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

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Luigi Ombrato[1,2]*, Emma Nolan[1], Diana Passaro[3,4], Ivana Kurelac[1,5,6], Victoria L. Bridgeman[1], Alexander Waclawiczek[3], Delfim Duarte[7,8], Cristina Lo Celso[7,8], Dominique Bonnet[3], Ilaria Malanchi[1]*.

1 Tumour-Host Interaction Laboratory, The Francis Crick Institute, London, UK.
2 Barts Cancer Institute, Queen Mary University of London, London, UK.
3 Haematopoietic Stem Cell Laboratory, The Francis Crick Institute, London, UK.
4 Leukemia and Niche Dynamics Laboratory, Cochin Institute, Paris, France.
5 Dipartimento di Scienze Mediche e Chirurgiche, Università di Bologna, Bologna, Italy.
6 Centro di Ricerca Biomedica Applicata (CRBA), Università di Bologna, Bologna, Italy
7 Department of Life Sciences, Imperial College London, London, UK.
8 The Francis Crick Institute, London, UK.
* e-mail: l.ombrato@qmul.ac.uk; ilaria.malanchi@crick.ac.uk

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,

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

Significant effort over the past few years has led to novel strategies to allow easier and more accurate analysis of spatially distinct niches within tissues. In one such strategy, the use of a mouse model expressing a photoactivable GFP protein[10] allows the fluorescent labelling of cells in a specific location within a tissue, enabling their isolation as live cells by flow cytometry to generate scRNA-seq data[11]. However, when using this system, the niche area in the whole tissue needs to be defined and targeted by two-photon irradiation. A recent approach to study intercellular cross-talk in vivo relies on the preservation of physical interactions after tissue dissociation[12]. Here, after staining with distinct fluorophore-conjugated antibodies, specific double-fluorescent doublets are isolated by flow cytometry and characterised by scRNA-seq. Next, an algorithm allows you to deconvolute the profile of the two different interacting cell types. However, the calibration of tissue dissociation protocols is critical and weaker interactions may still be missed. Finally, the algorithm needs to be finely tuned to exclude “contaminating” non-interacting doublets and successful deconvolution requires the two interacting cells to have very distinct transcriptional profiles.

Less unbiased methods have also been developed. One approach involved engineering of specific cell types to express two distinct peptide fragments on their surface. Upon adding a substrate in living mice, a fluorescent or biotin tag is transferred when the two cells are in contact, enabling the subsequent isolation of the acceptor cells by flow cytometry[13]. Although it overcomes the need to physically locate a specific tissue area, this method requires specific pairs of different cell types to be engineered and physically interact to generate a detectable signal. Thus, a prior knowledge of the cell types to identify in vivo is necessary. Another methodology that has been used to study intercellular cross-talk in metastasis, takes advantage of cancer cells engineered to express a highly brilliant fluorescent protein[14]. The efficacy of this method depends on the “blebbing” of the tumour cells that loose cellular particles and on the ability of the neighbouring cells to internalise them, therefore limiting its detection ability mostly to phagocytic myeloid immune cells in the TME.

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

types in the niche, including less well-represented components. This has the potential to identify new players in the TME, as we have recently shown[1].

OVERVIEW OF THE PROCEDURE

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

EXPERIMENTAL DESIGN

<u>Generation and isolation of labelling cells (phase 1; steps 1-17; Figure 2).</u>

This protocol has been used to generate labelling 4T1 mouse breast cancer cells. Other murine cancer cell lines such as renal carcinoma (Renca), colorectal cancer (CT26), or murine breast epithelial non-cancer cells (HC11), have been generated with a similar approach and their labelling ability in vivo has been confirmed[1].

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

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

<u>Testing the labelling efficiency in vitro (phase 2; steps 18-24; Figure 2).</u>

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

either co-culture with labelling cells or exposure to their *cm*, the recipient cells are analysed by flow cytometry (Figure 4a and 4c). Recipient cells are also analysed by microscopy to confirm the appearance of single mCherry$^+$ cells that are distinguishable from the double-positive labelling cells in the co-culture (Figure 4b and 4d).
The presence of some labelling in vitro (with at least 40% of all recipient cells labelled in co-culture and 5% with *cm*) and the appearance of some intracellular punctuated distribution of mCherry signal in recipient cells as shown in Figure 4d is sufficient to validate the system in vitro. Assessment of the in vitro labelling system should be considered as a *positive* or *negative* control test only, as the precise results do not directly correlate with the efficiency of in vivo labelling.

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

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

Testing the labelling efficiency in vivo (phase 3; step 25; Figure 2).
Cancer cells are injected as a single cell suspension to target the relevant organ. For instance, carcinoma cells can be injected intravenously via the tail-vein to target the lung or intra-splenic to target the liver. In contrast, tail-vein injected leukemic cells expand primarily in the bone marrow. Then, animals are sacrificed and the tissue of interest is collected, processed and analysed by histology to check for labelling via imaging on fresh or antibody-stained tissues. Two examples of settings are provided. When using breast cancer metastasis, we adopted an unbiased approach where labelling cancer cells expressed an endogenous GFP, while releasing sLP-mCherry. Here, cancer cells will appear GFP-mCherry double positive, and the labelled neighbouring cells will be mCherry positive but GFP negative (Figure 5a and 5b).

An alternative targeted approach involves injecting labelling leukemic cells expressing only the sLP-Cherry into transgenic animals where a type of bone mesenchymal cells is fluorescently labelled (Nestin-GFP). Using this strategy, Nestin-GFP cells within areas of leukemic growth can be specifically identified as GFP-mCherry double-positive cells. (Figure 5c).
Once the local tissue labelling is confirmed, a more accurate quantification of the labelled cells can be made by flow cytometry upon tissue dissociation. An example of the flow cytometry analysis, including gating strategy, is provided for lung (Figure 6a) and liver tissue (Figure 6b).
NOTE: In some experimental settings in vivo, the labelling cancer cells may downregulate the GFP expression. In these cases, we recommend using a stringent

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

We have previously shown that the ratio of labelled cells in the lung versus cancer cells in the 4T1 model is constant over the time of metastatic growth[1]. Dissociated tissue samples can also be stained with membrane markers to define the identity of the labelled cells by flow cytometry. Here, we provide an example of how to identify myeloid cells or epithelial cells among the mCherry positive or negative pool of lung cells (Supplementary Figure 2; gating strategy shown in Figure 6a). The efficiency of uptake of mCherry might vary in vivo due to the properties of the cell types, so the gate for the red channel should include all levels of mCherry intensity. The labelling system we developed relies on the modification of a red fluorescent protein (mCherry) that is not expected to induce functional changes in the sLP-Cherry-producing cells or the recipient cells. Here, we have included experiments to show that the expression of the sLP-Cherry in cancer (4T1) or non-cancer (HC11) cells does not influence their growth (Supplementary Figure 3a and 3b). Also, by using a well characterized cancer-specific fibroblast activation assay ex vivo[2,15], we show that the presence of the sLP-Cherry in culture media does not induce functional changes in the exposed cells (Supplementary Figure 3c-e). Finally, we show that the presence of the sLP-Cherry does not induce an additional inflammatory reaction in the lung (Supplementary Figure 3f and 3g), which corroborate our previously published data[1] (Ombrato et al., 2019 - Extended Data Figure 2d and 2e).

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

Once you have confirmed that the Cherry-niche system is working in vivo, labelled neighbouring cells can be freshly isolated by flow cytometry and used in downstream applications. Examples of these assays can be found in Ombrato et al., 2019[1], where we compared labelled vs unlabelled tissue cells either by bulk RNA sequencing or single cell sequencing, and demonstrated high-throughput analysis such as proteomic profiling. Moreover, as the isolated cells are viable, specific cell populations can be tested in ex vivo functional assays[1].

LIMITATIONS

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

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

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

MATERIALS

Biological materials

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

! CRITICAL

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

- Female Balb/c mice 6-12 weeks old (facility breeding or Jackson Laboratory, cat. no. 000651) housed in specific pathogen-free (SPF) cages. All animals were monitored daily for unexpected clinical signs following the P83B37B3C licence guidelines and the principles set out in the NCRI Guidelines for the Welfare and Use of Animals in Cancer Research (UK).
- NESTIN-GFP mice were a kind gift from Dr G. Enikolopov. NOD-SCID IL2Rgnull (NSG) strain mice were obtained from the Jackson Laboratory and bred at the Francis Crick Institute Biological Resources Facility in individually vented cages under Specific Pathogen Free (SPF) conditions. NSG-NESTIN-GFP mice were obtained by back-crossing the original lines into the NSG background (generation 8 or more). Animal experiments using human leukemic ML-1 cells were performed under the project license (PPL 70/8904) approved by the UK Home Office and in accordance with The Francis Crick institute animal ethics committee guidelines.

REAGENTS

For in vitro experiments

- DMEM (ThermoFisher Scientific, cat. no. 41965-039) used for 4T1 and HEK-293 cells
- RPMI medium 1640 + Glutamax (Gibco, 61870-010) used for ML-1 cells and HC11 cells
- IMDM (ThermoFisher Scientific, 12440-053) used for MS5 cells
- Penicillin-streptomycin (ThermoFisher Scientific, cat. no. 15140-122) used for 4T1, HEK-293 and HC11 cells
- Penicillin-streptomycin (Sigma Aldrich, P4333) used for ML-1 and MS5 cells
- FBS-Heat Inactivated (Labtech International Ltd, cat. no. FCS-sa/500) used for 4T1, HEK-293 and HC11 cells
- FBS (Sigma Aldrich, cat. no. F7524) used for ML-1 and MS5 cells
- Insulin (Sigma-Aldrich, cat. no. I9278) used for HC11 cells
- Epidermal growth factor (EGF; ThermoFisher Scientific, cat. no. PMG8041) used for HC11 cells
- Polybrene (hexadimethrine bromide; Sigma-Aldrich, cat. no. 107689)
- 0.25% Trypsin (ThermoFisher Scientific, cat. no. 25050-014)
- PBS (The Francis Crick Institute, Media Services)
- Lentiviral vector sLPmCherry (Ximbio, cat. no 155083)

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

<u>In vivo experiments</u>
- HBSS, -calcium, -magnesium (ThermoFisher Scientific, cat. no. 14175-053)
- Liberase TM (Roche, cat. no. 05401127001)
- Liberase TH (Roche, cat. no. 05401151001)
- DNAse I (Sigma, DN25-100mg)
- Red Blood Cell Lysis buffer (Miltenyi Biotech, cat. no. 130-094-183)
- Isofluorane (Zoetis, cat. no. 5690501) CAUTION Isoflurane is an anaesthetic and exposure to it can result in chronic or adverse health concerns. Perform the procedure in a well-ventilated hood.
- Meloxicam (Metacam 5 mg/ml; Boehringer Ingelheim, cat. no. 5012917011302)
- Liver Digestion Medium: (ThermoFisher Scientific, cat. no. 17703034)
- Pentobarbital (Pentoject, Animalcare limited)
- Intra-Epicaine (Decra)

- Antibodies used for IF/IHC: mCherry (Abcam, cat. no. ab183628); GFP antibody (Abcam, cat. no. ab6673); donkey anti goat AlexaFluor488 (Invitrogen, cat. no. A11055); donkey anti-rabbit AlexaFluor555 (Invitrogen, cat. no. A21432)
- Antibodies for flow cytometry: CD45 BV421 (Biolegend, cat. no. 103133); CD31 BV421 (Biolegend, cat. no. 102423); TER119 BV421 (Biolegend, cat. no. 116233); EPCAM APC (eBioscience, cat. no. 17-5791-81); CD45 APC780 (eBioscience, cat. no. 47-0451-82); CD11b APC (M1/70; Biolegend, cat. no. 101212); Ly-6A/E (Sca-1) PE-Cyanine (eBioscience, 725-5981-82); CD33 FITC (eBioscience, 11-0338-42); CD45 FITC (BD Bioscience 555482); CD45 APC-eFluor780 (BD Bioscience 47-0459-42).

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

- Eppendorf tubes (2ml; Eppendorf, cat. no. 616201)
- Pipettes (5, 10 and 25 ml; Falcon, cat. no. 356543, 356551 and 356525)
- Filter-tip pipettes (10, 20, 200, 1,000µl; STAR LAB cat. no. S1120-3810, S1120-1810, S1120-8810 and S1122-1830)
- 96-Well, 48-Well, 24-Well, 12-Well, 6-Well plates (Falcon, cat. no. 353072, 353078, 353047 and 353043)
- Cell culture Petri dishes 60mm (Falcon, cat. no. 353037) and 100mm (Corning, cat. no. 430167)
- CO_2 incubator (5% (vol/vol) CO2, 37 °C; Eppendorf New Brunswick, model no. Galaxy 170R)
- FACS tubes; polypropylene, polystyrene and filter top cap (Falcon, cat no. 352063, 352058 and 352235)
- Cell strainers 100µm and 40µm (Falcon, cat no. 352360 and 352340)
- Syringes 5ml, 20ml (BD Plastipak, SS+20ES1)
- Level 2 biosafety cabinet (Clean Air, model no. CA/REV4)
- Centrifuge (Eppendorf, model no. 5810R)
- Filters (0.45µm, sterile; VWR, cat. no. 514-0075)
- Fluorescence microscope (EVOS FL Auto Imaging System; Life Technologies, cat. no. AMAFD1000)
- Flow cytometer (Fortessa, BD Biosciences, model no. 339473)
- Cell sorters BD Biosciences FACSAria III and BD Influx™
- Syringes for intraveneous injection (1ml; Becton Dickinson, cat. no. 303172)
- Sterile scissors and forceps (B Braun Medical, cat. nos. BD313R and BC061R)
- Microtome (Leica, RM2235)
- Microtome blades, MX35 Ultra (ThermoFisher Scientific, cat. no. 3053835)
- Microscope slides, Superfrost Ultra Plus (ThermoFisher Scientific, cat. no.10417002)
- Coverslips (24 × 40mm; Menzel Gläser; VWR, cat. no. 631-1333)
- Confocal microscope (Zeiss, model no. 710 Upright microscope)
- Ultrasound machine (Visual Sonics, model no. VEVO 2100)

Software

- Image analysis software Fiji (https://imagej.net/Fiji)
- ZEN10 (Zeiss)
- FlowJo (BD Biosciences)
- FACS Diva (BD Biosciences)
- Imaris v 8.3.1

REAGENT PREPARATION

CRITICAL All reagents should be prepared in a sterile environment.

Growth medium

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

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

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

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

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

Calcium solution

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

Polybrene solution

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

Lung digestion solution

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

Liver Wash Buffer

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

Red Blood Cell Lysis

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

FACS buffer

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

DAPI

Dissolve 1mg of DAPI in 5mL of dH_2O for a 0.2mg/mL solution. This solution can be stored at 4 °C up to 6 months.

Citrate Buffer (pH6.0)

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

1.6% H_2O_2 in PBS

Add 13.3ml of 40% H_2O_2 to PBS until a final volume of 250ml. This solution should be prepared fresh each time.

0.4% Tween20 in PBS

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

Sudan Black solution

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

Haematoxylin solution

Add 120ml Mayer's Haematoxylin to 80ml of dH_2O. This solution can be stored at room temperature up to 1 week.

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.

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

Timing 9-14d.

! CAUTION

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

10. Seed $7.5x10^4$ tumour cells per well in a 6-well plate in 1ml of growth media.
11. The following day, remove the media and add 1ml of growth media plus varying amounts of media containing the sLP-Cherry lentivirus, from 1ml to 7ml, and Polybrene solution to a final concentration of 8µg/ml to enhance the binding of virus particles to the cells. Leave the plate at 37 °C in a CO_2 incubator overnight.
12. Remove the virus-containing medium, wash twice with 3ml of PBS and add 3ml of cell-type-specific growth medium.
13. At day 3 post-transduction, collect the cells and spin them at 300g for 5 min at room temperature.
14. Wash the pellet with PBS, spin at 300g for 5 min at room temperature.
15. Resuspend in 1ml of FACS buffer with DAPI 1:400, filter through a FACS tube with a 20µm strainer lid and process by FACS to isolate $mCherry^+$ cells (Figure 3a and 3b). TROUBLESHOOTING

    CRITICAL STEP 2-3 days post-infection is an ideal time to sort for $mCherry^+$ cells. After the cells have been infected, they will express the sLP-Cherry and begin releasing it into the media. Therefore, uninfected cells may uptake the protein from the media and become "transiently" $mCherry^+$ themselves.
16. Re-plate the sorted cells and allow them to recover and expand. For most cell lines, this usually requires 5-10 days. However, time in culture will vary depending on the recovery time and doubling time of each cell line.
17. Check the cells by FACS to confirm they are $mCherry^+$ and GFP^+ and freeze several aliquots. These are potential labelling-cells that will be tested by a combination of in vitro and in vivo approaches to determine their labelling efficiency.

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

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

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

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

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

ii) Plate $2\text{-}4x10^5$ labelling-cells ($mCherry^+GFP^+$) and $4x10^4$ unlabelled cells and co-culture them at 37 °C in a CO_2 incubator for 2 days (or until they are ≥90% confluent). For controls, keep one well with only labelling-cells and one well with only unlabelled cells.

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

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

i) Plate $2x10^6$ labelling-cells in a 10cm cell culture Petri dish with growth 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.

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.

iii) 7 days post-injection, cull the mice, collect the lungs and keep them on ice-cold PBS.

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)

v) Remove the PFA solution and add 5ml of 70% ethanol in water.

vi) Embed the lung lobes in paraffin blocks.

vii) Cut four-micrometre-thick tissue sections, deparaffinise and rehydrate using standard methods.

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.

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.

X) The following day, remove the solution and wash with PBS 3x 5 min at room temperature.

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).

Xii) Remove the solution, wash with PBS 3x 5 min at room temperature, then incubate the slides with Sudan Black B for 20 min.

CAUTION Filter the Sudan Black solution just before using it to remove precipitates that will affect the quality of the imaging.

Xiii) Mount the slides with Vectashield mounting medium with DAPI (Vector Laboratories) for imaging (Figure 5a). TROUBLESHOOTING

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).

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.

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

ii) Embed lung tissues in paraffin.

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

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

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

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.

iv) Place the head in a container plate under the Zeiss 710 NLO laser scanning multiphoton microscope with a 20x 1.0 NA water immersion lens. The microscope is equipped with a MaiTai "High Performance" fully automated 1-box 517 mode-locked Ti:Sapphire laser with DeepSee dispersion compensation (Spectra-Physics), tuned to 800nm excitation wavelength.

v) By using the microscope eyepiece, set the stage at the right level to visualize the tissue.

vi) Activate the 4 non-descanned detector NDDs to detect the desired fluorochromes. In this case, the collagen signal from bone (second harmonic generation, SHG) is collected at 380-485nm, the GFP signal from Nestin^{+} mesenchymal cells at 500-550nm, and the cherry signal at 640-690nm.

vii) Select 3D regions of interest (ROIs) and acquire the images (more details at PMID 28809828[17]) (Figure 5c).

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

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 the steps A i-iv.

i) Chop the tissue with scalpel and then with scissors until it becomes a smooth paste with no visible clumps.

ii) Place the chopped tissue in a 2ml eppendorf tube and add 1.5ml of Lung digestion solution.

iii) Incubate for 30 min at 37 °C, shaking at 120 rpm.

iv) Collect the cell suspension, filter through a 100μm cell strainer, then squeeze the filter by using a 5ml syringe plunger.

v) Add 4ml of DMEM with 10% FBS on top of the filter.

vi) Spin at 300g for 10 min at 4 °C. Carefully remove the supernatant without touching the pellet.

CRITICAL STEP Vacuum pumps should be used carefully to aspirate the supernatant, since a loose pellet can be aspirated if the pipette gets too close. If the pellet seems loose, we recommend spinning the tube again for another 5 min at 4 °C.

vii) Resuspend the pellet in 5ml of RBC Lysis buffer and leave at room temperature for 3 min.

viii) Spin at 300g for 10 min at 4 °C.

iX) Resuspend the pellet in 5ml of FACS buffer, filter it through a 40μm cell strainer, then squeeze the filter by using a 5ml syringe plunger.

X) Spin at 300g for 6 min at 4 °C, resuspend in 3ml of MACS buffer and filter it through a FACS tube with a 20μm strainer lid.

Xi) Spin at 1250 rpm for 5 min at 4 °C.

Xii) Resuspend the pellet in 1ml of FACS buffer with DAPI 1:400 and analyse the sample by FACS (Figure 6a). **Alternatively**, if staining is required, resuspend the pellet in 300μl of blocking solution and leave it at room temperature for 5 min.

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Xiii) Add the primary conjugated antibody mixture for FACS at the indicated concentration and incubate for 30 min on ice in the dark.

Xiv) Add 2ml of FACS buffer and spin at 300g for 5 min at 4 °C.

Xv) Remove the supernatant, wash with 2ml of FACS buffer and spin at 300g for 5 min at 4 °C.

Xvi) Resuspend the pellet in 1ml of FACS buffer with DAPI 1:400 and analyse the sample by FACS (Supplementary Figure 2).

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

i) Trypsinize and collect labelling 4T1 cells. Count and resuspend them in PBS at a final concentration of $2x10^7$ cells/ml.

ii) Perform ultrasound-guided splenic injections on 6-10 week old BALB/c mice, whilst under inhaled isoflourane anaesthesia (50μl / mouse). Sub-cutaneous analgesia (Metacam) should also be given prior to injection.

iii) Harvest liver 7 days post-injection. Metastatic lesions should be clearly visible on the surface of the spleen and liver.

iv) Inject mice intraperitoneally with a pentobarbitol/epicaine mix to induce terminal anaesthesia. Once mice are sufficiently anaesthetised, immediately open the peritoneal cavity and cannulate the portal vein with a 26-gauge needle. Successful cannulation will be immediately visible by blanching of the liver.

v) Perfuse the liver with 20ml of pre-warmed (37 °C) Liver Wash Buffer. Once the liver has started to swell, cut the inferior vena cava to allow fluid to drain.

vi) Perfuse the liver with 20ml pre-warmed Liver Digestion Media. A cotton bud should be used to regularly apply pressure on the vena cava to allow the liver to fill and swell with the digestion mix before removing to allow the liquid to drain out.

vii) Excise the liver, remove the gall-bladder and incubate in 5ml Liver Digestion Media for 10 min at 37 °C to improve tissue digestion. Cull mouse via cervical dislocation.

viii) After 10 min digestion, liberate the cells by tearing and shaking the liver with forceps in a 10cm dish.

iX) Filter the cell suspension through a 100μm cell strainer, liberating any extra cells by applying a 5ml plunger to the filter. Quench the digestion media with DMEM supplemented with 10% FBS, making up to 50ml volume.

X) Spin cell suspension at 100g for 10 min.

Xi) Resuspend the cell pellet in 10ml RBC Lysis buffer and incubate for 5 min at room temperature.

Xii) Quench in 20ml FACS buffer and spin down at 1000 rpm for 10 min.

Xiii) Resuspend in 8ml FACS buffer and filter through 2x FACS tubes (4ml per tube) containing a 20μm strainer lid.

Xiiii) Spin cell suspension at 100g for 10 min, resuspend in 4ml FACS buffer with DRAQ7 1:1000, filter through a strainer topped FACS tube and analyse by flow cytometry. Alternatively resuspend your sample in blocking solution for staining (Figure 6b).

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

27. Choose from a variety of OMICs approaches and ex vivo assays for downstream analysis (Figure 2), as demonstrated in our previous publication[1].

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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).

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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.

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

COMPETING INTERESTS

The authors declare no competing interests.

REFERENCES

1. Ombrato, L. *et al.* Metastatic-niche labelling reveals parenchymal cells with stem features. *Nature* **572**, 603–608 (2019).
2. del Pozo Martin, Y. *et al.* Mesenchymal Cancer Cell-Stroma Crosstalk Promotes Niche Activation, Epithelial Reversion, and Metastatic Colonization. *Cell Rep.* **13**, 2456–2469 (2015).
3. Quail, D. F. & Joyce, J. A. Microenvironmental regulation of tumor progression and metastasis. *Nat. Med.* **19**, 1423–1437 (2013).
4. Klemm, F. & Joyce, J. A. Microenvironmental regulation of therapeutic response in cancer. *Trends Cell Biol.* **25**, 198–213 (2015).
5. Barash, S., Wang, W. & Shi, Y. Human secretory signal peptide description by hidden Markov model and generation of a strong artificial signal peptide for secreted protein expression. *Biochem. Biophys. Res. Commun.* **294**, 835–842 (2002).
6. Flinterman, M. *et al.* Delivery of Therapeutic Proteins as Secretable TAT Fusion Products. *Mol. Ther.* **17**, 334–342 (2009).
7. Shaner, N. C., Steinbach, P. A. & Tsien, R. Y. A guide to choosing fluorescent proteins. *Nat. Methods* **2**, 905–909 (2005).
8. Espina, V. *et al.* Laser-capture microdissection. *Nat. Protoc.* **1**, 586–603 (2006).
9. Legres, L. G., Janin, A., Masselon, C. & Bertheau, P. Beyond laser microdissection technology: follow the yellow brick road for cancer research. 28.
10. Victora, G. D. *et al.* Germinal Center Dynamics Revealed by Multiphoton Microscopy with a Photoactivatable Fluorescent Reporter. *Cell* **143**, 592–605 (2010).
11. Medaglia, C. *et al.* Spatial reconstruction of immune niches by combining photoactivatable reporters and scRNA-seq. *Science* **358**, 1622–1626 (2017).
12. Giladi, A. *et al.* Dissecting cellular crosstalk by sequencing physically interacting cells. *Nat. Biotechnol.* (2020) doi:10.1038/s41587-020-0442-2.
13. Pasqual, G. *et al.* Monitoring T cell–dendritic cell interactions in vivo by intercellular enzymatic labelling. *Nature* **553**, 496–500 (2018).
14. Headley, M. B. *et al.* Visualization of immediate immune responses to pioneer metastatic cells in the lung. *Nature* **531**, 513–517 (2016).
15. Calvo, F. *et al.* Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. *Nat. Cell Biol.* **15**, 637–646 (2013).
16. Passaro, D. *et al.* Increased Vascular Permeability in the Bone Marrow Microenvironment Contributes to Disease Progression and Drug Response in Acute Myeloid Leukemia. *Cancer Cell* **32**, 324-341.e6 (2017).
17. Passaro, D., Abarrategi, A., Foster, K., Ariza-McNaughton, L. & Bonnet, D. Bioengineering of Humanized Bone Marrow Microenvironments in Mouse and Their Visualization by Live Imaging. *J. Vis. Exp.* 55914 (2017) doi:10.3791/55914.

Box 1: Validation of the labelling efficiency of labelling leukemic cells in vitro by FACS using media fractionation (Timing = 4 days).

1. Plate $5x10^5$ 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 $2.5x10^4$ 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 $5x10^5$ 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 defined[1]. The sLP-Cherry sequence can be found in Supplementary Figure 1 and it is also included as Supplementary Information in Ombrato et al., 2019[1].

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

labelling cells and sectioned to be analysed by microscopy after performing immunofluorescence (A) or immunohistochemistry (B) procedure. The mouse skull bone is collected after intravenous injection of leukemic labelling cells and analysed by two-photon microscopy (C). Phase 3: In vivo estimation of labelling is obtained by FACS. Mouse lung (D) or liver (E) tissue is collected after intravenous or intrasplenic injection, dissociated and analysed by FACS. The minimum ratio of labelled:labelling cells estimated by FACS needs to be 1:1 to meet the requirement. As an indication, the cancer cell lines we have tested 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. Phase 4: Downstream applications for isolated cells. Live cells can be isolated by FACS and analysed by comparing labelled cells from the niche and unlabelled cells from the distant tissue by OMICs (such as bulk RNA sequencing, single cell RNA sequencing and proteomic) or functional ex vivo assays.

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

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

Fig. 5 | Labelling detected via imaging on tissue sections. a, Representative immunofluorescence image of a lung tissue section from a Balb/c mouse harbouring labelling 4T1 metastases (white arrows, niche labelled cells (GFP^{-} mCherry^{+}); Green, GFP; Red, mCherry; Blue, DAPI; scalebar 50 μm). **b**, Representative immunohistochemistry images of lung tissue sections from a Balb/c mouse harbouring labelling 4T1 metastases (black arrows, niche labelled cells (GFP^{-}mCherry^{+}); upper panel GFP stained; lower panel mCherry stained; scalebar 50 μm). Lungs have been harvested from Balb/c 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].

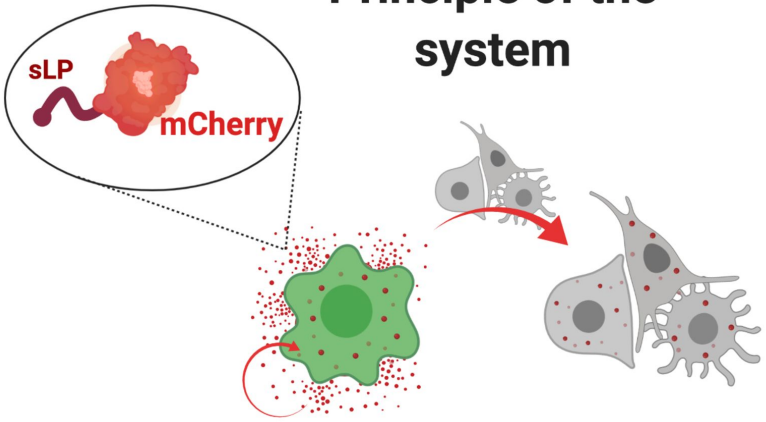
Suppl. Fig. 2 | Examples of gating strategy to define the identity of labelled cells in vivo. The gating strategy here follows doublets and cell death exclusion as described in Figure 6a. Gate sequence is indicated by red arrows. **a**, $CD45^+$ immune cells are gated distinctively from niche and distal 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

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

Suppl. Fig. 4 | Effect of permeabilization on mCherry detection. a-d, Representative images of labelling 4T1 cells by confocal microscopy. Labelling 4T1 cells (**a**) fixed in 4% PFA for 10 min or permeabilised for 7 min with (**b**) Saponin 0.1%, (**c**) Tween20 0.1% or (**d**) Triton X100 0.1% (**a**-**d** scalebar 10 μm). The use of strong detergents, such as Tween20 and Triton 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: 20-80µm*

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Phase 1 (Steps 1-17)

Generation of mCherry labelling cells

Phase 2 (Steps 18-24)

In vitro validation

Labelling occurs as punctuated red dots in recipient cells in:
≥40% cells in co-culture setting
or
≥5% cells in conditioned media setting

NO / YES

Phase 3 (Step 25)

In vivo validation

mCherry labelled cells are localised in proximity of labelling cells by imaging

NO / YES

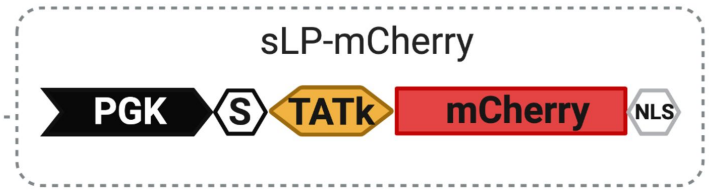
In vivo estimation of labelling

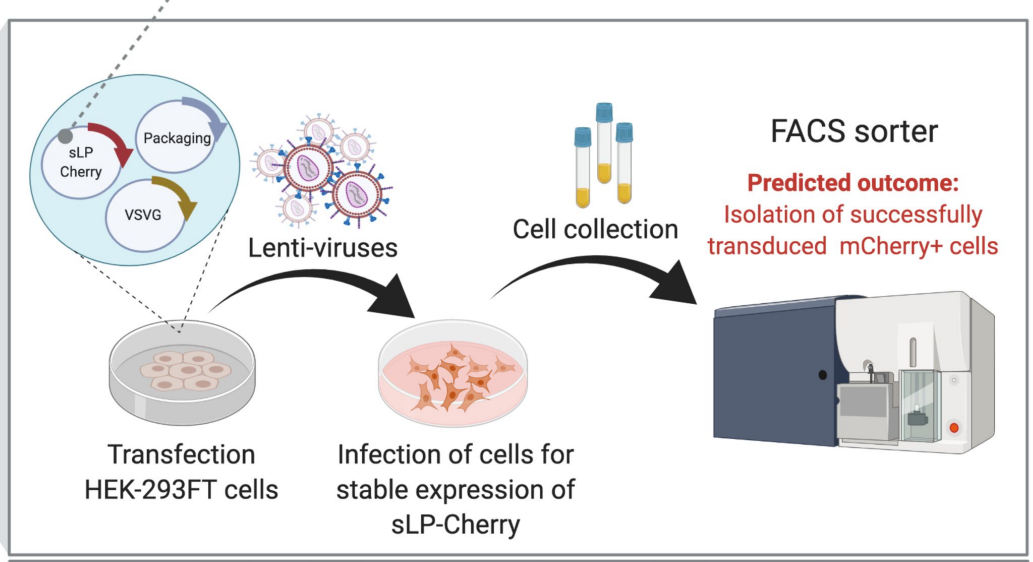
Estimation of the percentage of mCherry cells in the tissue:
Labelled : labelling cells ratio ≥1:1

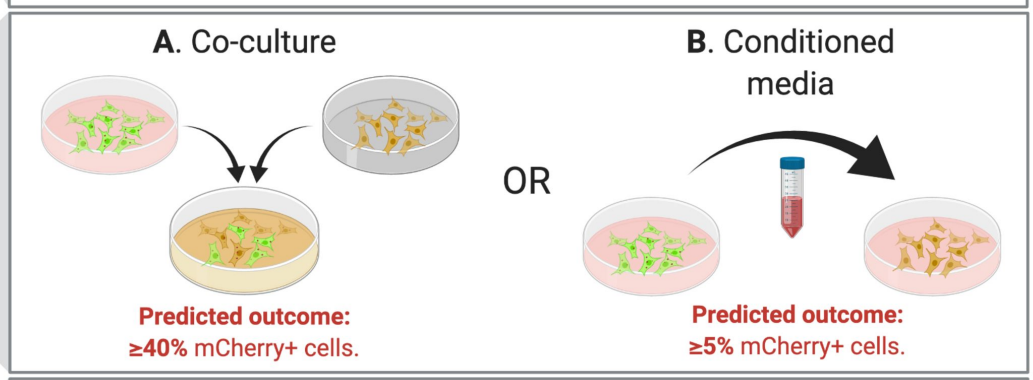
NO / YES

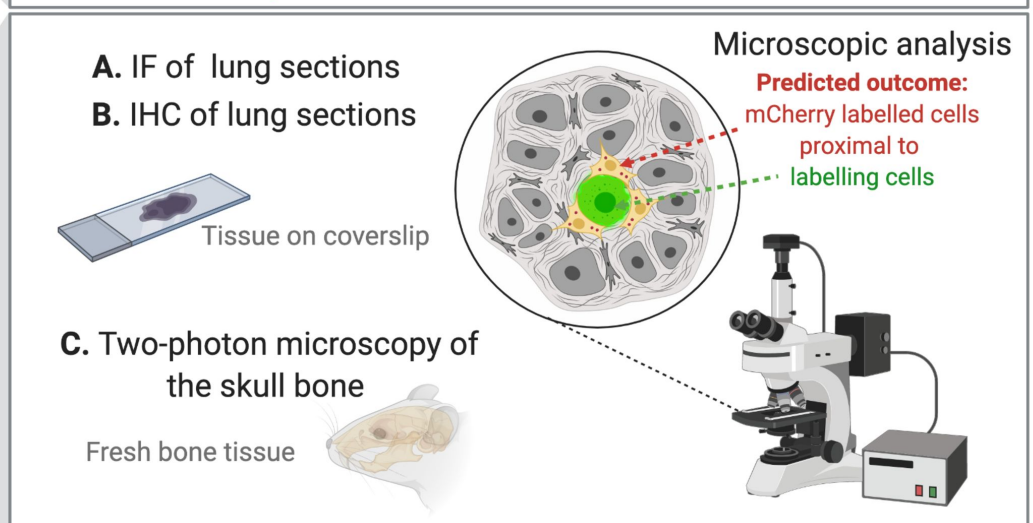
Phase 4 (Step 26)

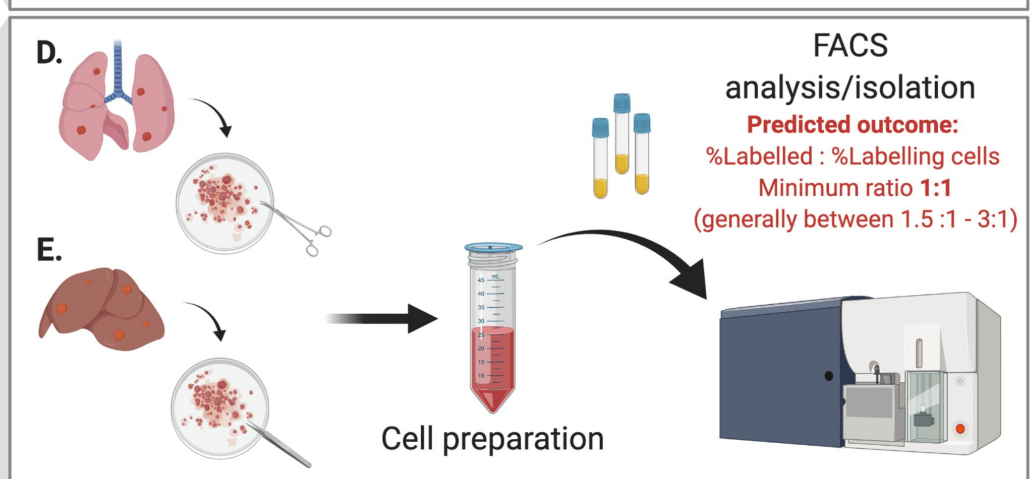
Protocol is successful. Cells can be isolated and used for downstream analysis

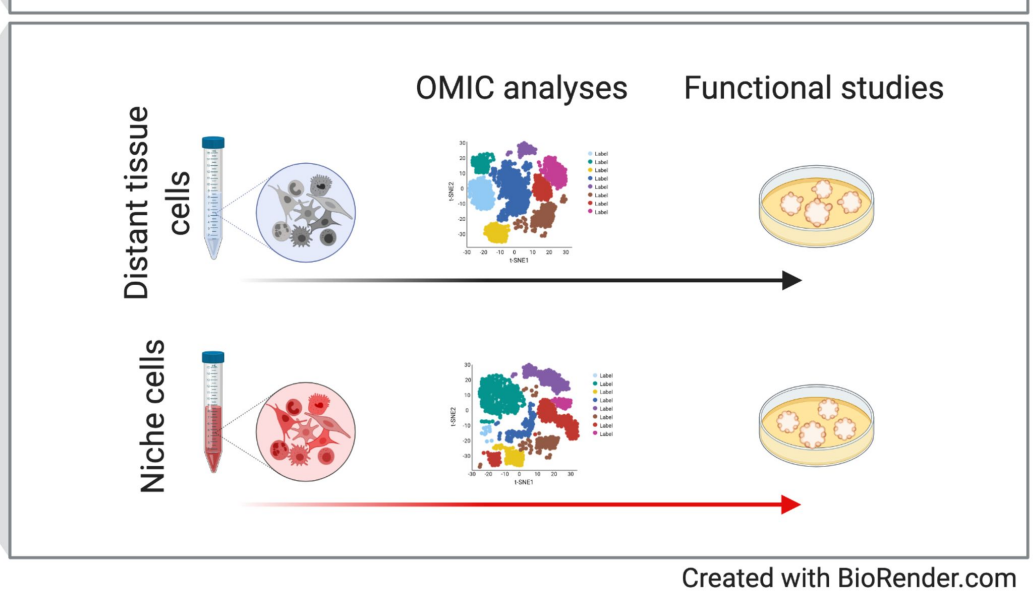












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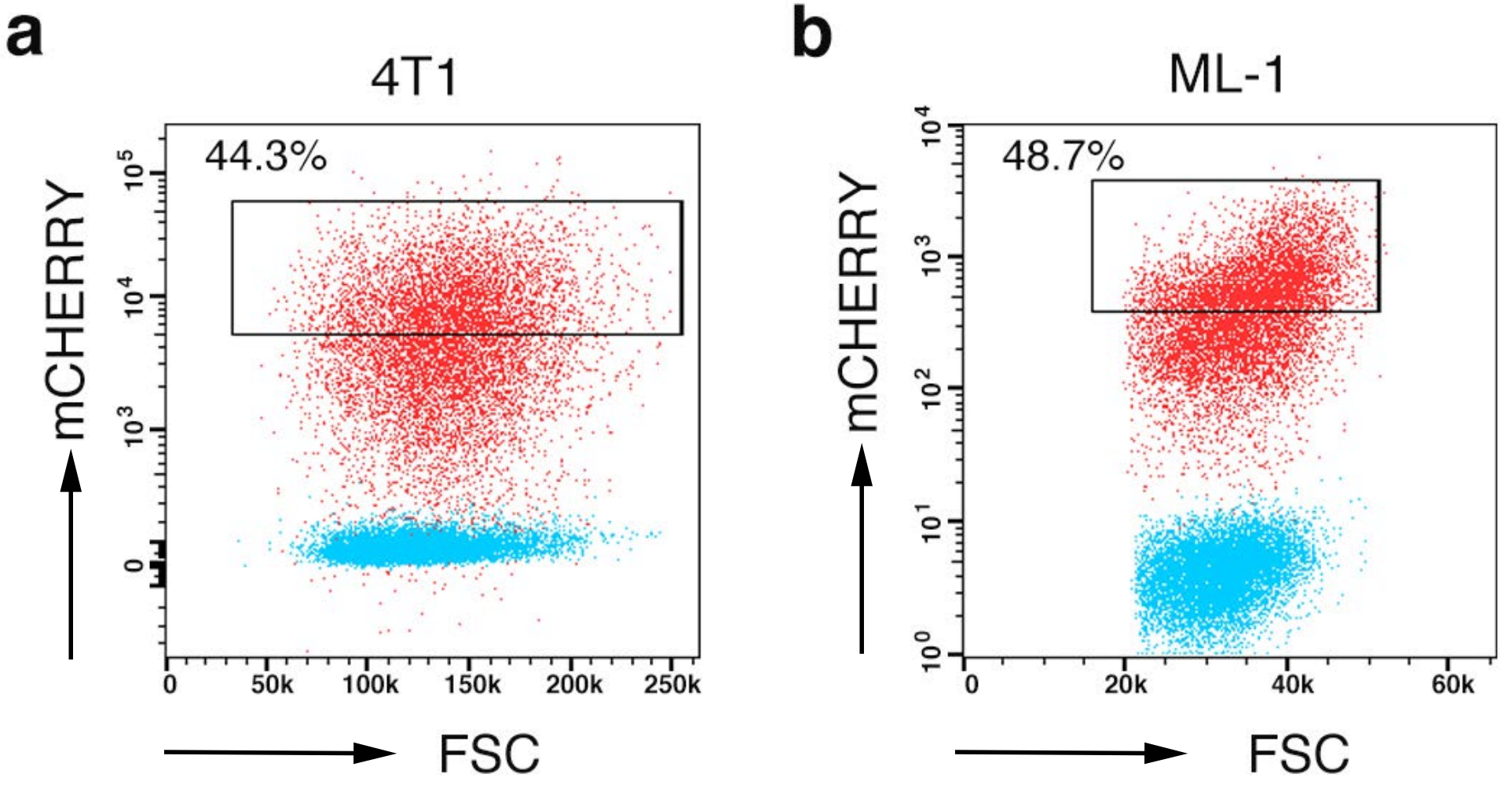
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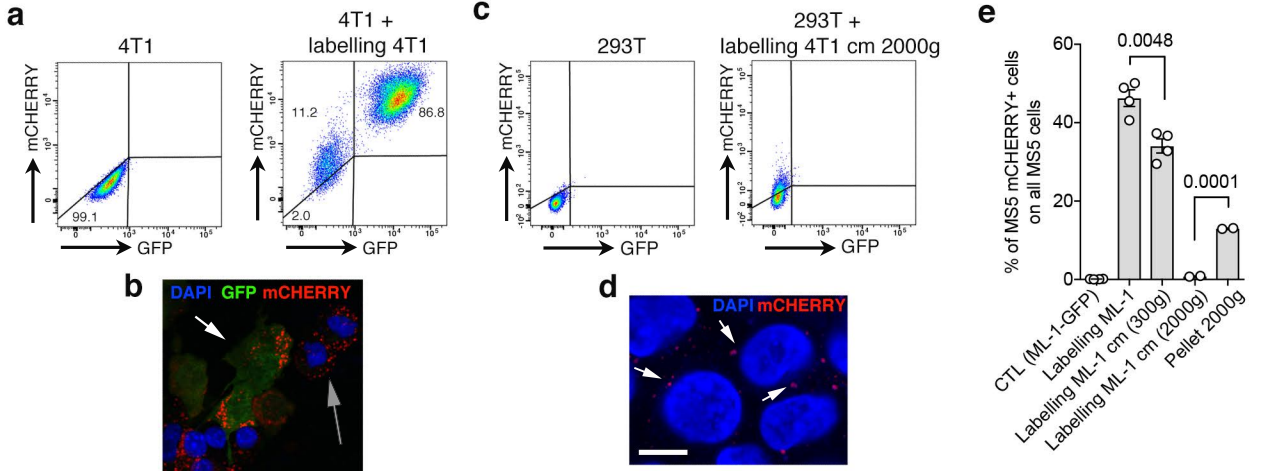
4T1
44.3%
mCHERRY
FSC
0
50k
100k
150k
200k
250k
b
ML-1
48.7%
mCHERRY
FSC
0
20k
40k
60k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4T1
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labelling 4T1
mCHERRY
GFP
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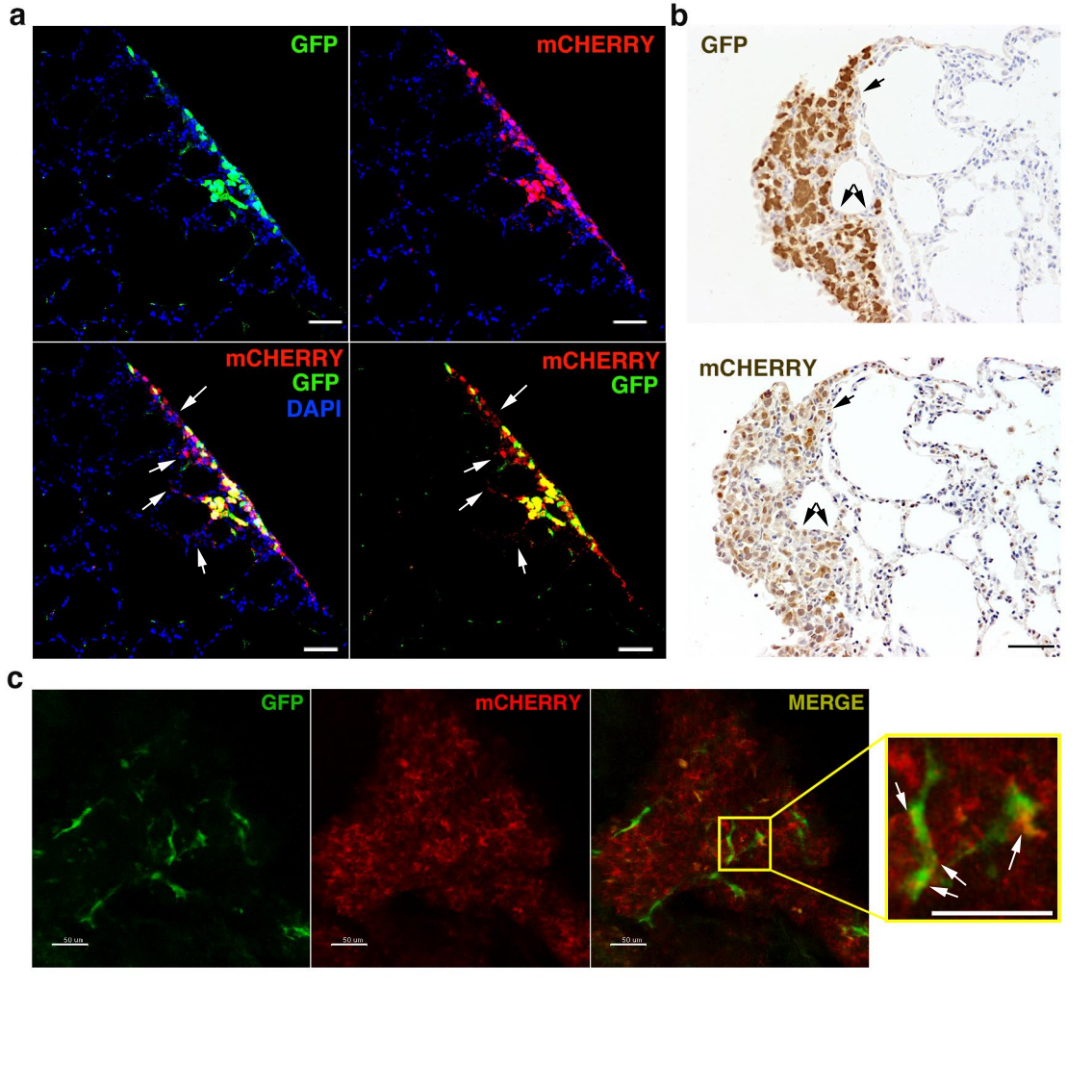
11.2
86.8
2.0
c
293T
293T +
labelling 4T1 cm 2000g
b
DAPI GFP mCHERRY
d
DAPI mCHERRY
e
% of MS5 mCHERRY+ cells
on all MS5 cells
0.0048
0.0001
CTL (ML-1-GFP)
Labelling ML-1
Labelling ML-1 cm (300g)
Labelling ML-1 cm (2000g)
Pellet 2000g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GFP
mCHERRY
mCHERRY
GFP
DAPI
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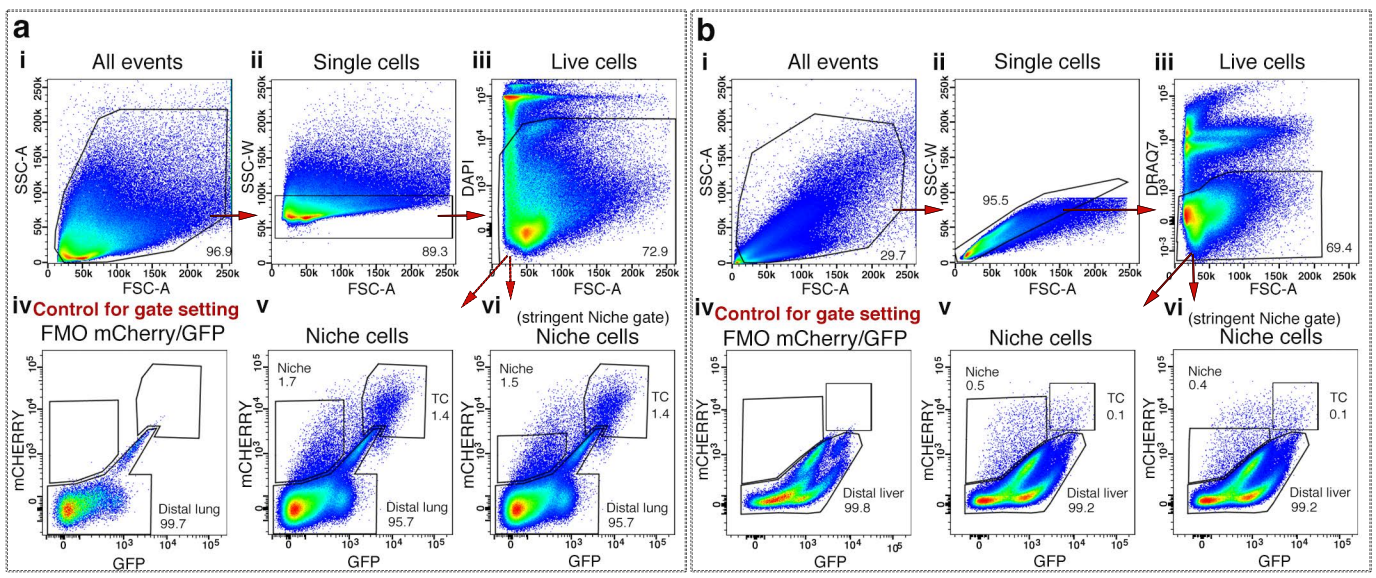
GFP
b
GFP
mCHERRY
c
GFP
mCHERRY
MERGE
50 um
50 um
50 um


a
i
All events
SSC-A
FSC-A
96.9
ii
Single cells
SSC-W
FSC-A
89.3
iii
Live cells
DAPI
FSC-A
72.9
iv
Control for gate setting
FMO mCherry/GFP
mCHERRY
GFP
Distal lung
99.7
v
Niche cells
Niche
1.7
TC
1.4
Distal lung
95.7
vi
(stringent Niche gate)
Niche cells
Niche
1.5
TC
1.4
Distal lung
95.7
b
i
All events
SSC-A
FSC-A
29.7
ii
Single cells
SSC-W
95.5
iii
Live cells
DRAQ7
69.4
iv
Control for gate setting
FMO mCherry/GFP
Distal liver
99.8
v
Niche cells
Niche
0.5
TC
0.1
Distal liver
99.2
vi
(stringent Niche gate)
Niche cells
Niche
0.4
TC
0.1
Distal liver
99.2