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

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ABSTRACT

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

INTRODUCTION

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

Development of the protocol

The principles underlying the strategy we have developed are summarised in Figure 1. We engineered breast cancer cells to stably express a modified version of a red fluorescent protein (mCherry) containing a secretory peptide[5] *"s"* and a TATk peptide[6] *"LP", sLP-Cherry*, which allows for its uptake into exposed cells. Upon secretion, cancer cells and their surrounding cells uptake the sLP-Cherry, which is then stored intracellularly in multi-vesicular bodies[1]. The internalised mCherry maintains a high fluorescence, likely due to its photostability at low pH[7], which shows a half-life in labelled cells in vitro of about 40 h[1]. We used the Cherry-niche system to engineer breast tumour cells and study the composition of their surrounding cells as they begin metastasising in the lung. We have estimated that the sLP-Cherry released by cancer cells could reach approximately five surrounding cell layers[1] (Figure 1), which can be isolated by flow cytometry. The sequence of the sLP-Cherry can be found in Supplementary Figure 1 (also found in our previous publication[1]).

We here supply a step-by-step protocol that has been used to generate and validate labelling 4T1 cells. We show how we used these cells to characterise the lung metastatic niche of breast cancer cells[1]. We also extend the protocol to show how the same labelling 4T1 cancer cells can be used to detect their surrounding niche in a different tissue, the liver. Moreover, we show how a similar approach can be used to generate a different type of labelling cancer cells, human leukemic cells (ML-1), for the study of their bone marrow niche. An overview of the procedure is provided in Figure 2.

APPLICATIONS OF THE METHOD

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

Besides tumour studies, we predict this method could be used to address other scientific questions aimed at understanding the local cellular composition of a cell of interest and the potential local interactions occurring in vivo. Indeed, in our previous study, we successfully generated a non-cancer mammary epithelial cell line expressing the sLP-Cherry and were able to mark their surrounding cells in vivo[1]. Further developments could be made in order to generate other types of modified proteins that could be delivered in neighbouring cells. However, the stability of the protein within the recipient cells upon uptake should be considered.

COMPARISON WITH OTHER METHODS

Laser capture microdissection (LCM) has been the technique traditionally used to isolate cells from the TME[8,9]. However, this technique has major limitations; in particular, the quality of the material isolated from frozen or paraffin-fixed tissue heavily depends on advanced technical skills to perform the microdissection itself. Moreover, downstream analysis is limited to bulk expression profiles of the collected tissue areas, without cellular resolution. For certain tissue such as the bone, LCM is 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 protein[10]
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 data[11]. 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 dissociation[12].
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 cytometry[13]. 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 protein[14]. 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 shown[1].
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 <u>Generation and isolation of labelling cells (phase 1; steps 1-17; Figure 2).</u>
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 confirmed[1].
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 <u>Testing the labelling efficiency in vitro (phase 2; steps 18-24; Figure 2).</u>
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 mCherry[1], 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 shown[1]. 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   <u>Testing the labelling efficiency in vivo (phase 3; step 25; Figure 2).</u>
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 growth[1]. 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 data[1]
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., 2019[1],
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 assays[1].
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　- Isofluorane (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-
403   1810, S1120-8810 and S1122-1830)
404   - 96-Well, 48-Well, 24-Well, 12-Well, 6-Well plates (Falcon, cat. no. 353072, 353078,
405   353047 and 353043)
406   - Cell culture Petri dishes 60mm (Falcon, cat. no. 353037) and 100mm (Corning, cat.
407   no. 430167)
408   - CO_2 incubator (5% (vol/vol) CO2, 37 °C; Eppendorf New Brunswick, model no.
409   Galaxy 170R)
410   - FACS tubes; polypropylene, polystyrene and filter top cap (Falcon, cat no. 352063,
411   352058 and 352235)
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.
418   no. AMAFD1000)
419   - Flow cytometer (Fortessa, BD Biosciences, model no. 339473)
420   - Cell sorters BD Biosciences FACSAria III and BD Influx™
421   - Syringes for intraveneous 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.
426   no.10417002)
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)
443   penicillin-streptomycin.
444   For ML-1 cells: RPMI 1640 with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-
445   streptomycin.
446   For HC11 cells: RPMI 1640 with 10% (vol/vol) FBS, 1% (vol/vol) penicillin-
447   streptomycin, 10ng/ml EGF and 5µg/ml insulin.
448   For MS5 cells: IMDM with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin.
449   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 dH$_2$O 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% H$_2$O$_2$ in PBS
492 Add 13.3ml of 40% H$_2$O$_2$ 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 dH_2O. This solution can be stored at
506 room temperature up to 1 week.
507
508

PROCEDURE

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

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

Timing 5d.

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

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

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

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

3. Prepare a 15ml transfection tube A by adding the following components and mix by pipetting up and down:
   - 10μg of sLPCherry plasmid
   - 6.5μg of pCMV delta R8.2 plasmid (packaging)
   - 3.5μg of pCMV-VSV-G
   - 62μg 2M Calcium Chloride solution
   - Up to 500μl with sterile water

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

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

6. After incubation, gently mix the solution and add dropwise to the HEK 293FT plate. Place the cells overnight at 37 °C in a CO_2 incubator.

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

8. On day 4, visualise the cells at the EVOS fluorescence microscope to estimate the efficiency of cell transfection: at least 80% of the cells should be mCherry[+]. If the transfection efficiency is high, collect the medium and replace it with 7ml of growth media. Centrifuge the collected media at 300g for 5 min and filter the supernatant by using a 10ml syringe and a 0.45μm filter to remove any cells or large debris from the media. Repeat this step the following day (day 5).
   CRITICAL STEP Lower HEK 293FT transfection efficiency may result in a low viral titre and consequently in a low infection efficacy of the viral preparation. TROUBLESHOOTING

9. Pool together the medium collected on day 4 and day 5. Make 6ml aliquots and store at -80 °C for up to 1 year or use it fresh on cultured cells.
   CRITICAL STEP Freeze and thaw will decrease the infective efficiency of the virus preparation. Therefore, the use of freshly prepared virus is recommended, particularly for cells that are difficult to infect. If cell-type-specific growth medium and/or higher viral titre is required, viral particles can be concentrated via ultracentrifugation and used immediately or stored at -80 °C for up to 12 months.

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 8μg/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 20μm 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 (mCherry$^+$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   ≥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.

ii) When the cells reach 80% confluence, remove the media and add 10ml of growth media.

iii) Allow the media to be conditioned for 48 h. Next, collect the media and spin at 300g for 10 min in a 15ml tube.

iv) Place the supernatant in another 15ml tube and spin at 2000g for 10 min. CRITICAL STEP Do not touch the pellet when removing the supernatant to avoid any contaminating labelling-cells to be present in the *cm*.

v) Remove the supernatant (this is the *cm*) and use it immediately or store at 4 °C for up to 2 days before use. The *cm* can also be fractionated to dissect the contribution of both the soluble fraction and the vesicles (Box 1).

vi) The day before collecting the *cm*, place 2 sterile glass coverslips per well in a 6-well plate and then plate $7.5x10^5$ of recipient cells and leave at 37 °C in a CO_2 incubator overnight.

vii) The following day, replace the media with 1ml of growth media 30 min to 3 hours before adding 7ml of *cm* and incubate for 24-36 h at 37 °C in a CO_2 incubator. Keep one well as a control where no *cm* is added.

viii) Remove the coverslips, place them in a well of a 12-well plate and add 1ml of PBS.

FACS validation

19. After moving the coverslips from the 6-well plate to a 12-well plate, trypsinize and collect the recipient cells from the 6-well plate, and pellet them at 300g for 5 min.

20. Wash 1x with PBS and pellet again at 300g for 5 min.

21. Resuspend the cells in FACS buffer with DAPI 1:400 and analyse by FACS, comparing either: the labelling-cells only, unlabelled cells only and the co-culture (if co-culture setting); or the recipient cells incubated with their own media and the recipient cells incubated with the *cm* (if conditioned media setting; see Figure 4c). TROUBLESHOOTING

Fluorescence imaging

22. Remove the PBS from each well of the 12-well plate and add 1ml of PFA 4% solution for 10 min at room temperature.

23. Remove the PFA 4% solution, wash 3x with 2ml of PBS and add 1ml of PBS with DAPI 1:250 and incubate for 5 min at room temperature.

24. Remove the coverslips and mount them for confocal imaging on a slide with a drop of mounting media (Figures 4b and 4d).

Phase 3. Validation of the labelling efficiency in vivo

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

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

i) Trypsinize and collect labelling 4T1 cells. Count and resuspend in PBS at a final concentration of $1x10^7$ cells per ml. Filter the cells using a 40μm cell strainer or a FACS tube with a strainer lid and keep the cells on ice.

CRITICAL STEP The cell suspension must be filtered to remove large cell aggregates before injection. Cell clumps injected intravenously may obstruct vessels, causing immediate mouse death. To minimise this risk, re-filter the cell suspension immediately before injection if there are any visible clumps or the cells have been kept on ice for more than 30 min before injection.

659 ii) Use 100μl of cell solution (10^6 labelling 4T1) to inject intravenously in the 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-cold PBS.
663 iv) Put the lungs in a 15ml tube, add 5x volume of 4% PFA in PBS and leave it at 4 °C overnight. Alternatively, separate the 5 lung lobes before adding the fixative solution: 1-2 lobes can be left in the 4% PFA in PBS as indicated and processed for immunofluorescence (steps A v-Xiii), 1-2 lobes can be left in the 4% PFA in PBS as indicated and processed for immunohistochemistry (steps 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 using standard methods.
673 viii) After heat-mediated antigen retrieval in pH6.0 citrate buffer, incubate the sections in a PBS solution with 1% BSA and 10% donkey serum for 1 h at room temperature.
676 iX) Remove the blocking solution, add the primary antibody mix (containing both 1:750 anti-mCherry and 1:300 anti-GFP in blocking solution) and incubate 4 °C overnight.
679 X) The following day, remove the solution and wash with PBS 3x 5 min at room temperature.
681 Xi) Incubate for 1 h at room temperature with the secondary antibody mix (containing both 1:400 donkey anti-goat Alexa Fluor 488-conjugated and 1:400 donkey anti-rabbit Alexa Fluor 555-conjugated antibodies in blocking solution).
685 Xii) Remove the solution, wash with PBS 3x 5 min at room temperature, then incubate the slides with Sudan Black B for 20 min.
687 CAUTION Filter the Sudan Black solution just before using it to remove precipitates that will affect the quality of the imaging.
689 Xiii) Mount the slides with Vectashield mounting medium with DAPI (Vector Laboratories) for imaging (Figure 5a). TROUBLESHOOTING

692 **B.** _Immunohistochemistry in a lung metastatic model_ (Figure 2). Timing = 2-3 days from harvesting the tissue (or 9-10 days starting from cancer cell intravenous injection).
695 CRITICAL Lung lobes from mice injected at step A ii can be used as indicated at step A iv. Alternatively, use different lung tissues harbouring cancer cells generated by repeating steps A i-iv.
698 i) After an overnight fixation, remove the PFA solution, wash with 5ml of PBS 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 temperature.
703 iii) Cut five-micrometre-thick tissue sections from the paraffin block to keep on 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.

v) Dewax the slides in xylene with 1x 3 min and then perform a 30 sec wash twice with xylene, twice with 100% ethanol, once with 70% ethanol and then rinse thoroughly with distilled water.

vi) For antigen retrieval, place a loosely covered container of pH6.0 Citrate Buffer in a 900W microwave and pre-heat for 8 min at full power, add the racked-sections and heat for a further 15 min at 80% power.

vii) Carefully remove the container from the microwave and place in a sink, under a trickling tap for 5 min. This will cool and dilute the buffer solution slowly, preventing salt crystallisation.

CAUTION After microwave heating, the solution will be hot. Please act carefully and use the necessary protective equipment such as protective glasses and lab coat and gloves.

viii) Discard the cooled buffer, and move the rack of washed slides to PBS.

iX) Incubate in 1.6% H_2O_2 in PBS for 10 min.

X) Terminate the H_2O_2 reaction by washing in distilled water for 5 min.

Xi) Carefully wipe excess fluid from slides and use a PAP (hydrophobic) pen to create a reagent barrier around the tissues.

Xii) Rinse in 0.4% Tween20 in PBS. This step will reduce slide surface tension, maximising reagent coverage.

Xiii) Remove the Tween20 solution and add blocking solution (10% donkey serum, 1% BSA in PBS) for 1 hour at room temperature.

Xiv) Remove the blocking solution and add the primary antibody diluted as required in 1%BSA/PBS (1:300 goat anti-GFP or 1:750 rabbit anti-mCherry). Incubate it at 4 °C overnight.

Xv) Perform a 5 min wash with PBS three times. Then, wash briefly in 0.4% Tween20 in PBS to maximise reagent coverage.

Xvi) Incubate with secondary antibody diluted as required in 1% BSA in PBS for 45 min, at room temperature (biotinylated donkey anti-goat 1:250 or biotinylated donkey anti-rabbit 1:250). During the incubation time, prepare ABC solution (according to manufacturer's directions) and leave to stand for 30 min.

Xvii) Wash three times in PBS (2-min washes). Then, wash briefly in 0.4% Tween20 in PBS to maximise reagent coverage.

Xviii) Incubate in ABC for 30 min, at room temperature.

Xix) Wash in PBS, 3 x 1 min, and wash briefly in PBS-Tween to maximise reagent coverage.

XX) Apply DAB solution and monitor microscopically.

Xxi) Wash in distilled water to terminate the development reaction.

Xxii) Counterstain with a light haematoxylin solution for 1 min then wash in tap water for 5 min, to 'blue' the haematoxylin.

Xxiii) Dehydrate with 70% ethanol and 100% ethanol, clear in xylene and coverslip/mount with DPX mountant for imaging (Figure 5b).

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

i) Collect ML-1 cells and spin at 300g for 5 min. Count and resuspend at a concentration of $1x10^7$ cells/ml.

ii) Inject 2 millions cells (200μl) into immunodeficient NSG–Nestin[GFP] mice[16].

iii) Fourteen days after injection, sacrifice mice, isolate the whole head and 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 28809828[17]) (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 $2x10^7$ cells/ml.
817   ii) Perform ultrasound-guided splenic injections on 6-10 week old BALB/c
818   mice, whilst under inhaled isoflourane anaesthesia (50µl / 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 100µm 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 20µm 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 publication[1].

855

856

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 (*cm*)	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

| 25A Xiii | Low or no mCherry signal detected via IF after intracellular staining on tissue sections | sLP-Cherry is liposoluble and easily lost after permeabilization | 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 |

TIMING

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

ANTICIPATED RESULTS

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

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

to obtain a more accurate estimation of the number of labelled cells, including cells that have been labelled at very low levels and may be missed by using microscopy.

It is worth noting that amplification of the mCherry signal by immunostaining may be particularly challenging. The sLP-Cherry is rapidly lost when using some permeabilising reagents (Supplementary Figure 4) due to its liposolubility (an essential feature mediating its secretion and uptake).

In this protocol, we describe how to confirm the presence of labelled cells in the lung and liver niche of 4T1 metastases and in the bone marrow niche of ML-1 leukemic cells. Imaging techniques can be used to confirm the presence of in vivo labelling and its confinement to the close proximity of the tumour bulk. Here, we imaged tissue sections from lungs in which labelling 4T1 metastases were growing as well as calvarium sections from mice engrafted with labelling ML-1 leukemic cells (Figure 5). Here, labelling ML-1 cells (mCherry[+] only) have been injected in Nestin-GFP[+] mice. This experiment shows a targeted approach where Nestin^{+} cells within the niche are double positive (GFP[+]mCherry[+]) and can be specifically identified.

A more accurate estimation of the number of labelled cells in the tissue can be obtained by FACS. As an indication, the cancer cell lines we have previously used[1] have the following labelling activity determined by the average ratio labelled:labelling cells in the lung: breast cancer cells 4T1 = 2.5:1, colon cancer cells CT26 = 1.7:1, renal cancer cells RENCA = 2.2:1.

We show a gating strategy to discriminate mCherry^{+} niche cells by flow cytometry in dissociated lung and liver tissues (Figure 6). Importantly, some tumour cells show a tendency in vivo to decrease the fluorescent signal intensity of some fluorophores, such as GFP. In this situation, a more stringent gating strategy to identify mCherry^{+} labelled cells can be used (Figure 6). A key advantage of using the Cherry-niche labelling system is the ability to identify and isolate specific populations of neighbouring cells. Examples of gating strategies to identify subpopulation of cells in the lung metastatic niche from 4T1 cells have been shown (Supplementary Figure 2).

AUTHOR CONTRIBUTIONS

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

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995   Box 1: <u>Validation of the labelling efficiency of labelling leukemic cells in vitro by</u>
996   <u>FACS using media fractionation</u> (Timing = 4 days).
997   1. Plate $5x10^5$ labelling ML-1 cells (without MS5 support) in a 24-well plate with
998   1ml of ML-1 media per well.
999   2. Plate as recipient cells $2.5x10^4$ MS5 cells per well in a 24-well plate with 1ml
1000   of MS5 media.
1001   3. Two days later, collect the media from labelling ML-1 cells and spin at 300g
1002   for 10 min.
1003   4. Use half of the supernatant as cm_300g and add to the MS5 recipient cells
1004   (1ml per well). Retain the other half of the supernatant and spin it down again
1005   at 2000g for 15 min.
1006   5. Use the supernatant as cm_2000g and add 1ml to the MS5 recipient cells.
1007   Resuspend the pellet in an equivalent amount of fresh media and add to the
1008   MS5 plated 2 days before.
1009   6. Collect labelling ML-1 cells, spin them down and resuspend at a concentration
1010   of $5x10^5$ cells per ml in ML-1 media and add 1ml to MS5 recipient cells.
1011   7. After 2 days of culture, remove the supernatant, wash once with PBS and
1012   then trypsinize for 5 min.
1013   8. Collect the trypsinized cells and spin them at 300g for 5 min.
1014   9. Resuspend the cells and stain for 15 min with 2% FBS in PBS with 1:400 anti-
1015   SCA1 (to detect MS5) and 1:25 anti-CD33 + 1:25 anti-CD45 at room temp.
1016   10. Spin the cells at 300g for 5 min and wash once with 2% FBS in PBS.
1017   11. Resuspend the cells in 2% FBS in PBS with DAPI 1:1000 and analyse by flow
1018   cytometry.

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1021
1022   **FIGURE LEGENDS**
1023   **Fig. 1 | Schematic of the labelling system.** GFP+ cancer cells have been
1024   engineered to express a modified mCherry protein (sLP-Cherry) containing a
1025   soluble peptide (s) and a TATk (LP). Note that other markers can also be
1026   used, instead of the GFP, to allow discrimination between labelling and
1027   labelled cells. The figure also highlights several key features of the system
1028   that have been previously defined[1]. The sLP-Cherry sequence can be found
1029   in Supplementary Figure 1 and it is also included as Supplementary
1030   Information in Ombrato et al., 2019[1].
1031
1032   **Fig. 2 | Schematic overview of the protocol.** <u>Phase 1</u>: First, labelling cells are
1033   generated using standard lentivirus production methods. HEK 293FT cells are
1034   transfected with a combination of lentiviral plasmids expressing the sLP-
1035   Cherry protein, the packaging proteins and the VSVG protein. The sLP-Cherry
1036   virus is then used to infect recipient cells. Next, the infected cells are collected
1037   and sorted according to levels of mCherry expression. The inset shows how
1038   the mCherry protein has been modified in the sLP-Cherry vector. VSVG,
1039   Vesicular stomatitis virus G. <u>Phase 2: In vitro validation</u> is performed using
1040   either co-culture between labelling cells and other cells (A), or culture of the
1041   recipient cells with labelling cell conditioned media (B). <u>Phase 3: In vivo</u>
1042   <u>validation</u> by microscopy is used to confirm proximity labelling in tissue
1043   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). <u>Phase 3: In vivo estimation of</u>
1048 <u>labelling</u> 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. <u>Phase 4</u>:
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 published[1], 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

4T1 cells in vivo (a,b) have been previously published[1], different examples from these experiments are shown here. **c**, Representative 3D reconstruction of two-photon imaging of the calvarium bone marrow from a NSG-Nestin-GFP mouse transplanted with labelling ML-1 cells and harvested 14 days after labelling ML-1 cells injection (white arrows, Nestin^{+} labelled cells (GFP[+]mCherry[+]); Green, GFP; Red, mCherry; scalebar 50 μm).

Fig. 6 | Validating labelling cells in vivo. a, **b**, FACS gating strategy to identify labelled cells in a dissociated metastatic lung (**a**) and liver (**b**). Metastatic tissues are collected 7 days after injection, dissociated and analysed by FACS. The gating hierarchy is shown by red arrows and the gates have been drawn according to the Full Minus One (FMO) controls. After gating for all events acquired (i), single cells (ii) and subsequently live cells (iii) are identified. Full Minus (FM) mCherry/GFP sample represents a dissociated lung or liver from a mouse that has not been injected with tumour cells. Single mCherry^{+} niche cells are gated according to the FMO mCherry/GFP sample (Control for gate setting). (iv). Live cells are discriminated on the mCherry/GFP gate (v) where double-positive tumour cells (TC), labelled cells (niche), and unlabelled cells (distal tissue) can be resolved (v). A more stringent niche gate (vi) reduces the possibility of including labelling 4T1 cells that may downregulate the GFP expression in vivo. Results from metastatic lungs using labelling 4T1 cells in vivo (a) have been previously published[1], different examples from these experiments are shown here.

SUPPLEMENTARY FIGURE LEGENDS
Suppl. Fig. 1 | Sequence of the mCherry protein (sLP-Cherry) containing a soluble peptide (s) and a TATk (LP). This can be also found in our previous publication[1].

Suppl. Fig. 2 | Examples of gating strategy to define the identity of labelled cells in vivo. The gating strategy here follows doublets and cell death exclusion as described in Figure 6a. Gate sequence is indicated by red arrows. **a**, CD45^{+} immune cells are gated distinctively from niche and distal lung cells, then CD11b^{+} myeloid cells are gated from CD45^{+} cells. **b**, Lineage^{−} (CD45^{−}CD31^{−}Ter119^{−}) cells are gated distinctively from niche and distal lung cells. The channel used for the Lin BV421 is plotted against the UV 450/50, which allows you to capture LIN^{−} cells that are auto-fluorescent (this strategy is only possible if DAPI^{+} cells have been previously excluded as shown in Figure 6). Next, epithelial Epcam^{+} cells are gated from Lin^{−} cells. Myeloid and epithelial cells from lungs harbouring labelling 4T1 cells have been identified using the same strategy and results have been previously published[1], using data generated from independent experimental replicates.

Suppl. Fig. 3 | Functional analysis to exclude specific mCherry-dependent effects on labelling and recipient cells. a, **b**, In vitro proliferation of 4T1 and HC11 cells (GFP or labelling). Representative images showing Crystal violet stained cells (**a**) and quantification (**b**). **c**-**e**, Gel contraction assay of fibroblasts co-cultured with HC11 and 4T1 cells (GFP or labelling): schematic images of co-culture (**c**); representative images (**d**); and quantification of gel 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 100μm. **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 ±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 μm). The use of strong detergents, such as Tween20 and Triton
1155   X-100, results in a large loss of the mCherry signal.

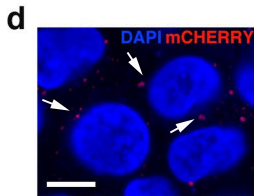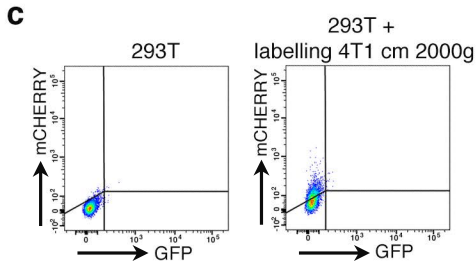**Principle of the system**

sLP

mCherry
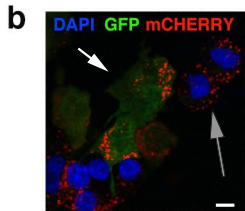
Cherry-Labelling GFP+ Cancer cell
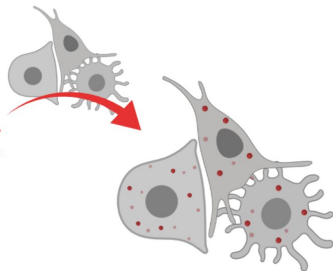
Labelled neighbouring cells

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: 20-80μm*

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a 4T1

mCHERRY axis (10^5, 10^4, 10^3, 0), FSC axis (50k, 100k, 150k, 200k, 250k)

44.3%

b ML-1

mCHERRY axis (10^4, 10^3, 10^2, 10^1, 10^0), FSC axis (20k, 40k, 60k)

48.7%

a

4T1

4T1 +
labelling 4T1

99.1

11.2   86.8

2.0

b

DAPI GFP mCHERRY

c

293T

293T +
labelling 4T1 cm 2000g

d

DAPI mCHERRY

e

0.0048

0.0001

% of MS5 mCHERRY+ cells
on all MS5 cells

CTL (ML-1-GFP)
Labelling ML-1
Labelling ML-1 cm (300g)
Labelling ML-1 cm (2000g)
Pellet 2000g

a

i All events
ii Single cells
iii Live cells
iv Control for gate setting — FMO mCherry/GFP
v Niche cells
vi (stringent Niche gate) Niche cells

b

i All events
ii Single cells
iii Live cells
iv Control for gate setting — FMO mCherry/GFP
v Niche cells
vi (stringent Niche gate) Niche cells