Video Article

Labeling of Breast Cancer Patient-derived Xenografts with Traceable Reporters for Tumor Growth and Metastasis Studies

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Abstract

The use of preclinical models to study tumor biology and response to treatment is central to cancer research. Long-established human cell lines, and many transgenic mouse models, often fail to recapitulate the key aspects of human malignancies. Thus, alternative models that better represent the heterogeneity of patients' tumors and their metastases are being developed. Patient-derived xenograft (PDX) models in which surgically resected tumor samples are engrafted into immunocompromised mice have become an attractive alternative as they can be transplanted through multiple generations,and more efficiently reflect tumor heterogeneity than xenografts derived from human cancer cell lines. A limitation to the use of PDXs is that they are difficult to transfect or transduce to introduce traceable reporters or to manipulate gene expression. The current protocol describes methods to transduce dissociated tumor cells from PDXs with high transduction efficiency, and the use of labeled PDXs for experimental models of breast cancer metastases. The protocol also demonstrates the use of labeled PDXs in experimental metastasis models to study the organ-colonization process of the metastatic cascade. Metastases to different organs can be easily visualized and quantified using bioluminescent imaging in live animals, or GFP expression during dissection and in excised organs. These methods provide a powerful tool to extend the use of multiple types of PDXs to metastasis research.

Video Link

The video component of this article can be found at https://www.jove.com/video/54944/

Introduction

The development of patient-derived tumor xenografts (PDXs), where surgically resected tumor samples are engrafted directly into immune-compromised mice, offers several advantages over standard cell-line xenograft models and represents a major advance in cancer research[1,2]. PDXs can be maintained and expanded by successive passages with minimal alteration of the genetic and biological characteristics of the tumor grown at the first passage; and more accurately reflect tumor heterogeneity than xenografts derived from human cancer cell lines[3-8]. These models are now extensively used as a platform for personalizing cancer therapeutics[9,10], as a preclinical platform in drug development[6,11] and as an experimental tool for studying cancer biology[4,12].

Most PDXs are implanted and propagated subcutaneously, which feasibly allows measurement of tumor growth over time using calipers. However, metastatic disease has been more difficult to model using PDXs. Specifically for breast cancer, xenografts with metastatic capacity to different organs have been described[3,5,13], but the frequency of spontaneous dissemination to metastatic sites is extremely low. Where reported, the identification and quantification of metastatic burden relies in laborious histological examination of target organs post-mortem. Cancer cell lines expressing bioluminescent (luciferase, Luc) or fluorescent (Green Fluorescent Protein, GFP) gene reporters are commonly used in experimental models of breast cancer metastases to brain, lung, bone and liver after intracardiac, tail-vein, intrafemoral and splenic injection[14-16]. While these models bypass dissemination from the primary tumors, they are valuable to study the mechanisms of organ tropism and metastatic colonization. However, cells derived from primary patient tumors and PDXs can have low transfection or transduction rates using standard procedures. One alternative is to establish PDX-derived cell lines *in vitro*[17], which can be then labeled using conventional tissue culture protocols. This approach however, is not suitable for labeling most PDXs, for which cell-line derivation is difficult and can change the phenotype of the cells. Here we present a protocol for transduction of PDX-dissociated tumor cells with lentiviral vectors suitable for *in vivo* imaging. In addition, we describe experimental metastasis using intracardiac injection of dissociated luc-GFP labeled PDX cells in immunocompromised mice.

A basic protocol for transduction of PDX-dissociated organoids with gene-reporter expressing lentivirus has previously been described [18]. In the current protocol we describe additional methods to enrich for human tumor cells and obtain near 100% transduction efficiency, as well as the use of labeled PDXs for detecting experimental breast cancer metastases. This protocol can be adapted for labeling multiple cancer types of PDXs with various luminescent and fluorescent markers as well as modulation of gene expression (*i.e.*, shRNA knockdown of genes of interest).

Protocol

All steps requiring the use of animals in this protocol follows the guidelines of University of Colorado animal research ethics committee (IACUC).

1. Preparation of Instruments, Culture Media and Other Reagents

1. Prepare 100 ml mammosphere media containing Dulbecco's Modified Eagle Medium and Han's F-12 medium (DMEM/F12) (1:1), basic Fibroblast Growth Factor (bFGF, 20 ng/ml), epidermal growth factor (EGF, 10 ng/ml), Heparin (4 µg/ml), 1x B27, Penicillin (100 U/ml), streptomycin (100 µg/ml). Make media in sterile conditions and store at 4 °C for up to 3 months.
2. Prepare epithelial enrichment buffer (EEB) containing PBS pH 7.2, 0.5% Bovine Serum Albumin (BSA), 2 mM EDTA. Filter sterilize and store at 4 °C for up to 6 months.
3. Make 5 ml aliquots of digestion buffer in sterile 15 ml conical tubes and store at -20 °C for months. The night before tumor digestion, thaw digestion buffer (5 ml per 500 mg tumor) on ice, at 4 °C. Add 1x antibiotic-antimycotic mix before use.
4. Autoclave (121 °C for 30 min) at least two forceps, scalpel and scissors for dissection of PDXs.
5. Make 500 ml wash buffer containing DMEM:F12 and 5% Fetal Bovine Serum (FBS). Store at 4 °C for months. Aliquot 10 ml per tumor the day of tumor dissection.
6. Prepare a polybrene stock at 4 µg/µl with sterile water. Filter and aliquot into 1.5 ml microcentrifuge tubes containing 100 µl each, store at -20 °C.

2. Generation of High Titer Lentiviral Particles Carrying Traceable Markers

1. Purchase or generate high titer lentiviral particles carrying a reporter gene (*i.e.*, GFP and Luc for *in vivo* tracking of tumor growth) [18,19].
   NOTE: A lentiviral titer of > 10^8 TU/ml (transduction units per milliliter) is recommended for successful transduction of PDXs. The lentiviral particles should be pseudotyped with an envelope G glycoprotein from Vesicular Stomatitis Virus (VSV-G), allowing transduction of a wide variety of mammalian cells.
   NOTE: The protocol used here for the generation and titration of high titer lentiviral particles is available at www.kottonlab.com. Vectors used in this protocol are pSIH1-H1-copGFP-T2A-puro and the dual promoter pHAGE-EF1aL-luciferase-UBC-GFP-W. This vector was generated by replacing dsRed with the luciferase gene downstream of EF1aL promoter in the pHAGE-EF1aL-dsRed-UBC-GFP-W vector, a kind gift of Darrell Kotton, Boston University.

3. Generation of Patient-derived Xenografts

1. Generate patient derived xenografts (PDX) from breast cancer by implanting primary or metastatic breast tumors in the mammary fat pad of immunocompromised mice[3,4]. Generate tumors at a maximum recommended size of 1 cm diameter as larger tumors are likely to contain necrotic cores.
   NOTE: Detailed methods for establishing and transplanting xenografts are described in DeRose *et al.* [18]. Growth of established-transplantable PDXs into > 1 cm-diameter tumors takes between 4 and 24 weeks after implantation in immunocompromised NOD/SCID/ILIIrg −/− (NSG), depending on intrinsic tumor growth rates. While this protocol describes transduction of breast cancer PDXs, it can be used for viral transduction of any tumor suitable to short-term *in vitro* passage. The sensitivity of PDXs to short-term (24 - 96 hr) *in vitro* survival is intrinsic to each tumor and must be determined experimentally.

4. Tumor Dissection and Dissociation of PDX-derived Tumor Cells

1. Euthanize mice carrying PDX using CO_2 inhalation followed by cervical dislocation (follow institutional guidelines of animal research ethics committee). Submerge mice in a 0.2% Chlorhexidine solution for 1 min. Set mouse on a supine position on top of a dissection board, and use pins to fix the extended upper and lower limbs to the board.
2. Using aseptic technique, use a scalpel to cut the skin around the tumor. Pull the skin with a set of forceps and separate it from the tumor using a clean scalpel until the tumor is completely exposed. Using a clean set of forceps and scalpel, remove the tumor and place it in 5 ml wash media on ice.
3. Take dissected tumor into a BLS2 cabinet for further processing. Remove media and rinse tumor with 10 ml of Hank's Balanced Salt Sodium Modified with 10 mM Hepes (HBSS/Hepes), twice.
4. Drop the tumor in a sterile pre-weighed 60 cm tissue culture plate. Weigh the plate containing the tumor to estimate tumor mass.
5. Using a scalpel, cut the tumor in half, then cut a 3 mm disc from one half and place it in a container with 10% formalin for 24 hr. Use this tissue to verify the heterogeneity of the tumor sample (parental PDX). Add 200 µl of HBSS/Hepes (enough to avoid the tissue to dry out) and mince the remaining tumor in the smallest possible pieces using forceps and a clean scalpel.
6. Transfer the minced tumor into a sterile 50 ml conical tube. Add at least 1 ml of digestion buffer containing 1x antimycotic-antibiotic per 100 mg of tumor.
   NOTE: Addition of antibiotics/antimycotic prevents contamination of tumor cells *in vitro*.
7. Digest tumor for 3 hr at 30 °C, shaking at 125 - 200 rpm.
   NOTE: Increasing temperature up to 37 °C increases the efficiency of the digestion (more single cells over time) but it is usually accompanied by increased cell death. For most tumors, digestion at 30 °C for 3 hr results in sufficient numbers of viable dissociated cells to proceed to the labeling step.
8. Stop the digestion adding 35 ml wash media (DMEM/F12 with 5% FBS). Filter through a 70 µm nylon mesh into a clean 50 ml conical tube to remove undigested tissue. Centrifuge the filtered media containing digested cells at 400 x g for 5 min.
9. Aspirate supernatant and resuspend pellet in 1 ml Red blood cell lysis buffer. Incubate at room temperature for 5 min.

10. Add 10 ml Wash buffer (DMEM:F12, 5% FBS) to stop lysis. Pass cells through a 40 µM nylon Mesh to remove clumps. Centrifuge at 400 x g for 5 min and remove supernatant, place on ice.
11. Resuspend digested cells in 5 ml wash buffer and count both viable and non-viable cells using trypan blue exclusion. Briefly, mix 50 µl of cells and 50 µl of trypan blue and count trypan-blue excluding cells using a hemocytometer.
NOTE: This crude digestion product will contain human tumor cells derived from the PDX, as well as mouse stromal cells (fibroblasts, blood cells, *etc.*). Digested cells can now be processed for labeling (step 6), or human cancer cells can be enriched using one of the procedures described in Ref 5.

5. Enrichment of Human Epithelial Cancer Cells

1. Deplete the mouse stromal cells using a lineage (Lin+) cell depletion kit[20].
NOTE: Recommended for highly vascularized tumors and/or tumors with high stromal content in which EpCAM expression is unknown or lost).
    1. Take up to 10^7 viable cells in a clean 5 ml polypropylene tube, add 2 ml EEB and centrifuge at 300 x g for 5 min. Pipette off supernatant completely.
    2. Resuspend cell pellet in 40 µl of ice-cold EEB per 10^7 cells.
    3. Add 10 µl of biotin-antibody cocktail per 10^7 cells, mix by gently pipetting and incubate at 4 °C for 10 min (cocktail contains antibodies to Lin+ mouse cells).
    4. Add 30 µl of cold EEB per 10^7 cells, mix by gently pipetting.
    5. Add 20 µl of anti-biotin microbeads per 10^7 cells mix by gently pipetting and incubate at 4 °C for 15 min.
    6. Wash cells with 3 ml cold EEB, centrifuge at 300 x g for 5 min at 4 °C. Carefully aspirate supernatant. Resuspend pellet in 500 µl of EEB and place on ice.
    7. Place column in the magnetic field of a magnetic separator. Rinse column with 0.5 ml epithelial enrichment buffer and allow it to drip through. Do not allow the column to dry out.
    8. Place a new polypropylene collection tube under the column. Slowly, add the 500 µl containing labeled cells over the column (Lin+ labeled cells will be retained in the magnetic column). Collect effluent as a fraction with unlabeled cells, representing the enriched lineage negative (tumor) cell fraction.
    9. Rinse the column 3 times with 500 µl of EBB and collect the effluent in the same tube as effluent of step 5.1.8. Keep effluent on ice and proceed to labeling step (6).
    10. Optional: Take the column outside of the magnetic field. Place a new polypropylene tube underneath and elute the LIN+ fraction by adding 500 µl of EEB, three times and using the provided plunger.

2. Enrichment of human EpCAM+ epithelial cancer cells (recommended for tumors known to express CD326+ and contain high mouse stromal content).
    1. Take up to 10^7 viable cells in a clean 5 ml polypropylene tube, add 2 ml cold EEB and centrifuge at 300 x g for 5 min. Pipette off supernatant completely.
    2. Resuspend cell pellet in 60 µl of ice-cold EBB per 10^7 cells.
    3. Add 20 µl of EpCAM microbeads per 10^7 cells, mix by gently pipetting and incubate at 4 °C for 30 min.
    4. Wash cells with 3 ml cold EEB, centrifuge at 300 x g for 5 min at 4 °C. Carefully aspirate supernatant. Resuspend pellet in 500 µl of EEB and put on ice.
    5. Place column in the magnetic field of a mini separator. Rinse column with 0.5 ml EEB and allow it to drip through. Do not allow the column to dry out.
    6. Place a new polypropylene collection tube under the column. Slowly, add the 500 µl containing labeled cells over the column (EpCAM+ labeled cells will be retained in the magnetic column). Collect effluent as a fraction with unlabeled cells, representing the mouse stromal cells.
    7. Rinse the column 4 times with 500 µl of ME Buffer and collect the effluent in the same tube as effluent of step 5.1.8.
    8. Take the column outside of the magnetic field. Place a new polypropylene tube underneath and elute the EpCAM+ fraction by adding 500 µl of ME buffer, three times. Flush out the magnetically labeled cells by firmly pushing the plunger into the column. Collect effluent containing the enriched EpCAM+ tumor cell fraction and keep on ice. Proceed to step 6.

6. Transduction of PDX-derived Tumor Cells

1. Centrifuge dissociated tumor cells at 300 g x 5 min and resuspend in 2 ml mammosphere media. Count viable cells using trypan blue exclusion as in 4.11.
2. Prepare 10 ml of mammosphere media containing 8 µg/ml polybrene (2 µl of stock polybrene per ml of media).
3. Determine the volume needed for 2 x 10^5 viable tumor cells. Centrifuge cells at 300 x g for 5 min, and resuspend pellet in 2 ml of polybrene containing media. Plate 2 x 10^5 viable tumor cells per well in a 6-well ultra-low adherence tissue culture plate.
NOTE: This is an optimal number of cells required for transduction with one lentiviral vector.
CAUTION! Lentiviral particles and all consumables used with lentiviral particles should be handled following institutional procedures for recombinant DNA biohazards.
NOTE: Lentiviral transduction of primary breast cells strongly favors myoepithelial cells which can result in poor labeling of luminal cells and selection of subpopulations of tumor cells during labeling[21].
4. Incubate the virus with 200 mU/ml neuraminidase at 37 °C for 1 hr prior to transduction to increase the binding of viral particles to different primary cell subpopulations and correct for this potential bias[21].
5. Add lentiviral particles at 10 MOI (2 x 10^6 TU for 2 x 10^5 viable cells) if PDX-dissociated cells are depleted from Lin+ mouse cells or enriched in EpCam+ cells. Add lentiviral particles at 30 MOI (6 x 10^6 TU for 2 x 10^5 viable cells) if using non-enriched PDX-dissociated cells.
NOTE: The increased MOI allows for efficient transduction even in the presence of large amounts of debris and dead cells in crude extracts. Keep one well with unlabeled cells to serve as a control for viability and transduction efficiency.

6. Swirl to mix virus with cells. Incubate at 37 °C, 5% CO_2 for up to 96 hr. Add 500 µl of fresh mammosphere media 24 hr after transfection. Cells will not attach to plates, do not aspirate media.

7. Evaluation of Transduction Efficiency and Re-implantation of Labeled Cells in Immunocompromised Mice

1. Monitor expression of the traceable marker (GFP) every 24 hr using a fluorescent microscope at 10X magnification.
   NOTE: GFP expression can be observed as early as 24 hr after infection but in most PDXs GFP expression is clearly visible after 72 hr (**Figure 1**).
2. Estimate the efficiency of transduction by evaluating the percentage of GFP+ cells within the total well.
   NOTE: Since cultures contain tumor cells, residual stromal cells, and dead cells at various degrees, wells with as low as 10% GFP+ cells can be implanted in a host mouse.
3. Transfer transduced cells from 6-well plates into a 15 ml conical tube. Add 1 ml of mammosphere media to the well to collect all the cells left behind. Place on ice.
4. Add 10 ml of HBSS/hepes to transduced cells and centrifuge at 300 x g for 5 min, 4 °C. Carefully aspirate supernatant and resuspend in 50 µl basement matrix extract (BME) on ice.
   NOTE: BME aliquots must be thawed on ice, 1 - 2 hr prior to use. BME will solidify at room temperature; keep on ice at all times.
5. Load BME-embedded cells into a 0.5 ml insulin syringe, keep on ice, and bring to the animal facility for reimplantation into NSG mice.
6. Anesthetize female 4 - 8 week old NSG mice, using 5% isoflurane/95% oxygen for 5min, followed by 2% isoflurane/98% oxygen or according to approved animal research ethics approved protocol.
7. When animal is unresponsive to pain stimuli (toe-pinch), clean the injection area with betadine followed by ethanol wipes, and inject the 50 µl of cells in the 4th mammary fat pad. Provide mice with analgesia to alleviate injection discomfort as indicated in approved protocol.
8. Allow cells to form tumors (2 - 12 weeks) and monitor GFP and/or luciferase activity using a tunable light system and/or *in vivo* luciferase imaging system (**Figure 2**).
   NOTE: Follow guidelines for anesthesia, analgesia, tumor burden measurements and euthanasia criteria as approved by institutional animal research ethics committee.

8. Quality Control of Labeled PDXs

1. Determine the efficiency of labeling by assessing luciferase and GFP expression in labeled tumors.
   NOTE: Follow institutional animal research ethics committee approved procedures for *in vivo* bioluminescence imaging (**Figures 2, 3***)*.
   1. Dissect tumors from euthanized mice when tumor size has reached 1.0 - 1.5 cm diameter (or before according to approved protocols, as described in 4). Dissection should be performed under a tunable light system (or an equivalent system that allows GFP fluorescence evaluation).
   2. From each tumor, qualitatively estimate the percentage of GFP+ tumor using microscopy. If less than 100%, dissect GFP+ portion only. Dissect a 3 mm disk and fix in 10% formalin for paraffin embedding and histological analysis, save a small piece for RNA or other desired analyses, and save as much of the tumor as possible in individual 1.5 ml cryotubes containing 90% FBS/10% DMSO for cryopreservation.
2. Re-implant pieces of labeled tumor into new recipient NSG mice[18] to expand and use in experimental metastasis studies.
3. Perform standard immunohistochemical staining of paraffin embedded tissue obtained from tumors prior to transduction and at each generation thereafter to verify that labeled PDXs remain histologically similar to the original PDXs.
   NOTE: The markers used for quality control vary with each PDXs. For breast cancer PDXs, expression of estrogen receptor, progesterone receptor, Epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor 1 (HER1 or EGFR), pan-cytokeratin (pan-CK) are recommended for initial screening.

9. Experimental Metastasis Models with Labeled PDXs

1. Following the steps described in section 4, dissociate tumor cells from a labeled PDX. If tumor is highly vascularized or rich in mouse stroma, perform enrichment of cancer cells as described in 4.5 or 4.6.
2. Count viable cells using trypan blue exclusion, and dilute 250,000 cells in 100 µl PBS (Ca^{2+}, Mg^{2+} free) per mice. Keep cells on ice. For injection of multiple mice, adjust the numbers accordingly (and prepare an excess of at least one extra injection to account for pipetting errors).
3. Bring cells on ice to the appropriate animal procedure room and proceed to intra-cardiac injection according to approved IACUC protocol.
   NOTE: Detailed protocols for intra-cardiac injection of cancer cells have been described elsewhere[22,23].
4. Track and quantify metastatic spread over time using bioluminescent imaging. Visualize macroscopic metastases at different organs using bioluminescence and/or GFP imaging of isolated organs at necropsy[24] (**Figure 6**).

Representative Results

This method describes the transduction of PDX-dissociated breast cancer cells using high titer lentiviral vectors pSIH1-H1-copGFP-T2A-puro and pHAGE-EF1aL-luciferase-UBC-GFP-W. These vectors express a fluorescent marker that allows estimating the efficiency of transduction *in vitro*, as early as 24 hr after infection (**Figure 1a**). For most PDXs, expression of GFP will be delayed up to 72 hr after infection (**Figure 1b**), at this time the formation of cell aggregates is commonly observed. Transduction can be achieved after enriching for human cancer cells (*i.e.,* using Lin+ cell depletion, **Figure 1a**) or in crude PDX-dissociated cells (**Figure 1b**). Enrichment of tumor epithelial cells is recommended for highly vascularized tumors were mouse blood cells represent a significant percentage of the total number of viable cells.

Once transduction efficiency is verified *in vitro*, labeled cells are collected and suspended in extracellular matrix and re-implanted in NSG mice. Labeled tumor cells regenerate tumors that can be tracked using bioluminescence or fluorescence (**Figure 2**). Transduction efficiency must be evaluated following tumor formation, and it will vary depending on the susceptibility of different PDXs to *in vitro* transduction. A successfully labeled PDX will be near 100% GFP+ at first generation post-transduction (**Figure 2b,c**) and will remain near 100% GFP+ in subsequent passages (**Figure 3**). However, some PDXs will show "patchy" distribution of GFP+ cells (**Figure 4**), indicating a suboptimal *in vitro* transduction. In these cases, tumors can be dissected under a fluorescence-viewing system to select GFP+ areas that can be re-implanted for expansion of the labeled subpopulation, or tumors can be dissociated and GFP+ cells can be isolated using FACS followed by re-implantation in NSG mice.

Since PDX dissociation and transduction can result in the selection of cell subpopulations within the tumor, investigators need to verify that labeled tumors closely resemble the parental tumors from which they were derived. PDXs should be stained by IHC at each generation to demonstrate that critical markers such EGFR, hormone receptors (*i.e.*, estrogen receptor) and pan-cytokeratins (PanCK, markers of epithelial cells) are conserved in labeled PDXs. **Figure 5** shows staining for EGFR and panCK in a triple negative breast cancer PDX (hormone receptor staining is not shown as this PDX lacks estrogen and progesterone receptor).

Labeled PDXs can be used to track spontaneous metastatic spread as well as experimental metastatic spread by seeding cells directly into the circulation. Spontaneous metastases from breast cancer PDXs occur less frequently, but have been reported by several groups[3,13,25]. Experimental models of metastases provide an alternative to study organ tropism and organ-colonization steps in the metastatic cascade. Cells dissociated from labeled tumors are injected intracardially and metastatic burden is tracked using bioluminescent imaging. As shown in **Figure 6**, a PDX transduced with pHAGE-EF1aL-luciferase-UBC-GFP-W injected at 250,000 cells/mouse formed metastases in lungs and liver that were easily identified with luciferase imaging *in vivo*, and GFP expression in organs *ex vivo*.

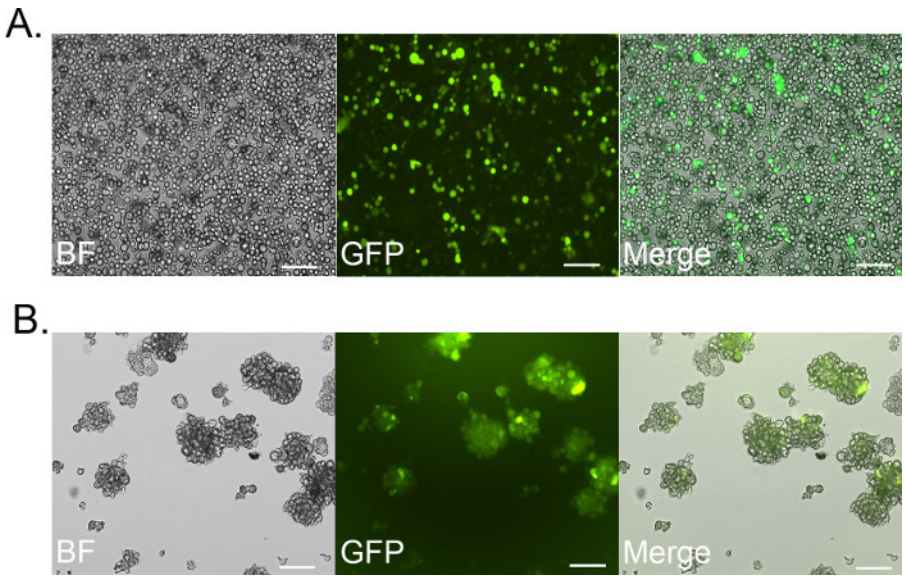

Figure 1:Tracking Transduction Efficiency in Dissociated PDX Cells. **A)** PDX-dissociated cells enriched for human epithelial cells (Lin+ depletion) 24 hr after transduction with pSIH1-H1-copGFP-T2A-puro lentiviral particles. **B)** PDX-dissociated cells from a separate experiment, without epithelial cell enrichment, 72 hr after transduction with pHAGE-EF1aL-luciferase-UBC-GFP-W. Both panels show live cells. BF: Bright field images, bar represents 50 µm. Please click here to view a larger version of this figure.

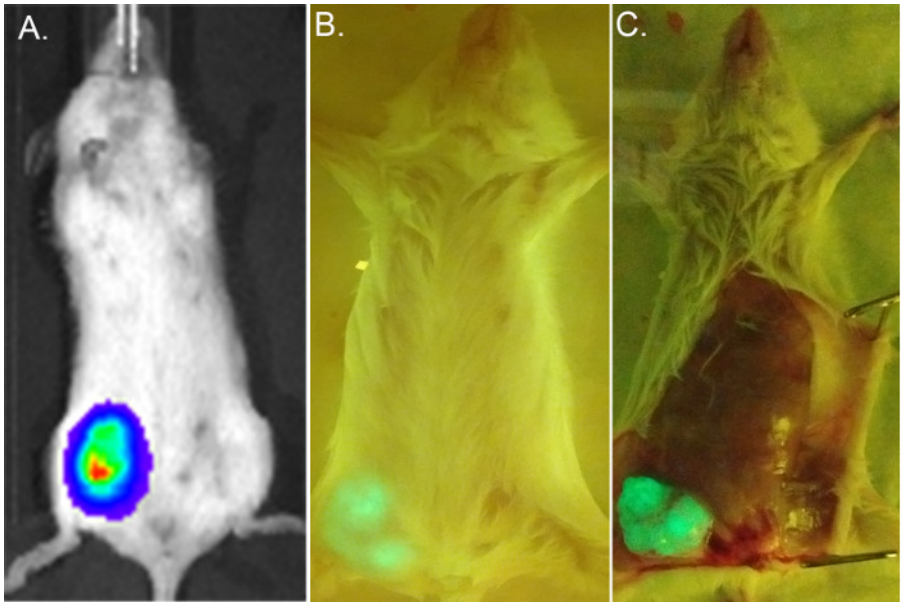

Figure 2: Establishment of Labeled PDXs in Host Mice. Cells transduced with pHAGE-EF1aL-luciferase-UBC-GFP-W for 72 hr were implanted in the mammary fat pad of a female NSG mouse. The tumor was allowed to grow for 14 weeks after injection. **A)** Luciferase reporter activity is assessed by *in vivo* imaging after intraperitoneal injection of luciferin. **B)** GFP expression can be observed through the intact skin using a fluorescence viewing system. **C)** GFP expression should also be verified at dissection to determine the extent of tumor labeling. This example shows ubiquitous GFP expressing tumor. Please click here to view a larger version of this figure.

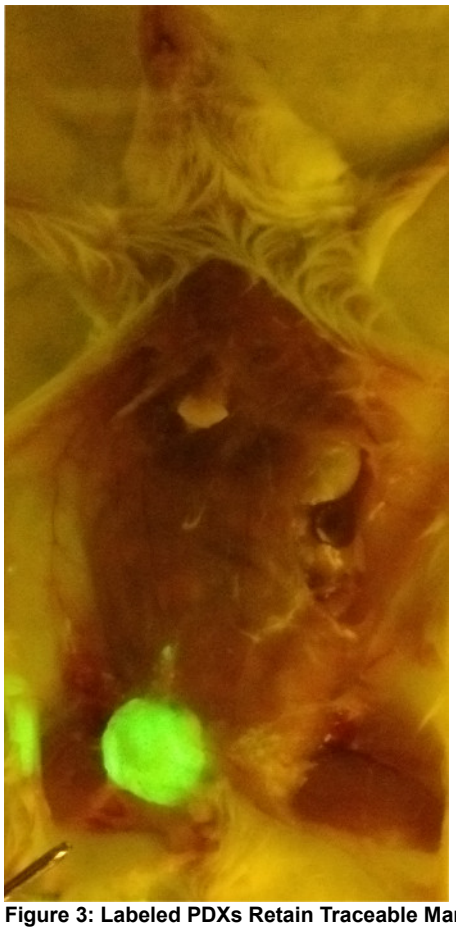

Figure 3: Labeled PDXs Retain Traceable Markers after Multiple Passages *In Vivo*. A breast cancer PDX labeled with pHAGE-EF1aL-luciferase-UBC-GFP-W is shown 3 passages after transduction. GFP expression remains at nearly 100% in the passaged tumor. Please click here to view a larger version of this figure.

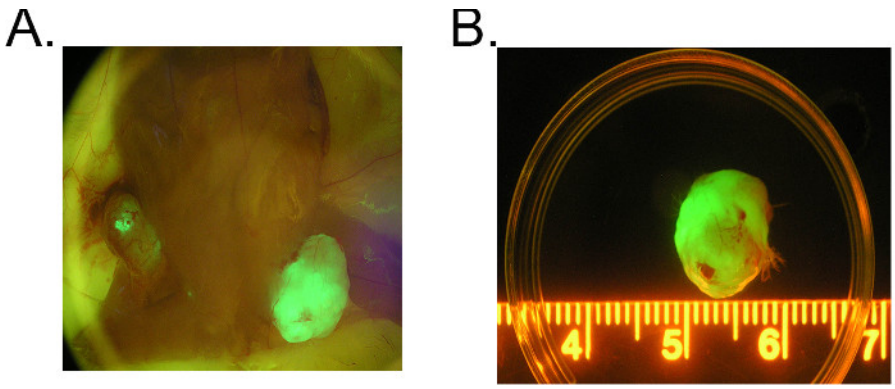

Figure 4. Example of a PDXs with Suboptimal Transduction Efficiency. A) Breast cancer PDX labeled with pHAGE-EF1aL-luciferase-UBC-GFP-W was passaged after a suboptimal transduction took place. The resulting tumors contain mixed populations of GFP+ and GFP- tumor cells. The left tumor is not suitable for further propagation. The right tumor can be propagated by dissecting the GFP+ regions under a fluorescence viewing system. **B)** Regions of GFP+ cells in mixed tumors can be easily identified under fluorescent light. Please click here to view a larger version of this figure.

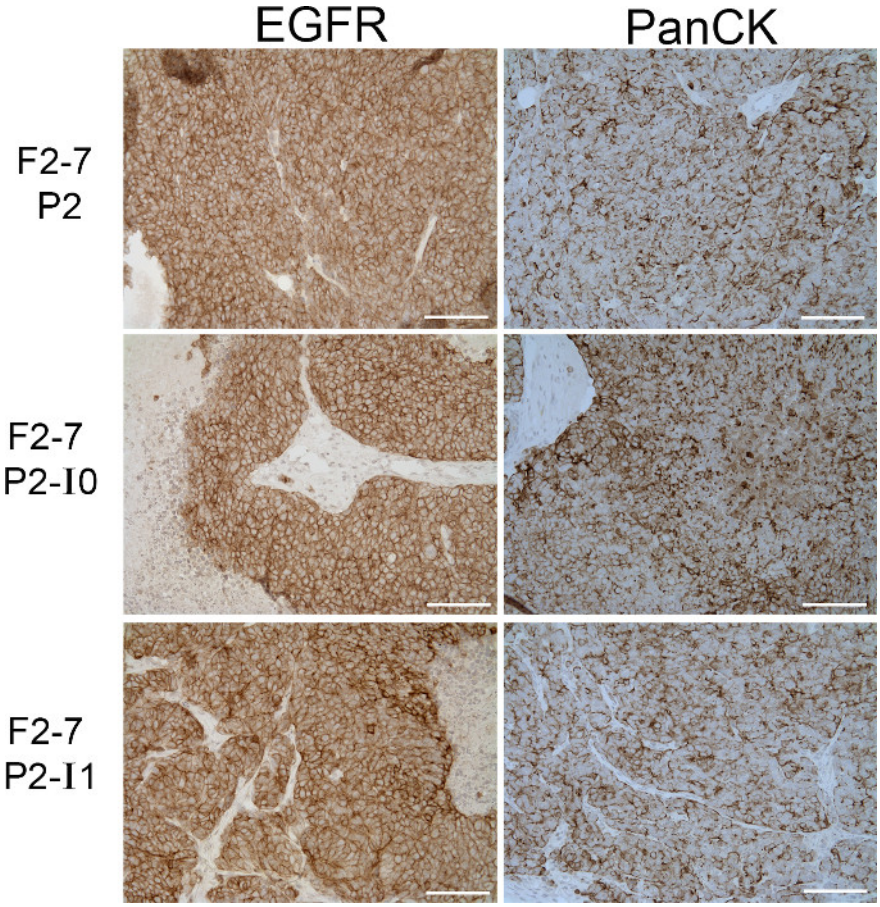

Figure 5. Quality Control of PDX Features after Transduction and Passaging. Expression of EGFR and Pan-cytokeratin (PanCK) in paraffin embedded tissue from a triple negative breast cancer PDX F2-7 at passage 2 (P2, prior to transduction), the tumor generated immediately after transduction (P2-i0), and the following passage (P2-i1). 20X images, Bars represents 100 µm. Please click here to view a larger version of this figure.

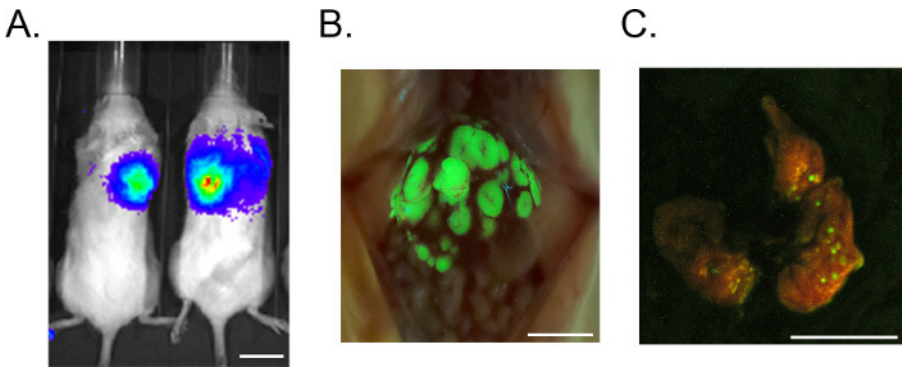

Figure 6. Experimental Metastasis Using Labeled PDXs. A PDX expressing pHAGE-EF1aL-luciferase-UBC-GFP-W was dissociated for 3 hr as described in 4, and 250,000 cells were injected in the left cardiac ventricle of NSG mice. **A)** Metastatic growth can be traced in live animals using luminescence imaging. Metastatic burden of this breast cancer PDX can be observed in **B)** liver and **C)** lungs using *ex-vivo* imaging of dissected organs under a fluorescence viewing system. Bars represents approximately 1cm. Please click here to view a larger version of this figure.

Discussion

Critical steps within the protocol:

The use of high titer lentiviral particles (>10^8 TU/ml) is a critical step in the success of this protocol, as allows careful control of the media composition during *in vitro* transduction. While multiple methods for production of high-titer viral particles have been well described[18,19]; this protocol uses lentiviral particles produced as described in detail at www.kottonlab.com. A gentle method for digestion of tumors and dissociation of cancer cells is critical for successful labeling and growth of labeled tumors. Extending the digestion time beyond three hours or increasing the digestion temperature to 37 °C increases the number of dissociated cells but decreases cell viability and success of labeling in most tumors.

Modifications and troubleshooting:

This protocol should be considered dynamic and requires adaptation for unique PDX with careful monitoring at each step. While this standard protocol works well for most xenografted tumors, small variations may be necessary to optimize labeling of different PDXs. For example, the abundance and composition of stroma and extracellular matrix usually differ among PDXs, which can affect the time needed for dissociation and the yield of tumor cells obtained thereafter. Large tumors that become necrotic and highly vascularized tumors can be difficult to label due to debris and excessive mouse blood cells. The use of Lin+ depletion and epithelial EpCam+ cell enrichment described in this protocol improves the efficiency of transduction in such PDXs. However, expression of EpCam+ cells varies even in tumors from epithelial origin, thus its expression must be verified in each PDX prior to use as method for cell enrichment.

Limitations of the technique:

Transduction of dissociated cells and their temporary culture *in vitro* could result in selection of cell subpopulations, which will then give rise to labeled tumors fundamentally different from the parental PDXs. The pre-incubation of lentiviral particles with neuraminidase is recommended to increase the binding of viral particles to cell subpopulations[21] difficult to transduce, thus decreasing the transduction bias that could be introduced at this step. In our experience, tumors reconstituted by transduced cells closely resemble the features of the parental PDXs not only histologically, but also using hierarchical clustering analysis of mRNA expression (RNA seq, data not shown). Quality control in terms of labeling efficiency and tumor fidelity should be performed for each PDX at every passage. Since PDX labeling requires expansion of the tumor *in vivo* and tumor drift could occur after repeated passaging, transduction should be performed at the earliest passage possible.

Significance of the technique:

This protocol describes how breast cancer PDXs can be fluorescently a bioluminescently labeled *in vitro* and re-implanted *in vivo* for tracking of orthotopic and metastatic tumor growth. While prior studies have used a similar strategy to label PDX-derived organoids[18], the current protocol includes variations in tumor digestion and labeling that result in a high transduction efficiency of multiple PDXs.

Future applications or directions after mastering this technique:

The ability to stably express traceable markers (GFP, luciferase), and to overexpress or knockdown specific genes (*i.e.,* pSIH1-H1-copGFP-T2A-puro can be used to deliver shRNAs) allows the use of PDXs to answer fundamental questions about tumor growth and metastasis in a similar fashion to established cell lines. Specifically, this protocol demonstrates the use of labeled PDXs in experimental metastasis models to study organ-colonization steps in the metastatic cascade. Metastases to different organs can be easily visualized and quantified using bioluminescent imaging in live animals, and GFP expression used to guided dissections and visualize metastases in excised organs. This represents a powerful tool to facilitate the use of PDXs for metastasis research.

Disclosures

The authors have nothing to disclose.

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