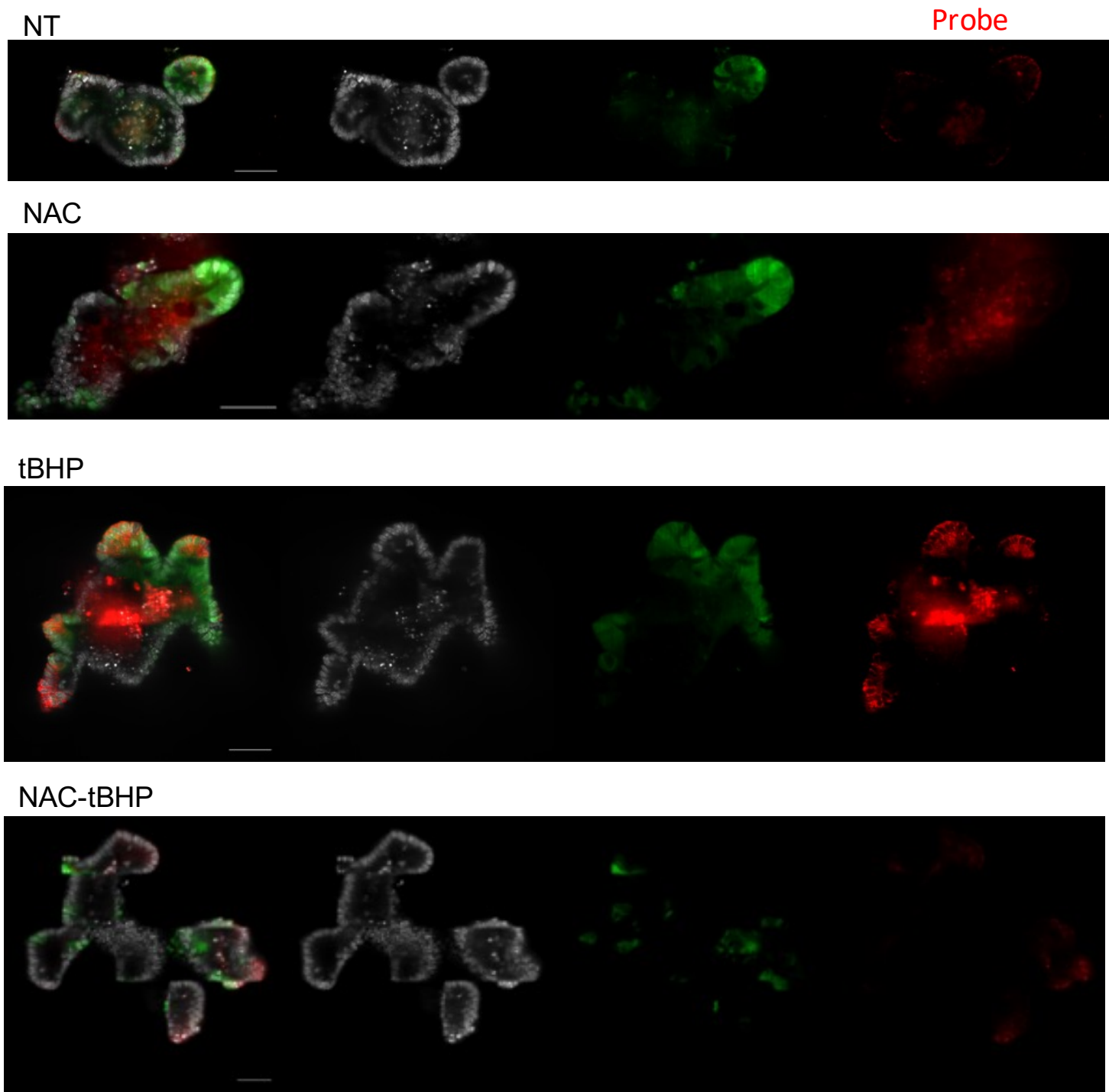
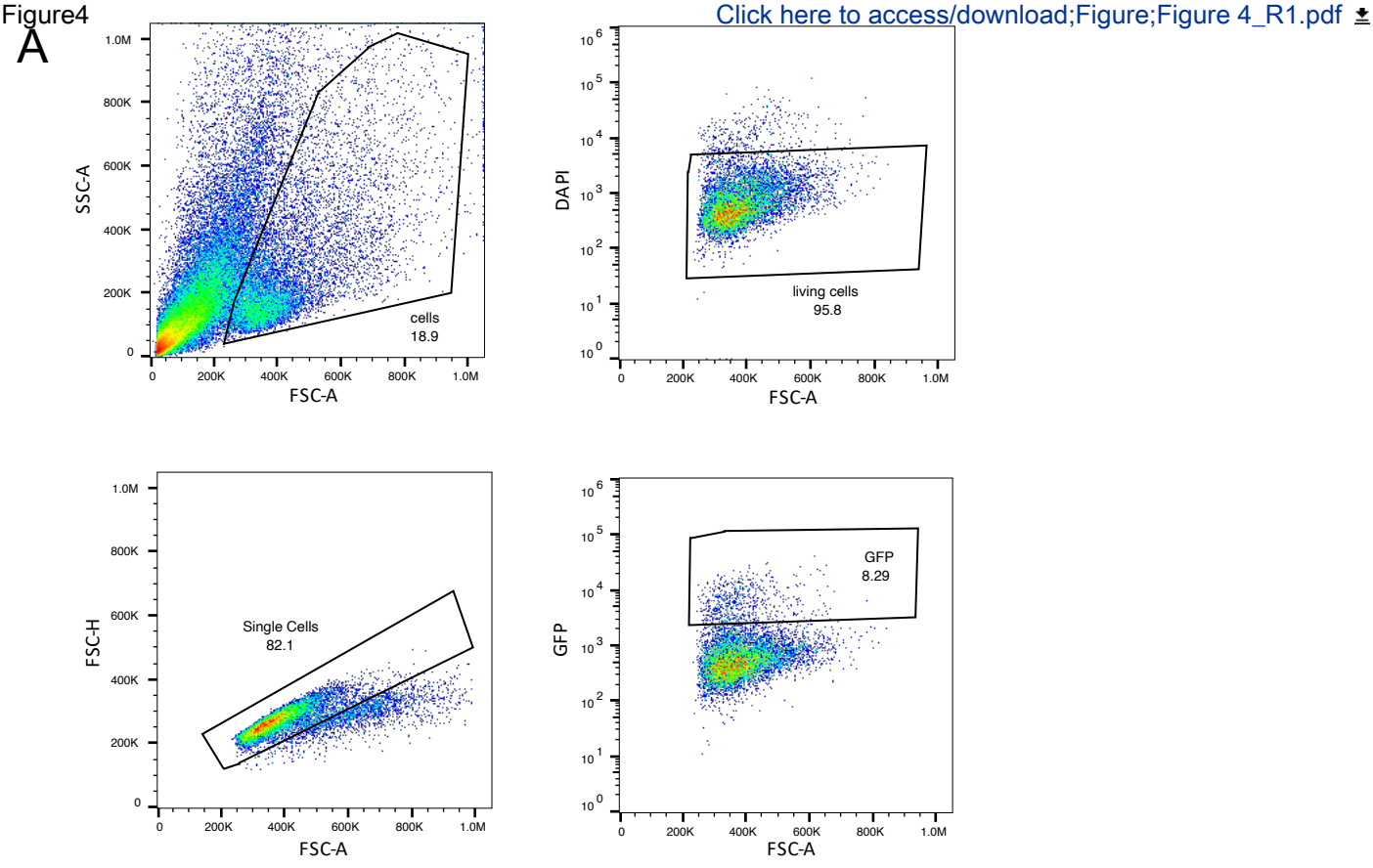
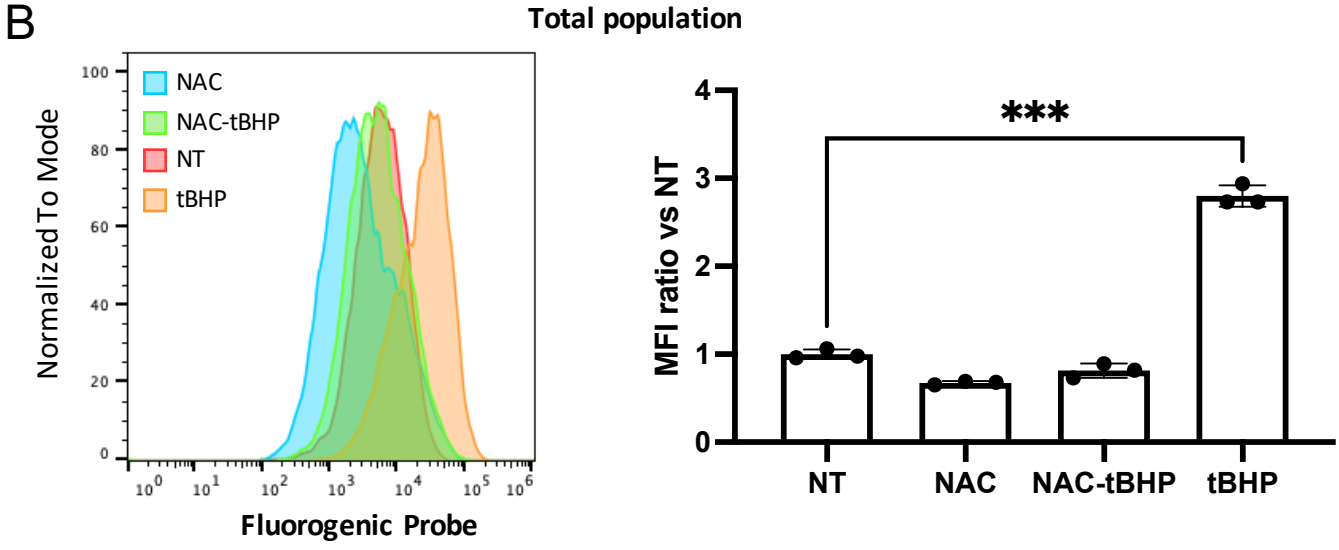
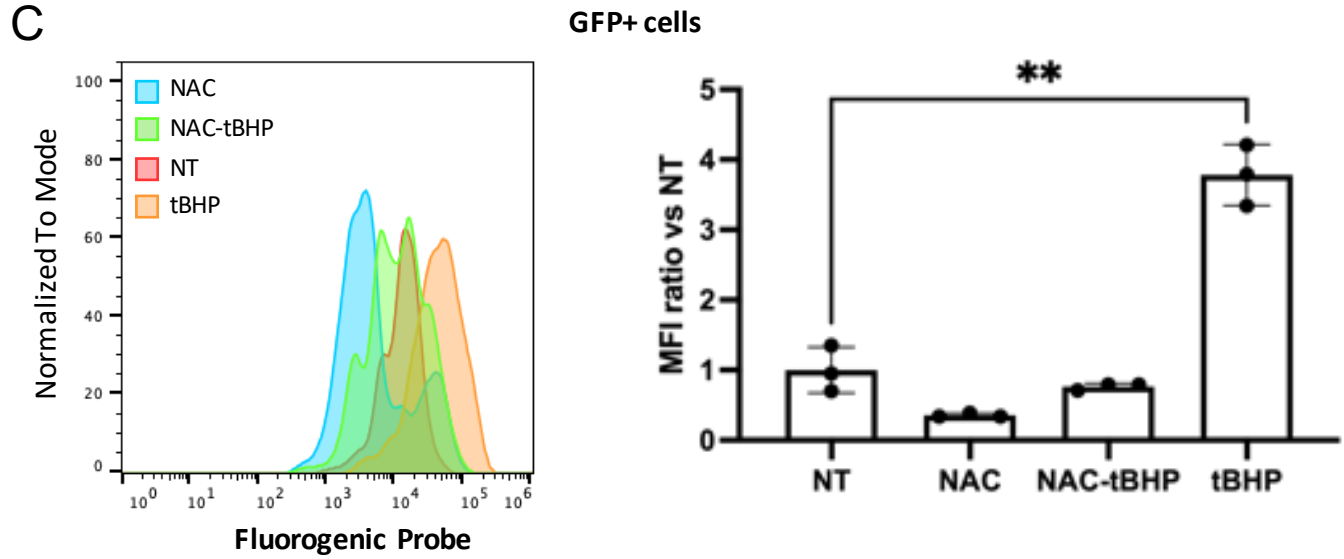


Figure4







Dear Editor,
First of all, we wish to thank the editorial board and the reviewers for the feedbacks in order to improve our manuscript. We have made several adjustments that reflect the detailed suggestions you have kindly provided. As major changes we have changed the title in order to be more coherent with the described protocol. We have introduced a new figure, to describe the experimental conditions, we have modified the original figures, and introduced a supplementary figure to answer to specific points raised by some reviewers.
We hope that our edits and the replies we provide below satisfactorily address all the issues and concerns you and the reviewers have pointed out.

Due to issues with language and grammar, the manuscript is not sufficiently coherent.

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Please revise the manuscript to thoroughly address the reviewers' concerns and all the editorial comments. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
We have corrected the spelling and grammar errors and defined all abbreviations accordingly.

2. Please avoid abbreviations in the title and revise your title to “Imaging and quantification of oxidative stress in murine intestinal organoids using a reactive oxygen species-sensitive fluorescent dye.”
We have modified the title to better clarify the described protocol, and we have removed the abbreviation as requested. The new title is “Analyzing oxidative stress in murine intestinal organoids using reactive oxygen species-sensitive fluorescent dye”

3. Please revise the following lines to avoid overlap with previously published work: 77-80.
We have revised the paragraph to circumvent overlapping issues.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (this includes the figures and the legends) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: GlutaMAX; Matrigel; FluoroBrite DMEM; TrypLe Express; FLowJo; TrypLE etc
We have modified the text accordingly but let the commercial name of the ROS probe used in the protocol as no generic terms can be used.

5. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
We have removed all personal pronouns from the protocol.

6. Instead of lines 221-225, consider a figure showing the plate format and refer to the figure here.
We have introduced the Figure 2 to describe the experimental conditions and the plate format.

7. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
We have removed all personal pronouns from the protocol.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
We have revised the protocol so that the protocol section is written at imperative tenses as asked.

9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step

performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

10. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. This will ensure that filming will be completed in one day. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

11. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.
We have revised the references to the requested format

12. Please sort the Materials Table alphabetically by the name of the material.
We have revised the materials table

Reviewers' comments:

Reviewer #1:

In the present work, Stedman and colleagues describe a protocol for the analysis of oxidative stress in murine intestinal organoids using confocal microscopy and flow cytometry. This is an interesting and valuable methodology for all researches in the area of redox biology, inflammatory bowel disease, colorectal carcinogenesis and CRC chemoprevention.

The methods are described in sufficient detail. Representative analysis by confocal imaging and flow cytometry are provided, showing the versatility of this approach. Possible limitations of the methodology and further applications are also mentioned.

Altogether, this work is of interest for the audience of JOVE. Nevertheless the are some points of concern as detailed below.
We thank the reviewer for his/her positive comment and his/her help in improving our manuscript.

- Abstract: "in the other hand" should read "on the other hand"
We have modified the text

- line 59: "expressing antioxidants" - do the authors mean expression of antioxidant enzymes or biosynthesis of molecules with antioxidant function (e.g. GSH, Vitamin C, etc.)?
We have modified the text as follow: expressing antioxidant enzymes and ROS scavengers

- Please add citations showing that ROS are required for stem cell proliferation (line 62)
We have introduced the reference: van der Post, S., Birchenough, G.M.H., Held, J.M. NOX1-dependent redox signaling potentiates colonic stem cell proliferation to adapt to the intestinal microbiota by linking EGFR and TLR activation. Cell Reports. 35 (1), 108949 (2021).

- Line 70 oxidative state that might vary (instead of variate)
We have modified the text

- Line 74 in live => in living cells/animals??
We have modified the text

- Line 96: please explain the term MDP
We have modified the text: muramyl-dipeptide (MDP)

- Line 118: DPBS-P please explain abbreviation
We have modified the text DPBS plus 1% penicillin-streptomycin solution (DPBS-P/S)

- Line 216: please also detail how to split organoids
We have added a protocol for organoids passaging: paragraph 3

- Line 273: are there any supplements in FluoroBrite DMEM?
We have specified in the text to use a DMEM without phenol red. The supplements are needed only if long-term acquisition is planned

- Line 273 ff: please include a part that describes how the confocal images are used for quantitative analysis and add information on the cLSM system used to acquire the images shown in Fig. 2
Information of the cLSM system has been added in the materials table. More details regarding lasers and filters have been added in the text and in the figure legend.
We have added the details for the quantitative analysis, but in the results section but we have decided to describe a confocal imaging approach to assess oxidative stress in organoids only qualitatively. The settings we used in this protocol make the acquisition long, and therefore the number of images per condition are limited and not enough to have statistically significant numbers to provide a quantification. We have added in the discussion section a small paragraph along this line.

- Line 312 ff: please include a part that describes the gating strategy and the quantitative evaluation (e.g. flow chart) and mention the equipment used to generate the data shown in Fig. 2
We have introduced a better description of the gating strategy in the text and in the figure legend. We have modified the text to better describe the quantitative evaluation. The information regarding the equipment has been added in the text, in the legend, and in the material table.

- Line 355: please explain the abbreviation MFI
We have modified the text: median fluorescent intensity (MFI)

- FACS setting: is it necessary to include unstained organoids with or without treatment?
We have modified the text and introduced a new figure to explain the samples needed for the unstained or monostained controls for the FACS settings

- Fig. 3A: top left panel: please comment on how the cell population was gated
Surprisingly, this population accounts for only about 19 % of the total population. Please explain.
We have modified the text and the legend to better explain the gating strategy. In the first gate we can isolate already a living population based on FSC and SSC parameters. Only 20% of the recorded events correspond to healthy living cells. One explanation lies in the fact that, comparably to the in vivo intestine, organoids present a high cellular turnover. Consequently, apoptotic cells and debris, that are shedded away from the epithelial layer, accumulating inside the organoids lumen, are released with the healthy cells upon organoids dissociation. Another reason why we get a high percentage of dead cells is that epithelial cells are highly sensitive to dissociation. To limit cell death, Rock inhibitors or other inhibitors of anoikis are generally added to the dissociation medium. However, since these might interfere with the ROS pathway, we don't use them in this particular protocol.

- Fig. 3b: what is difference between the two panels? => please label total pop vs. stem cells!
We have modified the figure and the legend and added a graph for the calculated ratio of MFI in stem cells

- Fig.2A: optical section or merged z-stack? Please also included a merged z-stack, if only a optical section is depicted.
Only optical sections of organoids are shown, and we have now specified it in the legend. 3D organoids are thick complex multicellular structures and we estimate that imaging whole organoids with a 63x objective would imply a too long acquisition time that might interfere with the probe fluorescence signal and introduce a bias when comparing ROS-levels between the different conditions. Moreover, to get the cellular resolution needed to differentiate between stem cells and neighboring cells from z projections of whole organoids would require the use of 3D segmentation tools that we don't provide in this protocol. For these reasons, we chose to acquire only few stacks, preferentially in the middle of the organoids depth in order to be able to visualize the different cell types in the same image. Therefore, we are not providing a maximal projection of the whole organoids that moreover we don't think will help the reader to visualize a single layer of cells.

- General question: which reactive oxygen species are actually detected by CellROX?
The CellRox probe is not specific to a particular ROS specie as it can detect Superoxide, Nitrite peroxyde and hydrogen peroxyde. For this reason, it is generally used to detect global oxidative stress. Although commercialized as a cytosolic only probe, CellRox Deep Red could be found to reach mitochondria (Bidaux et al, PNAS, 2015). As its specificity can vary between different cellular contexts, we suggest using in parallel other complementary approaches to measure ROS when possible. We have added this information in the discussion.

- Page 19: stock => which stocks are meant?
We think that this comment came from an issue with the generated PDF of the materials table. The Page with only stocks corresponds to the last column regarding the reagents that have been used.

- please highlight critical steps in the protocol and pitfalls
We have highlighted more critical steps and pitfalls in the discussion

Reviewer #2:
The manuscript is a protocol describeing organoid culturing and expressing a fluorescent reporter in a organoid line. Basically this review does not serve any purpose. How to culture organoids has been extensively described by those who developed this method/approach (e.g. Clevers group) and this review does not contribute any further understanding and is also written by adopters not developers. The same holds true for the fluorescent probe. This probe is highly debated iin the (redox) field and the general consensus is that it does not measure the acclaimed ROS. Taken together to me this review does not provide anything worthwhile nor does it contribute in deepening understanding, to what is already known and I fail to see the purpose of publishing.
We agree that organoids culture protocols already exist in the literature and that the approach we propose to study oxidative stress is not based on a new technological development, but rather on the adaptation of already existing methods. However, we provide an adapted version of organoids culture and manipulation that we feel are technically robust and are of interest to beginners in the field who wish to use organoids as a model system to evaluate ROS cellular management upon particular conditions. We think that comparing different protocols and results is the key to better defining technical bottlenecks and challenges. The CellRox probe is debated, in particular because it is not specific to a particular ROS specie as it can detect Superoxide, Nitrite peroxyde and hydrogen peroxyde. For this reason, it is generally used to detect global oxidative stress. Although commercialized as a cytosolic only probe, CellRox Deep Red could be found to reach mitochondria (Bidaux et al, PNAS, 2015). Moreover, as other ROS-sensitive dyes it is highly reactive and therefore can give different results in different contexts. That is why, in the discussion part, we stressed the importance of using in parallel other complementary approaches to measure ROS-levels.

Reviewer #3:
Manuscript Summary:
In this study the authors describe in detail a method to detect ROS levels in small intestinal murine organoids. For this they use both live imaging with confocal fluorescence microscopy and flow cytometry, using a commercial available fluorescent probe for ROS detection. ROS levels in specific cell types, as here exemplarily shown by the use of GFP expressing intestinal stem cells, can be monitored with this method.
We thank the reviewer for his/her positive comment and his/her help in improving our manuscript.

Major Concerns:
Figure 2: The authors only present a very limited number of organoids imaged by confocal microscopy. Please also include a representative 20x objective overview of your organoids under each condition tested (untreated, NAC; tBHP, and NAC+tBHP) and include additional high resolution organoid images of treatment with NAC only and untreated cells, to compare the results of the different treatments. At least three representative organoids each condition should be presented, to confirm reproducibility of your results. It is necessary, that these images come from independently performed experiments. It seems like the experiment has only been carried out once, which is not enough to conclude that these experiments are reproducible. What are the ROS levels in untreated organoids/stem cells compared to activated ones and within stem cells where ROS production is suppressed? How can this images be quantified, to have not only a visual read out?

We agree that reproducibility is essential when describing a protocol. That is why we have performed these experiments several times and imaged multiple organoids from each condition. One important change we noticed is the difference in absolute fluorescence intensity of the dye between experiments, therefore we insist in the protocol that a positive (incubation with a ROS-inducer) and a negative (incubation with an antioxidant) control should always be included in each experiment, and the relative fluorescence of the dye compared. However, we feel that providing at least 3 pictures of organoids at high resolution for each condition would make the figures too large and difficult to apprehend for the readers. We nonetheless provide new images from a different experiment in order to present the different conditions as requested and including additional pictures of organoids imaged at the 20x objectives that might support the reproducibility of the protocol.
We have added the details for the quantitative analyses. In this protocol, we have decided to describe a confocal imaging approach to qualitatively assess for oxidative stress in organoids, in addition to a more quantitative approach using FACS analysis. Quantitative analysis of confocal images is indeed feasible, we have added in the discussion section a small paragraph along this line, however we think the settings we used in this protocol do not allow us to obtain statistically analyzable images.
On the other hand, the FACS approach allowed us to conclude that the basal level of ROS in stem cells is decreased upon NAC treatment 3.5-fold and increased 4-fold upon induction by tBHP, versus the non-treated cells.

Line 273: To make it more clear for other scientist how to recapitulate the experiments, please describe the technical setup of your confocal microscope in more detail. Which microscope from which company has been

used. Which lasers have been used, at which emission wavelength have the single dyes been recorded? Which pinhole size was used? At what resolution have the images been recorded?

More details regarding lasers and filters, and the settings of the microscope have been added in the text and in the figure legend. Information regarding the microscope has been added in the methods table

Figure 3: Authors should also include a representative FACS image of the GFP gated stem cells on one axis and the CellRox Deep Red signal on the other axes as well as a representative image of all living cells on one axis and the CellRox Deep Red signal on the other axis. How many cells have been analyzed for the graphs in 3B? How does this histogram look like for GFP positive cells only? Please include an additional histogram image for GFP positive cells. Authors claim that with their protocol specific cell types, such as stem cells, can be analyzed for their ROS production, but this is not what they show finally. Similar, for Figure 3C please include also a graph presenting the GFP positive cells only, and how their MFI changes upon treatment, as well as state the number of cells analyzed. How many cells have been used to analyze the data in figure 3C? Also, it would be interesting to see a graph, where only GFP negative cells (no stem cells) are plotted for their MFI upon treatment. Do they react similar to the inhibitory or activating signal?

We have modified the Figure (now Figure 4) and the corresponding legend. We have also included more details regarding the number of events that should be acquired and the ones presented in the figure. We have now included a graph for the calculated MFI in GFP positive cells upon treatment.
In this protocol, we use GFP positive stem cells as a model to show that the effect of selected compounds on ROS in particular intestinal cell types can be addressed using confocal imaging and FACS. We indeed show that ROS-levels are modified in stem cells submitted to different treatments
We have implemented the discussion introducing some lines to better explain the mouse model we have used and how the GFP negative population is actually a mix of no stem cells and stem cells not expressing GFP.
“A caveat of this model is the selective silencing of the knocked-in allele, and the consequent mosaicism of the GFP expression, which can be absent in patches of stem cells or in entire crypts. During the imaging protocol, not all the organoids will present stem cells expressing GFP, therefore not all the organoids will be considered, unless it is possible to rely on the spatial position of the cells. Instead, this must be taken into consideration when analyzing the GFP negative cell population in the FACS protocol. Indeed, as it is not possible to rely on the spatial position, the GFP negative population will be composed of both non-stem cells and GFP-negative stem cells.”

Minor Concerns:
In the introduction of the manuscript, the authors speak of intestinal crypts and organoids. As they are working with small intestinal organoids only, this should be specified.
We have added in the introduction that we are describing a protocol applied to small intestinal organoids

Line 96: Please explain abbreviation: MDP.
We have modified the text: muramyl-dipeptide (MDP)

Line 178: and later on: Instead of dislodge, please use the word disrupt or homogenize the pellet or something similar.
We have replaced dislodge with disrupt

Line: 184: The authors should present a formula how they calculate the number of crypt being plated.
We have introduced a formula to explain how to calculate the volume of crypts.

Line 218: The authors should describe how to split organoids. This makes it easier for other researchers to perform their studies without the need of reading additional publications.
A protocol for organoids passaging has been added as step 3 in the new version of the manuscript

Line 367: Figure 2A represents the inhibited state and B the activated state. Please rewrite the figure legend.
We have corrected the figure legend

Line 344: It seems that the CellRox signal is only seen in LGR+ stem cells (and dead cells) in your representative image. Please rewrite your conclusions.
The CellROX signal is indeed higher in Lgr5 positive stem cells compared with other cells of the organoids, suggesting that stem cells have higher ROS-levels. The major difference in fluorescence intensity between non-stem cells, stem cells and dead cells present in the lumen, obliged us to apply acquisition settings that render the low CellROX signal present in non-stem cells invisible, unless we push the signal to the point that stem cells and

dead cells become saturated. The aim of this protocol is to compare ROS-level between different conditions. We have rewritten the conclusion to emphasize this aspect.

Line 355: Please explain abbreviation: MFI.
We have modified the text: median fluorescent intensity (MFI)

Line 314: Please state your setup for FACS analysis and which instrument (company) you have used to perform the analysis to make it easier for other researchers to adapt the protocol to their instrument.
We have added in the text more details regarding the FACS, and the instrument we have used in the Materials table.

Reviewer #4:
Manuscript Summary:
This manuscript describes a method to analyse cellular reactive oxygen species in intestinal epithelial organoids using both flow cytometry and microscopy. the first part of the methods describes how to establish murine intestinal epithelial organoids, which, although multiple such protocols exist, is clearly explained. The second part details how to measure ROS using a commercially available dye, CellRox. the method is well-described, and caveats are thoughtfully presented.
The protocol will be useful to many researchers interested in studying ROS in the intestinal epithelium, a key player in IBD.
We thank the reviewer for his/her positive comment and his/her help in improving our manuscript.

Major Concerns:
No major concerns

Minor Concerns:
the figures needed a bit more explanation.
We have modified the text of the representative results and the legends.

In Figure 2, it would be helpful if the colours were labelled on the figure.
Colors have been added on the figure.

I also think that A and B may have been swapped, because ROS is higher in B, which according to the figure legend has been treated with NAC. If the figures have not been swapped, can the authors please explain the result- as to why the signal for Cellrox is higher in the LGR5+ cells in B?
We have corrected the legend.

In Figure 3, what is the difference between the histograms plotted in the half graph and the right graph of B? It would be nice to know if on the GFP+ cells were gated on, would the graphs look different? or the GFP- cells?
We have corrected the legend to explain the graph in which we have presented the MFI in the GFP positive cells.
We have also introduced a new graph for the calculated MFI in this population.
We did not present the result after gating on the GFP negative cells as in the mouse line used for this study, the GFP expression is mosaic and therefore the GFP negative population still contains a lot of GFP negative stem cells.
We have implemented the discussion introducing some lines to better explain the mouse model we have used and how the GFP negative population is actually a mix of non-stem cells and stem cells not expressing GFP.
“A caveat of this model is the selective silencing of the knocked-in allele, and the consequent mosaicism of the GFP expression, with can be absent in patches of stem cells or in entire crypts. During the imaging protocol, not all the organoids will present stem cells expressing GFP, therefore not all the organoids will be considered, unless it is possible to rely on the spatial position of the cells. Instead, this must be taken into consideration when analyzing the GFP negative cell population in the FACS protocol. Indeed, as it is not possible to rely on the spatial position, the GFP negative population will be composed of both non-stem cells and GFP-negative stem cells.”

In the discussion, line 401, 'die' needs to replace 'dye'.
We have modified the text.

Also the rock inhibitor needs to be explained.
We have modified the text, explaining the use of the Rock inhibitor to counteract anoikis. Epithelial cells die following detachment from the surrounding matrix and upon dissociation to single cell.

Reviewer #5:
Manuscript Summary:
This manuscript provides a step-by-step protocol to quantitate the level of oxidative stress in specific cell

populations of cultured intestinal organoids using a ROS-sensitive fluorescent dye and analysis by confocal imaging or flow cytometry. Organoids are now widely used as state of the art model of the intestinal epithelium and ROS balance is a critical factor in normal epithelial function. Thus, this protocol should be of interest to other scientists in the field.

Major Concerns:
none

We thank the reviewer for his/her positive comment

Supplemental Figure1