Video Article

The Establishment of a Lung Colonization Assay for Circulating Tumor Cell Visualization in Lung Tissues

Tsung-Cheng Lin[1], Ying-Chih Liao[2], Wen-Tsan Chang[1,2], Cheng-Han Yang[1], Li-Hsin Cheng[1], Megan Cheng[3], Hung-Chi Cheng[1,2]

[1]The Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University

[2]Department of Biochemistry and Molecular Biology, College of Medicine, National Cheng Kung University

[3]Trauma Office, Children's National Health System

Correspondence to: Hung-Chi Cheng at hungchi@mail.ncku.edu.tw

URL: https://www.jove.com/video/56761
DOI: doi:10.3791/56761

Keywords: Cancer Research, Issue 136, Metastasis, Lung Colonization Assays, Lung Perfusion, Circulating Tumor Cells, Carboxyfluorescein Succinimidyl Ester, Polymeric Fibronectin, Extravasation

Date Published: 6/16/2018

Citation: Lin, T.C., Liao, Y.C., Chang, W.T., Yang, C.H., Cheng, L.H., Cheng, M., Cheng, H.C. The Establishment of a Lung Colonization Assay for Circulating Tumor Cell Visualization in Lung Tissues. *J. Vis. Exp.* (136), e56761, doi:10.3791/56761 (2018).

Abstract

Metastasis is the major cause of cancer death. The role of circulating tumor cells (CTCs) in promoting cancer metastasis, in which lung colonization by CTCs critically contributes to early lung metastatic processes, has been vigorously investigated. As such, animal models are the only approach that captures the full systemic process of metastasis. Given that problems occur in previous experimental designs for examining the contributions of CTCs to blood vessel extravasation, we established an *in vivo* lung colonization assay in which a long-term-fluorescence cell-tracer, carboxyfluorescein succinimidyl ester (CFSE), was used to label suspended tumor cells and lung perfusion was performed to clear non-specifically trapped CTCs prior to lung removal, confocal imaging, and quantification. Polymeric fibronectin (polyFN) assembled on CTC surfaces has been found to mediate lung colonization in the final establishment of metastatic tumor tissues. Here, to specifically test the requirement of polyFN assembly on CTCs for lung colonization and extravasation, we performed short term lung colonization assays in which suspended Lewis lung carcinoma cells (LLCs) stably expressing FN-shRNA (shFN) or scramble-shRNA (shScr) and pre-labeled with 20 μM of CFSE were intravenously inoculated into C57BL/6 mice. We successfully demonstrated that the abilities of shFN LLC cells to colonize the mouse lungs were significantly diminished in comparison to shScr LLC cells. Therefore, this short-term methodology may be widely applied to specifically demonstrate the ability of CTCs within the circulation to colonize the lungs.

Video Link

The video component of this article can be found at https://www.jove.com/video/56761/

Introduction

Metastasis is the major cause of cancer death[1,2]. Tumor cells derived from primary tissues enter the circulation in suspension and survive various hematogenous challenges, *e.g.*, anoikis, immune assaults, and damages due to shear stress from blood pressure or geometric constraints, before they are able to colonize distant organs, a key step dictating the success of metastasis[3,4,5,6]. Therefore, vigorous efforts have currently been made in characterizing circulating tumor cells (CTCs) and correlating these characteristics with tumor malignancy, metastasis, and survival rates of cancer patients[7,8,9]. Since the process of cancer metastasis specifically depicts an *in vivo* event, animal models are the only approach that captures the full systemic process of metastasis[10,11,12].

CTCs become metastatic tumor tissues through multiple cellular events including the colonization of distant organs[1,2]. However, the most commonly used metastasis assays[13,14,15] do not provide a way to observe CTC colonization of distant organs. Therefore, an *in vivo* assay design for CTC colonization visualization is urgently needed. Although several *in vivo* and *ex vivo* short term lung colonization assays have been designed, problems and disadvantages remain. For instance, while green fluorescence protein (GFP)-overexpressing tumor cells have been used in these assays[22,23], it takes time to stably transfect and clone tumor cells with sufficient GFP fluorescent intensity under the microscope. Similarly, although transient staining of tumor cells with the long term cell tracer CFSE has been employed to replace the GFP-expressing tumor cells, it remains difficult to judge whether the CFSE-labeled tumor cells are attached or merely present within the vasculature of the excised distant organs[16,17].

Polymeric fibronectin (polyFN) assembled on surfaces of CTCs has been found to critically contribute to the final establishment of metastatic tumor tissues[18,19,20,21,22]. Here, we performed short term lung colonization assays in which suspended Lewis lung carcinoma cells (LLCs) stably expressing FN-shRNA (shFN) or scramble-shRNA (shScr) and pre-labeled with CFSE were intravenously inoculated into C57BL/6 mice. After 2-3 days, mouse lungs were first perfused with phosphate buffered saline (PBS) to completely remove unattached CTCs within the vasculature before being subjected to confocal microscopy and quantification of lung-colonizing LLCs. We clearly showed that the numbers of lung-colonizing shFN LLCs and tumor nodules were significantly reduced in comparison with shScr LLCs,substantially corroborating the role of polyFN assembly

Copyright © 2018 Journal of Visualized Experiments

on CTCs in facilitating the colonization and growth of CTCs in the lungs. Our study warrants further investigation for the role of polyFN in cancer metastasis.

Protocol

All experiments on mice were performed according to the guidelines of our institute (the Guide for Care and Use of Laboratory Animals, NCKU Medical College).

1. Preparation of Instruments, Culture Media, and Dishes

1. Before starting the experimental protocols, obtain sterile surgical sutures, 1 mL syringes with 26G/0.5 cm needle (for tail vein injection), 3 mL syringes with 24G/3 cm needle (for lung perfusion), Dulbecco's Modified Eagle Media (DMEM), trypsin-EDTA solution (5 g/L trypsin and 0.53 mM), fetal bovine serum (FBS), 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PHO_4, and 1.8 mM KH_2PO_4), and 6 cm culture dishes.

2. Preparation and Recovery of Tumor Cells in Suspension

1. Prepare sterile Dulbecco's Modified Eagle Media (DMEM) containing 10% or 20% FBS and freshly add 2 mM L-glutamine, 1x PBS, and 0.05% Trypsin-EDTA.
2. Culture the cells in DMEM supplemented with 10% FBS at 37 °C and grow them until there is 70-80% confluence in the culture dish.
3. Remove the culture media (DMEM) from the dishes, followed by two washes with 2 mL of sterile 1x PBS.
4. Add 1 mL of 0.05% Trypsin-EDTA into the dishes to thoroughly cover all the cells. Immediately remove 800 µL of solution and leave only 200 µL in the dishes. Incubate the dishes at 37 °C for 30 s to 1 min (depending on cell type) and wait until the majority of cells are in the suspended state.
5. Add 1 mL of fresh DMEM containing 10% FBS directly into the dishes to stop the enzymatic activity of trypsin and vigorously pipette the cell suspension up and down with a 1000 µL pipette to prepare a single cell suspension.
6. Transfer the cells from the dish to a 1.5 mL microcentrifuge tube, and spin down the cells at room temperature at 162 x g for 3 min.
7. Aspirate the supernatant and resuspend the tumor cells in 1.5 mL DMEM containing 20% FBS and end-over-end rotate the cells in suspension at 37 °C for 2 h.
   NOTE: If the medium turns yellow during the recovery, exchange the old medium with fresh medium containing 20% FBS.

3. Fluorescence Staining and Analysis of Tumor Cells in Suspension With Carboxyfluorescein Succinimidyl Ester (CFSE)

1. After cell recovery in suspension, count the appropriate viable cell numbers with Trypan Blue in a Neubauer counting chamber for titrating the fluorescence staining doses and for tail vein injection.
2. Spin down the cells at 162 x g at room temperature for 3 min and resuspend the pelleted cells in 1 mL of sterilized 1x PBS, followed by room temperature centrifugation at 162 x g for 3 min.
3. Resuspend the pelleted cells in 500 µL of 1X PBS containing 10% FBS and 0, 5, 10, 20, or 40 µM CFSE for dose titration, and incubate the cell suspension at 37 ^{o}C for 10 min in the dark.
4. Spin down the cells at 162 x g at room temperature for 3 min and resuspend the pelleted cells with 4 mL of DMEM containing 1% FBS. Repeat this washing step three more times. Resuspend the pelleted cells with 4 mL of DMEM alone for the final wash.
5. Subject the CFSE-labeled cells to fluorescence-activated cell sorting (FACS) analysis to quantify the fluorescence intensity of a single cell, or tail vein injection.
   NOTE: Keep the cells on ice prior to performing the FACS analysis or tail vein injections.

4. Lung Colonization Assay

1. Prepare the cells labeled with 20 µM CFSE (refer to Steps 3.1 through 3.4).
   NOTE: Decide the working dose of CFSE for labelling tumor cells, based on the titration results from the FACS analysis (refer to Step 3.3).
2. Warm up the tails of 4-6 week-old male C57BL/6 mice (6 mice) with a halide lamp for 5-10 min on a mouse-by-mouse basis to dilate mouse tail veins.
3. Thoroughly mix and aspirate CFSE-labeled tumor cells with a 1 mL (26Gx1/2) syringe, avoiding any bubbles in the cell suspension (with a final cell density of 5 x 10^6 cells/mL).
4. Carefully inject 1 x10^6 CFSE-labeled tumor cells in 200 µL of DMEM into the mouse tail vein, which should be lodged in a mouse restrainer.
   NOTE: During the injection, if the needle is directly placed in the lumen of the tail vein, tumor cells should be easily delivered into the circulation without needing to push hard on the syringe. If it is hard to push the tumor cells through, they have most likely been injected outside of the tail vein, into the subcutaneous space.
5. Divide the mice that intravenously received the CFSE-labeled tumor cells into two groups: one group will undergo lung perfusion followed by confocal microscopy and ImageJ quantification in the lung colonization assay and the other one will be left alone for 4-5 weeks in the long term lung colonization assay.
6. At 20, 38, or 45 h and with 1x 10^6 tumor cells intravenously injected during a time course- and dose-dependent titration, anesthetize tumor-bearing mice with 50-75 mg/kg zoletil 50, which is a well-accepted and proper anesthetizer[23].
   NOTE: Use vet ointment on the mice's eyes to prevent dryness while under anesthesia.
7. Longitudinally cut the skin and subcutaneous tissues from the abdomen to the chest region with surgical scissors. Open the pleural cavity with surgical scissors and forceps to fully expose the heart and lungs.
   NOTE: Be careful to avoid cutting off blood vessels.

Copyright © 2018 Journal of Visualized Experiments

8. Tie the superior vena cava (SVC) and inferior vena cava (IVC) with sterile surgical sutures to prevent the backflow of perfusion solution during lung perfusion.
9. Cut the left ventricle wall with surgical scissors to open a fissure (about 2-4 mm) to drain the perfusion solution from the lungs.
10. Inject 1x PBS into the right ventricle with a 3 mL syringe and remove the drained solution with constant suction during lung perfusion until the lungs turn from a reddish color to completely pale.

5. Confocal Microscopic Imaging and Analysis of Lung-Colonized Tumor Cells

1. Remove the now-pale lungs from the pleural cavity and separate the five lobes by cutting away the trachea and connective tissue ligaments with surgical scissor and forceps[24].
2. Prepare a stretcher-like lung holder as shown in **Figure 3B** by winding a nylon suture around two metal rods to produce a reticulate texture with a space between the two rods for the placement of lung lobes. Set the lung holder in a 6 cm dish.
   NOTE: The lung holder will be used to stabilize the lung lobes underneath the objective lens of a confocal microscope.
3. Lodge and fix the lung lobes on the reticulate texture of the lung holder. Cover and moisten the lung lobes with 1x PBS.
4. Subject the 6-cm culture dish harboring the lung lobes on the lung holder to confocal microscopy to capture fluorescence images recording the lung-colonizing tumor cells.
5. For imaging, use objective lenses (5X, MPLN, NA: 0.1, WD: 20, Air).
6. Rotate the filter wheel to NIBA (excitation/emission; 470-495 nm/510-550 nm) and use a 488 nm laser to excite CFSE, to clearly visualize images of fluorescence dots in the lung lobes.
7. Rotate the filter wheel to R690 (for MaiTai HP DeepSee laser) to scan with 2.0 µs/pixel scanning speed and optimize the HV, Gain, and offset levels.
8. Capture fluorescence images (512 x 512 pixels) by using the microscope software with a 10 µs/pixel scanning speed.
9. Quantify and statistically analyze the microscopic images using ImageJ and GraphPad software as previously described[21].

6. Long term lung colonization assays

NOTE: Repeat steps 4.1 through 4.5.

1. Sacrifice the mice after tumor cell inoculation for 4-5 weeks and fix their lungs with Bouin's Fluid[25] for 1 to 2 days.
2. Quantify and statistically analyze the tumor nodules from the mice lungs with software[21].

Representative Results

Prior to performing the *in vivo* lung colonization assay as illustrated in **Figure 1A** to test whether polyFN on suspended tumor cells mediates lung colonization and/or extravasation in facilitating metastasis, we first titrated different concentrations of CFSE, a fluorescent compound that is cell permeable and covalently conjugates intracellular amine-containing molecules[26,27] in suspended tumor cells and remains in the cells for quite a long period of time. We found that tumor cells were effectively labeled with CFSE in a dose-dependent manner, as illustrated in **Figure 1B**. The CFSE-labeling in 6 x10^5 LLC cells/mL almost reached a plateau at 20 µM as illustrated in **Figure 1C**.

Owing to the abundant narrowed capillary system which might physically impede the flow of CTCs within the lung vasculature, we performed lung perfusion in mice bearing intravenously injected CFSE-labeled LLC cells to clear non-specifically trapped CTCs, as illustrated in **Figure 1A** prior to lung removal at each time point as depicted in **Figure 4**. Before perfusion, the lungs were clearly reddish due to the presence of blood, as illustrated in the **left panel of Figure 2**. The lungs became pale after perfusion with 10-15 mL of 1x PBS, as illustrated in the **right panel of Figure 2**.

Immediately after the perfused mouse lungs were removed from the pleural space, the lung lobes were separated and mounted on the lung holder placed in dishes as illustrated in **Figure 3A** with 1x PBS thoroughly covering the lung lobes. The lung lobes mounted on the lung holder as illustrated in **Figure 3B** were subjected to confocal fluorescence microscopy as illustrated in **Figure 3A**. The CFSE-labeled tumor cells colonizing the lungs were imaged as illustrated in the **upper panel of Figure 3C** and turned into black-and-white images as illustrated in the **lower panel of Figure 3C** to facilitate the quantification of lung colonization with Image J software.

We intravenously injected either shScr or shFN LLC cells that were labeled with 20 µM CFSE and waited for a time course from 24-72 h before performing the lung perfusions and confocal microscopic imaging. Quantification of the lung-colonizing tumor cells in 6 mice as illustrated in **Figure 4A** with ImageJ software revealed that significantly fewer shFN LLC cells than shScr LLC cells were counted at the 38 h and 45 h time points, as illustrated in **Figure 4B**. The reason why the numbers of both lung-colonized shScr and shFN LLC cells gradually diminished was very likely due to the continuous cytotoxicity exerted by the circulation's immunity even against the already colonized LLC cells. Altogether, these results suggest that polyFN assembled on CTCs is indeed required in mediating lung colonization by LLC cells, leading to complete metastasis in the lungs as revealed by the results in which some mice intravenously receiving aliquots of the CFSE-labeled shScr and shFN LLC cells for the lung colonization assays as illustrated in **Figure 4** were left aloneuntil lung tumor nodules were developed in the experimental tumor metastasis assay as illustrated in **Figure 5A and 5B.**

Copyright © 2018 Journal of Visualized Experiments

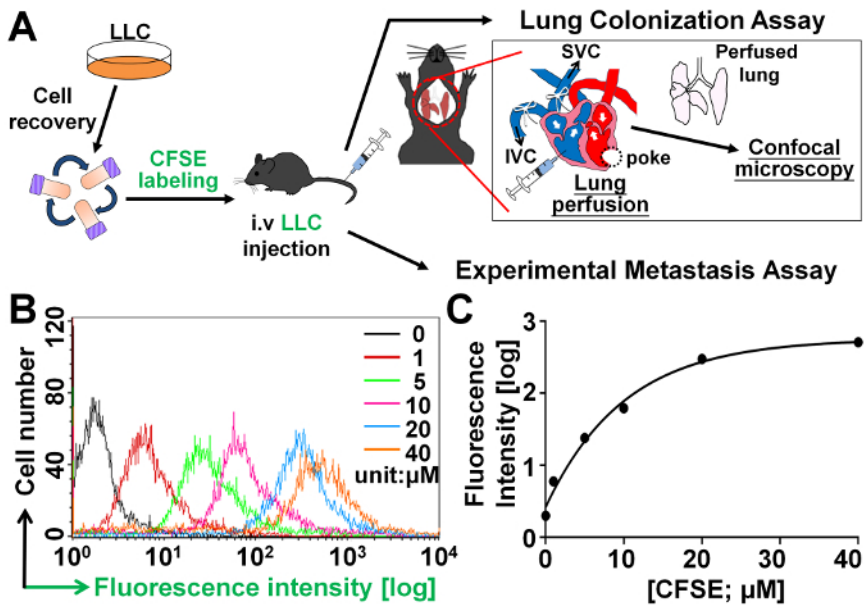

Figure 1: Titration of the CFSE concentrations for labeling tumor cells in the lung colonization assay. (A) Schematic illustration of procedures for the lung colonization assay and the experimental metastasis assay as detailed in the Protocols section. **(B)** FACS analysis for the CFSE fluorescence intensities of LLC cells that were stained with various concentrations of CFSE. **(C)** Fluorescence intensities were plotted as functions of various CFSE concentrations. Please click here to view a larger version of this figure.

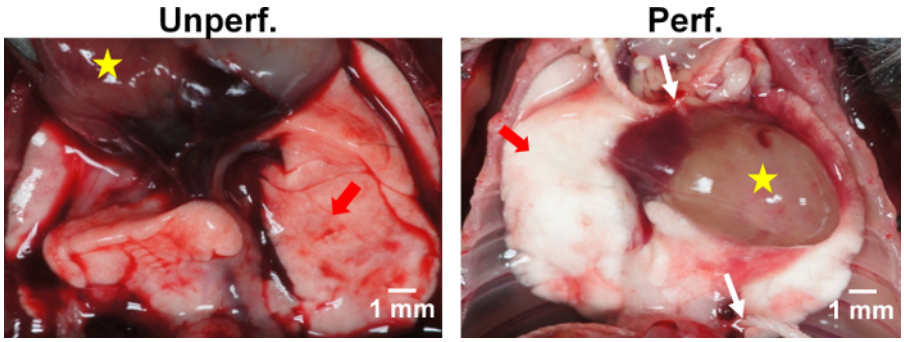

Figure 2: Lungs before and after perfusion. Representative images of lungs before (unperfused; Unperf.) and after (perfused; Perf.) performing lung perfusion. Gold star: heart. White arrow: the tie that closed the IVC/SVC. Red arrows: the lungs of the same mouse before and after perfusion. Please click here to view a larger version of this figure.

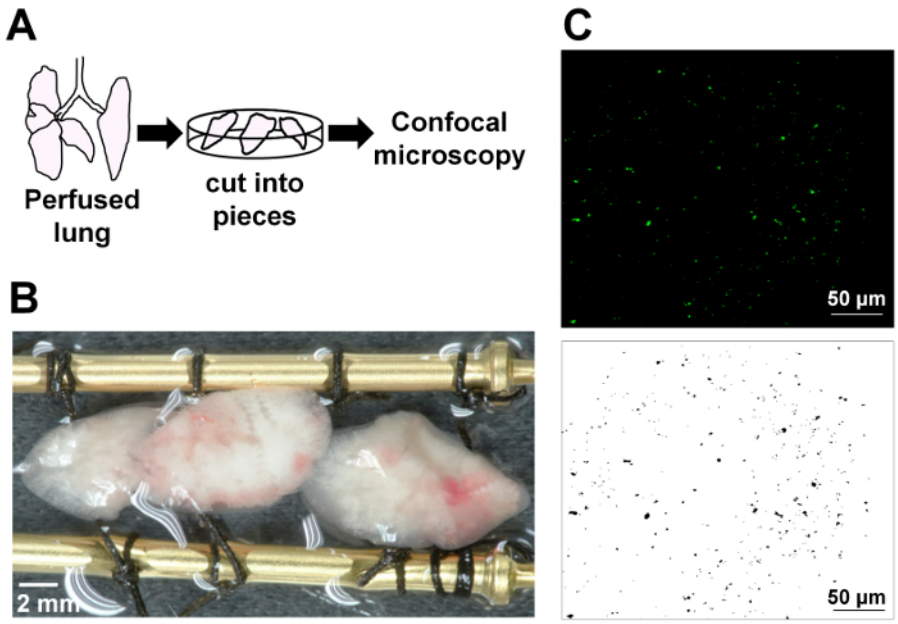

Figure 3: Mounting of the perfused lung lobes on the lung holder for fluorescence confocal microscopy. (A) Scheme of the lung-mounting on the lung holder for the confocal microscopy. **(B)** A representative image of 3 perfused lung lobes on the lung holder. **(C)** Representative images of lung-colonizing LLC cells with CFSE fluorescence for quantification by Image J software. Upper panel: regular imaging. Lower panel: reversed black-and-white imaging. Please click here to view a larger version of this figure.

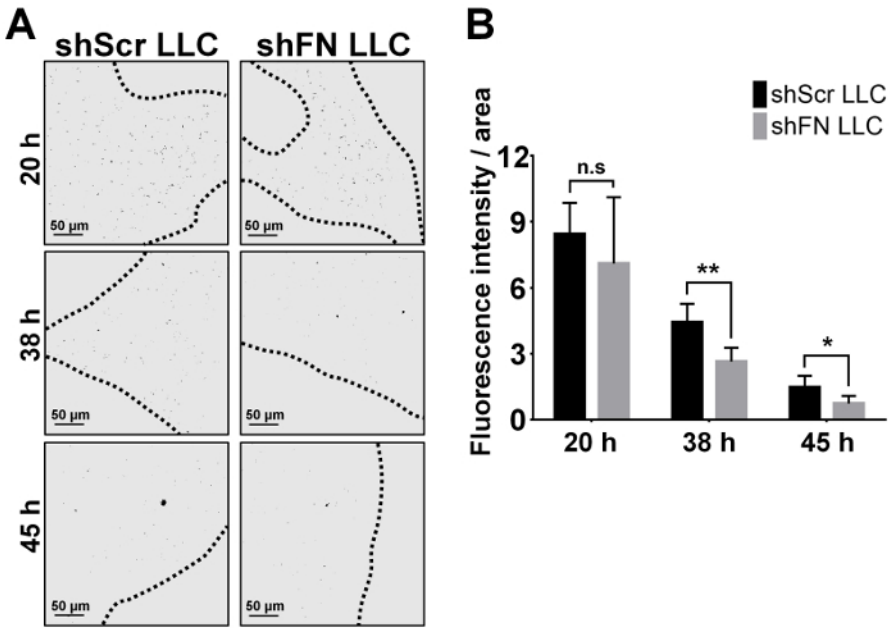

Figure 4: Time course imaging, quantification, and statistical analysis for the lung colonized shScr and shFN LLC cells after lung perfusion in the lung colonization assay. (A) Representative black-and-white images (converted from fluorescence images) of shScr and shFN LLC cells that were colonized in the lung vasculature at various time points as depicted after extensive lung perfusion. Dotted lines denote the edge of the in-focus confocal lung tissue areas. **(B)** Quantification and statistical analysis for the lung-colonizing LLC cells as visualized in (A) by averaging 5 absolute representative images/lung lobe/mouse. Note: Each bar indicates the averaged fluorescence intensity of each in-focus confocal area. Data were statistically analyzed from 6 mice and reported as mean ± SD; n.s. means nonsignificant; * means $p<0.05$; and ** means $p<0.01$; Two-way ANOVA. Please click here to view a larger version of this figure.

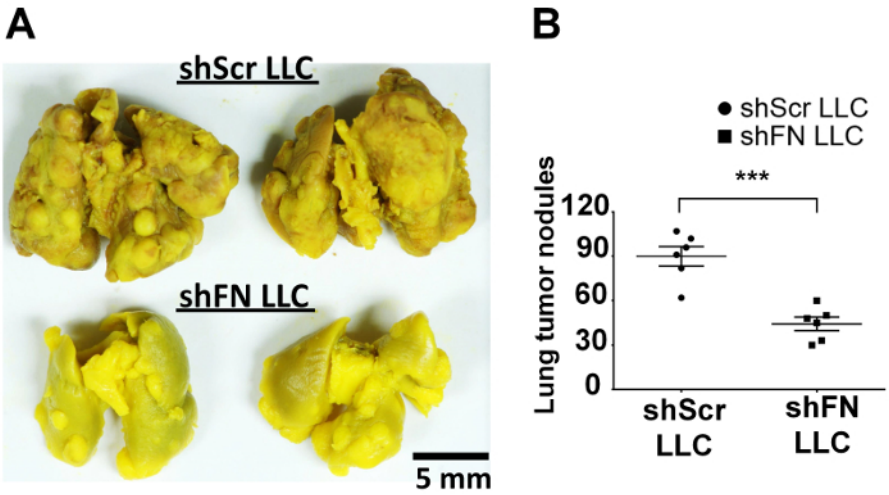

Figure 5: Silencing FN expression in CFSE-labeled LLC cells diminishes tumor nodules in the lungs in the long term in vivo CTC colonization assay. (A) Bouin's Fluid-fixed mouse lungs bearing tumor nodules derived from CFSE-labeled shScr or shFN LLC cells. **(B)** Quantification and statistical analysis for the tumor nodules in the lungs. Data are reported as mean ± SD; ***: p<0.001; n=6 per group; Unpaired t-test. Please click here to view a larger version of this figure.

Discussion

Together with long term lung colonization assays, the short term methodology we employed here to evaluate *in vivo* lung colonization by CTCs in distant organs clearly unveiled and differentiated the specific role of polyFN assembled on CTCs in colonizing the lungs, which then led to the extravasation and metastatic processes[18,19,20,21]. Although labeling cells with long term cell tracker CFSE allowed us to trace the intravenously injected CTCs for up to three days prior to the lung perfusion and retain sufficient green fluorescence for quantitative purposes, it was impossible to directly determine whether the tumor cells were located within the vessel lumen or had already extravasated from the blood vessels. To solve this problem, red fluorescent rhodamine-conjugated dextran which is able to non-specifically bind to lectins expressed on endothelia may be used to label the lung vasculature during lung perfusion[28,29,30]. Despite the fact that CFSE is a long term cell tracker, its fluorescence intensity is halved every cell division[27], limiting the potential of using it in labeling techniques. To circumvent this problem, it should be ascertained that the labeling dosage of CFSE is sufficient for cells to remain detectable for the entire duration of *in vivo* experiments and that any treatment applied to the cells has no effect on cell proliferation. Because the comparison and quantification of the lung-colonizing shScr and shFN CTCs were done in separate mice, it might be argued that the differences between the two groups were due to individual variation. To exclude such variation, a mixture of two types of tumor cells labeled with distinct fluoresce dyes (*e.g.,* CFSE/CM-dil) or stably transfected with GFP/dsRed may be concomitantly intravenously injected into the same animal in the lung colonization assay[31,32,33]. It is worth noting that cloning effects resulting from the cell cloning technology that is attempted to sort out stably fluorescent protein-transfected cell clones with sufficiently strong fluorescence[34,35,36,37] may make the cloned cells unrepresentative to represent the entire heterogeneous populations[38,39,40]. If a stable transfection of any fluorescent protein into tumor cells is desired for the lung colonization assays, elevating the transfection efficiency of the tumor cells as a whole should better preserve the original characteristics than cloning the cell populations with sufficient fluorescence[41,42].

The lung perfusion steps in the lung colonization assays are necessary in that some intravenously injected tumor cells, instead of specifically arresting to the lung capillary system, tend to be mechanically trapped and jammed within the capillary networks of lung tissues due to the averagely larger diameters of CTCs than the width of lung capillary lumens[43,44]. Quantification of the lung-colonizing tumor cells without perfusing the lungs may result in an overestimation by mistakenly counting the mechanically jammed cells[45,46,47]. Even if the lungs have been perfused, additional problems remain. For instance, it is still difficult to differentiate whether the lung-colonizing tumor cells are located on the lumenal endothelia or have already extravasated from the blood vessels. To resolve this issue, staining the blood vessels with Rhodamine-conjugated dextran as aforementioned may be useful[30,48,49]. Alternatively, if quantification of tumor extravasation is desired, calcium-chelator EDTA/EGTA or trypsin, a non-specific protease, can be used in the perfusion buffer to impede calcium-dependent and -independent tumor cell adhesion events[50,51,52]. Thus, the tumor cells remaining in the lung tissues may be considered as extravasated.

For quantification of the lung-colonizing tumor cells, the perfused lungs are often subjected to traditional confocal microscopy. However, the measurability of tissue depth is limited due in part to tissue autofluorescence and fluorescence scattering effects, rendering deeper tissues undetectable[53,54]. A two photon microscope with higher sensitivity than a traditional confocal microscope is suitable to resolve such problems in that the near-infrared radiation used in two-photon excitation with significantly less absorption by biological specimens than UV or blue-green light makes the technique more appropriate for imaging thick specimens[55,56,57]. Lung-colonizing tumor cells are counted by averaging tumor cell numbers in several representative confocal images, which do not include the entire number of lung-colonizing tumor cells in the whole lungs of an individual animal. To quantify the whole lungs in the lung colonization assays, tumor cells stably transfected with luciferase could be employed and the perfused lungs could then be subjected to IVIS imaging after intravenous inoculation of suspended tumor cells in the presence of luciferin[46,58,59]. Alternatively, the perfused lungs could be minced into pieces and subjected to enzymatic digestion with proteases, *e.g.*, collagenase, releasing single cells into suspension[60,61]. The tumor cells with fluorescence dyes or stably transfected with fluorescence proteins could then be quantified with fluorescence-activated cell sorting (FACS) analysis[56,62].

Copyright © 2018 Journal of Visualized Experiments

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors wish to thank Dr. Ming-Min Chang and Ms. Ya-Hsin Cheng for their technical support. This work was supported by Taiwan's Ministry of Science and Technology (MOST- 103-2325-B-006 -009, MOST- 104-2325-B-006-001, MOST- 105-2325-B-006-001, and MOST-106-2320-B-006-068-MY3) and the Ministry of Health and Welfare (MOHW106-TDU-B-211-144004). We are also grateful for the support from the Core Research Laboratory of the College of Medicine, National Cheng Kung University, for their multi-photon confocal microscope.

References

1. Massague, J., Obenauf, A.C. Metastatic colonization by circulating tumour cells. *Nature.* **529** (7586), 298-306 (2016).
2. Lambert, A.W., Pattabiraman, D.R., Weinberg, R.A. Emerging Biological Principles of Metastasis. *Cell.* **168** (4), 670-691 (2017).
3. Mohme, M., Riethdorf, S., Pantel, K. Circulating and disseminated tumour cells - mechanisms of immune surveillance and escape. *Nat Rev Clin Oncol.* **14** (3), 155-167 (2017).
4. Steinert, G. *et al.* Immune escape and survival mechanisms in circulating tumor cells of colorectal cancer. *Cancer Res.* **74** (6), 1694-1704 (2014).
5. Mahauad-Fernandez, W.D., Okeoma, C.M. Cysteine-linked dimerization of BST-2 confers anoikis resistance to breast cancer cells by negating proapoptotic activities to promote tumor cell survival and growth. *Cell Death Dis.* **8** (3), e2687 (2017).
6. Maheswaran, S., Haber, D.A. Circulating tumor cells: a window into cancer biology and metastasis. *Curr Opin Genet Dev.* **20** (1), 96-99 (2010).
7. Yu, M. *et al.* RNA sequencing of pancreatic circulating tumour cells implicates WNT signalling in metastasis. *Nature.* **487** (7408), 510-513 (2012).
8. Adams, D.L. *et al.* Mitosis in circulating tumor cells stratifies highly aggressive breast carcinomas. *Breast Cancer Res.* **18** (1), 44 (2016).
9. Baccelli, I. *et al.* Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. *Nat Biotechnol.* **31** (6), 539-544 (2013).
10. Malladi, S. *et al.* Metastatic Latency and Immune Evasion through Autocrine Inhibition of WNT. *Cell.* **165** (1), 45-60 (2016).
11. Spiegel, A. *et al.* Neutrophils Suppress Intraluminal NK Cell-Mediated Tumor Cell Clearance and Enhance Extravasation of Disseminated Carcinoma Cells. *Cancer Discov.* **6** (6), 630-649 (2016).
12. Pattabiraman, D.R. *et al.* Activation of PKA leads to mesenchymal-to-epithelial transition and loss of tumor-initiating ability. *Science.* **351** (6277), aad3680 (2016).
13. van der Weyden, L. *et al.* Genome-wide in vivo screen identifies novel host regulators of metastatic colonization. *Nature.* **541** (7636), 233-236 (2017).
14. Obenauf, A.C. *et al.* Therapy-induced tumour secretomes promote resistance and tumour progression. *Nature.* **520** (7547), 368-372 (2015).
15. Genovese, G. *et al.* Synthetic vulnerabilities of mesenchymal subpopulations in pancreatic cancer. *Nature.* **542** (7641), 362-366 (2017).
16. von Horsten, S. *et al.* Stereological quantification of carboxyfluorescein-labeled rat lung metastasis: a new method for the assessment of natural killer cell activity and tumor adhesion in vivo and in situ. *J Immunol Methods.* **239** (1-2), 25-34 (2000).
17. Reymond, N. *et al.* Cdc42 promotes transendothelial migration of cancer cells through beta1 integrin. *J Cell Biol.* **199** (4), 653-668 (2012).
18. Cheng, H.C., Abdel-Ghany, M., Elble, R.C., Pauli, B.U. Lung endothelial dipeptidyl peptidase IV promotes adhesion and metastasis of rat breast cancer cells via tumor cell surface-associated fibronectin. *J Biol Chem.* **273** (37), 24207-24215 (1998).
19. Huang, L. et al. Protein kinase Cepsilon mediates polymeric fibronectin assembly on the surface of blood-borne rat breast cancer cells to promote pulmonary metastasis. *J Biol Chem.* **283** (12), 7616-7627 (2008).
20. Chang, Y.H. *et al.* Secretomic analysis identifies alpha-1 antitrypsin (A1AT) as a required protein in cancer cell migration, invasion, and pericellular fibronectin assembly for facilitating lung colonization of lung adenocarcinoma cells. *Mol Cell Proteomics.* **11** (11), 1320-1339 (2012).
21. Wang, Y.J. *et al.* Pterostilbene prevents AKT-ERK axis-mediated polymerization of surface fibronectin on suspended lung cancer cells independently of apoptosis and suppresses metastasis. *J Hematol Oncol.* **10** (1), 72 (2017).
22. Cheng, H.C., Abdel-Ghany, M., Pauli, B.U. A novel consensus motif in fibronectin mediates dipeptidyl peptidase IV adhesion and metastasis. *J Biol Chem.* **278** (27), 24600-24607 (2003).
23. Hsu, Y.Y. *et al.* Thrombomodulin is an ezrin-interacting protein that controls epithelial morphology and promotes collective cell migration. *FASEB J.* **26** (8), 3440-3452 (2012).
24. Arlt, M.J., Born, W., Fuchs, B. Improved visualization of lung metastases at single cell resolution in mice by combined in-situ perfusion of lung tissue and X-Gal staining of lacZ-tagged tumor cells. *J Vis Exp.* (66), e4162 (2012).
25. Shi, Y., Parhar, R.S., Zou, M., Al-Mohanna, F.A., Paterson, M.C. Gene therapy of melanoma pulmonary metastasis by intramuscular injection of plasmid DNA encoding tissue inhibitor of metalloproteinases-1. *Cancer Gene Ther.* **9** (2), 126-132 (2002).
26. Parish, C.R. Fluorescent dyes for lymphocyte migration and proliferation studies. *Immunol Cell Biol.* **77** (6), 499-508 (1999).
27. Quah, B.J., Warren, H.S., Parish, C.R. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat Protoc.* **2** (9), 2049-2056 (2007).
28. Migone, F.F. *et al.* In vivo imaging reveals an essential role of vasoconstriction in rupture of the ovarian follicle at ovulation. *Proc Natl Acad Sci U S A.* **113** (8), 2294-2299 (2016).
29. Itkin, T. *et al.* Distinct bone marrow blood vessels differentially regulate haematopoiesis. *Nature.* **532** (7599), 323-328 (2016).
30. Jiang, M., Qin, C., Han, M. Primary breast cancer induces pulmonary vascular hyperpermeability and promotes metastasis via the VEGF-PKC pathway. *Mol Carcinog.* **55** (6), 1087-1095 (2016).
31. Aceto, N. *et al.* Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. *Cell.* **158** (5), 1110-1122 (2014).

Copyright © 2018 Journal of Visualized Experiments

32. Zheng, Y. *et al.* Expression of beta-globin by cancer cells promotes cell survival during blood-borne dissemination. *Nat Commun.* **8** 14344 (2017).
33. Chen, X. *et al.* Retinoic acid facilitates inactivated transmissible gastroenteritis virus induction of CD8(+) T-cell migration to the porcine gut. *Sci Rep.* **6** 24152 (2016).
34. Saranchova, I. *et al.* Discovery of a Metastatic Immune Escape Mechanism Initiated by the Loss of Expression of the Tumour Biomarker Interleukin-33. *Sci Rep.* **6** 30555 (2016).
35. Ahmed, M. *et al.* An Osteopontin/CD44 Axis in RhoGDI2-Mediated Metastasis Suppression. *Cancer Cell.* **30** (3), 432-443 (2016).
36. Hoffman, R.M. In vivo imaging of metastatic cancer with fluorescent proteins. *Cell Death Differ.* **9** (8), 786-789 (2002).
37. Mendoza, A. *et al.* Modeling metastasis biology and therapy in real time in the mouse lung. *J Clin Invest.* **120** (8), 2979-2988 (2010).
38. Meacham, C.E., Morrison, S.J. Tumour heterogeneity and cancer cell plasticity. *Nature.* **501** (7467), 328-337 (2013).
39. Wagenblast, E. *et al.* A model of breast cancer heterogeneity reveals vascular mimicry as a driver of metastasis. *Nature.* **520** (7547), 358-362 (2015).
40. Nguyen, D.X., Bos, P.D., Massague, J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer.* **9** (4), 274-284 (2009).
41. Ragelle, H. *et al.* Chitosan nanoparticles for siRNA delivery: optimizing formulation to increase stability and efficiency. *J Control Release.* **176** 54-63 (2014).
42. Chu, V.T. *et al.* Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nat Biotechnol.* **33** (5), 543-548 (2015).
43. Wirtz, D., Konstantopoulos, K., Searson, P.C. The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nat Rev Cancer.* **11** (7), 512-522 (2011).
44. Joyce, J.A., Pollard, J.W. Microenvironmental regulation of metastasis. *Nat Rev Cancer.* **9** (4), 239-252 (2009).
45. Sanchez-Laorden, B. *et al.* BRAF inhibitors induce metastasis in RAS mutant or inhibitor-resistant melanoma cells by reactivating MEK and ERK signaling. *Sci Signal.* **7** (318), ra30 (2014).
46. Torchiaro, E. *et al.* Peritoneal and hematogenous metastases of ovarian cancer cells are both controlled by the p90RSK through a self-reinforcing cell autonomous mechanism. *Oncotarget.* **7** (1), 712-728 (2016).
47. Wan, J. *et al.* Establishment of monoclonal HCC cell lines with organ site-specific tropisms. *BMC Cancer.* **15** 678 (2015).
48. Ye, Y., Liu, S., Wu, C., Sun, Z. TGFbeta modulates inflammatory cytokines and growth factors to create premetastatic microenvironment and stimulate lung metastasis. *J Mol Histol.* **46** (4-5), 365-375 (2015).
49. Morales, M. *et al.* RARRES3 suppresses breast cancer lung metastasis by regulating adhesion and differentiation. *EMBO Mol Med.* **6** (7), 865-881 (2014).
50. Stone, J.P. *et al.* Mechanical removal of dendritic cell-generating non-classical monocytes via ex vivo lung perfusion. *J Heart Lung Transplant.* **33** (8), 864-869 (2014).
51. Vettorazzi, S. et al. Glucocorticoids limit acute lung inflammation in concert with inflammatory stimuli by induction of SphK1. *Nat Commun.* **6** 7796 (2015).
52. Urade, Y., Yoshida, R., Kitamura, H., Hayaishi, O. Induction of indoleamine 2,3-dioxygenase in alveolar interstitial cells of mouse lung by bacterial lipopolysaccharide. *J Biol Chem.* **258** (10), 6621-6627 (1983).
53. Hong, G. *et al.* Through-skull fluorescence imaging of the brain in a new near-infrared window. *Nat Photonics.* **8** (9), 723-730 (2014).
54. Liu, Q., Guo, B., Rao, Z., Zhang, B., Gong, J.R. Strong two-photon-induced fluorescence from photostable, biocompatible nitrogen-doped graphene quantum dots for cellular and deep-tissue imaging. *Nano Lett.* **13** (6), 2436-2441 (2013).
55. Peti-Peterdi, J., Burford, J.L., Hackl, M.J. The first decade of using multiphoton microscopy for high-power kidney imaging. *Am J Physiol Renal Physiol.* **302** (2), F227-233 (2012).
56. Duda, D.G. *et al.* Malignant cells facilitate lung metastasis by bringing their own soil. *Proc Natl Acad Sci U S A.* **107** (50), 21677-21682 (2010).
57. Gupta, G.P. *et al.* Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. *Nature.* **446** (7137), 765-770 (2007).
58. Kosaka, N. *et al.* Neutral sphingomyelinase 2 (nSMase2)-dependent exosomal transfer of angiogenic microRNAs regulate cancer cell metastasis. *J Biol Chem.* **288** (15), 10849-10859 (2013).
59. Hoshino, A. *et al.* Tumour exosome integrins determine organotropic metastasis. *Nature.* **527** (7578), 329-335 (2015).
60. Jaskelioff, M. *et al.* Telomerase deficiency and telomere dysfunction inhibit mammary tumors induced by polyomavirus middle T oncogene. *Oncogene.* **28** (48), 4225-4236 (2009).
61. Si, L.L., Lv, L., Zhou, W.H., Hu, W.D. Establishment and identification of human primary lung cancer cell culture in vitro. *Int J Clin Exp Pathol.* **8** (6), 6540-6546 (2015).
62. Weng, D. *et al.* Metastasis is an early event in mouse mammary carcinomas and is associated with cells bearing stem cell markers. *Breast Cancer Res.* **14** (1), R18 (2012).

Copyright © 2018 Journal of Visualized Experiments