



**HAL**  
open science

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

Aline Stedman, Antonin Levy, Philippe Sansonetti, Giulia Nigro

► **To cite this version:**

Aline Stedman, Antonin Levy, Philippe Sansonetti, Giulia Nigro. Analyzing Oxidative Stress in Murine Intestinal Organoids using Reactive Oxygen Species-Sensitive Fluorogenic Probe. *Journal of visualized experiments: JoVE*, 2021, 175, 10.3791/62880 . pasteur-03584354

**HAL Id: pasteur-03584354**

**<https://pasteur.hal.science/pasteur-03584354>**

Submitted on 22 Feb 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **TITLE:**

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

4  
5 **AUTHORS AND AFFILIATIONS:**

6 Aline Stedman<sup>1,a</sup>, Antonin Levy<sup>1,b</sup>, Philippe J. Sansonetti<sup>1,2,c</sup>, Giulia Nigro<sup>1,d\*</sup>

7  
8 <sup>1</sup>Molecular Microbial Pathogenesis Unit, Institut Pasteur, INSERM U1202, 75015 Paris, France

9 <sup>2</sup>Chaire de Microbiologie et Maladies Infectieuses, Collège de France, 75231 Paris, France

10

11 Present address:

12 <sup>a</sup>Sorbonne Université, CNRS UMR7622, INSERM U1156, Institut de Biologie Paris Seine (IBPS) -  
13 Developmental Biology Unit, 75005 Paris, France.

14 <sup>b</sup>Molecular Radiotherapy, INSERM U1030, Gustave Roussy, Université Paris-Saclay, F-94805  
15 Villejuif, France

16 <sup>c</sup>The Center for Microbes, Development and Health, Institut Pasteur Shanghai and Chinese  
17 Academy of Sciences, Shanghai, China

18 <sup>d</sup>Microenvironment and Immunity Unit, Institut Pasteur, INSERM U1224, 75015 Paris, France

19

20 Email addresses of the authors:

21 Aline Stedman ([aline.stedman@sorbonne-universite.fr](mailto:aline.stedman@sorbonne-universite.fr))

22 Antonin Levy ([antonin.levy@gustaveroussy.fr](mailto:antonin.levy@gustaveroussy.fr))

23 Philippe J. Sansonetti ([philippe.sansonetti@ips.ac.cn](mailto:philippe.sansonetti@ips.ac.cn))

24 Giulia Nigro ([giulia.nigro@pasteur.fr](mailto:giulia.nigro@pasteur.fr))

25

26 \*Email address of the corresponding author:

27 Giulia Nigro ([giulia.nigro@pasteur.fr](mailto:giulia.nigro@pasteur.fr))

28

29 **KEYWORDS:**

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

32

33 **SUMMARY:**

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

38

39 **ABSTRACT:**

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

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

51

## 52 **INTRODUCTION:**

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

66

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

73

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

81

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

85

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

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

95

## 96 **PROTOCOL:**

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

100

### 101 **1. Preparation of reagents and materials for culturing intestinal organoids**

102

103 1.1. To prepare growth culture medium, mix advanced DMEM/F-12 supplemented with 1x  
104 glutamine, 1x penicillin/streptomycin (P/S) solution, 10 mM of HEPES, 50 ng/mL of murine EGF,  
105 20 µg/mL of murine Noggin 500 ng/mL of mouse R-Spondin1 (see **Table of Materials**). Leave the  
106 medium at room temperature (RT) during the crypt's extraction.

107

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

109

110 1.2. Fill a 50 mL tube with 40 mL of Advanced DMEM/F-12 and keep it on ice.

111

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

113

114 1.3. Pre-warm the cell culture plates (µ-Slide 8 well chambers and/or 96-well round bottom)  
115 in the incubator at 37 °C.

116

117 1.4. Thaw basement membrane matrix (BMM) (see **Table of Materials**) aliquots on ice (before  
118 starting the protocol or at least 1 h before plating crypts).

119

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

121

122 1.5. Prepare washing/flushing solution adding 1% penicillin-streptomycin solution to DPBS  
123 (DPBS-P/S).

124

125 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  
126 DPBS and label them from F1 to F6.

127

128 1.7. Prepare 30 mL of 10 mM EDTA solution by dilution from 0.5 M EDTA in DPBS. Fill two 15  
129 mL tubes with 10 mL of 10 mM EDTA, and label them E1 and E2.

130

131 1.8. Keep all solutions pre-cooled at 4°C and keep them on the ice during the procedure.

132

133 **2. Intestinal organoids culture**

134

135 2.1. Sacrifice a 8-10 weeks-old Lgr5-EGFP-IRES-creERT2 (Lgr5-GFP) mouse according to the  
136 national rules and regulations.

137

138 2.2. Collect 5-8 cm of the jejunum encompassing the region between the duodenum (5 cm  
139 from the stomach) and the ileum (10 cm from the cecum) and keep in cold DPBS-P/S on ice.

140

141 2.3. Clean the intestinal content by flushing with 5-10 mL of cold DPBS-P/S.

142

143 NOTE: Home-made flushing syringes can be obtained by plugging a 200  $\mu$ L tip onto a 10 mL  
144 syringe nozzle.

145

146 2.4. Open the intestine longitudinally using ball tip scissors (see **Table of Materials**) (to  
147 prevent damaging the tissue).

148

149 2.5. Using forceps, transfer the tissue into a petri dish containing cold DPBS-P/S at room  
150 temperature and shake it to rinse.

151

152 2.6. With a plastic Pasteur pipette, grab the intestine by aspiration and transfer it into a 15 mL  
153 tube labeled **E1** containing 10 mL cold 10 mM EDTA.

154

155 2.7. Invert the tube 3 times and incubate on ice for 10 min.

156

157 2.8. Using a plastic Pasteur pipette, transfer the tissue in tube **F1** containing 10 mL DPBS.  
158 Vortex for 2 min (on normal vortex, holding the tube by hand and ensuring that the intestine  
159 swirls nicely).

160

161 2.9. Put 10  $\mu$ L of the fraction in a petri dish and assess the quality of the fraction under a  
162 microscope.

163

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

166

167 2.10. With a plastic Pasteur pipette, grab the intestine by aspiration and transfer it in tube **F2**  
168 containing 10 mL DPBS and vortex for 2 min.

169

170 2.11. Repeat step 2.10, transferring the tissue in tube **F3** containing 10 mL DPBS and vortex for  
171 2 min.

172

173 2.12. Repeat EDTA incubation as in step 2.6, transferring the tissue in tube **E2** containing 10  
174 mM EDTA.

175

176 2.13. Invert the tube 3 times and incubate on ice for 5 min.

177  
178 2.14. Repeat step 2.10, transferring the tissue in tube **F4** containing 10 mL DPBS and vortex for  
179 3 min.

180  
181 2.15. Repeat step 2.14, transferring the tissue in tube **F5** containing 10 mL DPBS and vortex for  
182 3 min.

183  
184 2.16. Repeat step 2.15, transferring the tissue in tube **F6** containing 10 mL DPBS and vortex for  
185 3 min.

186  
187 2.17. Combine the best fractions filtering by gravity through a 70  $\mu\text{m}$  cell strainer into a 50 mL  
188 tube (on ice) to eliminate villi and significant debris.

189  
190 NOTE: Usually, F5 and F6 are the fractions containing numerous crypts and less debris.

191  
192 2.18. Spin the crypts at 150 x *g* at 4 °C for 3 min.

193  
194 2.19. Empty the tube, disrupt the pellet mechanically, and add 5 mL of cold DMEM/F12.

195  
196 2.20. Put 10  $\mu\text{L}$  of the suspension in a petri dish and count the number of crypts present in the  
197 aliquot manually under a microscope.

198  
199 NOTE: Do not count single cells or small debris.

200  
201 2.21. Calculate the volume (*V*) of crypts suspension required in  $\mu\text{L}$ , considering that 300 crypts  
202 are plated per well, *W* is the number of wells, and *N* is the number of crypts counted out of 10  
203  $\mu\text{L}$  of the suspension.

204  
205 NOTE:  $V = 300 \times W \times 10/N$ . Then transfer the solution to a new 15 mL tube. If a small volume is  
206 used in the planned experiment, a 1.5 mL centrifuge tube can be used.

207  
208 2.22. Spin the crypts at 200 x *g* at 4 °C for 3 min.

209  
210 2.23. Carefully remove the supernatant using a pipette.

211  
212 2.24. Mechanically disrupt the pellet and gently add growth culture medium to obtain a  
213 concentration of 90 crypts/ $\mu\text{L}$ .

214  
215 2.25. Add 2 volumes of undiluted BMM to have a final concentration of 30 crypts/ $\mu\text{L}$ . Carefully  
216 pipette up and down without introducing air bubbles into the mix.

217  
218 NOTE: Always keep the tube on ice to avoid BMM solidification.

219

220 2.26. Plate 10  $\mu$ L of the crypts/BMM mix into each well. For Flow cytometry analysis, use round-  
221 bottom 96-well plates. Distribute 10  $\mu$ L at the center of each well as a dome. For imaging, use  $\mu$ -  
222 slide 8 well (see **Table of Materials**) and deposit the 10  $\mu$ L as a thin layer.

223

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

225

226 2.27. Leave the plate for 5 min at RT to allow the BMM to solidify. Place the plate in the  
227 incubator at 37 °C and 5% CO<sub>2</sub> for 15 min.

228

229 2.28. Add 250  $\mu$ L of growth medium into each well, taking care not to detach the BMM.

230

231 2.29. Place the plates in the incubator at 37 °C and 5% CO<sub>2</sub>.

232

233 2.30. Perform the ROS analysis between days 4 and 6 of culture. Otherwise, change the medium  
234 and split the organoids after the appearance of several and long budding structures and when  
235 dead cells accumulate into the organoids lumens.

236

### 237 **3. Organoids passaging**

238

239 3.1. Start passaging small intestinal organoids from the 6<sup>th</sup> day of culture, when significant  
240 budding structures have formed, and the organoids lumens have become dark.

241

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

246

247 3.2. Fill a 15 mL tube with 4 mL of cold Advanced DMEM/F-12 and keep it on ice.

248

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

251

252 3.3. Carefully aspirate the medium with a pipette or a vacuum pump from the wells without  
253 touching the BMM domes, and discard it.

254

255 3.4. Add 100  $\mu$ L of cold Advanced DMEM/F-12 per well. Pipette up and down to break the  
256 BMM and transfer the content of the well into the 15 mL tube.

257

258 3.5. Wash the well with 200  $\mu$ L cold Advanced DMEM/F-12 and collect it in the same tube.

259

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

262

263 3.6. Spin the 15 mL collecting tube at 100 x g for 5 min at 4°C.

264  
265 3.7. Discard the supernatant and add 1mL of cold Advanced DMEM/F-12 to the pellet. Using  
266 a P1000 tip, take up a P10 tip (without filter) and pipette up and down at least 20 times.  
267  
268 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.  
269  
270 3.9. Aspirate the supernatant with a pipette or a vacuum pump without disturbing the pellet.  
271 Then, disrupt the pellet mechanically.  
272  
273 3.10. Add BMM diluted in growth culture medium (2:1 ratio). Carefully pipette up and down  
274 without introducing air bubbles into the mix.  
275  
276 3.11. Plate 10 µL of the crypts/BMM mix into each well.  
277  
278 3.12. Keep the plate for 5 min at RT to allow the BMM to solidify. Place the plate in the  
279 incubator at 37 °C and 5% CO<sub>2</sub> for 15 min.  
280  
281 3.13. Add 250 µL of growth medium into each well.  
282  
283 NOTE: Be careful not to detach the BMM.  
284  
285 3.14. Place the plates in the incubator at 37 °C and 5% CO<sub>2</sub>.  
286  
287 **4. Preparation of reagents and materials to assess oxidative stress in intestinal organoids**  
288  
289 4.1. Prepare a 250 mM stock solution of inhibitor N-acetylcysteine (NAC) (see **Table of**  
290 **Materials**), resuspend 10 mg with 245 µL of DPBS. Use at 1 mM final concentration.  
291  
292 4.2. Prepare a 50 mM stock solution of inducer Tert-butyl hydroperoxide (tBHP), 70% in water,  
293 dilute 3.22 µL with 496.8 µL of DPBS. Use at 200 µM final concentration.  
294  
295 4.3. For the Flow cytometry study, prepare a 250 µM working solution of a fluorogenic probe  
296 (see **Table of Materials**) by diluting the stock solution 1/10 in DMSO. Use at 1 µM final  
297 concentration.  
298  
299 NOTE: As indicated in the manufacturer's instructions, the fluorogenic probe is sensitive to light  
300 and oxygen. Stocks and aliquots should not be open and close too many times.  
301  
302 4.4. For the imaging study, prepare a 1.25 mM working solution of the fluorogenic probe by  
303 diluting the stock solution 1/2 in DMSO. Use at 5 µM final concentration.  
304  
305 4.5. Prepare a final solution of 0.1 µg/mL DAPI in DPBS, to be used for dead cell discrimination  
306 in the Flow cytometry assay.  
307



308 4.6. Dilute Hoechst 33342 to 1.25 mg/mL in DPBS. Use at 5 µg/mL final concentration to be  
309 used for nuclear staining in the imaging assay.

310

311 4.7. Warm DMEM without phenol red at 37 °C.

312

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

317

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

319

320 5.1. Take the organoids plated in the µ-Slide 8 well chambers and add 1 µL NAC stock solution  
321 in the corresponding wells to obtain a final concentration of 1 mM.

322

323 5.2. Incubate for 1 h at 37 °C and 5% CO<sub>2</sub>.

324

325 5.3. Add 1 µL tBHP stock solution in the corresponding wells to obtain a final concentration of  
326 200 µM.

327

328 5.4. Incubate for 30 min at 37 °C and 5% CO<sub>2</sub>.

329

330 5.5. Add 1 µL per well of the 1.25 mM dilution of the fluorogenic probe to obtain a final  
331 concentration of 5 µM.

332

333 5.6. Add 1 µL per well of the 1.25 mg/mL dilution of Hoescht to obtain a final concentration  
334 of 5 µg/mL.

335

336 5.7. Incubate for 30 min at 37 °C and 5% CO<sub>2</sub>.

337

338 5.8. Remove the medium without disturbing the BMM. Gently, add 250 µL of warm DMEM  
339 without phenol red.

340

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

343

344 5.9. Image the organoids using a confocal microscope equipped with a thermic chamber and  
345 gas supply that detects the fluorogenic probe (ROS).

346

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

351

352 5.10. Use the positive control to set up laser intensity and time exposure for the ROS signal and  
353 check that this signal is lower in the negative control.

354  
355 5.11. Using eyepiece screen the slide to identify the organoids expressing GFP and adjust laser  
356 intensity.

357  
358 NOTE: This step is manually performed. The eyepiece screens the slide to identify the GFP  
359 expressing organoids.

360  
361 5.12. Define positions to obtain a stitched image of the whole organoid. Setup a z-stack of 25  
362  $\mu\text{m}$  (step size 5  $\mu\text{m}$ ) to get a section of the organoids showing one layer of cells.

363  
364 NOTE: Refer to the microscope user manual to optimize the setup. Using living cells, the  
365 acquisition should be done within 1 h after the end of the incubation.

366  
367 5.13. Open the images in an open-source image processing software (see **Table of Materials**).

368  
369 5.14. Go through the z-stack and choose the section in which the middle of the organoids is  
370 well represented and create a new image with the selected area.

371  
372 5.15. Quantify the images as per steps 5.15.1 – 5.15.5.

373  
374 5.15.1. Select the freehand line tool.

375  
376 5.15.2. Draw a line following the nuclei.

377  
378 NOTE: Select only regions presenting GFP-positive cells if only stem cells are analyzed.

379  
380 5.15.3. Increase the line width to cover the cell layer with the line without including the luminal  
381 debris.

382  
383 5.15.4. Select the channel for the ROS signal and measure the fluorescence intensity in the  
384 selected region and annotate the values.

385  
386 5.15.5. Draw a line where there is no signal and measure the fluorescent intensity of the  
387 background that will be subtracted to the previous value to get the final intensity.

388  
389 **6. Quantification of the oxidative stress on the dissociated organoids using flow cytometer**

390  
391 6.1. Add 1  $\mu\text{L}$  NAC stock solution in the wells for negative controls to obtain a final  
392 concentration of 1 mM.

393  
394 NOTE: Use the organoids plated in the 96-well round-bottom plates.

395

396 6.2. Incubate for 1 h at 37 °C and 5% CO<sub>2</sub>.  
397  
398 6.3. Add 1 µL tBHP stock solution in the corresponding wells to obtain a final concentration of  
399 200 µM.  
400  
401 6.4. Incubate for 30 min at 37 °C and 5% CO<sub>2</sub>.  
402  
403 6.5. With a multichannel pipette, remove the medium without disturbing the attached BMM  
404 and transfer it to another 96-well round bottom plate. Keep this plate aside.  
405  
406 6.6. Add 100 µL of trypsin, and with a multichannel pipette, pipette up and down at least five  
407 times to destroy the BMM.  
408  
409 6.7. Incubate for not more than 5 min at 37 °C and 5% CO<sub>2</sub>.  
410  
411 6.8. With a multichannel pipette, pipette up and down at least five times to dissociate the  
412 organoids.  
413  
414 6.9. Spin at 300 x *g* for 5 min at RT.  
415  
416 6.10. Discard the supernatant by inverting the plate. Add back the medium collected in step 6.3  
417 to the corresponding wells and resuspend the cells by pipetting up and down 5 times.  
418  
419 6.11. Add the fluorogenic probe at the final concentration of 1 µM. Add 1 µL per well from the  
420 250 µM dilution and incubate for 30 min at 37 °C and 5% CO<sub>2</sub>.  
421  
422 NOTE: Do not add the fluorogenic probe to the wells needed for the instrument's settings (**Figure**  
423 **2B**).  
424  
425 6.12. Spin at 300 x *g* for 5 min at RT.  
426  
427 6.13. Resuspend the cells with 250 µL of 0.1 µg/mL DAPI solution. Transfer the samples in the  
428 proper Flow cytometry tubes, keep the tubes on ice, and proceed with the analysis.  
429  
430 NOTE: Add PBS instead of DAPI to the wells needed for the instrument's settings (**Figure 2B**).  
431  
432 6.14. Optimize the forward and side scatter voltage settings on unstained control and laser  
433 voltages for each fluorophore using mono-stained samples.  
434  
435 6.15. Using an appropriate gating strategy (**Figure 4A**), collect a minimum of 20,000 events.  
436  
437 NOTE: 50,000 events are preferred. Detailed acquisition settings vary according to the instrument  
438 used.  
439

440 **REPRESENTATIVE RESULTS:**

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

447  
448 **Figures 1A and 1B** show representative images of fractions F1 and F4 obtained during the crypts  
449 extraction procedure for the intestinal organoid culture. Each fraction must be checked under a  
450 microscope or binocular during the extraction procedure to follow crypts detachment and define  
451 those fractions enriched in crypts, rather than villi, single cells, or debris. The chosen fractions  
452 are then pooled together and passed through a 70 µm cell strainer to remove all the remaining  
453 fragments of villi and obtain a preparation with only crypts (**Figure 1C**). The crypts start to close  
454 within a few hours of embedding in BMM, and at D1, round organoids were observed (**Figure**  
455 **1D**). After 3-5 days, the organoids will appear with budding structures representing the "newly  
456 formed crypts." The organoids are ready for ROS analysis (**Figures 1E and 1F**).

457  
458 In the protocol of imaging oxidative stress by confocal microscopy, the slide containing the  
459 organoids, incubated with the probe, was imaged with a confocal fluorescence microscope  
460 equipped with lasers and filters to detect the Hoechst (ex/em: 361/486), the GFP (ex/em:  
461 488/510) and the fluorogenic probe (ex/em): 644/665) signals. A confocal microscope equipped  
462 with 20x air and 63x oil immersion objective allowed the visualization of ROS. In Lgr5-GFP mice,  
463 the GFP-positive cells are Lgr5-expressing intestinal stem cells. **Supplementary Figure 1** shows  
464 representative images obtained with the 20x objective providing an overview of the ROS in  
465 several organoids. **Figure 3** shows representative images, obtained with the 63x oil objective, of  
466 intestinal organoids expressing GFP, non-treated (NT), or pre-incubated or not with the ROS  
467 inhibitor NAC, and stimulated or not for 30 min with the ROS inducer tBHP.

468  
469 In the presence of the inhibitor, the only signal from the dead cells contained in the lumen of the  
470 organoid is visible. In the non-treated organoid, the basal ROS levels are shown, proving that  
471 stem cells produce higher ROS than differentiated cells (according to the microscope settings,  
472 the ROS signal might also be visualized in non-stem cells). GFP-positive cells present a more  
473 significant cytoplasmic signal with the inducer in the presence of the fluorogenic probe,  
474 demonstrating that ROS levels increase particularly in stem cells after treatment.

475  
476 **Figure 4** shows representative results obtained when analyzing ROS production in intestinal  
477 organoids stimulated or not with ROS inhibitor or inducer, using a Flow cytometer equipped with  
478 405 nm, 488 nm, and 630 nm lasers. The gating strategy presented in **Figure 4A** makes it possible  
479 to evaluate ROS production at the level of the whole organoids cell population, defining intact  
480 and living cells based on physical parameters and DAPI exclusion (SSC-A vs. FSC-A and DAPI vs.  
481 FSC-A) and FSC-H vs. FSC-A) or only in the intestinal stem cells, further gated on cells with GFP  
482 high signal. **Figure 4B** shows the ROS levels in the total population upon collection of 50,000  
483 events. Basal ROS levels in the non-treated (NT) cells decrease after stimulation with the inhibitor

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

494

#### 495 **FIGURE LEGENDS:**

496

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

503

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

510

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

519

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

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

532

533 **Supplementary Figure 1: Representative confocal images of ROS staining in organoids.** Stitched  
534 images were obtained with a confocal microscope equipped with a high-speed EMCCD Camera,  
535 20x objective, and slit 35  $\mu$ m, using the lasers 405, 488, 640, and filters 460/50, 535/50, 700/75  
536 to acquire Hoechst, GFP, and fluorogenic probe respectively. Confocal optical sections of  
537 organoids non-treated (NT), treated with the ROS-inhibitor (NAC), with the ROS-inducer (tBHP),  
538 or pre-treated with the ROS inhibitor and then stimulated with the ROS-inducer (NAC-tBHP). In  
539 grey, nuclei stained with Hoechst; in green, Lgr5-GFP cells; in red, fluorogenic probe (scale bar,  
540 100  $\mu$ m).

541

#### 542 **DISCUSSION:**

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

547

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

560

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

567

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

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

577  
578 Here a protocol is provided for the qualitative evaluation of ROS in intestinal organoids. A critical  
579 aspect for this part is linked to the working distance of the objectives that are used. The organoids  
580 are grown in BMM; they are not attached to the bottom of the well, introducing a distance from  
581 the objective focus plan. For this reason, it is critical to plate the organoids in a thin layer of BMM,  
582 to minimize this issue. Even in this optimized setting, not all the organoids will be in the correct  
583 position to be adequately imaged.

584  
585 A quantitative analysis of the images might be done using an appropriate image analysis  
586 software, evaluating the mean fluorescent intensity of the images in the ROS signal channel as  
587 described in the protocol. For this purpose, it is necessary to acquire a high number of images to  
588 get a sufficient number of events to be statistically significant. As mentioned before, using the  
589 Lgr5-GFP mice, not all the organoids will express GFP, requiring a considerable number of  
590 samples to be imaged.

591  
592 During the flow cytometry procedure, a critical step is the dissociation of the organoids into single  
593 cells. If the dissociation is too harsh, cells may die and release DNA. A rock-inhibitor, Y-27632, to  
594 counteract anoikis, and DNase may be added to the dissociation buffer if they do not interfere  
595 with the studied pathway. Trypsin dilution or reduced incubation times may be used.

596  
597 Finally, it is crucial to define the best time point to analyze ROS production after the different  
598 treatments (anti- or pro-oxidant) tested. In the case of drugs that rapidly induce ROS within  
599 minutes or hours, the imaging assay can be used to determine when there is the maximal  
600 induction by adding the fluorogenic probe before the tested compounds. The fluorescence  
601 intensity of the probe after organoids stimulation may vary between experiments performed on  
602 different days. Therefore, it is crucial always to calculate the ratio with the non-stimulated  
603 samples and add controls (oxidant /antioxidant) to verify the reactivity of the probe. NAC and  
604 tBHP were used as negative and positive controls as they gave the most conclusive results. Still,  
605 other reagents may be used, such as resveratrol as an antioxidant or paraquat/menadione as  
606 oxidants. Incubating cells for too long with the fluorogenic probe may be toxic and even modify  
607 the cell redox balance, so incubation times must also be tightly controlled. Cells stained with the  
608 probe may be fixed and analyzed a few hours after. In this case, for the flow cytometry analysis,  
609 the DAPI cannot discriminate between living and dead cells. Instead, a fixable dye for live/dead  
610 discrimination should be used before fixation.

611  
612 Organoids may also be grown for several days (more than 7), but this will increase the number of  
613 living and proliferative cells and the dead cells that accumulate in the organoids lumens,  
614 generating high background, particularly in the imaging assay. If an abnormal increase in the  
615 fluorogenic probe signal is observed, ensure that the solution used to resuspend stimulating

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

617

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

623

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

642

643

#### 644 **ACKNOWLEDGMENTS:**

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

647

#### 648 **DISCLOSURES:**

649 The authors have nothing to disclose.

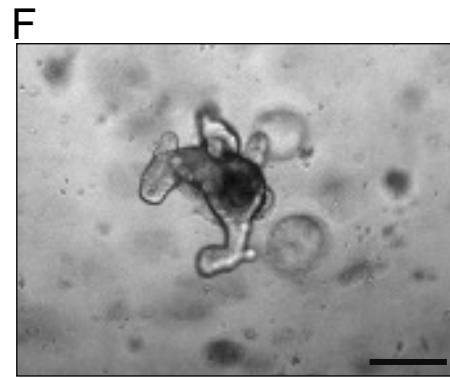
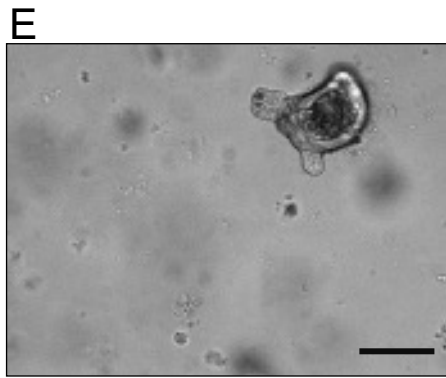
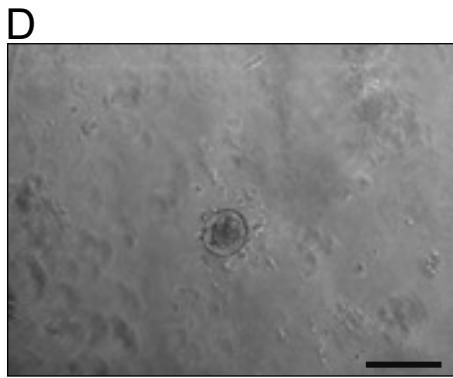
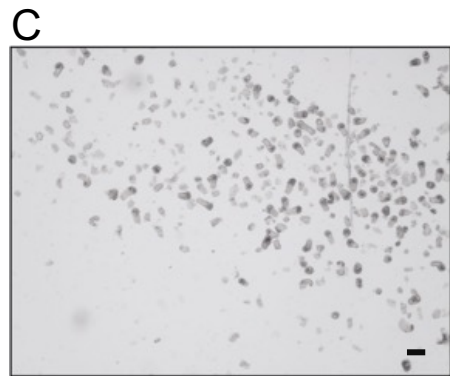
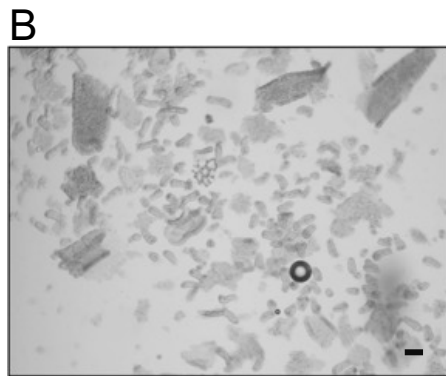
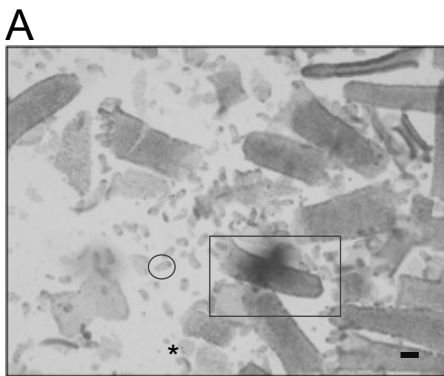
650

#### 651 **REFERENCES:**

- 652 1. Aviello, G., Knaus, U. G. NADPH oxidases and ROS signaling in the gastrointestinal tract  
653 review-article. *Mucosal Immunology*. **11** (4), 1011–1023 (2018).
- 654 2. Holmström, K. M., Finkel, T. Cellular mechanisms and physiological consequences of redox-  
655 dependent signalling. *Nature Reviews Molecular Cell Biology*. **15** (6), 411–421 (2014).
- 656 3. van der Post, S., Birchenough, G. M. H., Held, J. M. NOX1-dependent redox signaling  
657 potentiates colonic stem cell proliferation to adapt to the intestinal microbiota by linking  
658 EGFR and TLR activation. *Cell Reports*. **35** (1), 108949 (2021).
- 659 4. Schieber, M., Chandel, N. S. ROS function in redox signaling and oxidative stress. *Current*



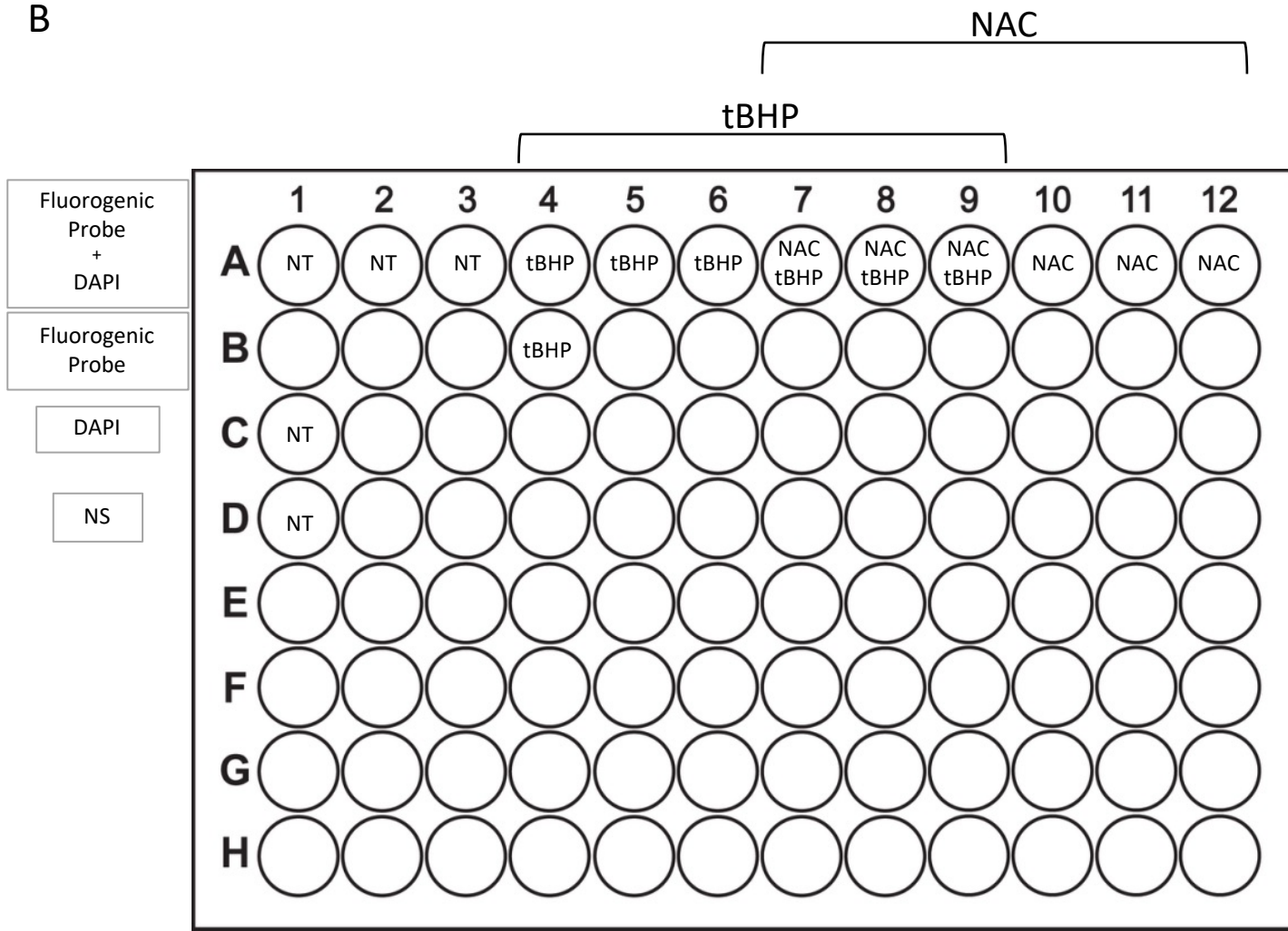
- 660 *Biology*. **24** (10), 453–462 (2014).
- 661 5. Myant, K. B. et al. ROS production and NF- $\kappa$ B activation triggered by RAC1 facilitate WNT-  
662 driven intestinal stem cell proliferation and colorectal cancer initiation. *Cell Stem Cell*. **12**  
663 (6), 761–773 (2013).
- 664 6. Juhasz, A. et al. NADPH oxidase 1 supports proliferation of colon cancer cells by modulating  
665 reactive oxygen species-dependent signal transduction. *Journal of Biological Chemistry*.  
666 **292** (19), 7866–7887 (2017).
- 667 7. Aviello, G., Knaus, U. G. ROS in gastrointestinal inflammation: Rescue Or Sabotage? *British*  
668 *Journal of Pharmacology*. **174** (12), 1704–1718 (2017).
- 669 8. Gomes, A., Fernandes, E., Lima, J. L. F. C. Fluorescence probes used for detection of  
670 reactive oxygen species. *Journal of Biochemical and Biophysical Methods*. **65** (2–3), 45–80  
671 (2005).
- 672 9. Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a  
673 mesenchymal niche. *Nature*. **459** (7244), 262–265 (2009).
- 674 10. Levy, A. et al. Innate immune receptor NOD2 mediates LGR5+ intestinal stem cell  
675 protection against ROS cytotoxicity via mitophagy stimulation. *Proceedings of the National*  
676 *Academy of Sciences*. **117** (4), 1994–2003 (2020).
- 677 11. Choi, H., Yang, Z., Weisshaar, J. C. Single-cell, real-time detection of oxidative stress  
678 induced in escherichia coli by the antimicrobial peptide CM15. *Proceedings of the National*  
679 *Academy of Sciences of the United States of America*. **112** (3), E303–E310 (2015).
- 680 12. Amri, F., Ghouili, I., Amri, M., Carrier, A., Masmoudi-Kouki, O. Neuroglobin protects  
681 astroglial cells from hydrogen peroxide-induced oxidative stress and apoptotic cell death.  
682 *Journal of Neurochemistry*. **140** (1), 151–169 (2017).
- 683 13. Ahn, H. Y. et al. Two-Photon Fluorescence Microscopy Imaging of Cellular Oxidative Stress  
684 Using Profluorescent Nitroxides. *Journal of the American Chemical Society*. **134** (10), 4721–  
685 4730 (2012).
- 686 14. Bidaux, G. et al. Epidermal TRPM8 channel isoform controls the balance between  
687 keratinocyte proliferation and differentiation in a cold-dependent manner. *Proceedings of*  
688 *the National Academy of Sciences*. **112** (26), E3345–E3354 (2015).
- 689 15. Van de Bittner, G. C., Dubikovskaya, E. A., Bertozzi, C. R., Chang, C. J. In vivo imaging of  
690 hydrogen peroxide production in a murine tumor model with a chemoselective  
691 bioluminescent reporter. *Proceedings of the National Academy of Sciences*. **107** (50),  
692 21316 LP – 21321 (2010).
- 693 16. Rabbani, P. S., Abdou, S. A., Sultan, D. L., Kwong, J., Duckworth, A., Ceradini, D. J. In vivo  
694 imaging of reactive oxygen species in a murine wound model. *Journal of Visualized*  
695 *Experiments*. **141**, e58450 (2018).
- 696 17. Sato, T. et al. Long-term expansion of epithelial organoids from human colon, adenoma,  
697 adenocarcinoma, and Barrett's epithelium. *Gastroenterology*. **141** (5), 1762–1772 (2011).
- 698
- 699



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



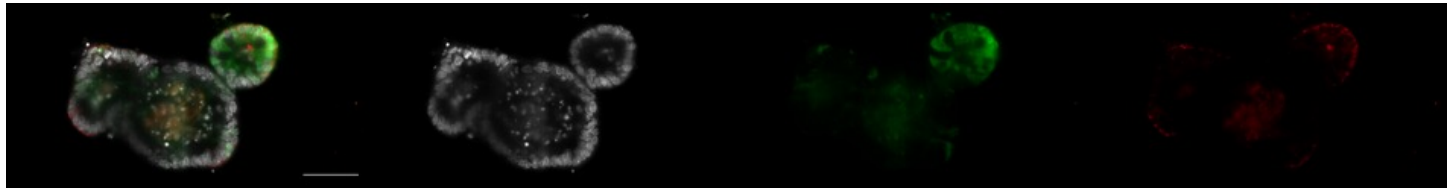
Merge

Nuclei

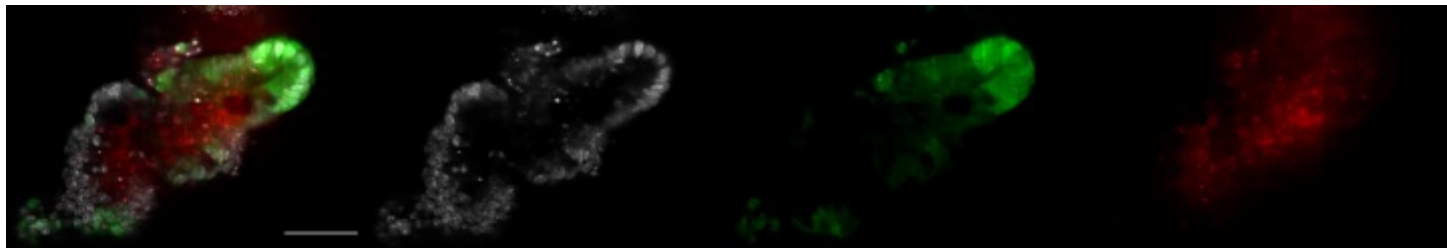
Lgr5-GFP

Fluorogenic  
Probe

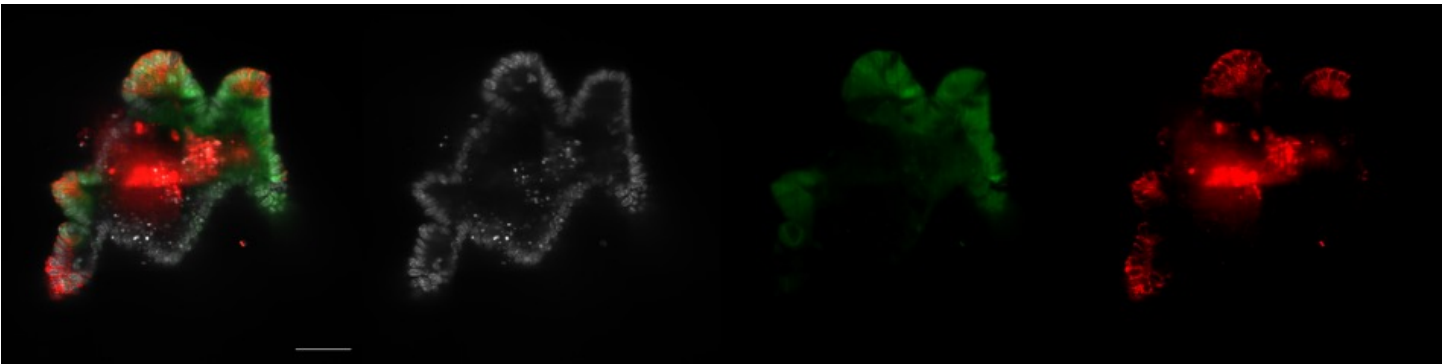
NT



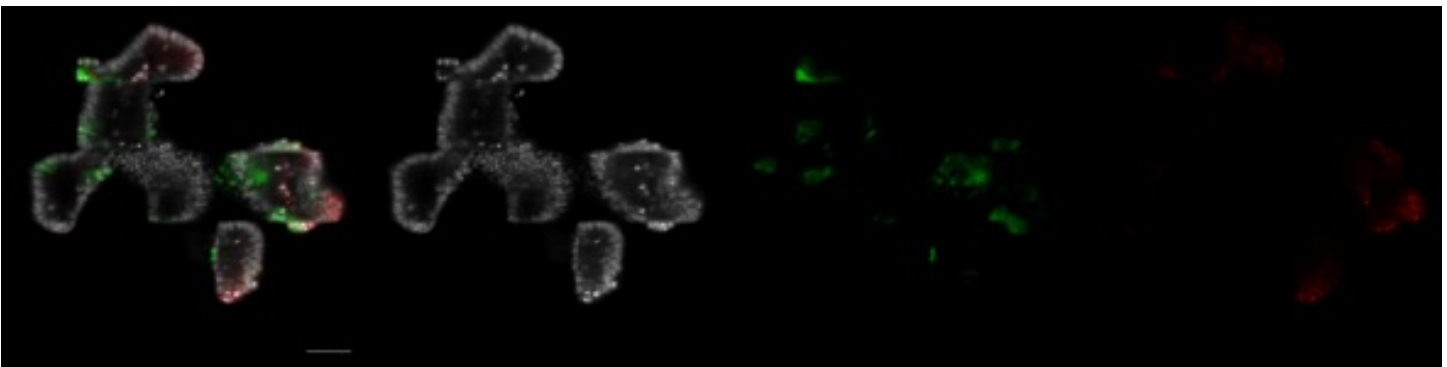
NAC

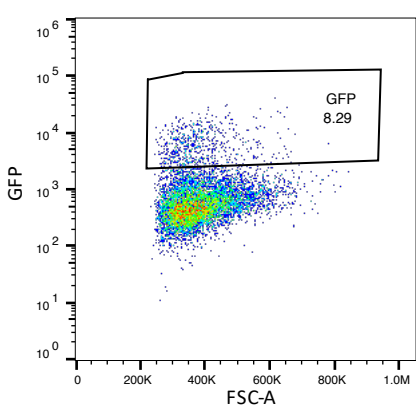
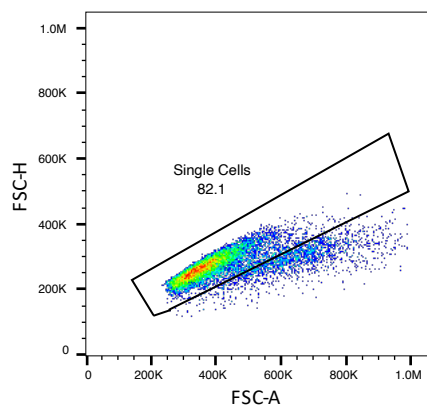
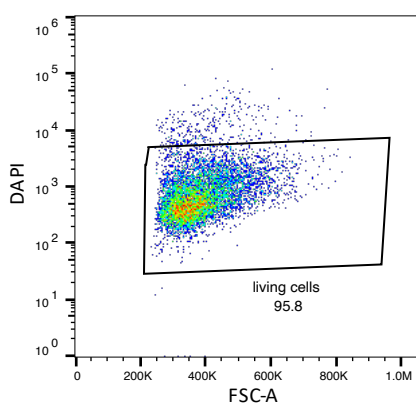
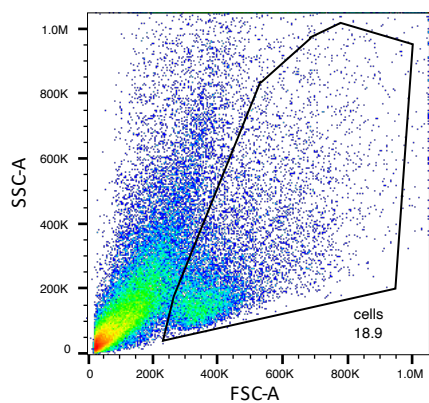
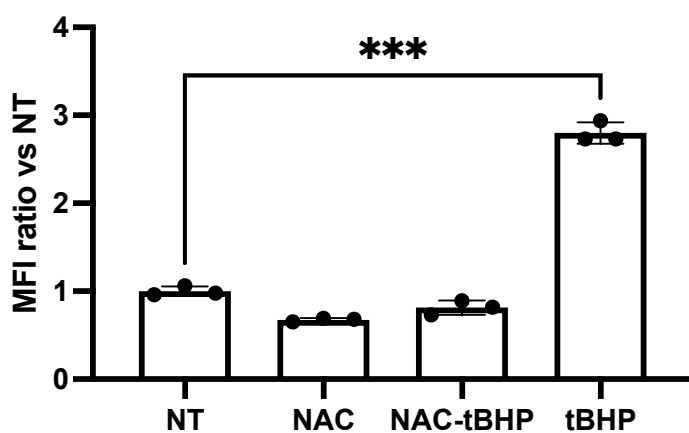
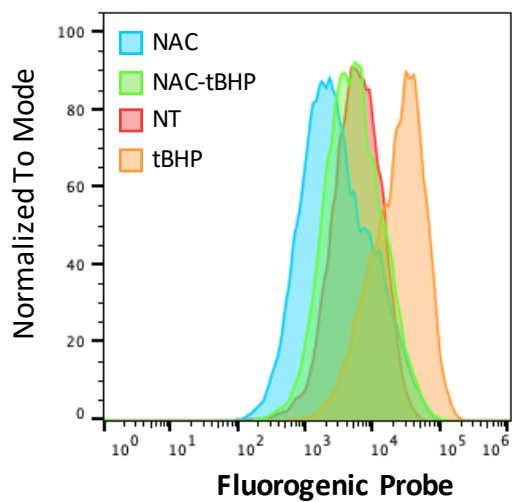
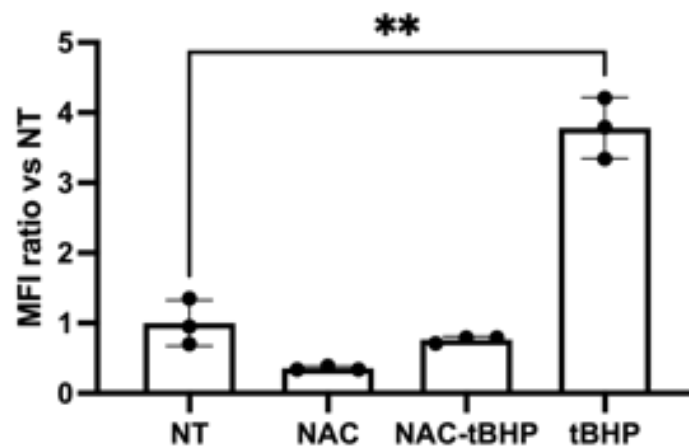
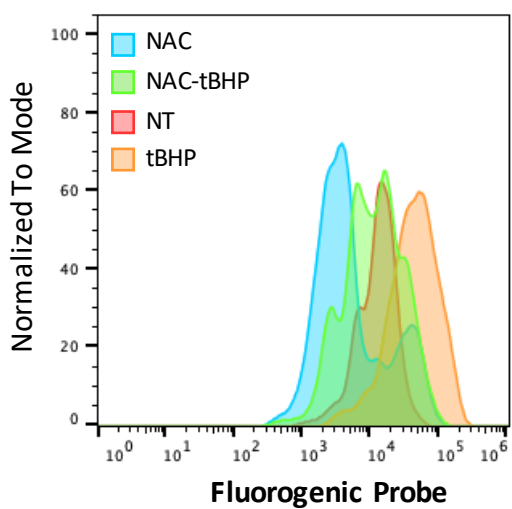


tBHP



NAC-tBHP



**A****B****Total population****C****GFP+ cells**

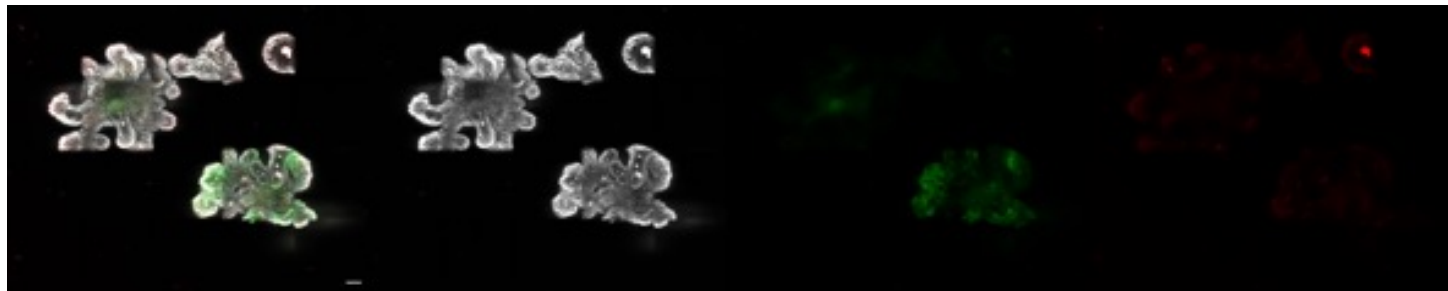
Merge

Nuclei

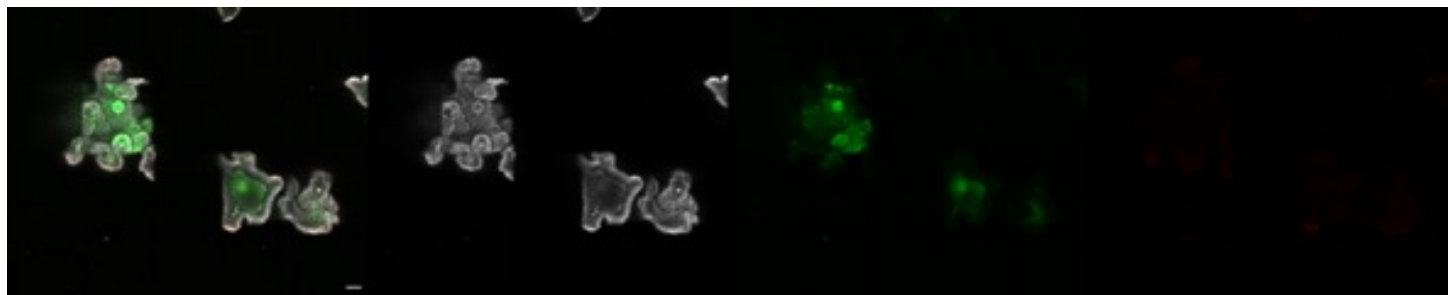
Lgr5-GFP

Fluorogenic  
Probe

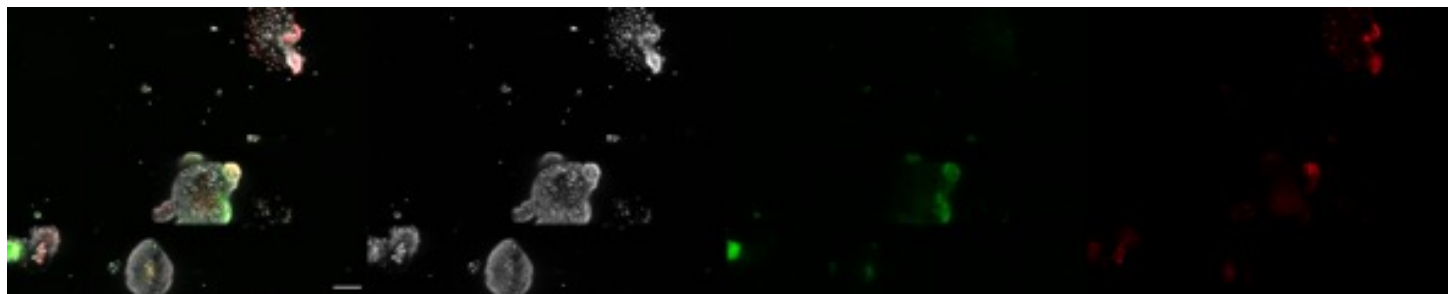
NT



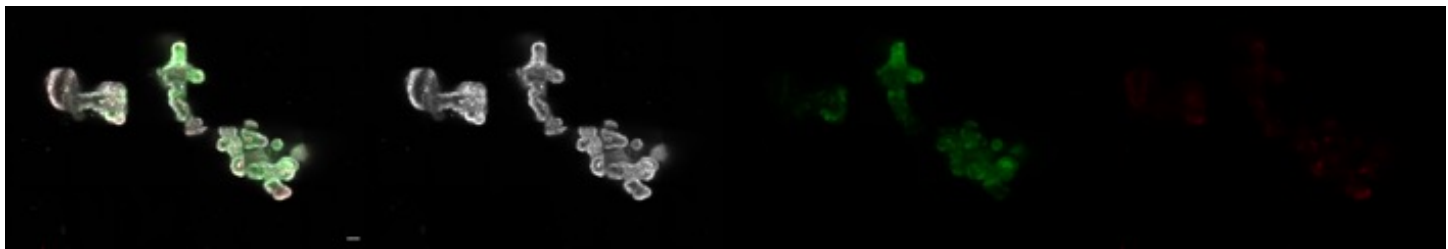
NAC



tBHP



NAC-tBHP



Mice	Company	Catalog Number	Comments/Description
Lgr5-EGFP-IRES-creERT2 (Lgr5-GFP)	The Jackson Laboratory+A3:B3		
Growth culture medium	Company	Catalog Number	Stock Concentration
Advanced DMEM F12 (DMEM/F12)	ThermoFisher	12634010	
B-27 Supplement, minus vitamin A	ThermoFisher	12587010	stock: 50X
GlutaMAX ( <i>glutamine</i> )	ThermoFisher	35050038	stock: 100x
Hepes	ThermoFisher	15630056	stock: 1M
Murine EGF	R&D	2028-EG-200	stock: 500 µg/mL in PBS
murine Noggin	R&D	1967-NG/CF	stock: 100 µg/mL in PBS
Murine R-spondin1	R&D	3474-RS-050	stock: 50 µg/mL in PBS
N-2 Supplement	ThermoFisher	17502048	stock: 100x
Penicillin-Streptomycin (P/S)	ThermoFisher	15140122	stock: 100x (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin)

Name of Material	Company	Catalog Number	Comments/Description
70 µm cell strainer	Corning	352350	
96-well round bottom	Corning	3799	
ball tip scissor	Fine Science Tools GMBH	14086-09	
CellROX® Deep Red Reagent	ThermoFisher	C10422	
DAPI (4',6-diamidino-2-phénylindole, dichlorhydrate) ( <i>fluorogenic probe</i> )	ThermoFisher	D1306	stock at 10 mg/mL
DPBS 1x no calcium no magnesium (DPBS)	ThermoFisher	14190144	
FLuoroBrite DMEM ( <i>DMEM no phenol red</i> )	ThermoFisher	A1896701	
Hoechst 33342	ThermoFisher	H3570	stock at 10 mg/mL
Matrigel Growth Factor Reduced, Phenol Red Free ( <i>Basement Membrane Matrix</i> )	Corning	356231	once received thaw o/n in the fridge, keep for 1h on ice and, make 500 µL aliquots and store at -20°C
µ-Slide 8 Well chambers	Ibidi	80826	
N-acetylcysteine (NAC)	Sigma	A9165	
tert-Butyl hydroperoxide (tBCHP)solution (70%wt. In H <sub>2</sub> O <sub>2</sub> )	Sigma	458139	
TrypLE Express Enzyme (1X), no phenol red ( <i>trypsin</i> )	ThermoFisher	12604013	
UltraPure 0.5 M EDTA, pH8.0 Y-27632	ThermoFisher Sigma	15575020 Y0503	Rock-inhibitor to be used to minimize cell death upon tissue dissociation

#### Programs and Equipment

		Comments/Description
Attune NxT (Flow Cytometer)	ThermoFischer	Flow cytometer analyzer
Fiji/ImageJ	<a href="https://imagej.net/software/fiji/downloads">https://imagej.net/software/fiji/downloads</a>	images generation
FlowJo	BD Bioscience	FACS analysis
Observer.Z1	Zeiss	
Opterra (swept-field confocal)	Bruker	confocal system
high speed EMCCD Camera Evolve Delta 512	Photometrics	
Prism	GraphPad Software	statistical analysis