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Generation of neighbor-labeling cells to study intercellular interactions in vivo

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1 The generation of neighbour-labelling cells to study intercellular 2 interactions in vivo.

3
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16 17 **ABSTRACT**

18 Understanding cell-cell interactions is critical in most, if not all, research fields in
19 biology. Nevertheless, studying intercellular crosstalk in vivo remains a significant
20 challenge, mainly due to the difficulty in spatially locating the surroundings of
21 particular cells in the tissue. Cherry-niche is a powerful new method that allows the
22 cells expressing it to label their surrounding cells, facilitating their specific isolation
23 from the whole tissue as live cells. We previously applied Cherry-niche in cancer
24 research to study the tumour microenvironment (TME) in metastasis¹. Here, we
25 describe how to generate cancer cells with the ability to label their neighbouring cells
26 (within the tumour niche) by transferring a liposoluble fluorescent protein. Live niche
27 cells can be isolated and compared to cells distant from the tumour bulk, in a variety
28 of ex vivo approaches. As previously shown, this system has the potential to identify
29 novel components in the TME, and improve our understanding of their local
30 interactions. Importantly, Cherry-niche can also be applied to study potential cell-cell
31 interactions due to in vivo proximity in research fields beyond cancer. This protocol
32 takes 2-3 weeks to generate the labelling cells and 1-2 weeks to test their labelling
33 ability.

34 35 **INTRODUCTION**

36 Cells are continuously cross-talking with each other and these interactions underlie
37 many physiological as well as pathological processes. Many interactions will occur
38 between cells that are physically located in close proximity within the tissue structure.
39 In particular, cancer cells are known to require perturbation within the tissue to
40 create a suitably supportive tumour microenvironment (TME), which is a crucial
41 factor in cancer biology^{1,2}. The TME is made of a variety of different cellular and non-
42 cellular components surrounding the cancer cells, and is known to influence key
43 aspects in cancer development, such as tumour growth and therapeutic response^{3,4}.
44 However, a complete understanding of the TME heterogeneity and the mechanisms
45 by which it supports tumour initiation and progression is yet to be achieved. As such,
46 the ability to spatially locate and isolate the cells surrounding a cancer cell-of-interest
47 in vivo could help to elucidate the complex changes involved in the creation of the
48 TME.

49 50 ***Development of the protocol***

51 The principles underlying the strategy we have developed are summarised in Figure
52 1. We engineered breast cancer cells to stably express a modified version of a red
53 fluorescent protein (mCherry) containing a secretory peptide⁵ “s” and a TATk
54 peptide⁶ “LP”, *sLP-Cherry*, which allows for its uptake into exposed cells. Upon
55 secretion, cancer cells and their surrounding cells uptake the sLP-Cherry, which is
56 then stored intracellularly in multi-vesicular bodies¹. The internalised mCherry
57 maintains a high fluorescence, likely due to its photostability at low pH⁷, which shows
58 a half-life in labelled cells in vitro of about 40 h¹. We used the Cherry-niche system to
59 engineer breast tumour cells and study the composition of their surrounding cells as
60 they begin metastasising in the lung. We have estimated that the sLP-Cherry
61 released by cancer cells could reach approximately five surrounding cell layers¹
62 (Figure 1), which can be isolated by flow cytometry. The sequence of the sLP-Cherry
63 can be found in Supplementary Figure 1 (also found in our previous publication¹).
64 We here supply a step-by-step protocol that has been used to generate and validate
65 labelling 4T1 cells. We show how we used these cells to characterise the lung
66 metastatic niche of breast cancer cells¹. We also extend the protocol to show how
67 the same labelling 4T1 cancer cells can be used to detect their surrounding niche in
68 a different tissue, the liver. Moreover, we show how a similar approach can be used
69 to generate a different type of labelling cancer cells, human leukemic cells (ML-1),
70 for the study of their bone marrow niche. An overview of the procedure is provided in
71 Figure 2.

72

73 **APPLICATIONS OF THE METHOD**

74 The Cherry-niche system is extremely valuable in cancer research, particularly in the
75 context of metastatic seeding, where it allows the precise identification of cells within
76 the local surrounding of cancer cells infiltrating the tissue, including less represented
77 niche components that may be otherwise difficult to detect¹. It is well known that the
78 cellular composition of the metastatic niche is distinct from the normal tissue³, so the
79 possibility to isolate live cells from the niche using flow cytometry and compare them
80 with the tissue cells isolated from the same organ is a significant advance. Once
81 isolated, these cells can be studied using a variety of approaches, such as ex vivo
82 3D co-cultures, proteomics, bulk RNA sequencing and single cell RNA sequencing
83 (scRNA-seq)¹.

84 Besides tumour studies, we predict this method could be used to address other
85 scientific questions aimed at understanding the local cellular composition of a cell of
86 interest and the potential local interactions occurring in vivo. Indeed, in our previous
87 study, we successfully generated a non-cancer mammary epithelial cell line
88 expressing the sLP-Cherry and were able to mark their surrounding cells in vivo¹.
89 Further developments could be made in order to generate other types of modified
90 proteins that could be delivered in neighbouring cells. However, the stability of the
91 protein within the recipient cells upon uptake should be considered.

92

93 **COMPARISON WITH OTHER METHODS**

94 Laser capture microdissection (LCM) has been the technique traditionally used to
95 isolate cells from the TME^{8,9}. However, this technique has major limitations; in
96 particular, the quality of the material isolated from frozen or paraffin-fixed tissue
97 heavily depends on advanced technical skills to perform the microdissection itself.
98 Moreover, downstream analysis is limited to bulk expression profiles of the collected
99 tissue areas, without cellular resolution. For certain tissue such as the bone, LCM is
100 technically challenging, especially for the isolation of the endosteal area. Importantly,

101 histological identification of the dissection area is required, implying that the portion
102 of the tumour mass has to be correctly visualised. This can be particularly difficult, as
103 the microdissection is performed in absence of multicolour staining. Therefore, when
104 cell isolation is required from very small lesions, such as early metastases, it
105 becomes extremely challenging.

106
107 Significant effort over the past few years has led to novel strategies to allow easier
108 and more accurate analysis of spatially distinct niches within tissues. In one such
109 strategy, the use of a mouse model expressing a photoactivable GFP protein¹⁰
110 allows the fluorescent labelling of cells in a specific location within a tissue, enabling
111 their isolation as live cells by flow cytometry to generate scRNA-seq data¹¹. However,
112 when using this system, the niche area in the whole tissue needs to be defined and
113 targeted by two-photon irradiation. A recent approach to study intercellular cross-talk
114 in vivo relies on the preservation of physical interactions after tissue dissociation¹².
115 Here, after staining with distinct fluorophore-conjugated antibodies, specific double-
116 fluorescent doublets are isolated by flow cytometry and characterised by scRNA-seq.
117 Next, an algorithm allows you to deconvolute the profile of the two different
118 interacting cell types. However, the calibration of tissue dissociation protocols is
119 critical and weaker interactions may still be missed. Finally, the algorithm needs to
120 be finely tuned to exclude “contaminating” non-interacting doublets and successful
121 deconvolution requires the two interacting cells to have very distinct transcriptional
122 profiles.

123
124 Less unbiased methods have also been developed. One approach involved
125 engineering of specific cell types to express two distinct peptide fragments on their
126 surface. Upon adding a substrate in living mice, a fluorescent or biotin tag is
127 transferred when the two cells are in contact, enabling the subsequent isolation of
128 the acceptor cells by flow cytometry¹³. Although it overcomes the need to physically
129 locate a specific tissue area, this method requires specific pairs of different cell types
130 to be engineered and physically interact to generate a detectable signal. Thus, a
131 prior knowledge of the cell types to identify in vivo is necessary. Another
132 methodology that has been used to study intercellular cross-talk in metastasis, takes
133 advantage of cancer cells engineered to express a highly brilliant fluorescent
134 protein¹⁴. The efficacy of this method depends on the “blebbing” of the tumour cells
135 that loose cellular particles and on the ability of the neighbouring cells to internalise
136 them, therefore limiting its detection ability mostly to phagocytic myeloid immune
137 cells in the TME.

138
139 The Cherry-niche system presented here, whereby cancer cells directly label their
140 neighbouring cells, represents an important addition to the toolbox to study the TME.
141 As with other techniques, no dedicated equipment or special expertise is required
142 except for access to standard fluorescence-activated cell sorting (FACS). These
143 cells can be freshly isolated from the tissue as live cells and functionally tested ex
144 vivo and analysed as a pool or at single-cell level. Importantly, Cherry-niche
145 overcomes key limitations of the current methodologies: spatial visualisation of
146 tumour nodules is not required and an accurate isolation of cancer-surrounding cells
147 is possible, starting from the early stages of metastatic disease, with no previous
148 knowledge of the niche composition required. The labelling relies on the liposolubility
149 of the sLP-Cherry, allowing an unbiased marking of potentially all the different cell

150 types in the niche, including less well-represented components. This has the
151 potential to identify new players in the TME, as we have recently shown¹.

152

153 **OVERVIEW OF THE PROCEDURE**

154 Here, we present a step-by-step description of how to generate and validate labelling
155 cells (Figure 2). First, sLP-Cherry viral particles are prepared using standard virus
156 production methods and used to infect the cells of interest; shortly after infection,
157 mCherry-expressing cells are isolated by flow cytometry (phase 1). After being
158 expanded, the sorted cells are tested for their labelling ability in vitro by validating the
159 mCherry uptake in recipient cells' vesicular structures (phase 2). The cells are then
160 injected into mice and tested for their labelling ability in vivo (phase 3); the cells need
161 to be visualized in the tissue to validate their ability to transfer mCherry to
162 neighbouring cells. The system can then be used to isolate the neighbouring cells of
163 the tumour by flow cytometry upon tissue dissociation. And, finally, cells isolated by
164 flow cytometry are subject to downstream analysis (phase 4).

165

166 **EXPERIMENTAL DESIGN**

167 Generation and isolation of labelling cells (phase 1; steps 1-17; Figure 2).

168 This protocol has been used to generate labelling 4T1 mouse breast cancer cells.
169 Other murine cancer cell lines such as renal carcinoma (Renca), colorectal cancer
170 (CT26), or murine breast epithelial non-cancer cells (HC11), have been generated
171 with a similar approach and their labelling ability in vivo has been confirmed¹.

172 Here, we describe the generation and use of labelling 4T1 mouse breast cancer cells
173 (Figure 3a) and human ML-1 leukemia cell line (Figure 3b) as examples. However,
174 this protocol is in principle suitable for all cell lines. The labelling efficiency of the
175 generated cells, both in vitro and in vivo, may be different and be dependent on the
176 intrinsic secretory ability of the cells. In general, highly secretive cells are expected to
177 release more sLP-Cherry, which should then be transferred to more of the
178 surrounding cells, presumably resulting in increased detection. However, this theory
179 has not been proven and these features should be tested on a case-by-case basis.

180 To produce the sLP-Cherry virus, we used a 2nd generation lentiviral packaging
181 system VSVG pseudotyped. However, a 3rd generation lentiviral system is also
182 suitable. The production of another virus expressing a different fluorescence marker
183 (for example, GFP) or a distinctive membrane marker epitope may be required
184 depending on the experimental setting. If needed, infection with the two viruses (e.g.,
185 the sLP-Cherry and the GFP-expressing viruses) can be concomitant or sequential.
186 This will generate double-positive cells (labelling cells) that can be easily
187 discriminated from the single mCherry⁺ labelled cells in following applications. We
188 recommend isolating the double-positive cells by flow cytometry 48-72h after
189 infection with the sLP-Cherry virus. The sorted cells are then plated back in culture
190 and amplified.

191

192 Testing the labelling efficiency in vitro (phase 2; steps 18-24; Figure 2).

193 Imaging analysis is used to visualise labelling as punctuated red dots in recipient
194 cells, which, in our experience, is a measure of efficient labelling activity. In vitro
195 labelling can be performed either as co-culture or using conditioned media (*cm*). It is
196 only necessary to perform validation with one of these two systems. With the co-
197 culture system, sLP-Cherry-infected cells are plated in co-culture with the same or a
198 different type of unlabelled cell line. Alternatively, conditioned media (*cm*) from sLP-
199 Cherry-infected cells can be collected and used to culture unlabelled cells. Following

200 either co-culture with labelling cells or exposure to their *cm*, the recipient cells are
201 analysed by flow cytometry (Figure 4a and 4c). Recipient cells are also analysed by
202 microscopy to confirm the appearance of single mCherry⁺ cells that are
203 distinguishable from the double-positive labelling cells in the co-culture (Figure 4b
204 and 4d).

205 The presence of some labelling in vitro (with at least 40% of all recipient cells
206 labelled in co-culture and 5% with *cm*) and the appearance of some intracellular
207 punctuated distribution of mCherry signal in recipient cells as shown in Figure 4d is
208 sufficient to validate the system in vitro. Assessment of the in vitro labelling system
209 should be considered as a *positive* or *negative* control test only, as the precise
210 results do not directly correlate with the efficiency of in vivo labelling.

211
212 As an optional step, the *cm* can be fractionated to separate the soluble fraction and
213 the vesicles, a portion of which also contain the mCherry¹, and assess whether one
214 or both fractions are responsible for the labelling in vitro (Box 1). This process
215 depends on the specific properties of the labelling cells, as well as the way they
216 interact with their neighbouring cells. Depending on the scientific question the
217 labelling cells are required to address, it may be relevant to assess these fractions.
218 The labelling efficiency of the 4T1 cells *cm* was previously shown¹. Here, an
219 example of labelling media efficiency from leukemic ML-1 cells is shown in Figure 4e.

220
221 We anticipate that co-culture represents the most effective approach to confirm the
222 labelling capacity of the generated cells in vitro. However, since the ultimate goal is
223 using these sLP-Cherry engineered cancer cells in vivo, testing their labelling ability
224 in vivo is crucial and strictly necessary.

225
226

227 Testing the labelling efficiency in vivo (phase 3; step 25; Figure 2).

228 Cancer cells are injected as a single cell suspension to target the relevant organ. For
229 instance, carcinoma cells can be injected intravenously via the tail-vein to target the
230 lung or intra-splenic to target the liver. In contrast, tail-vein injected leukemic cells
231 expand primarily in the bone marrow. Then, animals are sacrificed and the tissue of
232 interest is collected, processed and analysed by histology to check for labelling via
233 imaging on fresh or antibody-stained tissues. Two examples of settings are provided.
234 When using breast cancer metastasis, we adopted an unbiased approach where
235 labelling cancer cells expressed an endogenous GFP, while releasing sLP-mCherry.
236 Here, cancer cells will appear GFP-mCherry double positive, and the labelled
237 neighbouring cells will be mCherry positive but GFP negative (Figure 5a and 5b).

238

239 An alternative targeted approach involves injecting labelling leukemic cells
240 expressing only the sLP-Cherry into transgenic animals where a type of bone
241 mesenchymal cells is fluorescently labelled (Nestin-GFP). Using this strategy,
242 Nestin-GFP cells within areas of leukemic growth can be specifically identified as
243 GFP-mCherry double-positive cells. (Figure 5c).

244 Once the local tissue labelling is confirmed, a more accurate quantification of the
245 labelled cells can be made by flow cytometry upon tissue dissociation. An example
246 of the flow cytometry analysis, including gating strategy, is provided for lung (Figure
247 6a) and liver tissue (Figure 6b).

248 **NOTE:** In some experimental settings in vivo, the labelling cancer cells may
249 downregulate the GFP expression. In these cases, we recommend using a stringent

250 niche gate (as shown in Figure 6a and Figure 6b) to reduce a possible contamination
251 of cancer cells among the mCherry⁺GFP⁻ (niche) cells. Moreover, if there is doubt of
252 a possible contamination, staining on niche sorted cells can be performed to check
253 the possible presence of cancer cells within the niche cells, as well as ex vivo
254 cultures and functional analysis to reveal if a significant cancer cell contamination is
255 present.

256 We have previously shown that the ratio of labelled cells in the lung versus cancer
257 cells in the 4T1 model is constant over the time of metastatic growth¹. Dissociated
258 tissue samples can also be stained with membrane markers to define the identity of
259 the labelled cells by flow cytometry. Here, we provide an example of how to identify
260 myeloid cells or epithelial cells among the mCherry positive or negative pool of lung
261 cells (Supplementary Figure 2; gating strategy shown in Figure 6a). The efficiency of
262 uptake of mCherry might vary in vivo due to the properties of the cell types, so the
263 gate for the red channel should include all levels of mCherry intensity. The labelling
264 system we developed relies on the modification of a red fluorescent protein (mCherry)
265 that is not expected to induce functional changes in the sLP-Cherry-producing cells
266 or the recipient cells. Here, we have included experiments to show that the
267 expression of the sLP-Cherry in cancer (4T1) or non-cancer (HC11) cells does not
268 influence their growth (Supplementary Figure 3a and 3b). Also, by using a well
269 characterized cancer-specific fibroblast activation assay ex vivo^{2,15}, we show that the
270 presence of the sLP-Cherry in culture media does not induce functional changes in
271 the exposed cells (Supplementary Figure 3c-e). Finally, we show that the presence
272 of the sLP-Cherry does not induce an additional inflammatory reaction in the lung
273 (Supplementary Figure 3f and 3g), which corroborate our previously published data¹
274 (Ombrato et al., 2019 - Extended Data Figure 2d and 2e).

275

276 Downstream analysis (phase 4; step 26; Figure 2)

277 Once you have confirmed that the Cherry-niche system is working in vivo, labelled
278 neighbouring cells can be freshly isolated by flow cytometry and used in downstream
279 applications. Examples of these assays can be found in Ombrato et al., 2019¹,
280 where we compared labelled vs unlabelled tissue cells either by bulk RNA
281 sequencing or single cell sequencing, and demonstrated high-throughput analysis
282 such as proteomic profiling. Moreover, as the isolated cells are viable, specific cell
283 populations can be tested in ex vivo functional assays¹.

284

285 **LIMITATIONS**

286 The Cherry-niche's ability to reveal the microenvironment relies on the capacity of
287 the sLP-Cherry-expressing cells to secrete it. Thus, the more labelling Cherry that is
288 released, the greater the chances of effectively detecting all surrounding cells.
289 Therefore, cells that are highly secretive (such as most cancer cells) may be
290 particularly suitable for this system.

291 Once released, the Cherry labelling is taken up by the cells in the local tissue
292 environment and potentially all cell types are able to uptake the sLP-Cherry.
293 However, different cells have different ability to internalise the fluorescent label; for
294 instance, phagocytic cells may be brighter than other labelled cells. Therefore, the
295 intensity of the mCherry signal does not precisely reflect the proximity to the labelling
296 cells and all labelled mCherry⁺ cells should be considered in the analysis. If required,
297 the ability of different cellular components to internalize the mCherry can be tested in
298 each labelling cell experimental model. Notably, the detection level by flow cytometry
299 exceeds the level of mCherry that can be visualized by staining in the tissue sections.

300 The processes of secretion and uptake of the sLP-Cherry rely on the solubility of the
301 modified mCherry, which, due to its nature, is promptly released when the tissue
302 sample is permeabilized with detergents (Supplementary Figure 4a-d). This is not an
303 issue when working with live tissue cells, but it needs to be considered when
304 immunofluorescence experiments requiring permeabilization are performed.

305

306 **MATERIALS**

307 Biological materials

308 - Cells: 4T1 cells (ATCC® CRL-2539™ or Cell Services unit at the Francis Crick
309 Institute), HEK-293FT cells (ThermoFisher Scientific R700-07), ML-1 cells (ECACC
310 88113007 or Cell Services unit at the Francis Crick Institute), HC11 cells (ATCC®
311 CRL-3062™ or Cell Services unit at the Francis Crick Institute), MS5 cells (Cell
312 Services unit at the Francis Crick Institute).

313 ! CRITICAL

314 Ensure that the cell lines used are regularly checked for authenticity and tested for
315 mycoplasma infection.

316 - Female Balb/c mice 6-12 weeks old (facility breeding or Jackson Laboratory, cat.
317 no. 000651) housed in specific pathogen-free (SPF) cages. All animals were
318 monitored daily for unexpected clinical signs following the P83B37B3C licence
319 guidelines and the principles set out in the NCRI Guidelines for the Welfare and Use
320 of Animals in Cancer Research (UK).

321 - NESTIN-GFP mice were a kind gift from Dr G. Enikolopov. NOD-SCID IL2Rgnull
322 (NSG) strain mice were obtained from the Jackson Laboratory and bred at the
323 Francis Crick Institute Biological Resources Facility in individually vented cages
324 under Specific Pathogen Free (SPF) conditions. NSG-NESTIN-GFP mice were
325 obtained by back-crossing the original lines into the NSG background (generation 8
326 or more). Animal experiments using human leukemic ML-1 cells were performed
327 under the project license (PPL 70/8904) approved by the UK Home Office and in
328 accordance with The Francis Crick institute animal ethics committee guidelines.

329

330 **REAGENTS**

331 For in vitro experiments

332 - DMEM (ThermoFisher Scientific, cat. no. 41965-039) used for 4T1 and HEK-293
333 cells

334 - RPMI medium 1640 + Glutamax (Gibco, 61870-010) used for ML-1 cells and HC11
335 cells

336 - IMDM (ThermoFisher Scientific, 12440-053) used for MS5 cells

337 - Penicillin-streptomycin (ThermoFisher Scientific, cat. no. 15140-122) used for 4T1,
338 HEK-293 and HC11 cells

339 - Penicillin-streptomycin (Sigma Aldrich, P4333) used for ML-1 and MS5 cells

340 - FBS-Heat Inactivated (Labtech International Ltd, cat. no. FCS-sa/500) used for 4T1,
341 HEK-293 and HC11 cells

342 - FBS (Sigma Aldrich, cat. no. F7524) used for ML-1 and MS5 cells

343 - Insulin (Sigma-Aldrich, cat. no. I9278) used for HC11 cells

344 - Epidermal growth factor (EGF; ThermoFisher Scientific, cat. no. PMG8041) used
345 for HC11 cells

346 - Polybrene (hexadimethrine bromide; Sigma-Aldrich, cat. no. 107689)

347 - 0.25% Trypsin (ThermoFisher Scientific, cat. no. 25050-014)

348 - PBS (The Francis Crick Institute, Media Services)

349 - Lentiviral vector sLPmCherry (Ximbio, cat. no. 155083)

- 350 - Second-generation lentiviral vectors (pCMV delta R8.2 and pCMV-VSV-G;
 351 Addgene, cat. nos. 12263 and 8454)
 352 - HBS 2X solution (Sigma, cat. no. H1012)
 353 - Calcium Chloride Dihydrate (Sigma, cat. no. C5080)
 354 - Bovine Serum Albumin (BSA; Sigma, cat. no. A7906)
 355 - Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA; Sigma, cat. no.
 356 E5134)
 357 - Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; Sigma,
 358 cat. no. E3889)
 359 - Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D8418)
 360 - HEPES (ThermoFisher Scientific, cat. no. 15630056)
 361 - Paraformaldehyde (PFA; 16% (w/v), Alfa Aesar, cat. no. 43368). CAUTION PFA is
 362 toxic if inhaled and can cause irritation of the skin and eyes. Use PFA in a fume hood
 363 and wear appropriate laboratory protective equipment.
 364 - Saponin (Sigma, cat. no. 47036-50G-F)
 365 - Tween20 (Sigma, cat. no. P2287-500ml)
 366 - Triton X100 (Sigma, cat. no. X100-500ml)
 367 - DAPI (Sigma, cat. no. D9542)
 368 - DRAQ7 (BD Pharmingen, cat. no. S1-9011172)
 369 - Sudan Black (Sigma, cat. no. 199664-25g)
 370 - Virkon (Bio Services, cat. no. 100-150)
 371 - Distel (Tristel Solutions Ltd, cat. no. MED/SOL/628/1)

372

373 In vivo experiments

- 374 - HBSS, -calcium, -magnesium (ThermoFisher Scientific, cat. no. 14175-053)
 375 - Liberase TM (Roche, cat. no. 05401127001)
 376 - Liberase TH (Roche, cat. no. 05401151001)
 377 - DNase I (Sigma, DN25-100mg)
 378 - Red Blood Cell Lysis buffer (Miltenyi Biotech, cat. no. 130-094-183)
 379 - Isoflurane (Zoetis, cat. no. 5690501) CAUTION Isoflurane is an anaesthetic and
 380 exposure to it can result in chronic or adverse health concerns. Perform the
 381 procedure in a well-ventilated hood.
 382 - Meloxicam (Metacam 5 mg/ml; Boehringer Ingelheim, cat. no. 5012917011302)
 383 - Liver Digestion Medium: (ThermoFisher Scientific, cat. no. 17703034)
 384 - Pentobarbital (Pentoject, Animalcare limited)
 385 - Intra-Epicaine (Decra)
 386
 387 - Antibodies used for IF/IHC: mCherry (Abcam, cat. no. ab183628); GFP antibody
 388 (Abcam, cat. no. ab6673); donkey anti goat AlexaFluor488 (Invitrogen, cat. no.
 389 A11055); donkey anti-rabbit AlexaFluor555 (Invitrogen, cat. no. A21432)
 390 - Antibodies for flow cytometry: CD45 BV421 (Biolegend, cat. no. 103133); CD31
 391 BV421 (Biolegend, cat. no. 102423); TER119 BV421 (Biolegend, cat. no. 116233);
 392 EPCAM APC (eBioscience, cat. no. 17-5791-81); CD45 APC780 (eBioscience, cat.
 393 no. 47-0451-82); CD11b APC (M1/70; Biolegend, cat. no. 101212); Ly-6A/E (Sca-1)
 394 PE-Cyanine (eBioscience, 725-5981-82); CD33 FITC (eBioscience, 11-0338-42);
 395 CD45 FITC (BD Bioscience 555482); CD45 APC-eFluor780 (BD Bioscience 47-
 396 0459-42).

397

398 **EQUIPMENT**

- 399 - Conical 50- and 15-ml tubes (Falcon, cat. no. 352070 and 352096)

- 400 - Eppendorf tubes (2ml; Eppendorf, cat. no. 616201)
- 401 - Pipettes (5, 10 and 25 ml; Falcon, cat. no. 356543, 356551 and 356525)
- 402 - Filter-tip pipettes (10, 20, 200, 1,000µl; STAR LAB cat. no. S1120-3810, S1120-1810, S1120-8810 and S1122-1830)
- 403
- 404 - 96-Well, 48-Well, 24-Well, 12-Well, 6-Well plates (Falcon, cat. no. 353072, 353078, 353047 and 353043)
- 405
- 406 - Cell culture Petri dishes 60mm (Falcon, cat. no. 353037) and 100mm (Corning, cat. no. 430167)
- 407
- 408 - CO₂ incubator (5% (vol/vol) CO₂, 37 °C; Eppendorf New Brunswick, model no. Galaxy 170R)
- 409
- 410 - FACS tubes; polypropylene, polystyrene and filter top cap (Falcon, cat no. 352063, 352058 and 352235)
- 411
- 412 - Cell strainers 100µm and 40µm (Falcon, cat no. 352360 and 352340)
- 413 - Syringes 5ml, 20ml (BD Plastipak, SS+20ES1)
- 414 - Level 2 biosafety cabinet (Clean Air, model no. CA/REV4)
- 415 - Centrifuge (Eppendorf, model no. 5810R)
- 416 - Filters (0.45µm, sterile; VWR, cat. no. 514-0075)
- 417 - Fluorescence microscope (EVOS FL Auto Imaging System; Life Technologies, cat. no. AMAFD1000)
- 418
- 419 - Flow cytometer (Fortessa, BD Biosciences, model no. 339473)
- 420 - Cell sorters BD Biosciences FACSAria III and BD Influx™
- 421 - Syringes for intravenous injection (1ml; Becton Dickinson, cat. no. 303172)
- 422 - Sterile scissors and forceps (B Braun Medical, cat. nos. BD313R and BC061R)
- 423 - Microtome (Leica, RM2235)
- 424 - Microtome blades, MX35 Ultra (ThermoFisher Scientific, cat. no. 3053835)
- 425 - Microscope slides, Superfrost Ultra Plus (ThermoFisher Scientific, cat. no.10417002)
- 426
- 427 - Coverslips (24 × 40mm; Menzel Gläser; VWR, cat. no. 631-1333)
- 428 - Confocal microscope (Zeiss, model no. 710 Upright microscope)
- 429 - Ultrasound machine (Visual Sonics, model no. VEVO 2100)

430

431 Software

- 432 - Image analysis software Fiji (<https://imagej.net/Fiji>)
- 433 - ZEN10 (Zeiss)
- 434 - FlowJo (BD Biosciences)
- 435 - FACS Diva (BD Biosciences)
- 436 - Imaris v 8.3.1

437

438 **REAGENT PREPARATION**

439 CRITICAL All reagents should be prepared in a sterile environment.

440

441 Growth medium

442 For 4T1 and HEK 293FT cells: DMEM with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin.

443 For ML-1 cells: RPMI 1640 with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin.

444 For HC11 cells: RPMI 1640 with 10% (vol/vol) FBS, 1% (vol/vol) penicillin-streptomycin, 10ng/ml EGF and 5µg/ml insulin.

445 For MS5 cells: IMDM with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin.

446 All these media can be stored at 4 °C up to two weeks.

450

451 Calcium solution

452 Dissolve 18.37g of Calcium Chloride in 50ml sterile distilled water. Sterile filter
453 through a 0.33µm syringe filter. This solution can be stored at room temperature up
454 to 1 year.

455

456 Polybrene solution

457 Dissolve 1g of Polybrene in 1.25ml of sterile distilled water for a stock solution of
458 800mg/ml. Dilute 1ml of this stock in 99ml of sterile distilled water to obtain an
459 8mg/ml working solution. Store at -20 °C in 1ml aliquots for up to 1 year.

460

461 Lung digestion solution

462 Prepare 5mg/ml stock solutions in HBSS (-Ca, -Mg) for Liberase TM , Liberase TH
463 and 5mg/ml stock solution in HBSS (-Ca, -Mg) of DNase 1. Aliquot and store at -
464 20 °C for up to 1 year. To prepare digestion solution, put 4823µl of HBSS (-Ca,-Mg)
465 in a 15ml Falcon tube and bring the volume to 5ml by adding 76µl of Liberase TM,
466 76µl of Liberase TH and 25µl of DNase from the stock solutions. This solution
467 should be prepared fresh each time.

468

469 Liver Wash Buffer

470 Hank's Balanced Salt Solution (HBSS) supplemented with 190mg/l EGTA and 25mM
471 HEPES (pH7.4 at 37 °C). This solution can be stored at room temperature up to 1
472 month.

473

474 Red Blood Cell Lysis

475 Dilute Red Blood Cell Lysis buffer 1:10 in sterile distilled water. This solution should
476 be prepared fresh each time.

477

478 FACS buffer

479 Dissolve 2.5g BSA and 372mg EDTA in 500ml of PBS. This solution can be stored at
480 4 °C up to 6 months.

481

482 DAPI

483 Dissolve 1mg of DAPI in 5mL of dH₂O for a 0.2mg/mL solution. This solution can be
484 stored at 4 °C up to 6 months.

485

486 Citrate Buffer (pH6.0)

487 Dissolve 2.94g tri-sodium citrate plus 18ml 0.2M HCl in 800ml of distilled water.
488 Bring to 1L final volume with distilled water and check for the pH. This solution can
489 be stored at room temperature up to 6 months.

490

491 1.6% H₂O₂ in PBS

492 Add 13.3ml of 40% H₂O₂ to PBS until a final volume of 250ml. This solution should
493 be prepared fresh each time.

494

495 0.4% Tween20 in PBS

496 Add 2ml of Tween20 to 500ml PBS. This solution can be stored at room temperature
497 up to 1 month.

498

499 Sudan Black solution

500 Sudan Black B 0.1% in 70% ethanol. This solution can be stored at room
501 temperature up to 6 months. Filter the solution on every use through filter papers
502 (GE Healthcare cat. No. 1202-320).

503

504 Haematoxylin solution

505 Add 120ml Mayer's Haematoxylin to 80ml of dH₂O. This solution can be stored at
506 room temperature up to 1 week.

507

508

509 **PROCEDURE**

510 **Phase 1. Generation of mCherry labelling cells (steps 1-17)**

511 **Generation of sLP-Cherry lentivirus (Steps 1-9; Figure 2)**

512 Timing 5d.

513 ! CRITICAL All lentiviral work should be carried out in designated CL2 areas. All
514 material and areas that have been in contact with lentivirus should be
515 decontaminated with antiviral disinfectants such as Distel or Virkon.

516 CRITICAL Before starting the procedure, all cell lines should be verified for
517 authenticity and tested for mycoplasma infection.

518 1. On day 1, split 1:4 a 100%-confluent 10cm Petri dish of HEK 293FT cells into
519 a new 10cm Petri dish in growth medium.

520 2. On day 2 (or when the cells are 80% confluent), remove the medium, replace
521 it with 9ml of growth medium and place the cells back in the incubator. Media
522 replacement should be done from 30 min to 6 h before the transfection. HEK
523 293FT cells tend to easily detach when manipulated, so media should always
524 be added slowly to the border of the well to avoid or reduce the number of
525 cells that detach.

526 3. Prepare a 15ml transfection tube A by adding the following components and
527 mix by pipetting up and down:

- 528 - 10µg of sLPCherry plasmid
- 529 - 6.5µg of pCMV delta R8.2 plasmid (packaging)
- 530 - 3.5µg of pCMV-VSV-G
- 531 - 62µg 2M Calcium Chloride solution
- 532 - Up to 500µl with sterile water

533 4. Prepare a 15ml transfection tube B by adding 0.5ml of HBS 2X solution.

534 5. Slowly vortex the solution in tube B while adding dropwise the solution in tube
535 A and incubate for 15 min at room temperature (25 °C).

536 6. After incubation, gently mix the solution and add dropwise to the HEK 293FT
537 plate. Place the cells overnight at 37 °C in a CO₂ incubator.

538 7. On day 3, remove the transfection medium, wash with 5ml of PBS and add
539 7ml of growth media.

540 8. On day 4, visualise the cells at the EVOS fluorescence microscope to
541 estimate the efficiency of cell transfection: at least 80% of the cells should be
542 mCherry⁺. If the transfection efficiency is high, collect the medium and
543 replace it with 7ml of growth media. Centrifuge the collected media at 300g
544 for 5 min and filter the supernatant by using a 10ml syringe and a 0.45µm
545 filter to remove any cells or large debris from the media. Repeat this step the
546 following day (day 5).

547 CRITICAL STEP Lower HEK 293FT transfection efficiency may result in a low
548 viral titre and consequently in a low infection efficacy of the viral preparation.

549 **TROUBLESHOOTING**

550 9. Pool together the medium collected on day 4 and day 5. Make 6ml aliquots
551 and store at -80 °C for up to 1 year or use it fresh on cultured cells.

552 CRITICAL STEP Freeze and thaw will decrease the infective efficiency of the
553 virus preparation. Therefore, the use of freshly prepared virus is
554 recommended, particularly for cells that are difficult to infect. If cell-type-
555 specific growth medium and/or higher viral titre is required, viral particles can
556 be concentrated via ultracentrifugation and used immediately or stored at -
557 80 °C for up to 12 months.

558

559 Cell culture preparation using sLP-Cherry lentivirus (Steps 10-17) (Figure 2)

560 Timing 9-14d.

561 ! CAUTION

562 All lentiviral work should be carried out in designated CL2 areas. All material
563 areas that have been in contact with lentivirus should be decontaminated with
564 antiviral disinfectants such as Distel or Virkon.

565 10. Seed 7.5×10^4 tumour cells per well in a 6-well plate in 1ml of growth media.

566 11. The following day, remove the media and add 1ml of growth media plus
567 varying amounts of media containing the sLP-Cherry lentivirus, from 1ml to
568 7ml, and Polybrene solution to a final concentration of $8 \mu\text{g/ml}$ to enhance the
569 binding of virus particles to the cells. Leave the plate at 37°C in a CO_2
570 incubator overnight.

571 12. Remove the virus-containing medium, wash twice with 3ml of PBS and add
572 3ml of cell-type-specific growth medium.

573 13. At day 3 post-transduction, collect the cells and spin them at 300g for 5 min
574 at room temperature.

575 14. Wash the pellet with PBS, spin at 300g for 5 min at room temperature.

576 15. Resuspend in 1ml of FACS buffer with DAPI 1:400, filter through a FACS
577 tube with a $20 \mu\text{m}$ strainer lid and process by FACS to isolate mCherry^+ cells
578 (Figure 3a and 3b). TROUBLESHOOTING

579 CRITICAL STEP 2-3 days post-infection is an ideal time to sort for mCherry^+
580 cells. After the cells have been infected, they will express the sLP-Cherry and
581 begin releasing it into the media. Therefore, uninfected cells may uptake the
582 protein from the media and become “transiently” mCherry^+ themselves.

583 16. Re-plate the sorted cells and allow them to recover and expand. For most cell
584 lines, this usually requires 5-10 days. However, time in culture will vary
585 depending on the recovery time and doubling time of each cell line.

586 17. Check the cells by FACS to confirm they are mCherry^+ and GFP^+ and freeze
587 several aliquots. These are potential labelling-cells that will be tested by a
588 combination of in vitro and in vivo approaches to determine their labelling
589 efficiency.

590 CRITICAL STEP If the cells are not at least $>97\%$ double positive, you may
591 wish to consider re-sorting them by FACS or repeating this protocol from step
592 10 onwards.

593

594 Phase 2. Validation of the labelling efficiency in vitro (Figure 2)

595 18. Test labelling efficiency in vitro by FACS and fluorescence imaging of co-
596 cultured labelled and unlabelled cells (option A) or culture with conditioned
597 media (option B)

598 A. *Co-culture setting*. Timing 3 days.

599 i) Place 2 sterile glass coverslips into each well of a 6-well plate.

600 ii) Plate $2-4 \times 10^5$ labelling-cells ($\text{mCherry}^+\text{GFP}^+$) and 4×10^4 unlabelled cells
601 and co-culture them at 37°C in a CO_2 incubator for 2 days (or until they are
602 $\geq 90\%$ confluent). For controls, keep one well with only labelling-cells and one
603 well with only unlabelled cells.

604 iii) Remove the coverslips from the wells and place each into one well of a 12-
605 well plate and add 1ml of PBS.

606 B. *Conditioned media setting*. Timing 6-8 days.

607 i) Plate 2×10^6 labelling-cells in a 10cm cell culture Petri dish with growth
608 media.

- 609 ii) When the cells reach 80% confluence, remove the media and add 10ml of
610 growth media.
611 iii) Allow the media to be conditioned for 48 h. Next, collect the media and
612 spin at 300g for 10 min in a 15ml tube.
613 iv) Place the supernatant in another 15ml tube and spin at 2000g for 10 min.
614 CRITICAL STEP Do not touch the pellet when removing the supernatant to
615 avoid any contaminating labelling-cells to be present in the *cm*.
616 v) Remove the supernatant (this is the *cm*) and use it immediately or store at
617 4 °C for up to 2 days before use. The *cm* can also be fractionated to dissect
618 the contribution of both the soluble fraction and the vesicles (Box 1).
619 vi) The day before collecting the *cm*, place 2 sterile glass coverslips per well
620 in a 6-well plate and then plate 7.5×10^5 of recipient cells and leave at 37 °C in
621 a CO₂ incubator overnight.
622 vii) The following day, replace the media with 1ml of growth media 30 min to 3
623 hours before adding 7ml of *cm* and incubate for 24-36 h at 37 °C in a CO₂
624 incubator. Keep one well as a control where no *cm* is added.
625 viii) Remove the coverslips, place them in a well of a 12-well plate and add
626 1ml of PBS.

627 FACS validation

- 628 19. After moving the coverslips from the 6-well plate to a 12-well plate, trypsinize
629 and collect the recipient cells from the 6-well plate, and pellet them at 300g
630 for 5 min.
631 20. Wash 1x with PBS and pellet again at 300g for 5 min.
632 21. Resuspend the cells in FACS buffer with DAPI 1:400 and analyse by FACS,
633 comparing either: the labelling-cells only, unlabelled cells only and the co-
634 culture (if co-culture setting); or the recipient cells incubated with their own
635 media and the recipient cells incubated with the *cm* (if conditioned media
636 setting; see Figure 4c). TROUBLESHOOTING

637 Fluorescence imaging

- 638 22. Remove the PBS from each well of the 12-well plate and add 1ml of PFA 4%
639 solution for 10 min at room temperature.
640 23. Remove the PFA 4% solution, wash 3x with 2ml of PBS and add 1ml of PBS
641 with DAPI 1:250 and incubate for 5 min at room temperature.
642 24. Remove the coverslips and mount them for confocal imaging on a slide with a
643 drop of mounting media (Figures 4b and 4d).
644

645 **Phase 3. Validation of the labelling efficiency in vivo**

- 646 25. Validate labelling efficiency in vivo either by immunofluorescence in a lung
647 metastatic model (option A), immunohistochemistry in a lung metastatic
648 model (option B), two-photon microscopy (option C), FACS in a lung
649 metastatic model (option D) or FACS in a liver metastatic model (option E).

650 A. Immunofluorescence in a lung metastatic model (Figure 2): Timing 9-10 days.

- 651 i) Trypsinize and collect labelling 4T1 cells. Count and resuspend in PBS at a
652 final concentration of 1×10^7 cells per ml. Filter the cells using a 40µm cell
653 strainer or a FACS tube with a strainer lid and keep the cells on ice.
654 CRITICAL STEP The cell suspension must be filtered to remove large cell
655 aggregates before injection. Cell clumps injected intravenously may obstruct
656 vessels, causing immediate mouse death. To minimise this risk, re-filter the
657 cell suspension immediately before injection if there are any visible clumps or
658 the cells have been kept on ice for more than 30 min before injection.

659 ii) Use 100µl of cell solution (10⁶ labelling 4T1) to inject intravenously in the
660 tail-vein of 6-10 week old Balb/c mice.
661 iii) 7 days post-injection, cull the mice, collect the lungs and keep them on ice-
662 cold PBS.
663 iv) Put the lungs in a 15ml tube, add 5x volume of 4% PFA in PBS and leave it
664 at 4 °C overnight. Alternatively, separate the 5 lung lobes before adding the
665 fixative solution: 1-2 lobes can be left in the 4% PFA in PBS as indicated and
666 processed for immunofluorescence (steps A v-Xiii), 1-2 lobes can be left in the
667 4% PFA in PBS as indicated and processed for immunohistochemistry (steps
668 B i-Xxiii), the remaining lobes can be analysed by flow cytometry (steps C i-vii)
669 v) Remove the PFA solution and add 5ml of 70% ethanol in water.
670 vi) Embed the lung lobes in paraffin blocks.
671 vii) Cut four-micrometre-thick tissue sections, deparaffinise and rehydrate
672 using standard methods.
673 viii) After heat-mediated antigen retrieval in pH6.0 citrate buffer, incubate the
674 sections in a PBS solution with 1% BSA and 10% donkey serum for 1 h at
675 room temperature.
676 ix) Remove the blocking solution, add the primary antibody mix (containing
677 both 1:750 anti-mCherry and 1:300 anti-GFP in blocking solution) and
678 incubate 4 °C overnight.
679 X) The following day, remove the solution and wash with PBS 3x 5 min at
680 room temperature.
681 Xi) Incubate for 1 h at room temperature with the secondary antibody mix
682 (containing both 1:400 donkey anti-goat Alexa Fluor 488-conjugated and
683 1:400 donkey anti-rabbit Alexa Fluor 555-conjugated antibodies in blocking
684 solution).
685 Xii) Remove the solution, wash with PBS 3x 5 min at room temperature, then
686 incubate the slides with Sudan Black B for 20 min.
687 CAUTION Filter the Sudan Black solution just before using it to remove
688 precipitates that will affect the quality of the imaging.
689 Xiii) Mount the slides with Vectashield mounting medium with DAPI (Vector
690 Laboratories) for imaging (Figure 5a). TROUBLESHOOTING
691

692 **B. Immunohistochemistry in a lung metastatic model (Figure 2).** Timing = 2-3
693 days from harvesting the tissue (or 9-10 days starting from cancer cell
694 intravenous injection).

695 CRITICAL Lung lobes from mice injected at step A ii can be used as indicated at
696 step A iv. Alternatively, use different lung tissues harbouring cancer cells
697 generated by repeating steps A i-iv.

698 i) After an overnight fixation, remove the PFA solution, wash with 5ml of PBS
699 and add 5x volume of ethanol 70%.

700 ii) Embed lung tissues in paraffin.

701 PAUSE POINT Paraffin block can be stored for several years at 4 °C or room
702 temperature.

703 iii) Cut five-micrometre-thick tissue sections from the paraffin block to keep on
704 a slide.

705 PAUSE POINT Slides can be stored for several years at 4 °C.

706 iv) Place the slides into chemical resistant, microwavable plastic racks.

707 v) Dewax the slides in xylene with 1x 3 min and then perform a 30 sec wash
708 twice with xylene, twice with 100% ethanol, once with 70% ethanol and then
709 rinse thoroughly with distilled water.
710 vi) For antigen retrieval, place a loosely covered container of pH6.0 Citrate
711 Buffer in a 900W microwave and pre-heat for 8 min at full power, add the
712 racked-sections and heat for a further 15 min at 80% power.
713 vii) Carefully remove the container from the microwave and place in a sink,
714 under a trickling tap for 5 min. This will cool and dilute the buffer solution
715 slowly, preventing salt crystallisation.
716 CAUTION After microwave heating, the solution will be hot. Please act
717 carefully and use the necessary protective equipment such as protective
718 glasses and lab coat and gloves.
719 viii) Discard the cooled buffer, and move the rack of washed slides to PBS.
720 ix) Incubate in 1.6% H₂O₂ in PBS for 10 min.
721 X) Terminate the H₂O₂ reaction by washing in distilled water for 5 min.
722 Xi) Carefully wipe excess fluid from slides and use a PAP (hydrophobic) pen
723 to create a reagent barrier around the tissues.
724 Xii) Rinse in 0.4% Tween20 in PBS. This step will reduce slide surface
725 tension, maximising reagent coverage.
726 Xiii) Remove the Tween20 solution and add blocking solution (10% donkey
727 serum, 1% BSA in PBS) for 1 hour at room temperature.
728 Xiv) Remove the blocking solution and add the primary antibody diluted as
729 required in 1%BSA/PBS (1:300 goat anti-GFP or 1:750 rabbit anti-mCherry).
730 Incubate it at 4 °C overnight.
731 Xv) Perform a 5 min wash with PBS three times. Then, wash briefly in 0.4%
732 Tween20 in PBS to maximise reagent coverage.
733 Xvi) Incubate with secondary antibody diluted as required in 1% BSA in PBS
734 for 45 min, at room temperature (biotinylated donkey anti-goat 1:250 or
735 biotinylated donkey anti-rabbit 1:250). During the incubation time, prepare
736 ABC solution (according to manufacturer's directions) and leave to stand for
737 30 min.
738 Xvii) Wash three times in PBS (2-min washes). Then, wash briefly in 0.4%
739 Tween20 in PBS to maximise reagent coverage.
740 Xviii) Incubate in ABC for 30 min, at room temperature.
741 Xix) Wash in PBS, 3 x 1 min, and wash briefly in PBS-Tween to maximise
742 reagent coverage.
743 XX) Apply DAB solution and monitor microscopically.
744 Xxi) Wash in distilled water to terminate the development reaction.
745 Xxii) Counterstain with a light haematoxylin solution for 1 min then wash in tap
746 water for 5 min, to 'blue' the haematoxylin.
747 Xxiii) Dehydrate with 70% ethanol and 100% ethanol, clear in xylene and
748 coverslip/mount with DPX mountant for imaging (Figure 5b).

749 **C. Two-photon microscopy (Figure 2) Timing 14 days.**

751 i) Collect ML-1 cells and spin at 300g for 5 min. Count and resuspend at a
752 concentration of 1x10⁷ cells/ml.
753 ii) Inject 2 millions cells (200µl) into immunodeficient NSG–Nestin^{GFP} mice¹⁶.
754 iii) Fourteen days after injection, sacrifice mice, isolate the whole head and
755 remove the skin.

- 756 iv) Place the head in a container plate under the Zeiss 710 NLO laser
757 scanning multiphoton microscope with a 20x 1.0 NA water immersion lens.
758 The microscope is equipped with a MaiTai “High Performance” fully
759 automated 1-box 517 mode-locked Ti:Sapphire laser with DeepSee
760 dispersion compensation (Spectra-Physics), tuned to 800nm excitation
761 wavelength.
762 v) By using the microscope eyepiece, set the stage at the right level to
763 visualize the tissue.
764 vi) Activate the 4 non-descanned detector NDDs to detect the desired
765 fluorochromes. In this case, the collagen signal from bone (second harmonic
766 generation, SHG) is collected at 380-485nm, the GFP signal from Nestin⁺
767 mesenchymal cells at 500-550nm, and the cherry signal at 640-690nm.
768 vii) Select 3D regions of interest (ROIs) and acquire the images (more details
769 at PMID 28809828¹⁷) (Figure 5c).
770

771 **D. FACS in a lung metastatic model (Figure 2)** Timing 1 day (on the same day of
772 tissue harvesting; or 7 days starting from cancer cell intravenous injection).

773 CRITICAL Lung lobes from mice injected at step A ii can be used as indicated at
774 step A iv. Alternatively, use different lung tissues harbouring cancer cells
775 generated by repeating the steps A i-iv.

- 776 i) Chop the tissue with scalpel and then with scissors until it becomes a
777 smooth paste with no visible clumps.
778 ii) Place the chopped tissue in a 2ml eppendorf tube and add 1.5ml of Lung
779 digestion solution.
780 iii) Incubate for 30 min at 37 °C, shaking at 120 rpm.
781 iv) Collect the cell suspension, filter through a 100µm cell strainer, then
782 squeeze the filter by using a 5ml syringe plunger.
783 v) Add 4ml of DMEM with 10% FBS on top of the filter.
784 vi) Spin at 300g for 10 min at 4 °C. Carefully remove the supernatant without
785 touching the pellet.
786 CRITICAL STEP Vacuum pumps should be used carefully to aspirate the
787 supernatant, since a loose pellet can be aspirated if the pipette gets too close.
788 If the pellet seems loose, we recommend spinning the tube again for another
789 5 min at 4 °C.
790 vii) Resuspend the pellet in 5ml of RBC Lysis buffer and leave at room
791 temperature for 3 min.
792 viii) Spin at 300g for 10 min at 4 °C.
793 ix) Resuspend the pellet in 5ml of FACS buffer, filter it through a 40µm cell
794 strainer, then squeeze the filter by using a 5ml syringe plunger.
795 X) Spin at 300g for 6 min at 4 °C, resuspend in 3ml of MACS buffer and filter it
796 through a FACS tube with a 20µm strainer lid.
797 Xi) Spin at 1250 rpm for 5 min at 4 °C.
798 Xii) Resuspend the pellet in 1ml of FACS buffer with DAPI 1:400 and analyse
799 the sample by FACS (Figure 6a). **Alternatively**, if staining is required,
800 resuspend the pellet in 300µl of blocking solution and leave it at room
801 temperature for 5 min.

802 26.

- 803 Xiii) Add the primary conjugated antibody mixture for FACS at the indicated
804 concentration and incubate for 30 min on ice in the dark.
805 Xiv) Add 2ml of FACS buffer and spin at 300g for 5 min at 4 °C.

806 Xv) Remove the supernatant, wash with 2ml of FACS buffer and spin at 300g
807 for 5 min at 4 °C.
808 Xvi) Resuspend the pellet in 1ml of FACS buffer with DAPI 1:400 and analyse
809 the sample by FACS (Supplementary Figure 2).
810

811 **E. FACS in a liver metastatic model (Figure 2).** Timing 1 week. CRITICAL This
812 stage of the protocol has been optimised for the isolation of liver hepatocytes. If
813 the isolation of other cell types, such as breast metastatic cells, is needed,
814 adaptation of the protocol may be required.

815 i) Trypsinize and collect labelling 4T1 cells. Count and resuspend them in
816 PBS at a final concentration of 2×10^7 cells/ml.
817 ii) Perform ultrasound-guided splenic injections on 6-10 week old BALB/c
818 mice, whilst under inhaled isoflourane anaesthesia (50 μ l / mouse). Sub-
819 cutaneous analgesia (Metacam) should also be given prior to injection.
820 iii) Harvest liver 7 days post-injection. Metastatic lesions should be clearly
821 visible on the surface of the spleen and liver.
822 iv) Inject mice intraperitoneally with a pentobarbitol/epicaine mix to induce
823 terminal anaesthesia. Once mice are sufficiently anaesthetised, immediately
824 open the peritoneal cavity and cannulate the portal vein with a 26-gauge
825 needle. Successful cannulation will be immediately visible by blanching of the
826 liver.
827 v) Perfuse the liver with 20ml of pre-warmed (37 °C) Liver Wash Buffer. Once
828 the liver has started to swell, cut the inferior vena cava to allow fluid to drain.
829 vi) Perfuse the liver with 20ml pre-warmed Liver Digestion Media. A cotton
830 bud should be used to regularly apply pressure on the vena cava to allow the
831 liver to fill and swell with the digestion mix before removing to allow the liquid
832 to drain out.
833 vii) Excise the liver, remove the gall-bladder and incubate in 5ml Liver
834 Digestion Media for 10 min at 37 °C to improve tissue digestion. Cull mouse
835 via cervical dislocation.
836 viii) After 10 min digestion, liberate the cells by tearing and shaking the liver
837 with forceps in a 10cm dish.
838 ix) Filter the cell suspension through a 100 μ m cell strainer, liberating any
839 extra cells by applying a 5ml plunger to the filter. Quench the digestion media
840 with DMEM supplemented with 10% FBS, making up to 50ml volume.
841 X) Spin cell suspension at 100g for 10 min.
842 Xi) Resuspend the cell pellet in 10ml RBC Lysis buffer and incubate for 5 min
843 at room temperature.
844 Xii) Quench in 20ml FACS buffer and spin down at 1000 rpm for 10 min.
845 Xiii) Resuspend in 8ml FACS buffer and filter through 2x FACS tubes (4ml per
846 tube) containing a 20 μ m strainer lid.
847 Xiiii) Spin cell suspension at 100g for 10 min, resuspend in 4ml FACS buffer
848 with DRAQ7 1:1000, filter through a strainer topped FACS tube and analyse
849 by flow cytometry. Alternatively resuspend your sample in blocking solution for
850 staining (Figure 6b).
851

852 **Phase 4. Analysis of the labelled tissue cells isolated by FACS**

853 27. Choose from a variety of OMICs approaches and ex vivo assays for
854 downstream analysis (Figure 2), as demonstrated in our previous publication¹.
855

Table 1 Troubleshooting table			
Step	Problem	Possible reason	Solution
8	Cells have not been efficiently infected	Viral titre is low	Ensure that at least 80% of HEK 293FT producing cells have been efficiently transfected. If not, make a new plasmid preparation and repeat the transfection
15	Cells have not been efficiently infected	Some cell lines or primary cells can be difficult to infect	Make sure mycoplasma-free cells have been used. Concentrate the viral supernatant via ultracentrifugation. This allows the addition of more virus per well and increases the infection efficiency
21	No labelled cells are detected when co-cultured with labelling cells	The secretory ability of the labelling cells may be low	First, confirm the presence of sLP-Cherry protein in the supernatant of labelling-cells via Western blotting. Then, use a higher ratio of sLP-Cherry expressing cells (labelling) vs unlabelled cells and/or keep the cells in co-culture for longer before analysing them by FACS
21	No labelled cells are detected when co-cultured with labelling cells	The recipient cells used may not uptake the sLP-Cherry	You may use HEK 293FT cells as recipients, to confirm that the absence of uptake is not dependent on the secretory ability of the labelling cells. If other cells can uptake, try to use a higher ratio of labelling vs unlabelled cells and/or keep the cells in co-culture for longer before analysing them by FACS.
24	No labelled cells are detected when cultured with labelling cells conditioned media (<i>cm</i>)	Direct cell-cell contact may be required to label the recipient cells	Recipient cells may internalise the sLP-Cherry via different routes. Direct co-culture is the most efficient approach to test labelling activity in vitro. If no labelling is observed in direct co-culture, different recipient cells should be tested
21, 24	Low uptake efficiency in vitro	The level of mCherry expression in the labelling cells may be low	Perform a second round of infection with the sLP-Cherry virus and analyse the infected cells by FACS. Sort labelling cells with different levels of mCherry expression (low, medium and high) and test them separately to identify the most efficient labelling pool

<p>25A Xiii</p>	<p>Low or no mCherry signal detected via IF after intracellular staining on tissue sections</p>	<p>sLP-Cherry is easily lost after permeabilization</p>	<p>liposoluble and after intracellular staining on tissue sections</p>	<p>Unfixed lung tissue can be cut at the microtome after embedding in agarose and the slices imaged via confocal microscopy. The mCherry signal should be clearly visible, at least in the labelling cells. The slices can then be shortly fixed in PFA 4% solution and different permeabilization reagents and conditions tested in case intracellular staining is required</p>
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857
858
859

TIMING

- 860 • Steps 1-9, generation of sLP-Cherry viral particles: 5 days
- 861 • Steps 10-12, cell line transduction: 3 days
- 862 • Steps 13-17, isolation and amplification of mCherry expressing cells: 7-10
- 863 days (depending on the duplication rate of the cells in vitro after sorting)
- 864 • Steps 18-24, testing the labelling of the generated cells in vitro (this includes
- 865 co-culture with labelling 4T1 cells (steps A i-iii, 19-24), culture with conditioned
- 866 media from labelling 4T1 cells (steps B i-viii, 19-24), co-culture with labelling
- 867 ML-1 cells and culture with conditioned media from labelling ML-1 cells (box
- 868 1): 3-8 days
- 869 • Step 25 A-B, validation of the labelling of 4T1 cells in the lung (by
- 870 immunofluorescence (steps A i-Xiii), by immunohistochemistry (steps B i-XXiii):
- 871 7-10 days when labelling cancer cells are used (this time varies according to
- 872 the in vivo growing rate of the different cells).
- 873 • Step 25 C, testing the labelling of ML-1 cells in the bone marrow by two-
- 874 photon microscopy (steps C i-vii): 14 days
- 875 • Step 25 D-E, validation of the labelling efficiency in vivo by flow cytometry in
- 876 the lung (steps D i-Xvi) or in the liver (steps E i-Xiv): 7-10 days when labelling
- 877 cancer cells are used (this time varies accordingly to the in vivo growing rate
- 878 of the different cells)

879

ANTICIPATED RESULTS

880 Here, we have shown that sLP-Cherry viral particles, generated using a standard
881 lentivirus production procedure, can be used to infect breast tumour cells (4T1) and
882 leukemic cells (ML-1). After infection, the cells are processed via FACS to isolate
883 mCherry⁺ cells (Figure 3). We anticipate that most cell lines can be successfully
884 engineered using this procedure. However, the protocol may need to be adapted for
885 some cell lines to obtain a high percentage of infected cells and high level of
886 mCherry expression.

887 After sorting, mCherry expressing cells are amplified in culture. Their labelling ability
888 is tested in vitro, either by co-culturing them with unlabelled cells or by using their
889 conditioned media to culture mCherry⁻ cells (Figure 4). Using double-labelled cells
890 (e.g. labelling 4T1 cells that are mCherry⁺GFP⁺), allows you to easily identify
891 recipient labelled cells (mCherry⁺GFP⁻) in co-culture. In vitro labelling can be
892 evaluated by flow cytometry and microscopy. We recommend using flow cytometry
893

894 to obtain a more accurate estimation of the number of labelled cells, including cells
895 that have been labelled at very low levels and may be missed by using microscopy.
896 It is worth noting that amplification of the mCherry signal by immunostaining may be
897 particularly challenging. The sLP-Cherry is rapidly lost when using some
898 permeabilising reagents (Supplementary Figure 4) due to its liposolubility (an
899 essential feature mediating its secretion and uptake).
900 In this protocol, we describe how to confirm the presence of labelled cells in the lung
901 and liver niche of 4T1 metastases and in the bone marrow niche of ML-1 leukemic
902 cells. Imaging techniques can be used to confirm the presence of in vivo labelling
903 and its confinement to the close proximity of the tumour bulk. Here, we imaged
904 tissue sections from lungs in which labelling 4T1 metastases were growing as well
905 as calvarium sections from mice engrafted with labelling ML-1 leukemic cells (Figure
906 5). Here, labelling ML-1 cells (mCherry⁺ only) have been injected in Nestin-GFP⁺
907 mice. This experiment shows a targeted approach where Nestin⁺ cells within the
908 niche are double positive (GFP⁺mCherry⁺) and can be specifically identified.
909 A more accurate estimation of the number of labelled cells in the tissue can be
910 obtained by FACS. As an indication, the cancer cell lines we have previously used¹
911 have the following labelling activity determined by the average ratio labelled:labelling
912 cells in the lung: breast cancer cells 4T1 = 2.5:1, colon cancer cells CT26 = 1.7:1,
913 renal cancer cells RENCA = 2.2:1.
914 We show a gating strategy to discriminate mCherry⁺ niche cells by flow cytometry in
915 dissociated lung and liver tissues (Figure 6). Importantly, some tumour cells show a
916 tendency in vivo to decrease the fluorescent signal intensity of some fluorophores,
917 such as GFP. In this situation, a more stringent gating strategy to identify mCherry⁺
918 labelled cells can be used (Figure 6). A key advantage of using the Cherry-niche
919 labelling system is the ability to identify and isolate specific populations of
920 neighbouring cells. Examples of gating strategies to identify subpopulation of cells in
921 the lung metastatic niche from 4T1 cells have been shown (Supplementary Figure 2).

922

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937

938 **AUTHOR CONTRIBUTIONS**

939 L.O. designed the protocol, performed most of the experiments, analysed the data
940 and wrote the manuscript. E.N. and V.L.B. performed the experiments on liver
941 metastasis and analysed the data. D.P. and A.W. generated the leukemic labelling
942 ML-1 cells and performed the experiments reported with those cells. I.K. performed
943 the proliferation and the gel contraction experiments and analysed the data. D.D.

944 and C.L.C. have run pilot experiments to validate the labelling system which helped
945 with the troubleshooting, and critically read the manuscript. D.B. supervised the
946 experiments with the leukemic cells. I.M. supervised the study and critically revised
947 the manuscript.

948

949 **COMPETING INTERESTS**

950 The authors declare no competing interests.

951

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954 stem features. *Nature* **572**, 603–608 (2019).
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Box 1: Validation of the labelling efficiency of labelling leukemic cells in vitro by FACS using media fractionation (Timing = 4 days).

1. Plate 5×10^5 labelling ML-1 cells (without MS5 support) in a 24-well plate with 1ml of ML-1 media per well.
2. Plate as recipient cells 2.5×10^4 MS5 cells per well in a 24-well plate with 1ml of MS5 media.
3. Two days later, collect the media from labelling ML-1 cells and spin at 300g for 10 min.
4. Use half of the supernatant as cm_300g and add to the MS5 recipient cells (1ml per well). Retain the other half of the supernatant and spin it down again at 2000g for 15 min.
5. Use the supernatant as cm_2000g and add 1ml to the MS5 recipient cells. Resuspend the pellet in an equivalent amount of fresh media and add to the MS5 plated 2 days before.
6. Collect labelling ML-1 cells, spin them down and resuspend at a concentration of 5×10^5 cells per ml in ML-1 media and add 1ml to MS5 recipient cells.
7. After 2 days of culture, remove the supernatant, wash once with PBS and then trypsinize for 5 min.
8. Collect the trypsinized cells and spin them at 300g for 5 min.
9. Resuspend the cells and stain for 15 min with 2% FBS in PBS with 1:400 anti-SCA1 (to detect MS5) and 1:25 anti-CD33 + 1:25 anti-CD45 at room temp.
10. Spin the cells at 300g for 5 min and wash once with 2% FBS in PBS.
11. Resuspend the cells in 2% FBS in PBS with DAPI 1:1000 and analyse by flow cytometry.

FIGURE LEGENDS

Fig. 1 | Schematic of the labelling system. GFP+ cancer cells have been engineered to express a modified mCherry protein (sLP-Cherry) containing a soluble peptide (s) and a TATk (LP). Note that other markers can also be used, instead of the GFP, to allow discrimination between labelling and labelled cells. The figure also highlights several key features of the system that have been previously defined¹. The sLP-Cherry sequence can be found in Supplementary Figure 1 and it is also included as Supplementary Information in Ombrato et al., 2019¹.

Fig. 2 | Schematic overview of the protocol. Phase 1: First, labelling cells are generated using standard lentivirus production methods. HEK 293FT cells are transfected with a combination of lentiviral plasmids expressing the sLP-Cherry protein, the packaging proteins and the VSVG protein. The sLP-Cherry virus is then used to infect recipient cells. Next, the infected cells are collected and sorted according to levels of mCherry expression. The inset shows how the mCherry protein has been modified in the sLP-Cherry vector. VSVG, Vesicular stomatitis virus G. Phase 2: In vitro validation is performed using either co-culture between labelling cells and other cells (A), or culture of the recipient cells with labelling cell conditioned media (B). Phase 3: In vivo validation by microscopy is used to confirm proximity labelling in tissue sections. The mouse lung tissue is collected after intravenous injection of

1044 labelling cells and sectioned to be analysed by microscopy after performing
1045 immunofluorescence (A) or immunohistochemistry (B) procedure. The mouse
1046 skull bone is collected after intravenous injection of leukemic labelling cells
1047 and analysed by two-photon microscopy (C). Phase 3: In vivo estimation of
1048 labelling is obtained by FACS. Mouse lung (D) or liver (E) tissue is collected
1049 after intravenous or intrasplenic injection, dissociated and analysed by FACS.
1050 The minimum ratio of labelled:labelling cells estimated by FACS needs to be
1051 1:1 to meet the requirement. As an indication, the cancer cell lines we have
1052 tested have the following labelling activity determined by the average ratio
1053 labelled:labelling cells in the lung: breast cancer cells 4T1 = 2.5:1, colon
1054 cancer cells CT26 = 1.7:1, renal cancer cells RENCA = 2.2:1. Phase 4:
1055 Downstream applications for isolated cells. Live cells can be isolated by FACS
1056 and analysed by comparing labelled cells from the niche and unlabelled cells
1057 from the distant tissue by OMICs (such as bulk RNA sequencing, single cell
1058 RNA sequencing and proteomic) or functional ex vivo assays.
1059

1060 **Fig. 3 | Isolation of labelling cells by FACS.** FACS plots showing the gating
1061 strategy to sort high-mCherry⁺-expressing cells after infection for 4T1 breast
1062 tumour cell line (a) and ML-1 human acute myeloid leukemic cells (b).
1063 mCherry expression is analysed with a 561-nm laser and a 610/20 filter (blue
1064 dots show control cells before viral infection; red dots show cells 2-3 days
1065 after infection; the percentage refers to the proportion of gated cells for all the
1066 infected cells).
1067

1068 **Fig. 4 | Validating labelling cells in vitro.** a, FACS plots showing 4T1 alone and in
1069 co-culture with labelling 4T1 cells. b, Representative fluorescence image
1070 (white arrow, labelling cell; grey arrow, recipient cell; Green, GFP; Red,
1071 mCherry; Blue, DAPI; scalebar 10 μm). c, FACS plots showing 293T cells
1072 alone and in culture with labelling 4T1 cell cm after 2000g spin. d,
1073 Representative fluorescence image (white arrows, internalised mCherry; Red,
1074 mCherry; Blue, DAPI; scalebar 10 μm). Results for co-culture and culture with
1075 conditioned media using labelling 4T1 cells (a-d) have been previously
1076 published¹, different examples from these experiments are shown here. e,
1077 Quantification of MS5 stromal cells uptaking the mCherry analysed by FACS.
1078 MS5 in a 1:1 co-culture with ML-1 GFP is cultured with labelling ML-1 cm after
1079 300g or 2000g spin, pellet from 2000g spin (box 1). Data are represented as
1080 mean ±SEM and statistical analysis are performed by unpaired two-tailed t-
1081 test. a, c, e, mCherry expression is analysed with a 561-nm laser and a
1082 610/20 filter; GFP expression is analysed with a 488-nm laser and a 530/30
1083 filter.
1084

1085 **Fig. 5 | Labelling detected via imaging on tissue sections.** a, Representative
1086 immunofluorescence image of a lung tissue section from a Balb/c mouse
1087 harbouring labelling 4T1 metastases (white arrows, niche labelled cells (GFP⁻
1088 mCherry⁺); Green, GFP; Red, mCherry; Blue, DAPI; scalebar 50 μm). b,
1089 Representative immunohistochemistry images of lung tissue sections from a
1090 Balb/c mouse harbouring labelling 4T1 metastases (black arrows, niche
1091 labelled cells (GFP⁻mCherry⁺); upper panel GFP stained; lower panel
1092 mCherry stained; scalebar 50 μm). Lungs have been harvested from Balb/c
1093 mice 7 days after labelling 4T1 cells injection (a,b). Results using labelling

1094 4T1 cells in vivo (a,b) have been previously published¹, different examples
1095 from these experiments are shown here. **c**, Representative 3D reconstruction
1096 of two-photon imaging of the calvarium bone marrow from a NSG-Nestin-GFP
1097 mouse transplanted with labelling ML-1 cells and harvested 14 days after
1098 labelling ML-1 cells injection (white arrows, Nestin⁺ labelled cells
1099 (GFP⁺mCherry⁺); Green, GFP; Red, mCherry; scalebar 50 μ m).

1100
1101 **Fig. 6 | Validating labelling cells in vivo. a, b**, FACS gating strategy to identify
1102 labelled cells in a dissociated metastatic lung (**a**) and liver (**b**). Metastatic
1103 tissues are collected 7 days after injection, dissociated and analysed by FACS.
1104 The gating hierarchy is shown by red arrows and the gates have been drawn
1105 according to the Full Minus One (FMO) controls. After gating for all events
1106 acquired (i), single cells (ii) and subsequently live cells (iii) are identified. Full
1107 Minus (FM) mCherry/GFP sample represents a dissociated lung or liver from
1108 a mouse that has not been injected with tumour cells. Single mCherry⁺ niche
1109 cells are gated according to the FMO mCherry/GFP sample (Control for gate
1110 setting). (iv). Live cells are discriminated on the mCherry/GFP gate (v) where
1111 double-positive tumour cells (TC), labelled cells (niche), and unlabelled cells
1112 (distal tissue) can be resolved (v). A more stringent niche gate (vi) reduces
1113 the possibility of including labelling 4T1 cells that may downregulate the GFP
1114 expression in vivo. Results from metastatic lungs using labelling 4T1 cells in
1115 vivo (a) have been previously published¹, different examples from these
1116 experiments are shown here.

1117 1118 **SUPPLEMENTARY FIGURE LEGENDS**

1119 **Suppl. Fig. 1 |** Sequence of the mCherry protein (sLP-Cherry) containing a soluble
1120 peptide (s) and a TATk (LP). This can be also found in our previous
1121 publication¹.

1122
1123 **Suppl. Fig. 2 | Examples of gating strategy to define the identity of labelled**
1124 **cells in vivo.** The gating strategy here follows doublets and cell death
1125 exclusion as described in Figure 6a. Gate sequence is indicated by red
1126 arrows. **a**, CD45⁺ immune cells are gated distinctively from niche and distal
1127 lung cells, then CD11b⁺ myeloid cells are gated from CD45⁺ cells. **b**, Lineage⁻
1128 (CD45⁻CD31⁻Ter119⁻) cells are gated distinctively from niche and distal lung
1129 cells. The channel used for the Lin BV421 is plotted against the UV 450/50,
1130 which allows you to capture LIN⁻ cells that are auto-fluorescent (this strategy
1131 is only possible if DAPI⁺ cells have been previously excluded as shown in
1132 Figure 6). Next, epithelial Epcam⁺ cells are gated from Lin⁻ cells. Myeloid and
1133 epithelial cells from lungs harbouring labelling 4T1 cells have been identified
1134 using the same strategy and results have been previously published¹, using
1135 data generated from independent experimental replicates.

1136
1137 **Suppl. Fig. 3 | Functional analysis to exclude specific mCherry-dependent**
1138 **effects on labelling and recipient cells. a, b**, In vitro proliferation of 4T1 and
1139 HC11 cells (GFP or labelling). Representative images showing Crystal violet
1140 stained cells (**a**) and quantification (**b**). **c-e**, Gel contraction assay of
1141 fibroblasts co-cultured with HC11 and 4T1 cells (GFP or labelling): schematic
1142 images of co-culture (**c**); representative images (**d**); and quantification of gel
1143 area (**e**). **f**, Representative IHC on lung tissue sections stained with S100a9 to

1144 detect neutrophils from Balb/c mice injected with either labelling 4T1 or 4T1
1145 cells. Scale bar 100 μ m. **g**, CD45⁺ cell frequency in lungs from Balb/c mice
1146 injected with control media or conditioned media from 4T1, labelling HC11
1147 and labelling 4T1 cells by FACS. **b,e,g**, Data are represented as mean \pm SEM
1148 and statistical analysis are performed by unpaired two-tailed t-test.

1149

1150 **Suppl. Fig. 4 | Effect of permeabilization on mCherry detection. a-d,**
1151 Representative images of labelling 4T1 cells by confocal microscopy.
1152 Labelling 4T1 cells (**a**) fixed in 4% PFA for 10 min or permeabilised for 7 min
1153 with (**b**) Saponin 0.1%, (**c**) Tween20 0.1% or (**d**) Triton X100 0.1% (**a-d**
1154 scalebar 10 μ m). The use of strong detergents, such as Tween20 and Triton
1155 X-100, results in a large loss of the mCherry signal.

Principle of the system

Key features from
Ombrato et al., 2019

mCherry stored in CD63+
vesicular bodies upon
internalization*

Intracellular half life:
about 40h* (*in vitro*)

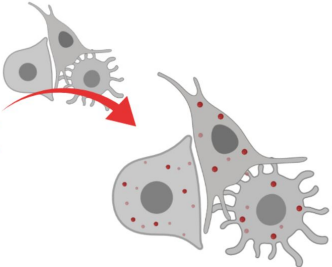
Max distance of labelled
lung cells from labelling
cells in the tissue:
20-80 μ m*



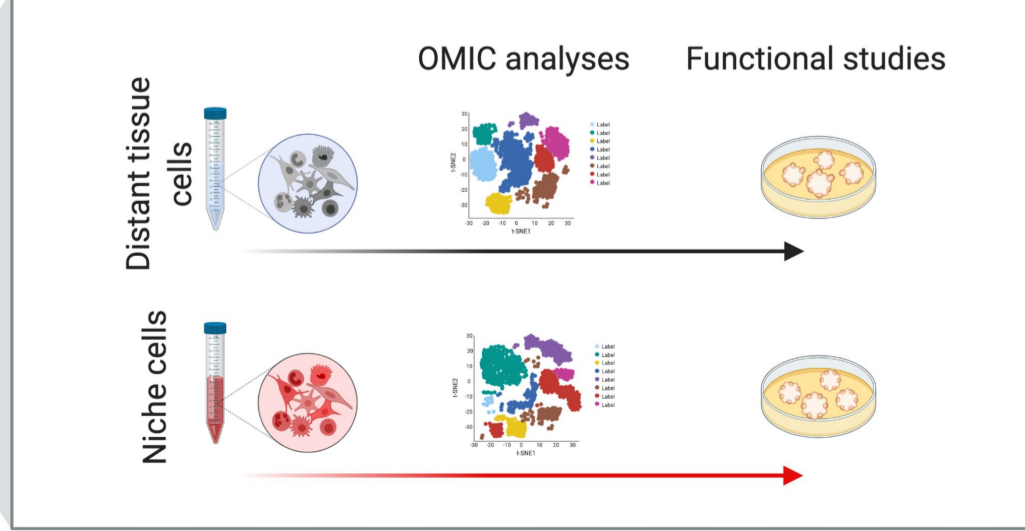
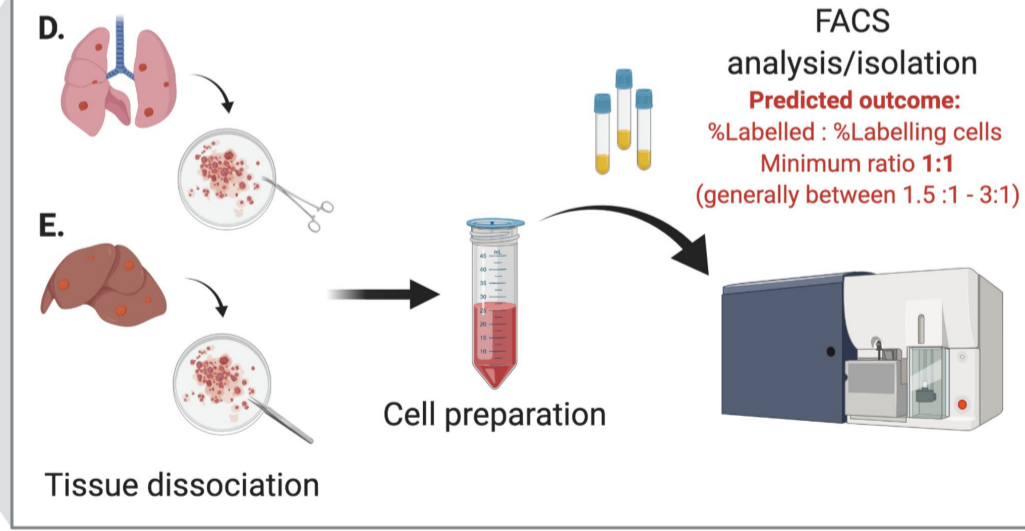
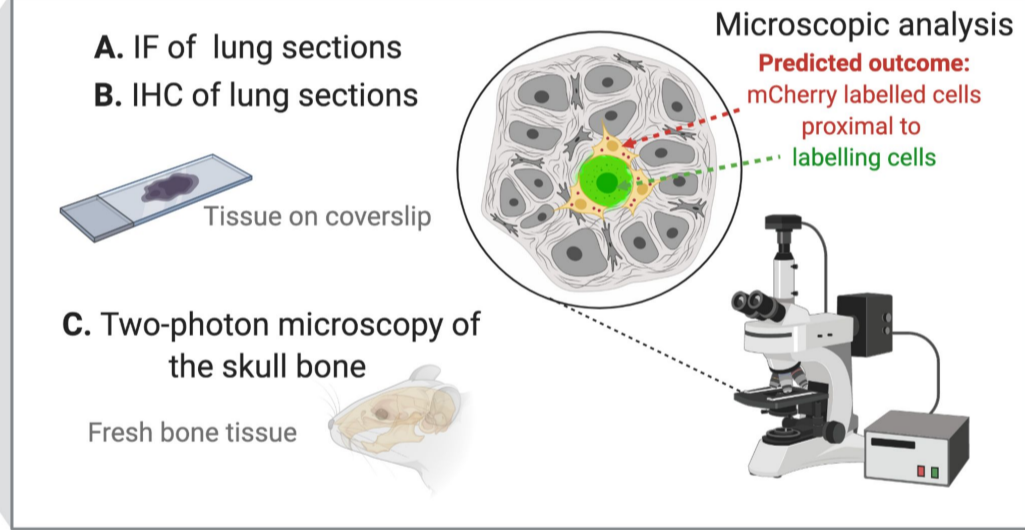
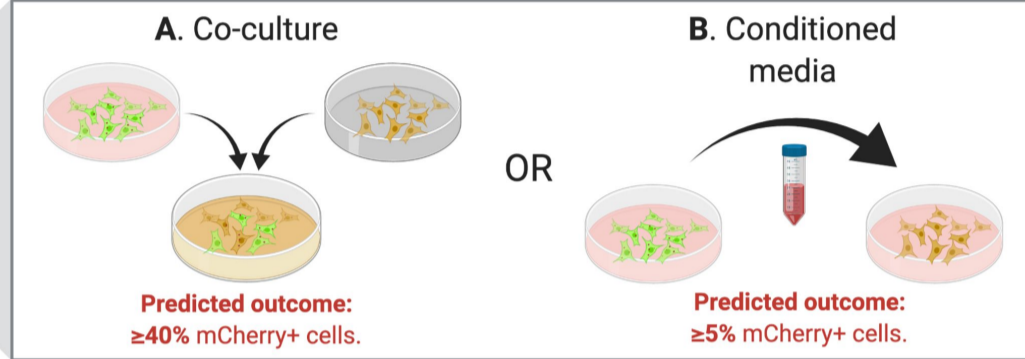
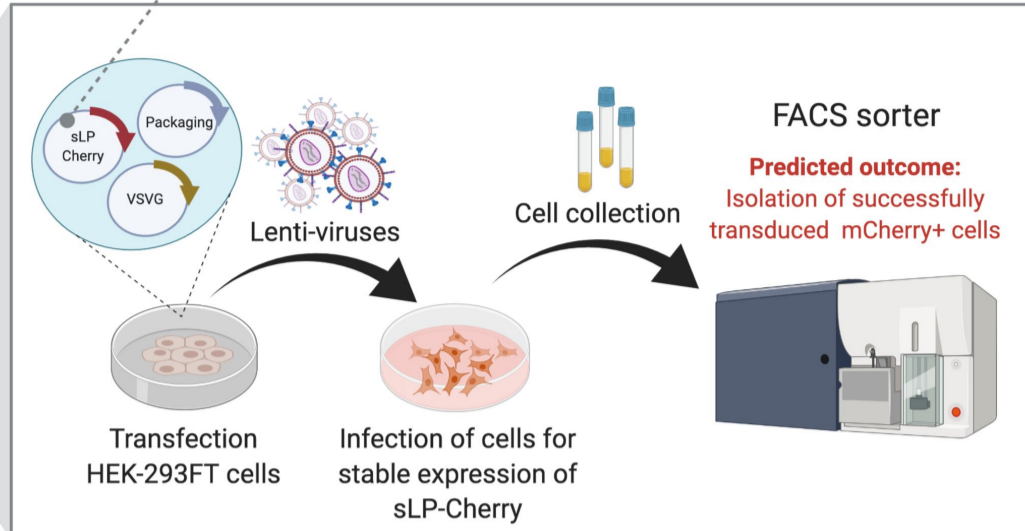
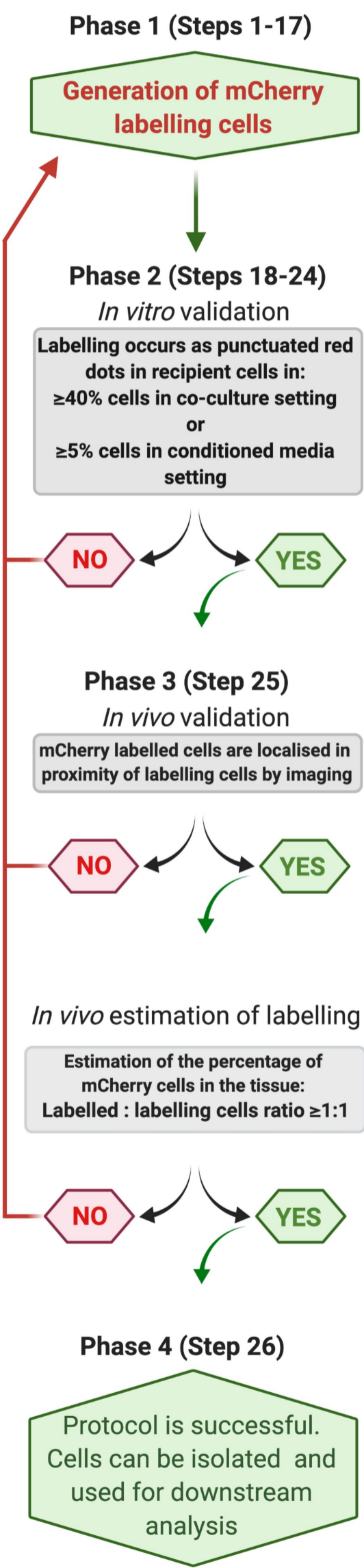
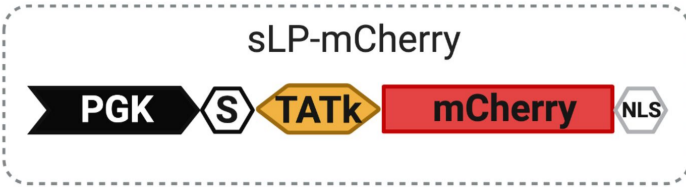
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mCherry

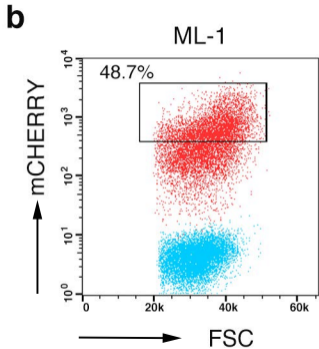
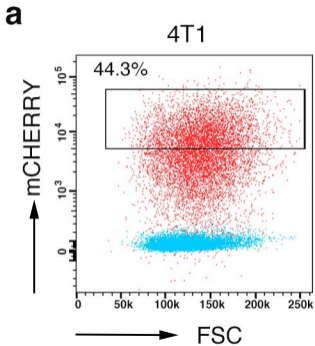


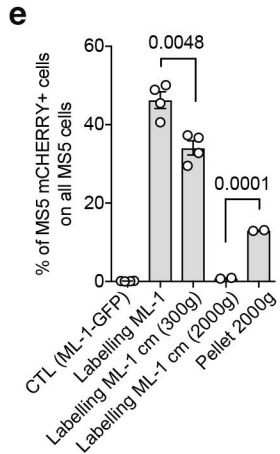
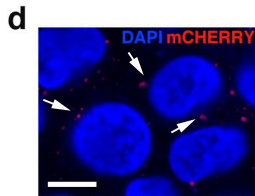
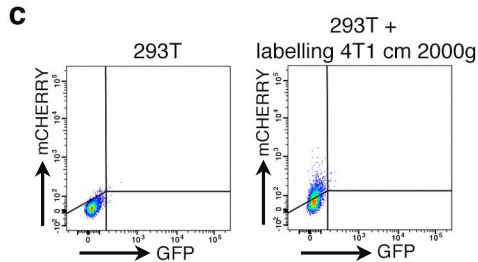
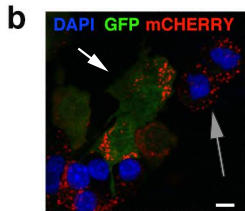
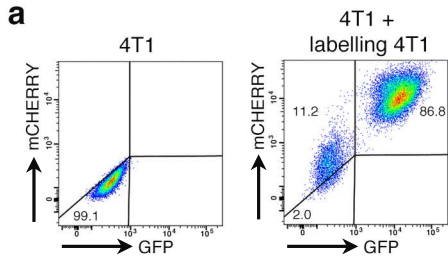
Cherry-Labelling
GFP+ Cancer cell

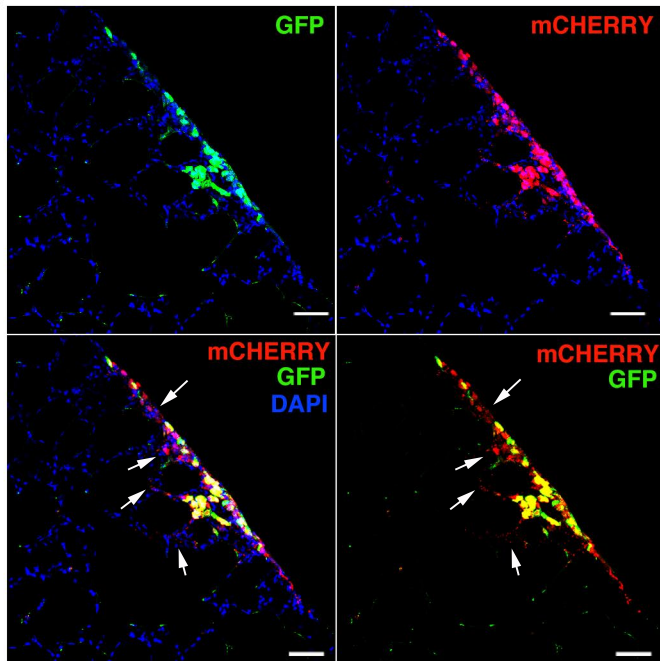
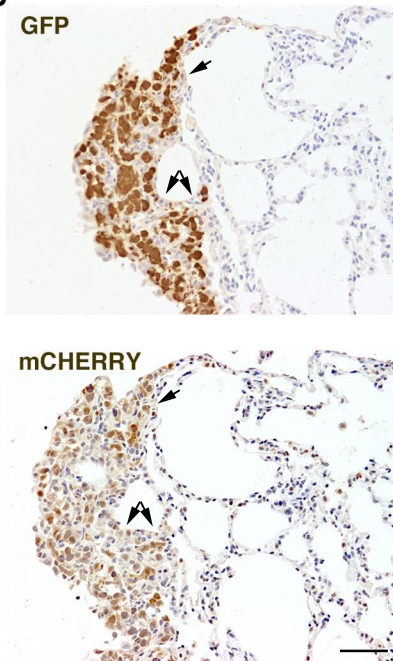


Labelled
neighbouring cells







a**b****c**