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1 **TITLE:**

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

4
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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 peroxide¹. By expressing antioxidant enzymes and ROS scavengers, cells can finely tune their
57 redox balance, thereby protecting tissue homeostasis². Although ROS can be highly toxic to the
58 cells and damage DNA, proteins, and lipids, they are crucial signaling molecules². In the intestinal
59 epithelium, moderate ROS levels are required for stem and progenitor cell proliferation³; high
60 ROS levels lead to their apoptosis⁴. 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^{5,6}. 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 better⁷.

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^{4,7}. 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 models⁸.

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 Clevers⁹, 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)¹⁰, 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 μ 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 μ 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 μm 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 μL 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 μL , 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 μL 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/ μL .

214
215 2.25. Add 2 volumes of undiluted BMM to have a final concentration of 30 crypts/ μL . 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 μ L of the crypts/BMM mix into each well. For Flow cytometry analysis, use round-
221 bottom 96-well plates. Distribute 10 μ L at the center of each well as a dome. For imaging, use μ -
222 slide 8 well (see **Table of Materials**) and deposit the 10 μ 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% CO₂ for 15 min.

228

229 2.28. Add 250 μ 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% CO₂.

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 6th 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 μ 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 μ 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% CO₂ 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% CO₂.
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% CO₂.

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% CO₂.

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% CO₂.

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 μm (step size 5 μm) 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 μL 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% CO₂.
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% CO₂.
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% CO₂.
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% CO₂.
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 cells¹⁰ 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 μ 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 μ 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 Ca^{2+} and 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 peroxide¹¹⁻¹³.
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 mitochondria¹⁴.
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 used¹⁰. A repertoire of
634 chemiluminescent probes was also developed to detect specific ROS species with high sensitivity,
635 such as luciferin-based probes^{15, 16}. 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 accordingly¹⁷. 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:**

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646 Stress).

647

648 **DISCLOSURES:**

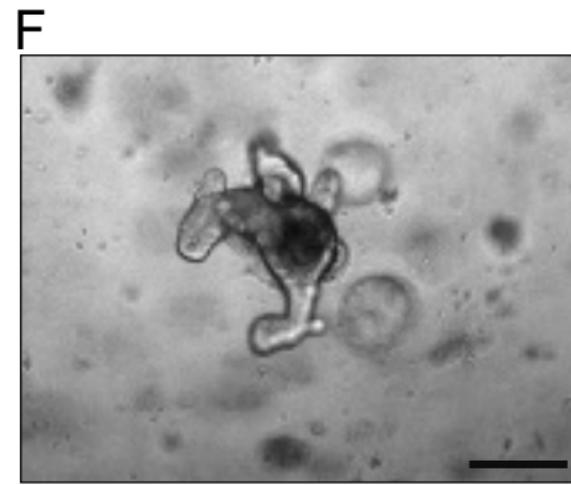
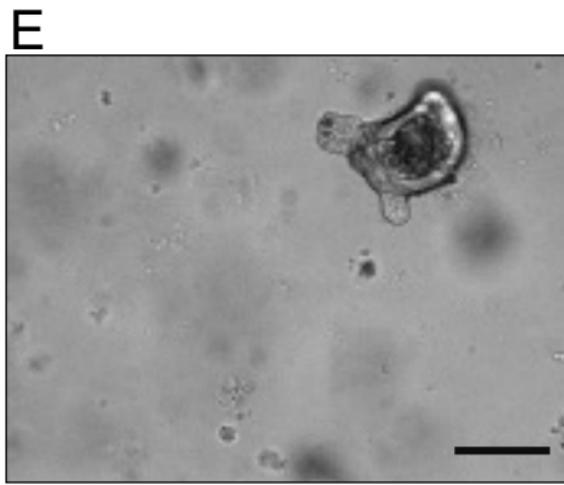
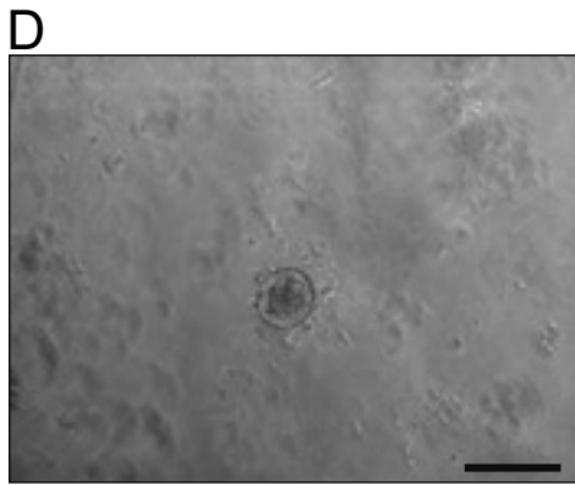
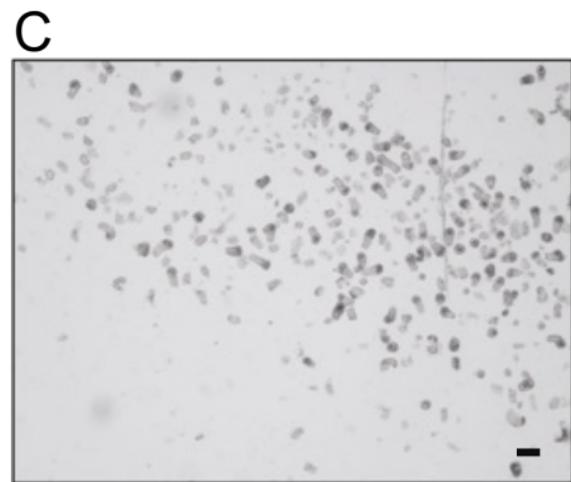
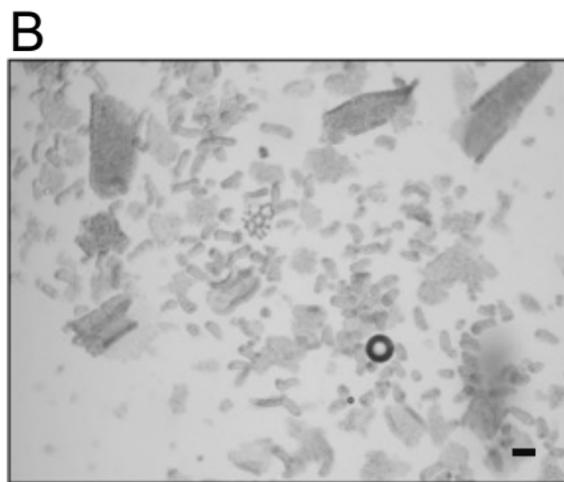
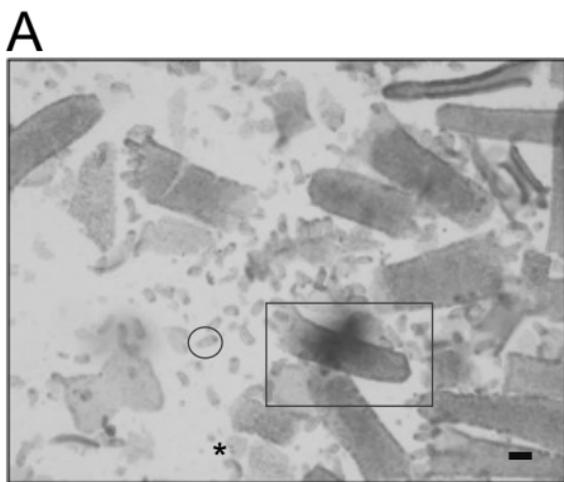
649 The authors have nothing to disclose.

650

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653 review-article. *Mucosal Immunology*. **11** (4), 1011–1023 (2018).
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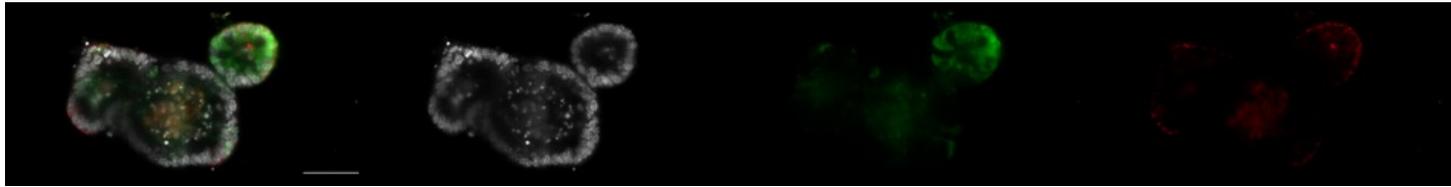
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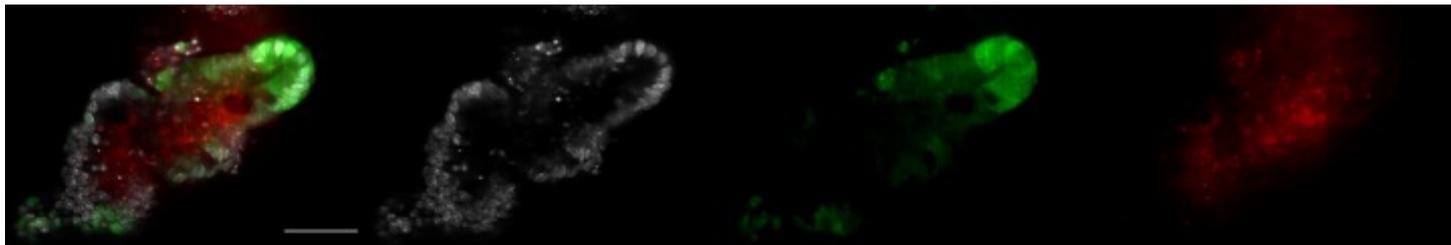
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Fluorogenic
Probe

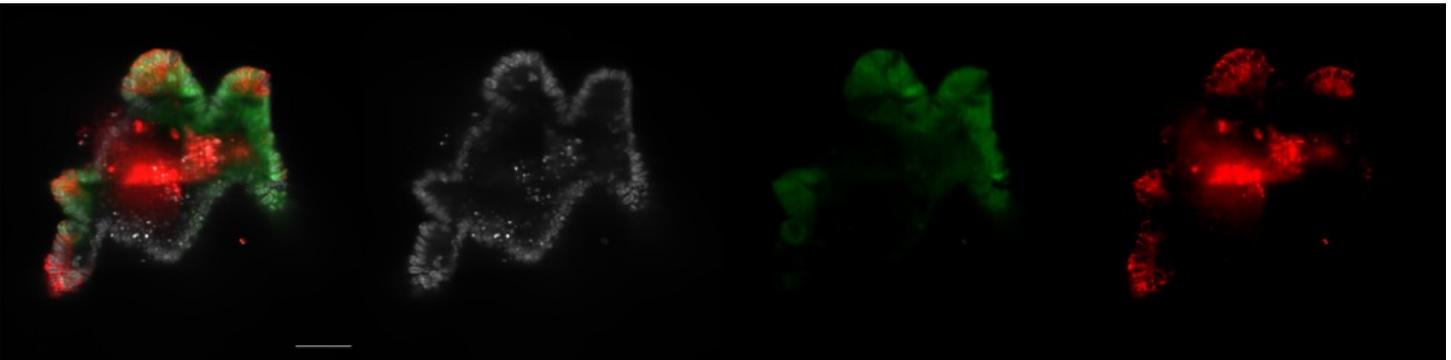
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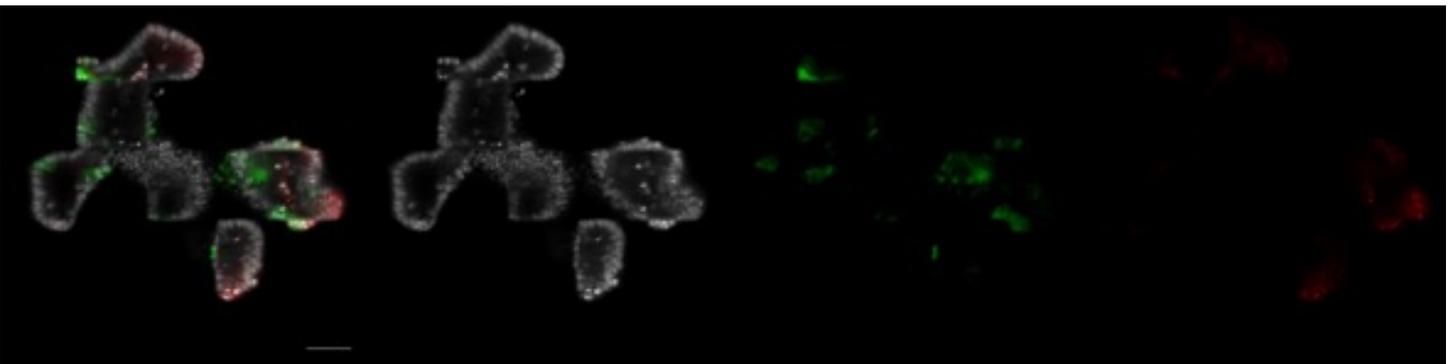
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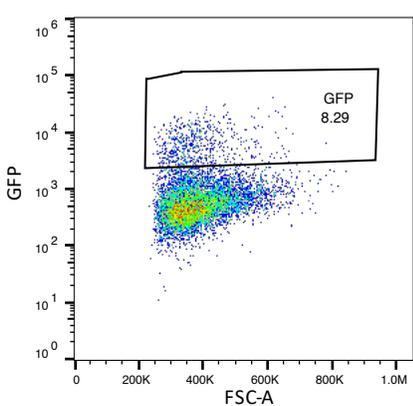
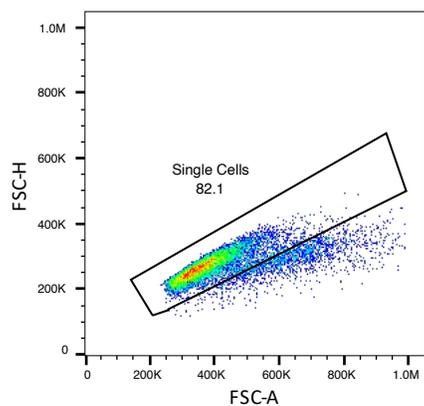
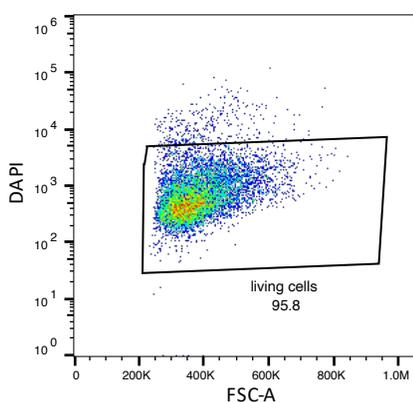
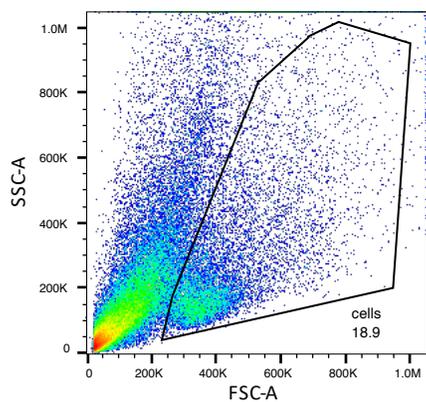
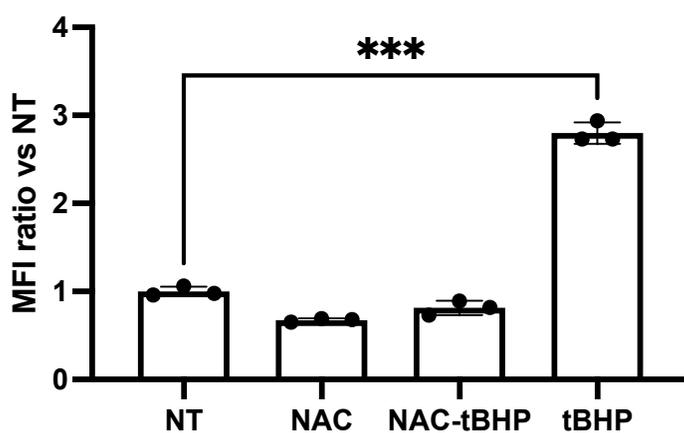
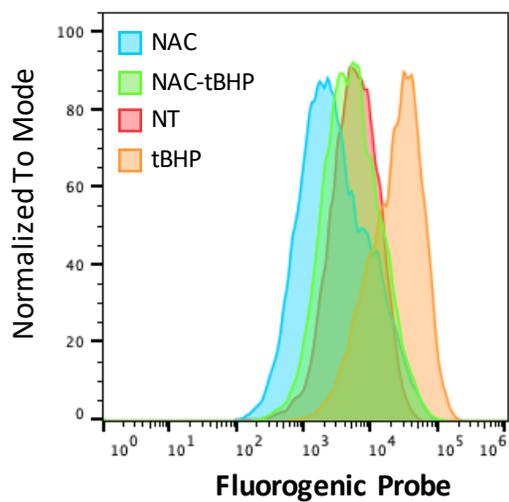
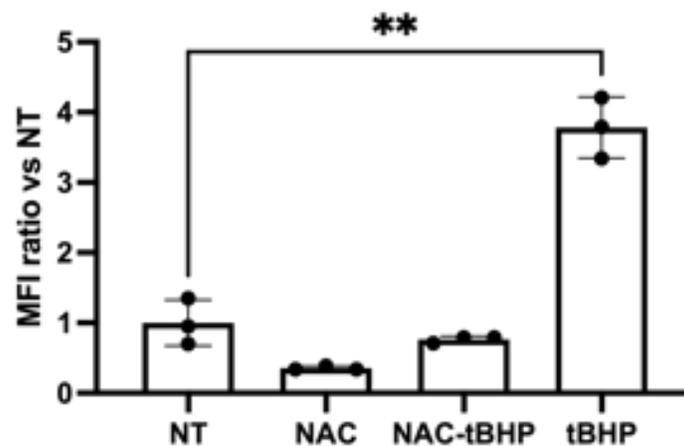
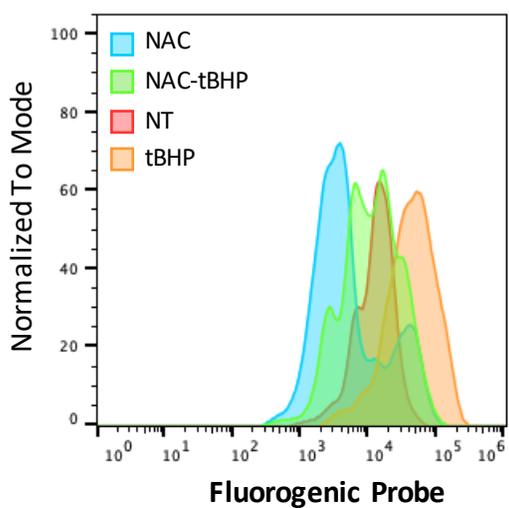


tBHP



NAC-tBHP



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

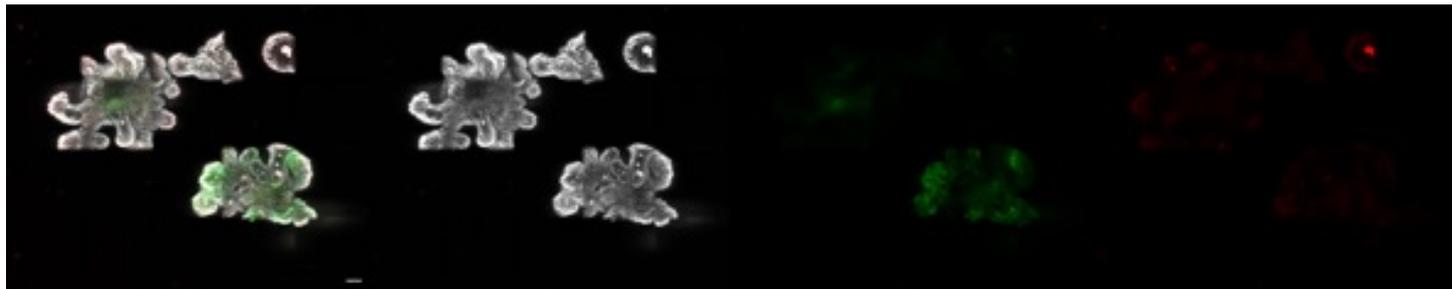
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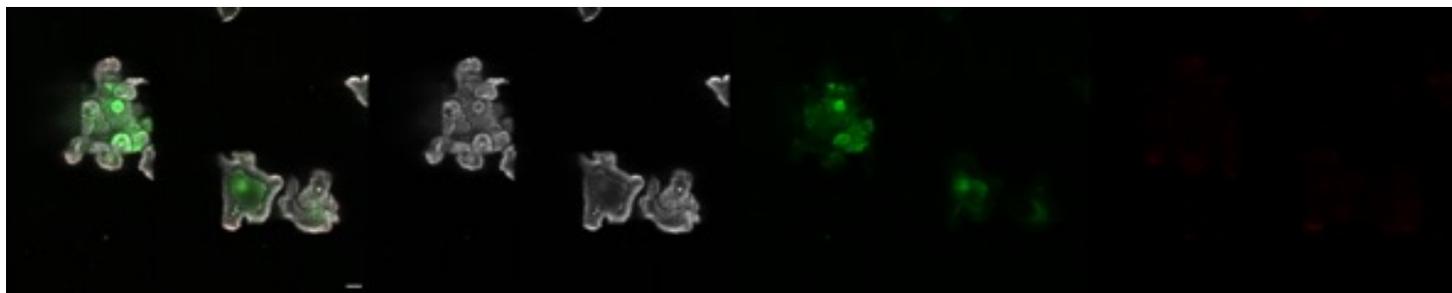
Lgr5-GFP

Fluorogenic
Probe

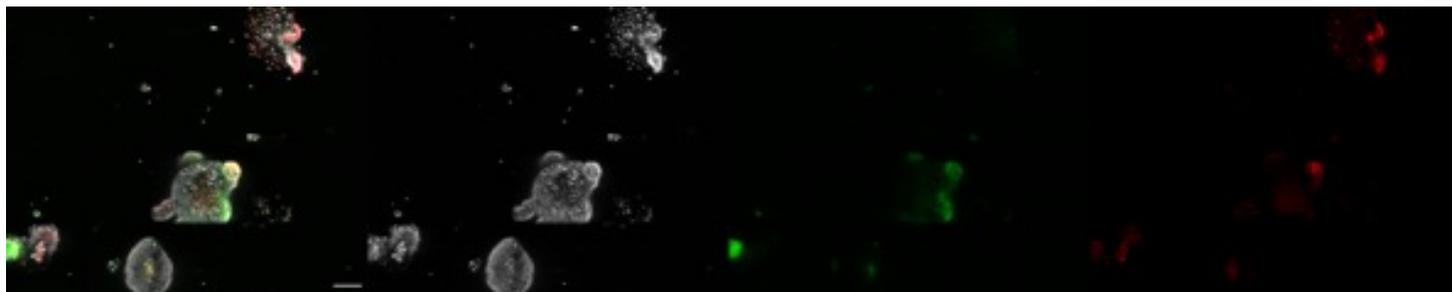
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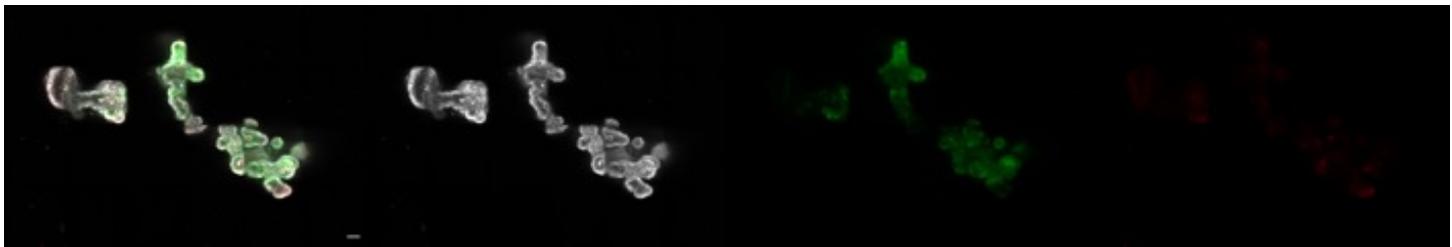
NAC



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 ₂ O ₂)	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	https://imagej.net/software/fiji/downloads	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