Generation of *in vivo* labelling tumour cell lines

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Method Article

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Abstract

The characterization of the tumour microenvironment is highly desirable in order to get a better understating on how the tumour cells exploit their neighbours to support their own growth. In *Ombrato et al., Nature 2019* we describe a novel strategy that allows 4T1 breast tumour cells to label their lung metastatic niche *in vivo*. The labelled cells surrounding the tumour cells can be freshly isolated within the all tissue and used for *ex vivo* assays.

Here, we describe how we generated Labelling-4T1 cells. The same procedure can be used to generate labelling cells from other cell lines, but it might require optimization. This protocol is a suggested guide.

Introduction

The Protocol described here has been used to generate Labelling-4T1 cells in *Ombrato et al., Nature 2019*.

Reagents

Equipment

Procedure

Details on how the sLP-Cherry plasmid was generated are in the methods of *Ombrato et al., Nature 2019.*

The 2nd generation lentiviral system was used to prepare sLP-Cherry viral particles. However, the 3rd generation system could be used. In the original work a GFP lentiviral construct was used to mark the producing cells, however any other cell-retained marker can be used.

Lentivirus production

Plate 293FT (mycoplasma free) cells in P100 plates (10 cm Petri dishes).

Transfect the cells when they are about 80% confluent.

Change the media 30 min to 3 h before the transfection (add 9 ml of fresh DMEM media plus 10% FBS plus Penicillin/Streptomycin).

Prepare the transfection solution A (Lentiviral plasmid (either sLP-Cherry or GFP plasmid) 10 ug, pCMV-dR8.2 plasmid 6.5 ug, pCMV-VSV-G plasmid 3.5 ug, $CaCl_2$ 2M 62 uL; up to 500 ul with water).

Prepare the transfection solution B (2x HEPES buffered saline 500 ul) and slowly add the solution A to B. Mix and leave at room temperature for 15 min.

Add the A/B mixed solution to the 293FT cells drop by drop (1 ml per P100 plate).

18 h after the transfection wash 2X with PBS and add 6 ml of fresh medium (DMEM with 10% FBS and Penicillin/Streptomycin).

1st virus collection (48 h after the transfection): collect medium from the plates (and add 6 ml of fresh medium for the next day collection); spin 5 min at 1200 rpm at 4 degrees; filter the supernatant through 0.45 uM filters; aliquot and store at -80 degrees.

2nd virus collection (72 h after the transfection): same as described in step 9.

Note: if required, the virus supernatant can be ultracentrifuged to obtain a higher viral titre.

Lentiviral infection of 4T1 cells

1. Plate 75,000 4T1 cells per well in a 6-well-plate.

2. The day after, infect the cells by adding the same amount of supernatant (or concentrated viruses) containing sLP-Cherry and GFP lentiviral particles plus polybrene 8 ug/ml and leave them in incubator overnight.

Notes: a) the optimal amount of virus to use depends on the quality of each preparation; therefore, every virus preparation should be tested and titre in advance; b) polybrene can be toxic to some cell lines, in that case it should be avoided.

3. Wash 2X with PBS and add fresh media.

4. Three days after the infection, collect your cells and isolate the high expressing mCherry-GFP double positive cells by FACS.

Note: if you sort your infected cells later than 3 days after the infection, you may collect cells that are mCherry+ because they are uptaking the mCherry and not because they have been infected with the sLP-Cherry virus.

5. Amplify the sorted cells and check their purity. If necessary, repeat the purification by FACS.

Check for labelling ability:

a. Uptake *in vitro* by FACS

a1. Co-culture

Plate $5x10^5$ wild type 4T1 (or other recipient cells) and $2x10^6$ Labelling-4T1 cells in a P100 (1:4 ratio).

The day after change the media.

3. Collect the cells on day 3 (or anytime when they reach at least 90% confluence) and check for mCherry uptake by FACS.

a2. Culture with conditioned media (CM)

(Note: this assay will be less efficient, cells might be labelling in the co-culture test, but show poor labelling by supernatant transfer, this will depend on the general secretion level of the specific cell type)

1. Plate Labelling-4T1 cells in a P100.

2. When the cells are 80% confluent add 10 ml of fresh medium (DMEM with 10% FBS and Penicillin/Streptomycin).

3. Collect CM after 48 h in culture.

4. Spin 10 min at 750 rpm at 4 degrees.

5. Collect the supernatant and spin 10 min at 2500 rpm at 4 degrees.

6. Collect the supernatant: this is the CM ready-to-use.

7. Add 8 ml of CM to recipient cells (50,000 cells per well on a 6-well plate, seeded the day before). 293T, 4T1 or other cell lines of interest can be used as recipient.

8. Collect the recipient cells 48 h after and check for mCherry uptake by FACS.

b. Uptake *in vivo* by FACS

Note: different tissues will have different levels of autofluorescence, the guide below is referred to the lungs.

1. Inject 100,000-1,000,000 Labelling 4T1 cells intravenously in Balb/c mice.

2. Collect the lungs and process them as indicated in the methods of *Ombrato et al., Nature 2019*.

3. Identify by FACS labelled tissue cells by using a mCherry-vs-GFP gate (as shown in Fig. 1f, *Ombrato et al., Nature 2019*). Use FMOs control stainings to exclude autofluorescent cells (usually on the diagonal in the gate) from mCherry+ cells.

Note: some cells could specifically lose the cell-retained marker (such as GFP) *in vivo*. In this case, the mCherry+GFP- gate should be lowered to exclude the highest mCherry+ cells: by doing this, potential tumour cells will be excluded (the majority of labelled tissue cells uptaking the sLP-Cherry are not as bright as the Labelling-4T1 cells).

c. Uptake *in vitro / in vivo* by Immunofluorescence

Upon uptake, the sLP-Cherry mostly localizes in vesicular structures. When permeabilising, the up-taken protein will be in part lost.

To overcome this issue, you may consider different options when performing immunofluorescence:

i. stain live cells or live tissue sections by using membrane markers to avoid permeabilisation with detergents;

ii. where intracellular staining is required, isolate the cells of interest by FACS (discriminating mCherry+ and mCherry- cells) and stain them after sorting.

iii. Test multiple detergents to test if some are better in retaining the up-taken fluorescent signal.

Troubleshooting

Low uptake efficiency *in vitro*: the number of cells uptaking the sLP-Cherry depend on how much sLP-Cherry is released by the producing cells (this varies among cells lines).

If the uptake is low, you may consider different options to increase the labelling efficiency:

a. add more sLP-Cherry-containing CM to fewer recipient cells (**note**: the co-culture would be the most effective option to check if the labelling system is working in your cells).

b. isolate by FACS producing cells with different levels of mCherry expression (low, medium and high) and test them separately to identify the most efficient labelling pool; use this pool for *in vivo* experiments.

c. perform a second round of infection with the sLP-Cherry virus and sort the cells by FACS in order to increase the level of mCherry expression in sLP-Cherry producing cells.

Time Taken

Anticipated Results

References

Acknowledgements

Figures

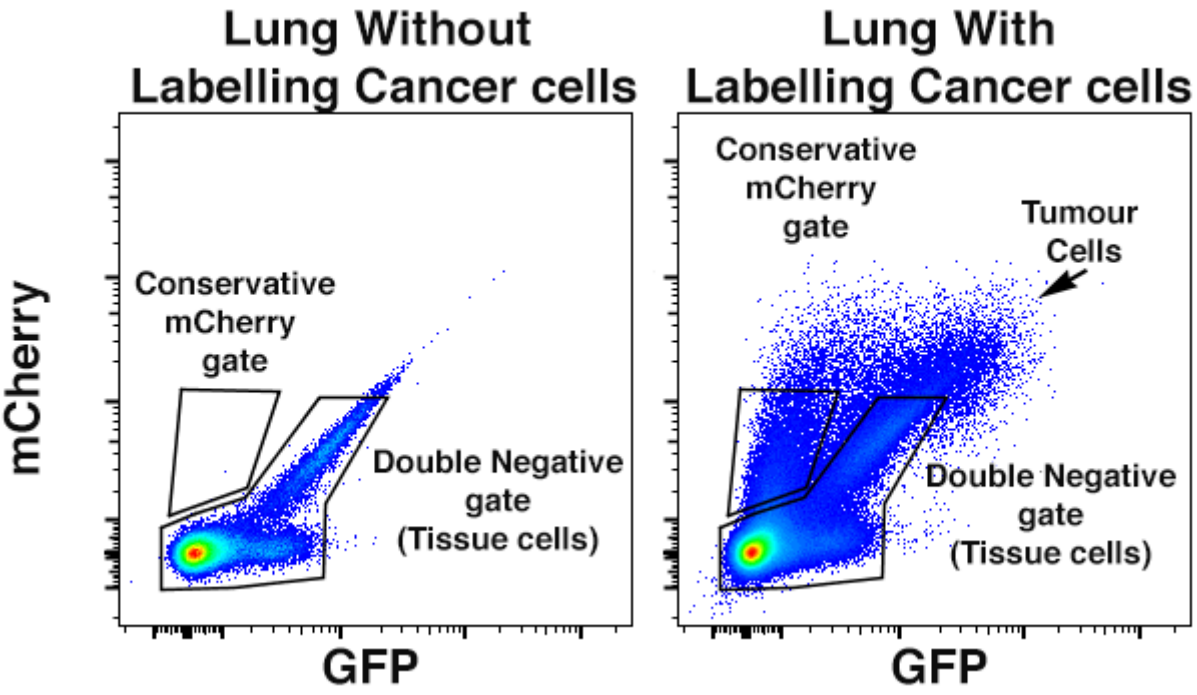

Figure 1

FACS plots showing live cells from dissociated Balb/c mouse lung tissue.