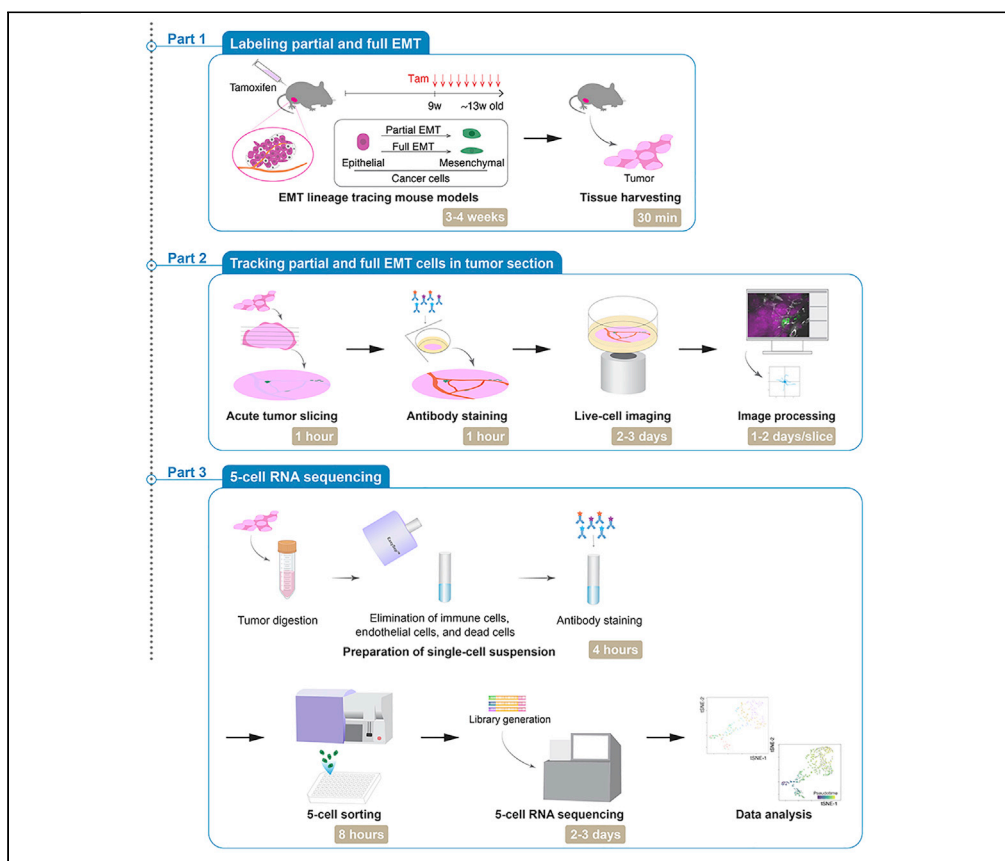


Protocol

Tracking and characterization of partial and full epithelial-mesenchymal transition cells in a mouse model of metastatic breast cancer



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Highlights

A dual recombinase lineage tracing system for cancer cells undergoing EMT

Enables live imaging of cancer cells undergoing early or late EMT

Supports flow-cytometry-mediated isolation of cancer cells in various stages of EMT

Enables single-cell transcriptomic studies of cancer cells undergoing EMT

The various stages of epithelial-mesenchymal transition (EMT) generate phenotypically heterogeneous populations of cells. Here, we detail a dual recombinase lineage tracing system using a transgenic mouse model of metastatic breast cancer to trace and characterize breast cancer cells at different EMT stages. We describe analytical steps to label cancer cells at an early partial or a late full EMT state, followed by tracking their behavior in tumor slice cultures. We then characterize their transcriptome by five-cell RNA sequencing.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Lüönd et al., STAR Protocols 3, 101438
 June 17, 2022 © 2022 The Author(s).
<https://doi.org/10.1016/j.xpro.2022.101438>



Protocol

Tracking and characterization of partial and full epithelial-mesenchymal transition cells in a mouse model of metastatic breast cancer

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SUMMARY

The various stages of epithelial-mesenchymal transition (EMT) generate phenotypically heterogeneous populations of cells. Here, we detail a dual recombinase lineage tracing system using a transgenic mouse model of metastatic breast cancer to trace and characterize breast cancer cells at different EMT stages. We describe analytical steps to label cancer cells at an early partial or a late full EMT state, followed by tracking their behavior in tumor slice cultures. We then characterize their transcriptome by five-cell RNA sequencing. For complete details on the use and execution of this protocol, please refer to Luond et al. (2021).

BEFORE YOU BEGIN

Epithelial-mesenchymal transition (EMT) is a transient, reversible process where cancer cells transit between various stages of the EMT continuum, such as fully epithelial, partial EMT, and full mesenchymal (full EMT) cell states. Due to the dynamic nature of this process, identification and molecular characterization of cells that are undergoing an EMT or have ever undergone an EMT and tracking the morphological and behavioral dynamics of these cells in tumors are technically challenging. This protocol describes the use of Tamoxifen-inducible dual recombinase lineage tracing systems for visualizing cancer cells undergoing partial EMT or full EMT in the MMTV-PyMT transgenic mouse model of metastatic breast cancer as described by Lüönd and Sugiyama et al. (Luond et al., 2021). By employing mesenchymal-specific Cre driver mouse lines of choice, this EMT lineage tracing strategy can be adapted to target specific cells of interest during breast cancer progression. Moreover, the protocols can be adapted to visualize, track, and characterize normal cells of interest undergoing an EMT during embryonic development, organogenesis, or tissue repair and cancer cells undergoing an EMT during progression of other cancer types.

Animals

Note: Authorization to perform animal experimentation must be obtained in advance. In this study, mouse experiments were carried out in accordance with the guidelines of the Swiss Federal Veterinary Office and the Cantonal Veterinary Office of Basel-Stadt (License numbers 1878, 1907, 1908).



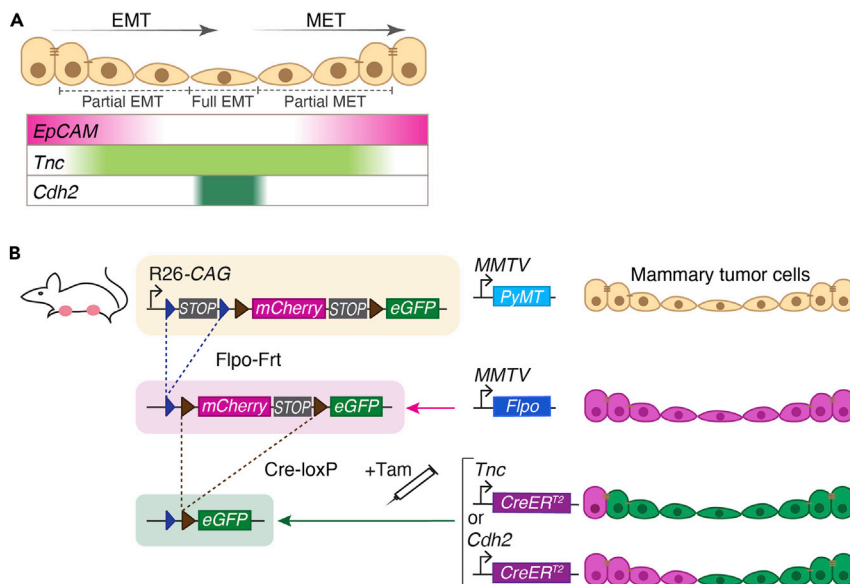


Figure 1. Dual recombinase lineage tracing of partial and full EMT during MMTV-PyMT breast tumor progression

(A) Schematic representation of *EpCAM*, *Tenascin C (Tnc)*, and *N-cadherin (Cdh2)* gene expression during EMT and MET progression. *EpCAM* expression is gradually downregulated during EMT, while *Tnc* is upregulated at early partial EMT and *Cdh2* is expressed at late EMT during the step-wise progression of EMT.

(B) Schematic representation of the EMT-lineage tracing mouse lines. Combination of the Flp/Frt and Cre/loxP responsive RC::FrePe reporter allele with MMTV-Flpo, MMTV-PyMT, and either *Tnc*-CreER^{T2} or *Cdh2*-CreER^{T2} leads to Flp-activated mCherry expression specifically in all mammary cancer cells and, upon application of Tamoxifen, to a CreER^{T2}-mediated irreversible switch to GFP expression in cancer cells undergoing early partial EMT in *Tnc*-CreER^{T2} mice and late full EMT in *Cdh2*-CreER^{T2} mice. Tam, Tamoxifen.

To visualize and trace partial and full EMT in breast cancer, we generated a dual recombinase-specific fluorescent color-switching system in the MMTV-PyMT transgenic mouse model of metastatic breast cancer. Hemizygous MMTV-PyMT females develop multifocal mammary tumors that metastasize to the other organs including lungs by the age of 13 weeks on average. Mammary tumors in MMTV-PyMT mice correspond to the aggressive luminal B subtype of human breast cancer and this strain has been widely used to study breast cancer progression and metastasis (Attalla et al., 2021; Guy et al., 1992).

To trace EMT during tumor progression, the following transgenic mouse lines are combined to generate composite transgenic mice (Figures 1A and 1B): Transgenic mice carrying the flippase (Flp) and Cre recombinase-responsive fluorescence reporter allele RC::FrePe in the *Rosa26* locus (Bang et al., 2012; Engleka et al., 2012), commercially available at The Jackson Laboratory (Bar Harbor, Maine, JAX:029486) in C57BL/6J background. The MMTV-Flpo mouse line (Luond et al., 2019), available from the lead contact by full MTA from University of Basel, expressing murine-optimized Flp recombinase in mammary epithelial cells. The MMTV-PyMT transgenic mouse line (Guy et al., 1992), commercially available at The Jackson Laboratory (Bar Harbor, Maine, JAX:002374), expressing polyoma middle T antigen in mammary luminal cells and driving malignant transformation of mammary epithelial cells. One of two mesenchymal-specific Tamoxifen-inducible Cre lines, either *Tnc*-CreER^{T2} to drive expression of CreER^{T2} in partial EMT cells or *Cdh2*-CreER^{T2} leading to the expression of CreER^{T2} in full EMT cells (Luond et al., 2021). These mouse lines are available from the lead contact by full MTA from University of Basel.

Note: Tenascin C (*Tnc*) expression occurs early, while N-cadherin (*Cdh2*) expression occurs late during the stagewise progression of EMT in mammary tumor cells (Figure 1A).

Combination of RC::FrePe, MMTV-Flpo, MMTV-PyMT and either Tnc-CreER^{T2} or Cdh2-CreER^{T2} in female mice leads to labeling of all breast cancer cells by mCherry expression and - upon Tamoxifen administration to the mice - an irreversible switch to GFP in cancer cells undergoing a partial EMT (Tnc-CreER^{T2}) or a full EMT (Cdh2-CreER^{T2}) (Figures 1A and 1B). For a detailed description of these two mouse strains, please refer to Lüönd and Sugiyama et al. (Luond et al., 2021). To induce CreER^{T2} activity required for mCherry to GFP color-switching of cells undergoing an EMT, mice are treated with 2 mg Tamoxifen injected intraperitoneally twice per week, i.e., Monday and Friday, at the onset of tumor growth at around 8–9 weeks of age and continuing until sacrifice when humane endpoints are reached by 13–14 weeks of age (Attalla et al., 2021; Guy et al., 1992) (Figure 1B).

Breeding of transgenic EMT lineage tracing mouse model

⌚ **Timing: 6–8 months**

To obtain quadruple transgenic females, we recommend the following breeding strategy using heterozygous/hemizygous mice as young as 6 weeks of age for all strains and breeding trios of one male and two females resulting in litters of 10 pups on average:

1. Cross the following strains to obtain various double-transgenic mice:
 - a. Cross MMTV-PyMT⁺ males with RC::FrePe⁺ females.

Note: 1/8 of the offspring will be MMTV-PyMT⁺/RC::FrePe⁺ males. We recommend initially setting-up 4 matings to obtain 4–8 MMTV-PyMT⁺/RC::FrePe⁺ males for breeding in 1–2 breeding cycle.

- b. Cross the MMTV-Flpo strain with the Tnc-CreER^{T2} or Cdh2-CreER^{T2} strain.

Note: 1/8 of the offspring will be MMTV-Flpo⁺/Tnc-CreER^{T2+} or MMTV-Flpo⁺/Cdh2-CreER^{T2+} females, respectively. We recommend initially setting-up 4 matings each to obtain 4–8 MMTV-Flpo⁺/CreER^{T2+} females in 1–2 breeding cycle.

2. Combine all four alleles by crossing MMTV-PyMT⁺/RC::FrePe⁺ males × MMTV-Flpo⁺/Tnc-CreER^{T2+} or MMTV-Flpo⁺/Cdh2-CreER^{T2+} females.

Note: 1/32 of the offspring will be Tnc-CreER^{T2+}/MMTV-Flpo⁺/RC::FrePe⁺/MMTV-PyMT⁺ or Cdh2-CreER^{T2+}/MMTV-Flpo⁺/RC::FrePe⁺/MMTV-PyMT⁺ females that can be used for lineage tracing experiments. We recommend setting up at least 2–4 matings in parallel to obtain one quadruple transgenic female in 1–2 breeding cycles.

3. After these crosses, in addition to the matings described in step 2, the following crosses can be set-up: MMTV-PyMT⁺/MMTV-Flpo⁺/Tnc-CreER^{T2+} or MMTV-PyMT⁺/MMTV-Flpo⁺/Cdh2-CreER^{T2+} males × RC::FrePe⁺ females.

Note: 1/32 of the offspring will be quadruple positive females that can be used for lineage tracing experiments. Once the lines are established, we recommend keeping 4–6 breeding pairs or trios per line and replacing them every 3–6 months.

Note: For genotyping, please refer to Lüönd and Sugiyama et al. (Luond et al., 2021).

Note: The MMTV-PyMT allele must be maintained by crossing male mice, as MMTV-PyMT⁺ females develop mammary tumors at the onset of puberty. MMTV-PyMT⁺ males occasionally develop salivary gland tumors between 20–30 weeks of age.

Note: Offsprings with other allele combinations can be mated and we have done so sporadically in the past. However, it is advisable to keep the CreER^{T2} and Flpo alleles separately from the RC::FrePe allele to exclude the possibility of rare germline recombination.

Note: Tnc-CreER^{T2} and Cdh2-CreER^{T2} are knock-in alleles resulting in heterozygous loss of Tnc and Cdh2, respectively, and should be kept strictly heterozygous. While the RC::FrePe allele (ROSA26 locus) can be bred homozygous, the integration sites of MMTV-PyMT and MMTV-Flpo are unknown. Therefore, we kept all alleles heterozygous/hemizygous. After breeding the mice for more than 10 generations, we did not observe any striking differences in mating efficiency and outcome between mice with different allele combinations.

Labeling partial and full EMT in vivo

⌚ **Timing:** 3–4 weeks

4. Starting at 6 weeks of age monitor onset of tumor growth at least twice per week.
5. At 9 weeks of age – or before if tumor size reaches 200 mm³ – initiate Tamoxifen treatment twice per week:
 - a. Prepare Tamoxifen and/or warm up to 37°C.
 - i. Weigh 20 mg of Tamoxifen in a 2 mL Eppendorf tube, add 100 µL of ethanol, briefly vortex at max. intensity and spin down.
 - ii. Add 900 µL of sunflower seed oil and vortex briefly at maximum intensity. Sunflower seed oil was chosen for best availability and reproducibility. Other food oils may work as well.

Note: Tamoxifen is highly lipophilic and therefore dissolved in oil. Corn oil and peanut oil (available e.g., from Sigma-Aldrich) are suitable alternatives for sunflower seed oil.

- iii. Mix at 1000 rpm in a horizontal shaker at 37°C until Tamoxifen is completely dissolved. Vortex intermittently to speed up the process.

Note: Store Tamoxifen solution protected from light at 4°C for up to one week. Warm-up to 37°C before injection to reduce viscosity.

Note: Tamoxifen is a hazardous substance. Review the MSDS, wear PPE and dispose of left-over or accidental spills appropriately.

- b. Inject 100 µL Tamoxifen solution intraperitoneally.

Note: Tamoxifen solution is viscous, do not exert too much pressure. We recommend using 26 G needles and 1 mL syringes with low friction.

- c. Monitor tumor growth daily and prepare to sacrifice when humane endpoints (here max. cumulative tumor size of 1500 mm²) are reached, usually between 13–14 weeks of age of the mice.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Purified anti-mouse CD16/32 (1:100 dilution)	BioLegend	Cat# 101302, RRID: AB_312801
Biotin anti-mouse CD31 (1:100 dilution)	BioLegend	Cat# 102404, RRID: AB_312899
Biotin anti-mouse CD45 (1:100 dilution)	BioLegend	Cat# 103104, RRID: AB_312969

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biotin anti-mouse TER-119/Erythroid Cells (1:100 dilution)	BioLegend	Cat# 116204, RRID: AB_313705
APC anti-mouse CD326 (Ep-CAM) (1:100 dilution)	BioLegend	Cat# 118213, RRID: AB_1134105
Brilliant Violet 650™ Streptavidin (1:100 dilution)	BioLegend	Cat# 405232
Alexa Fluor 647 anti-mouse CD31 (1:50 dilution)	BioLegend	Cat# 102416, RRID: AB_493410
Chemicals, peptides, and recombinant proteins		
Tamoxifen	Sigma	Cat# T5648
Sunflower seed oil	Sigma	Cat# S5007
Ethanol	Sigma	Cat# 51976
DMEM/F12	Thermo Fisher Scientific	Cat# 11039021
DMEM/F12	Sigma	Cat# D6421
DMEM high glucose	Sigma	Cat# D5671
Glutamine	Sigma	Cat# G7513
Penicillin-Streptomycin	Sigma	Cat# P4333
10 × Trypsin/EDTA	Sigma	Cat# T4174
FBS	Sigma	Cat# F7524
NU Serum	BD Biosciences	Cat# 355104
Antibiotic-antimycotic	Thermo Fisher Scientific	Cat# 15240062
Gentamicin	Sigma	Cat# G1272
Collagenase D	Roche	Cat# 11088858001
DNaseI	Roche	Cat# 11284932001
Streptavidin-conjugated Dynabeads	Thermo Fisher Scientific	Cat# 11047
Draq7	BioStatus	Cat# DR71000
PBS	Sigma	Cat# D8537
4% paraformaldehyde (PFA)	Roth AG	Cat# P087.1
Tissue-Tek O.C.T compound	Sakura	Cat# 4583
Low temperature gelling agarose	Sigma	Cat# A9045
RNase ZAP	Sigma	Cat# R2020
DNA decontamination reagent	Sigma	Cat# 43944
Triton X-100	Sigma	Cat# T9284
Recombinant RNase Inhibitor	Takara Bio	Cat# 2313B
RNasin Plus (40 U/ μL)	Promega	Cat# N2615
Nuclease free water	Invitrogen	Cat# 750023
dNTP mix (10 mM each)	Thermo Fisher Scientific	Cat# R0192
Betaine	Sigma	Cat# 61952
Magnesium chloride	Sigma	Cat# M8266
DTT	Sigma	Cat# D9779
AMPure XP beads	Beckman Coulter	Cat# A63881
EB solution	QIAGEN	Cat# 19086
PicoGreen	Thermo Fisher Scientific	Cat# P7581
Critical commercial assays		
EasySep™ Dead Cell Removal (Annexin V) Kit	STEMCELL Technologies	Cat# 17899
SuperScript II Reverse Transcriptase	Thermo Fisher Scientific	Cat# 18064014
KAPA HiFi HotStart ReadyMix	Roche	Cat# KK2601
Agilent HS NGS Fragment Kit	Agilent Technologies	Cat# NF-474-0500
Nextera XT DNA library preparation kit	Illumina	Cat# FC-131-1096
Oligonucleotides		
TSO: Biot-AAGCAGTGGTATCAACGCAGAGTACAT rGrG+G (rG=riboinosine; +G=LNA-modified guanosine)	(Picelli et al., 2014)	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligo-dT30VN: Biot-AAGCAGTGGTATCAACGCAGA GTACT30VN	(Picelli et al., 2014)	N/A
ISPCR oligo: Biot-AAGCAGTGGTATCAACGCAGAGT	(Picelli et al., 2014)	N/A
Deposited data		
Raw and analyzed data	(Luond et al., 2021)	GEO database under GSE171940 (https://www.ncbi.nlm.nih.gov/geo/browse/)
Script for drift correction	This paper	DOI: 10.5281/zenodo.6103102
Experimental models: Organisms/strains		
Mouse: Tg(MMTV-PyVT)634Mul	(Guy et al., 1992)	JAX:002374
Mouse: B6;129S6 - Gt(ROSA)26Sortm8(CAG-mCherry,-EGFP)Dym/J	(Bang et al., 2012; Engleka et al., 2012)	JAX:029486
Mouse: MMTV-Flpo FVB/N-Tg(MMTV-Flpo)9Gcr	(Luond et al., 2019)	N/A *
Mouse: Tnc-CreERT2 129S6-Tnctm1(cre/ERT2)Gcr	(Luond et al., 2021)	N/A *
Mouse: Cdh2-CreERT2 129S6-Cdh2tm1(cre/ERT2)Gcr	(Luond et al., 2021)	N/A *
Mouse: Tnc-CreERT2/MMTV-Flpo/RC::FrePe/MMTV-PyMT (FVB/N background)	(Luond et al., 2021)	N/A *
Mouse: Cdh2-CreERT2/MMTV-Flpo/RC::FrePe/MMTV-PyMT (FVB/N background)	(Luond et al., 2021)	N/A *
Experimental models: Cell lines		
Cell line: mCherry ⁺ Tnc-CreERT2/MMTV-Flpo/RC::FrePe/MMTV-PyMT Recommended passage number: below 20.	(Luond et al., 2021)	N/A *
Cell line: GFP ⁺ Tnc-CreERT2/MMTV-Flpo/RC::FrePe /MMTV-PyMT (GFP-expression induced by in-vitro 4-hydroxytamoxifen and TGFβ treatment) Recommended passage number: below 20.	(Luond et al., 2021)	N/A *
Software and algorithms		
ImageJ software	(Schneider et al., 2012)	https://imagej.nih.gov/ij/
Arivis Vision4D software	arivis AG	https://imaging.arivis.com/en/imaging-science/arivis-vision4d
VisiView software	Visitron Systems	https://www.visitron.de/products/visiviewr-software.html
Other		
Peel-A-Way histology square mold	Ted Pella, Inc.	Cat# 27110
Millicell Cell Culture inserts, PTFE, pore size 0.4μm	Millipore	Cat# PICM01250
24-well imaging plate	ibidi	Cat# 82406
Vibrating blade microtome	Leica	VT1200S
Spinning disk confocal microscope	Visitron Systems	N/A
Yokogawa Spinning Disk	Yokogawa	CSU-W1-T2
Stage top incubator	Okolab	N/A
Centrifuge	Eppendorf	5702
Rotor for centrifuge	Eppendorf	A-4-38
BD FACS Aria™ Illu sorter	BD Biosciences	Aria™ Illu
BD falcon cell strainers 70 μm	Falcon	Cat# 352350
EasySep Magnet	STEMCELL Technologies	Cat# 18000
Round-bottom polystyrene test tubes with cap	Falcon	Cat# 352054
Round-bottom polypropylene test tubes with cap	Falcon	Cat# 352063
MicroAmp® Fast 96-Well Reaction Plate	Thermo Fisher Scientific	Cat# 4346906

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
ABsolute qPCR Plate Seals	Thermo Fisher Scientific	Cat# AB1170
Mosquito HV liquid-handling robot	SPT Labtech	N/A
Agilent 2100 Bioanalyzer Instrument	Agilent	N/A
Illumina NovaSeq 6000 system	Illumina	N/A
Magnetic stand 96	Ambion	Cat# AM10027

* Mouse lines and cell lines will be shared by the [lead contact](#) upon request under a completed MTA with the University of Basel.

MATERIALS AND EQUIPMENT

DMEM/F12 medium

Reagent	Final concentration	Amount
DMEM/F12 (Sigma)	N/A	500 mL
Glutamine	2 mM	5 mL
Penicillin-Streptomycin	100 U/mL/100 µg/mL	5 mL
Total		510 mL

Prepare in a sterile environment, store at 4°C for up to 6 months.

DMEM/F12 medium for breast tumor slice tissue (stock medium)

Reagent	Final concentration	Amount
DMEM/F12 (Thermo Fisher Scientific)	N/A	500 mL
Glutamine	2 mM	5 mL
Penicillin-Streptomycin	100 U/mL/100 µg/mL	5 mL
Total		510 mL

Prepare stock medium without FBS, store at 4°C for up to 6 months.

DMEM/F12 medium for breast tumor slice tissue (completed medium)

Reagent	Final concentration	Amount
Stock medium		45 mL
FBS	10%	5 mL
Total		50 mL

On the day of the experiment, FBS is added to the medium. The completed medium is stored at 4°C for up to 1 week.

Note: To avoid the background signal during imaging, Phenol red-free DMEM/F12 medium (Thermo Fisher Scientific, Cat# 11039021) is used for slice tissue culture.

DMEM high glucose medium

Reagent	Final concentration	Amount
DMEM high glucose	N/A	500 mL
Glutamine	2 mM	5 mL
Penicillin-Streptomycin	100 U/mL/ 100 µg/mL	5 mL
FBS	10%	50 mL
Total		560 mL

Prepare in a sterile environment, store at 4°C for up to 4 months.

Collagenase/trypsin solution

Reagent	Final concentration	Amount
DMEM/F12 medium	N/A	9.1 mL
Collagenase D	1 mg/mL	100 μ L
Trypsin/EDTA	4%	400 μ L
NU Serum	2.5%	250 μ L
Gentamicin	10 μ g/mL	10 μ L
Antibiotic-antimycotic	1%	100 μ L
Total		10 mL

Prepare in a sterile environment before use.

DNase solution

Reagent	Final concentration	Amount
DMEM/F12 medium	N/A	9.8 mL
DNaseI	0.1 mg/mL	100 μ L
Gentamycin	10 μ g/mL	10 μ L
Antibiotic-antimycotic (100 \times)	1 \times	100 μ L
Total		10 mL

Prepare in a sterile environment before use.

FACS buffer

Reagent	Final concentration	Amount
PBS	N/A	48.8 mL
FBS	2%	1 mL
EDTA (0.5 M stock solution)	2 mM	200 μ L
Total		50 mL

Prepare in a sterile environment, store at 4°C for up to 1 month.

Dead Cell Removal buffer

Reagent	Final concentration	Amount
PBS	N/A	4.9 mL
FBS	2%	100 μ L
CaCl ₂ (1 M stock solution)	1 mM	5 μ L
Total		5 mL

Prepare in a sterile environment, store at 4°C for up to 1 month.

Lysis buffer

Reagent	Final concentration	Amount
PBS	N/A	100 mL
TX-100	0.2%	200 μ L
Total		100 mL
PBS/TX-100 solution	N/A	475 μ L
RNase inhibitor	2 U/ μ L	25 μ L
Total		500 μL

Prepare TX-100/PBS solution in a sterile environment, autoclave, aliquot at 475 μ L and store at 4°C for up to 6 months. Before use, add RNase inhibitor and keep on ice.

STEP-BY-STEP METHOD DETAILS

Part 1

Tissue harvesting

⌚ Timing: 30 min

This section details the collection of mammary tumors for individual downstream analysis.

1. Sterilize surfaces, forceps, scalpels, and surgical scissors with 70% ethanol.
2. Euthanize the mouse using CO₂
3. Dissect mammary tumors:
 - a. Place mouse on its back, stretch-out and pin-down on all four limbs.
 - b. Spray the abdomen with 70% ethanol and wipe-off excess liquid.
 - c. Use forceps and a surgical scissor to make an incision extending from the bottom of the abdomen to the thorax. Extend the incision along the limbs to create an x-shape.

Note: Avoid piercing the peritoneum as the mammary tumors are located between the skin and the peritoneum.

- d. Separate the skin from the peritoneum to expose the tumors. Using two forceps, gently pull the skin to the sides and use a scalpel to separate it from the abdomen if necessary. Pin down the stretched-out skin.
- e. Using a scalpel, dissect all individual mammary tumors and the surrounding mammary fat pad.

△ CRITICAL: Early-stage nodules may be peeled off the skin by gentle pulling. If tumors have grown into the skin, do not forcefully detach them, but collect a wider range of tissue to contain disseminated cancer cells.

Note: A tail or ear biopsy may be collected from the animal to verify the genotyping results.

4. Continue with downstream processing:
 - a. Prepare paraffin and/or OCT frozen tissue blocks with 1–2 pieces (3–5 mm in diameter) of tumors from individual mammary tumors as described in the study by Lüönd and Sugiyama et al. (Luond et al., 2021). These blocks can be stored at room temperature (around 25°C) and at –80°C, respectively, and used for staining later.
 - b. For the tumor slice culture and/or single-cell isolation, follow the protocols described below in [part 2](#) and [part 3](#), respectively.

Note: MMTV-PyMT mice develop multiple tumors in all mammary fat pads. Early and late-stage lesions will be present at harvest. To account for tumor heterogeneity, collect tumors from multiple locations (e.g., center and periphery as well as solid and soft areas) for sliced tissue culture and for 5 cell RNAseq.

Part 2

Tumor slicing

⌚ Timing: 1 h

This section describes the preparation of mammary tumor slices for live-cell imaging using a vibrating blade microtome instrument such as the Leica VT 1200S vibratome ([Figure 2](#)).

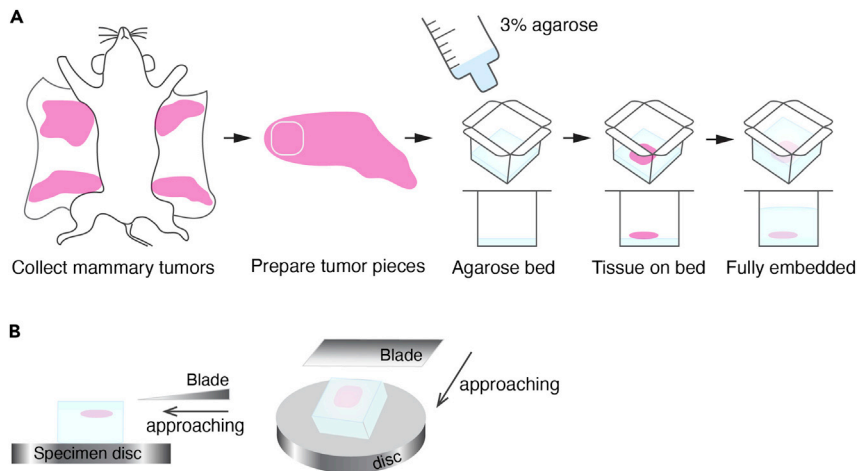


Figure 2. Preparation of agarose blocks for tissue slicing using a vibratome

(A) Schematic illustration of the preparation of fresh tissue-embedded agarose blocks for tumor slicing. Mammary tumors are collected from transgenic EMT lineage tracing mouse models. 3–5 tumor pieces (around $5 \times 5 \times 5$ mm in size) are collected from individual tumors and kept on ice. Using a 10 mL syringe, 3% agarose solution is poured into a Peel-A-Way histology mold to make a thin layer of agarose (agarose bed). Once agarose has solidified, a piece of tumor is placed on the agarose bed and agarose solution is added over the top of tumor piece in the mold until the tissue is entirely covered (fully embedded).

(B) Schematic illustration of the relative positions of the sample on specimen disc and a blade as well as approaching direction of the blade for tissue slicing using a vibratome. Of note, the location of the tumor piece embedded in the agarose gels should be in close proximity to the blade to increase the stability during slicing.

5. Using a scalpel, collect 3 to 5 small pieces of tumors (3–5 mm diameter) from individual mammary tumors. Gently remove excess adipose tissue from the tumor pieces and keep them in DMEM/F12 medium placed on ice.
6. Embed tumors in 3% low-melting agarose (Figure 2A):
 - a. Using a 10 mL syringe, put warm ($\approx 37^\circ\text{C}$) 3% low-melting agarose solution (in sterile PBS, $\approx 1\text{--}2$ mL) in a Peel-A-Way histology mold to make a thin layer of agarose.
 - b. Place the mold on ice. Agarose will solidify in 5 min.
 - c. Once agarose has solidified, place one piece of tumor on top of agarose. The mold should be kept on ice.
 - d. Add agarose solution ($\approx 5\text{--}6$ mL) over the top of tumor piece in the mold until the tissue is entirely covered.
 - e. Cover with aluminum foil and keep on ice until slicing.
7. Set up the Leica VT 1200S vibrating blade microtome:
 - a. Put a blade into blade holder with an Allen wrench.
 - b. Using an Allen wrench, rotate the blade to the clearance angle -18° .
 - c. Optimize the position of the blade using the VibroCheck.
 - d. After the optimization, secure the blade into the blade holder.
8. Pop the embedded tissue out of the mold.
9. Spray the specimen disc and buffer tray of the Leica VT 1200S vibratome with 70% ethanol and wipe-off excess liquid.
10. Glue the face of the upper side of the agarose block directly to the specimen disc. The embedded tumor that was at the bottom of the mold will now be on top (Figure 2B). The glue dries in 3–5 min.
11. Place the buffer tray into the ice holder of the Leica VT 1200S vibratome. Add ice into the ice holder and fill the buffer tray with ice-cold sterile PBS.
12. Once the sample embedded in agarose has completely adhered to the specimen disc, place the disc into the buffer tray.

13. Set the ice tray onto the vibratome stand and lock the tray in place by moving the lever to the right.
14. Using an Allen wrench, rotate the blade into cutting position with the clearance angle -18° .
15. Using the control pad, raise the stage up until the blade touches the surface of the agarose gel (Figure 2B).
16. Set the cutting conditions using the control pad:
 - a. Set cutting window.
 - b. Set section thickness (auto feed, 250–350 μm), amplitude (1–2 mm) and speed (0.1–1.0 mm/s).
17. Until the blade reaches the tissue, run the vibratome at 1.0 mm/s speed.
18. Adjust settings on the vibratome depending on the consistency and integrity of the tissue. In most cases, cut the tumors at amplitude 2 mm, speed 0.1 mm/s.
19. Once uniform slices are obtained, gently scoop them out of the bath using spatulas or forceps. Clean spatulas and forceps with 70% ethanol prior to use.
20. Transfer them into a 6-well plate containing 2–3 mL of ice-cold DMEM/F12 medium and place the plate on ice. Slices from one piece of tumor can be collected into the same well.

Note: Sliced tumors can be stored for up to 3 h on ice. However, it is recommended to keep them on ice for as short as possible.

Staining of the slices

⌚ Timing: 1 h

This section describes the staining of mammary tumor slices with fluorophore-conjugated antibodies for live-cell imaging.

21. Scoop up one slice from each piece of tumor using a sterile spatula. Place each one slice into one well of a 24-well plate.
22. Add 300 μL of pre-warmed DMEM/F12 medium for breast tumor slice tissue containing Alexa Fluor-conjugated antibody (for example, Alexa Fluor 647 anti-mouse CD31, 1:50) and incubate for 30 min at 37°C .

Note: Tissues can be incubated with a combination of fluor-conjugated antibodies based on your needs. We have not tested indirect immunofluorescence staining so far.

23. During the incubation time, prepare a plate for imaging the slice tissue culture. Place one Millipore Cell Culture insert into each well of a 24-well imaging plate using sterile forceps.
24. Pipette 300 μL of DMEM/F12 medium for breast tumor slice tissue into the well and 50 μL into the insert.
25. After staining, wash the tissue slices by transferring them twice through separate wells containing 500 μL of pre-warmed DMEM/F12 medium for breast tumor slice tissue using forceps.
26. Using forceps, gently transfer one slice onto one cell culture insert. Once transferred, use a spatula to gently spread the slice to ensure it lays completely flat on the cell insert membrane.
27. If necessary, remove excess media from the insert to the outer well to avoid floating and folding of the slice in the insert. Media will eventually equilibrate on its own. The tissue slices are kept at an interface of air and culture media.

⚠ CRITICAL: To avoid evaporation during imaging, use the middle rows in the plate for the slice tissue cultures and add sterile water or PBS to one row of wells on each of the four surrounding sides. If more than 8 wells are needed for culture, fill the spaces between wells with sterile water or PBS.

Live-cell imaging

⌚ Timing: 2–3 days

This section describes live-cell imaging of mammary tumor slices using a spinning disc confocal microscope (Visitron Systems) to track morphological and behavioral dynamics of partial and full EMT cells within the tumor microenvironment.

28. Switch on all components of the spinning disc confocal microscope and turn on the computer.
29. Set up the incubation chamber (Okolab) at 37°C with 5% CO₂, 90 ± 5% humidity at least 30 min prior to imaging.
30. Switch on the individual lasers by turning on the keys.
31. Start VisiView Premier Image acquisition Software.
32. Place the imaging plate containing the tissue culture inserts on the microscope stage.
33. Set up the live-cell imaging using a spinning disc confocal microscope with a CFI Plan Apo 20×, 0.75 NA objective, a Hamamatsu Flash 4.0 V2 CMOS camera, a Yokogawa Spinning Disk.
 - a. Open “Acquisition” panel, set up the exposure time for every channel and pixel binning as “2”.
 - b. Go to “Wavelength” tab and tick “Wavelength series” box. Select the number of channels and lasers that should be active during acquisition.
 - c. Go to “Time-lapse” tab and tick “time-lapse series” box. Set up the time interval and the duration of the acquisition. For tissue slices, we set up 1–2.5 h interval for up to 3 days.
 - d. Turn on the Perfect Focus System (PFS) controller.
 - e. To maintain the focus, tick “Autofocus always on” box and select “Autofocus on during waiting” option.

Note: PFS automatically maintains microscope focus on the region of interest during live-cell imaging. However, it can interfere with the acquisition of Z-stacks as it will start racing after the piezo stage if it is active during Z-stack acquisition. Therefore, PFS should be active before scanning each setting point and off during Z-stack acquisition.

- f. Go to “Z-series” tab and tick the “Z-plane series” box. Manually define a plane of interest to set “Home position” and then set “Top Offset” and “Bottom Offset” for region of interest.
- g. Go to “Stage” tab and set multiple XYZ points. We recommend to set 2–4 positions per slice to obtain images of different regions of the tumor.
- h. Save the setting for multidimensional live-cell imaging. These can be re-loaded for use at a later time.
34. Click “sequence” to start live-cell imaging of the slices.

⚠ CRITICAL: During long-term imaging of the tissue, pause the image acquisition at every 24 h and carefully replace the medium in the wells with fresh pre-warmed medium using a 200 µL pipette with long tips. Do not touch the slices in the cell culture inserts.

Note: Examples of live cell imaging of early and late EMT mammary tumor cells are shown in [Methods video S1](#) (Representative time lapse movie of mammary tumor slice tissue culture with migrating GFP⁺ partial EMT tumor cells) and [Methods video S2](#) (Representative time lapse movie of mammary tumor slice tissue culture with migrating GFP⁺ full EMT tumor cells).

Image processing

⌚ Timing: 1–2 days for one slice

This section describes image processing and data analysis of live-cell imaging using ImageJ (ImageJ, U. S. National Institutes of Health) and Arivis Vision4D software (arivis AG). Timings of image processing are for one tumor slice for an experienced researcher.

35. Open the script (DOI: [10.5281/zenodo.6103102](https://doi.org/10.5281/zenodo.6103102)) to correct three-dimensional positions on ImageJ software.
36. Press "Run".
37. Browse the stk file for drift correction. Set up Z spacing (μm), number of timepoints, number of slices, first position (S) to process, number of positions, name of the different channels, and name of the channel of reference for the registration. We used mCherry as a reference channel for drift correction.
38. Press "OK" to start the correction. The script corrects the focus drift and, at the same time, makes a movie for each XYZ position. The movies are automatically saved in the folder where the original files are.
39. Open the movies on Arivis Vision4D software.
40. Visually inspect the XYZ position and manually adjust it using "drift correction" from "Data" sub-menu if necessary.
41. Track the migration of individual GFP-expressing cells that have undergone either partial or full EMT in tumors.
 - a. Crop the region of interest using "transformation gallery" from "Data" sub-menu.
 - b. Activate the "Track Cells and Particles" pipeline from Analysis panel.
 - c. Select GFP channel for tracking in "Input ROI" panel.
 - d. Set up diameter, probability threshold, and split sensitivity in "Blob finder" panel. Check the values by executing the pipeline using step-by-step tool.
 - e. Define the minimum volume of the blob filter.
 - f. In "Tracking" panel, select "Brownian motion (Centroid)" in "Motion type". Set max distance $15 \mu\text{m}$ per hour. Tick "Fusions" and "Divisions" boxes to follow a cell even if this cell is fused with another cell or divides simultaneously.
 - g. Check the all settings using step-by-step tool.
 - h. Run the pipeline.
 - i. Open "Object" panel. Select the features which should be considered during tracking.
 - j. Export the results as excel file.
42. Plot the trajectories of GFP⁺ cells in tumors on excel. Make a scatter plot with smooth line chart using values of X and Y displacement to start (μm).

Part 3

Preparation of single-cell suspension for flow cytometry

⌚ Timing: 4 h

This section describes the enzymatic digestion of the tumor samples to obtain single cell suspensions from murine mammary tumors. Timings of the protocols in [part 3](#) are adjusted for processing the pooled tissue of all mammary tumors and the adjacent mammary fat pad from one mouse (1–2 mg wet weight). If processing samples from multiple mice, the timing will increase accordingly.

Note: We suggest selecting tumor pieces from multiple individual tumors with different morphology to account for tumor heterogeneity for sliced tissue culture and frozen tissue/paraffin embedding. The remaining tumor tissue and surrounding mammary fat pad is then pooled for digestion and single cell isolation for RNA sequencing. Hence several tumor samples are being used per mouse to generate sufficient single cells for RNA sequencing.

Note: The maximum number of mice to handle at the same time is 2 for an experienced researcher to avoid compromising the quality and viability of the cells.

△ CRITICAL: For negative controls and single stain control samples required for compensation, collect the mammary tumors from MMTV-PyMT, MMTV-PyMT/RC::FrePe/Tnc-CreER^{T2} or MMTV-PyMT/RC::FrePe/Cdh2-CreER^{T2} mice and prepare single-cell suspensions. In these mice, none of the cells express mCherry or GFP. Negative control cells can be frozen in FBS containing 10% DMSO as cryomedium and stored in liquid nitrogen.

43. Prepare 10 mL collagenase/trypsin solution and DNase solution per mouse and store it on ice.

Note: Once tumor pieces were selected for sliced tissue culture and OCT/paraffin block preparation, pool all remaining mammary tumors from an individual mouse. Do not remove adipose tissue (mammary fat pad) surrounding tumors as it may contain early-stage lesions and disseminated cells.

44. Place the mammary tumor tissue on the lid of a sterile petri dish and cut it into small pieces (<1 mm) using a scalpel with a sterile blade.
45. Transfer the chopped-up tissue into a 50 mL Falcon tube containing 10 mL of collagenase/trypsin solution and incubate at 37°C while shaking at 200 rpm for 1 h.
46. Centrifuge the tube at 200 × g for 5 min at room temperature (RT).
47. Discard the supernatant by carefully decanting the content of the tube.
48. Add 10 mL of DNase solution and tilt the tube up and down few times for 5 min at RT.
49. Centrifuge the tube at 200 × g for 5 min at RT.
50. Discard the supernatant by decanting the content of the tube.
51. Suspend the pellet in 20–40 mL of ice-cold FACS buffer and filtrate into a 50 mL Falcon tube using a 70 μm sterile cell strainer.

Note: After digestion, the cell suspension will still contain small pieces of tumors. Using a plunger of a 5 mL syringe, smash the pieces of tumor on the strainer and wash the strainer with FACS buffer several times.

52. Determine the total number of cells manually using a hemocytometer.
53. Centrifuge the tube at 200 × g for 5 min at RT.
54. Discard the supernatant and prepare single-cell suspension at 1×10^6 – 1×10^7 cells/mL in ice-cold FACS buffer.
55. Remove immune cells, endothelial cells and red blood cells:
 - a. Add blocking antibody (CD16/32, 1:100) and incubate for 5 min on ice.
 - b. Add 5 mL of ice-cold FACS buffer.
 - c. Centrifuge the tube at 200 × g for 5 min at RT.
 - d. Discard the supernatant and resuspend the pellet in ice-cold FACS buffer maintaining the cell suspension at 1×10^6 – 1×10^7 cells/mL.
 - e. Add biotin-conjugated CD31, CD45, and Ter-119 antibodies (1:100 dilution for each) and incubate on ice for 20 min.
 - f. Add 5 mL of ice-cold FACS buffer.
 - g. Centrifuge the tube at 200 × g for 5 min at RT.
 - h. Discard the supernatant and resuspend the pellet in ice-cold FACS buffer to reach a cell concentration of 1×10^7 cells/mL. Transfer the cell suspension into a 5 mL polystyrene tube.
 - i. Wash 100 μL of Dynabeads biotin binder in 1 mL of FACS buffer, place into an EasySep Magnet and discard the supernatant. The beads are then resuspended in 100 μL of FACS buffer.
 - j. Add 100 μL pre-washed Dynabeads per up to 10^7 total cells. If cell number is bigger than 10^7 cells, scale up all reagent volumes accordingly.
 - k. Mix well and incubate on ice for 30 min.
 - l. Place the tube into an EasySep Magnet for 3 min and collect the supernatant by pouring it into a 15 mL falcon tube.
 - m. Centrifuge the tube at 200 × g for 5 min at RT.

56. Remove dead cells using EasySep™ Dead Cell Removal (Annexin V) Kit:
 - a. Remove the supernatant and resuspend the pellet in ice-cold Dead Cell Removal buffer at 1×10^8 cells/mL. Transfer the suspension into a 5 mL polystyrene tube.
 - b. Add 50 μ L of Dead Cell Removal (Annexin V) Cocktail and 50 μ L of Biotin Selection Cocktail to 1 mL of cell suspension. If the amount of cell suspension is bigger than 1 mL, scale up all reagent volumes accordingly.
 - c. Incubate for 3 min at room temperature.
 - d. During the incubation, vortex RapidSpheres™ for 30 s.
 - e. Add 100 μ L of RapidSpheres per 1 mL of sample and immediately add Dead Cell Removal buffer to top up the sample to 2.5 mL.
 - f. Place the tube into the EasySep Magnet for 3 min.
 - g. Pour the live-cell enriched supernatant into a 15 mL Falcon tube.
 - h. Centrifuge the tube at $200 \times g$ for 5 min at RT.

Note: Steps 55 and 56 can be switched.

57. Stain with Fluorophore-conjugated antibodies.
 - a. Remove the supernatant and resuspend the pellet in FACS buffer to adjust the cell suspension to 1×10^6 cells/100 μ L.
 - b. Add 1 μ L each of APC anti-mouse CD326 (EpCAM) and Brilliant Violet 650™ (BV650) Streptavidin per 100 μ L suspension. If the amount of cell suspension is bigger than 100 μ L, scale up antibody volumes accordingly.
 - c. Incubate for 20 min on ice.
 - d. Add 1 mL of FACS buffer and centrifuge at $200 \times g$ for 5 min.
 - e. Remove the supernatant and resuspend the pellet in 5–15 mL FACS buffer.

Note: Consult with the FACS facility or operator regarding the concentration and volumes of the samples.

- f. Store the samples on ice in the dark until FACS sorting.

58. At the same time, prepare the experimental control samples.
 - a. Prepare experimental control samples in the following tubes:
 - i. Non-stained control: unstained sample cells.
 - ii. Draq7: sample cells stained with Draq7.
 - iii. APC-FMO: sample cells stained with BV650 Streptavidin and Draq7.
 - iv. BV650-FMO: sample cells stained with APC EpCAM and Draq7.

Note: Draq7 is added to the samples immediately before loading onto the FACS sorter to stain dead/damaged cells.

- b. Aliquot 50 μ L of sample cell suspension for each control samples.
- c. Add 0.5 μ L of Brilliant Violet 650™ Streptavidin or APC anti-mouse CD326 (Ep-CAM) in tube iii and tube iv, respectively.
- d. Incubated on ice for 20 min.
- e. Add 1 mL of FACS buffer to tubes i-iv and centrifuge at $200 \times g$ for 5 min. Remove the supernatant and resuspend the pellet in 200 μ L FACS buffer and store on ice in the dark until sorting.

59. Prepare compensation control samples.
 - a. Prepare compensation control samples in the following tubes:
 - i. Negative control: unstained negative control cells.
 - ii. Draq7: negative control cells stained with Draq7.

- iii. APC: negative control cells stained with APC EpCAM.
- iv. BV650: negative control cells stained with BV650 Streptavidin.
- v. mCherry: single-cell suspension prepared from a mCherry⁺ cell line.
- vi. GFP: single-cell suspension prepared from a GFP⁺ cell line.
- b. Prepare single-cell suspension of negative control cells at 1×10^6 cells/100 μ L and aliquot 50 μ L suspension into four microcentrifuge tubes (i-iv). Single-cell suspension of negative control cells prepared from the mammary tumors of MMTV-PyMT, MMTV-PyMT/RC::FrePe/Tnc-CreERT2 or MMTV-PyMT/RC::FrePe/Cdh2-CreERT2 mice should be used. These cells should not contain any mCherry or GFP-expressing cells.
- c. Add 0.5 μ L of APC anti-mouse CD326 (Ep-CAM) or Brilliant Violet 650™ Streptavidin in tubes iii and iv, respectively.
- d. Incubate on ice for 20 min.
- e. Add 1 mL of FACS buffer to tubes i-iv and centrifuge at $200 \times g$ for 5 min at RT. Remove the supernatant and resuspend the pellet in 200 μ L FACS buffer and store on ice in the dark until sorting.
- f. For tube v and vi trypsinize mCherry⁺ and GFP⁺ cell lines to prepare single-cell suspension.

Note: These cell lines were previously established by isolating mCherry⁺ and GFP⁺ breast cancer cells derived from tumors in Tnc-CreERT2/MMTV-Flpo/RC::FrePe/MMTV-PyMT mice. Wash cells once with PBS, add trypsin and incubate at 37°C until cells detach.

- g. After trypsinization, add 10 mL of complete DMEM high glucose medium and centrifuge at $200 \times g$ for 5 min at RT.
- h. Remove the supernatant and resuspend the pellet in 200 μ L–1 mL of FACS buffer depending on the size of the pellet.
- i. Store tube v and vi on ice in the dark until sorting.

Note: Draq7 is added to the samples immediately before loading onto the FACS sorter to stain dead/damaged cells.

5-Cell sorting of partial and full EMT subpopulations using flow cytometry

⌚ Timing: 4–8 h

This section describes the process of 5-cell sorting of subpopulations of partial and full EMT cells using Becton-Dickinson's FACS Aria IIIc. In our partial and full EMT lineage tracing mouse models, mCherry labels breast cancer cells and GFP labels breast cancer cells that have ever undergone either partial or full EMT. Cells expressing both mCherry and GFP are cells just undergoing an EMT (transitioning cells). In this protocol, based on the expression of GFP or mCherry in combination with the epithelial marker EpCAM expression, we focus on the sorting of breast cancer cells in different stages of the EMT spectrum for further RNA sequencing.

Note: All the steps from this section should be performed at 4°C.

⚠ CRITICAL: The time from tissue harvesting to 5-cell sorting should be kept as short as possible. Total processing times will depend on the abundance of the target populations, but we recommend to process no longer than 12 h for harvesting healthy cells for RNA sequencing.

- 60. Filtrate the experimental sample and all control samples through a 40 μ m mesh into 5 mL polystyrene tubes.

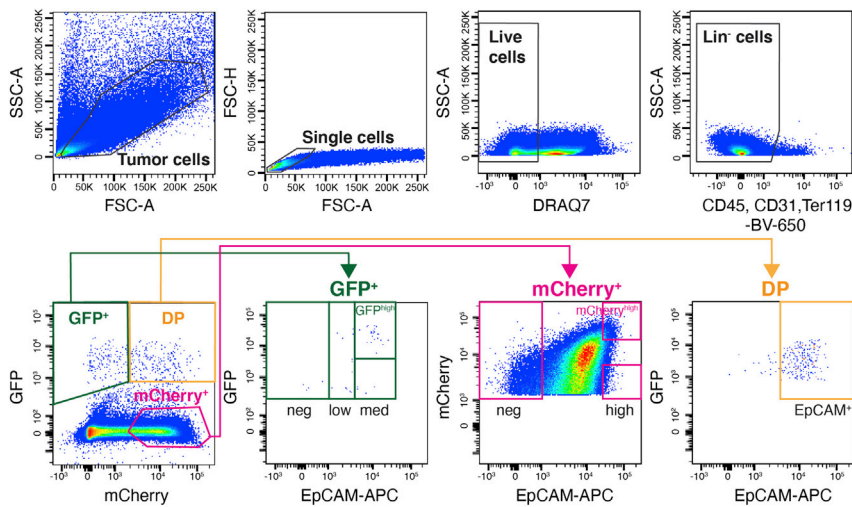


Figure 3. Gating strategy for the sorting of GFP⁺ partial and full EMT cells derived from mammary tumors by flow cytometry

Schematic representation of the gating strategy for flow cytometry used to sort GFP⁺ tumor cells from Tnc-CreER^{T2} and Cdh2-CreER^{T2} mice treated with Tamoxifen. Tumor cells are separated from debris by Forward Scatter Area (FSC-A)/Side Scatter Area (SSC-A), and doublets and aggregates are excluded by FSC-A and FSC-height (FSC-H) dot plots. Living Lin⁻ cells are selected by gates on the negative fractions for Draq7 and BV650 Streptavidin (biotinylated-CD31, CD45, and Ter119). Further setting the gates, 4 populations of GFP⁺ cells (GFP⁺EpCAM⁺, GFP⁺EpCAM^{low}, GFP⁺EpCAM^{med} and GFP^{high}EpCAM^{med}), 3 populations of mCherry⁺ cells (mCherry⁺EpCAM⁺, mCherry⁺EpCAM^{high} and mCherry^{high}EpCAM^{high}), and 1 population of double-positive cells (mCherry⁺GFP⁺EpCAM⁺) are isolated.

61. Add Draq7 to the experimental sample and Draq7 containing controls. 1 μ L of Draq7 is added per 200 μ L of cell suspension.
62. Start the FACSaria IIIc sorter.
63. Choose a nozzle size of 100 μ m.
64. Set up the side stream and optimize the position of the side streams for 4-way sort using the voltage sliders.
65. Set up the laser and the following filters:
 - a. Draq7 (Laser: Red 633 nm, Mirror: 735LP, Filter: 780/60).
 - b. APC (Laser: Red 633 nm, Filter: 660/20).
 - c. Brilliant Violet 650 (Laser: Violet 405 nm, Mirror: 635LP, Filter: 670/30).
 - d. mCherry (Laser: Yellow/Green 561 nm, Mirror: 600LP, Filter: 610/20).
 - e. GFP (Laser: Blue 488 nm, Mirror: 502LP, Filter: 530/30).
66. Apply negative control (compensation tube step 59a (i)) to set baselines for all channels.
67. Analyze compensation controls steps 59a (ii)–(vi) to select appropriate laser power and outputs for each of the fluorophores used. Calculate and apply compensation.
68. Set up the sorting gates using experimental control samples (Figure 3):
 - a. Load non-stained experimental control step 58a (i) onto the FACSaria IIIc and verify that the sample cells contain mCherry and GFP-expressing cells.
 - i. Set up the gating by selecting the cellular compartment in the Forward Scatter Area (FSC-A) versus Side Scatter Area (SSC-A) dot plot.
 - ii. Exclude doublets and aggregates using FSC-A and FSC-height (FSC-H).
 - iii. Set up gating for the negative fraction for Draq7.
 - iv. Set up a gate for the negative cells for BV650 Streptavidin (biotinylated-CD31, CD45, and Ter119).
 - b. Analyze Draq7 control (experimental control step 58a (ii)) to ensure the selection of live cells.
 - c. Analyze APC-FMO control (experimental control step 58a (iii)) to ensure that the gates for live Lin⁻ cells are set correctly.

- i. Set up gates for mCherry⁺, GFP⁺ and double positive (mCherry⁺ GFP⁺) cells.
- ii. Set up the gating for the EpCAM⁻ cells in each mCherry⁺, GFP⁺ and double positive populations.
- d. Analyze BV650-FMO control (experimental control step 58a (iv)) to ensure the gates for EpCAM⁻ cells.
 - i. Set up the gates for 3 populations of mCherry⁺ cells (mCherry⁺EpCAM⁻, mCherry⁺EpCAM^{high} and mCherry^{high}EpCAM^{high}), 4 populations of GFP⁺ cells (GFP⁺EpCAM⁻, GFP⁺EpCAM^{low}, GFP⁺EpCAM^{med} and GFP^{high}EpCAM^{med}) and 1 population of double-positive cells (mCherry⁺ GFP⁺EpCAM⁺) (Figure 3).
69. Open the sort collection chamber door and install 4 tubes of 5 mL polypropylene collection tubes containing 1 mL of FACS buffer on 4-way chamber.
70. Insert the experimental sample in the sample injection chamber of the sorter.
71. Start acquisition and verify that the gate settings are correct.
72. Set up the desired number of events to collect. Proceed with the sorting of 5K cells each for the 3 mCherry⁺ populations and the 1 mCherry⁺GFP⁺ double-positive population.

△ CRITICAL: Since GFP⁺ cells are very rare in the mammary tumors, collect as many cells as possible for each of the 4 populations of GFP⁺ cells from the rest of the sample.

Note: The sorted cells are further applied to 5-cell sorting. We assume that more than 50% of the sorted cells will be lost during this 2nd sorting step. In addition, the number of cells returned from the sorter could differ from the actual number of sorted cells. We recommend to set up the number of events for sorting 5–10 times bigger than of what is aimed at for collection of 5-cell pools, if possible.

Note: In the mammary tumors, we estimate only 0.13% and 0.002% of cancer cells to be partial and full EMT cells, respectively, that are marked as GFP⁺ cells. Due to the low number of GFP⁺ cells, around 3–6 h of sorting is needed for each population of GFP⁺ breast cancer cells.

73. After collecting the cells in polypropylene collection tubes, centrifuge at 200 × g for 5 min.
74. Remove the supernatant, but keep approximately 200 μL of liquid. Resuspend the cells in the collection tubes and keep them on ice in the dark.

Note: Consult with the FACS facility or operator regarding the volumes of the samples.

75. Optimize the position of the side streams for 96-well plates.
 - a. Set a MicroAmp® Fast 96-Well Reaction Plate with a transparent lid on the stage in the sorting chamber.
 - b. Select Sort > Home Device to access the Device Setup dialog.
 - c. Select the desired collection device. In this protocol, “96 well-Falcon”.
 - d. Click “Go to Home”. The stage moves to the pre-programmed Home position.
 - e. Start test sort. Inspect the plate lid to see where a drop was deposited.
 - f. Wipe the lid dry and place it back onto the stage.
 - g. Adjust the Home location if the drop did not hit the center of the well.
 - h. Repeat e to g until the drop is centered appropriately.
 - i. Click arrow buttons to move the tray.
 - j. Set up new Home position and check the location of drop deposition as above. We recommend to check one row of wells on each of the four sides of the plate.

Note: Large arrows move the tray by five steps. Small arrows move the tray by one step.

76. Clean a tissue culture hood with RNaseZap and DNA decontamination reagent before preparing a MicroAmp® Fast 96-Well Reaction Plate for 5-cell sorting. Clean pipettes with RNaseZap. Wipe-off excess liquid.
77. Pipette 2.3 μL of 0.2% TX-100 in PBS containing 2 U/ μL RNase inhibitor into each well of a 96-well plate and store it on ice. Cover plate with RNase free plate seal.

Note: Choose a type of 96-well plate that is compatible with robot for RNA sequencing library preparation.

78. Clean the stage in the sorting chamber of FACSAria IIIc with RNaseZap.
79. Install the plate with well A1 toward the front of the stage.

Note: Sorting starts on the front left corner of the device (A1), and proceeds from front to back, and then from left to right, sorting in a serpentine motion. Thus, for a plate, sorting proceeds from well A1–A12, B12–B1, C1–C12, and so on.

80. Insert the sample tube in the sample injection chamber of the sorter. In this protocol, 3 tubes for mCherry⁺, 4 tubes for GFP⁺ and 1 tube for double-positive populations will be sorted consecutively.
81. Select target wells for 5-cell sorting.
82. Start acquisition and sort 5 cells per well of the MicroAmp® Fast 96-Well Reaction Plate.
83. Repeat for every pre-sorted cell population.
84. After sorting, seal the plate with an adhesive plate seal and spin down.
85. Place the plate in a box containing dry ice. Store the plate at -80°C as soon as possible until preparing libraries for RNA sequencing.

Note: One sorting droplet for one cell is ranging from 0.001 μL to 0.005 μL . Therefore, total volume in one well would remain around 2.3 μL after sorting.

5-Cell RNA sequencing

⌚ **Timing:** 2–3 days

This section describes the major steps for the preparation of RNA sequencing libraries according to the Smart-seq2 method (Picelli et al., 2014) using the low-volume liquid-handling robot Mosquito HV system (SPT Labtech).

86. Spray the 96-well plate containing 5-cell pools and a cooling rack with RNaseZap and remove the seal. Place the plate on the rack on ice.
87. Prepare the annealing mixture by combining (per 96-well plate) 90 μL H₂O, 10 μL oligo-dT primer (10 μM) and 100 μL dNTP mix (10 mM each). Add 2 μL annealing mix to the 5-cell pool in lysis buffer per well using the Mosquito HV liquid-handling robot.
88. Cover the plate with an adhesive seal, vortex briefly and spin down.
89. Incubate the plate at 72°C for 3 min. Centrifuge at $700 \times g$ for 1 min. Transfer the plate back on ice immediately afterward.
90. Prepare reverse transcription (RT) reaction mix.

Reverse Transcription Reaction Mix

Reagent	Final concentration	Volume per well (μL)
SuperScript II reverse transcriptase (200 U/ μL)	100 U/ μL	0.50
RNasin Plus (40 U/ μL)	0.5 U	0.125
Superscript II first-strand buffer (5 \times)	1 \times	2.00

(Continued on next page)

Continued		
Reagent	Final concentration	Volume per well (μL)
DTT (100 mM)	5 mM	0.50
Betaine (5 M)	1 M	2.00
MgCl ₂ (1 M)	6 mM	0.06
TSO (100 μM)	1 μM	0.10
Nuclease-free water	N/A	0.415
Total	N/A	5.7

91. Add 5.7 μL of RT mix to the samples. The final reaction volume per well is 10 μL.
92. Cover the plate with an adhesive seal, vortex briefly and spin down.
93. Run the RT reaction following the program below:

Reverse Transcription Program			
Steps	Temperature	Time	Cycles
RT and template switching	42°C	90 min	1
Unfolding of RNA secondary structures	50°C	2 min	10
RT and template-switching	42°C	2 min	
Enzyme inactivation	70°C	15 min	1
Hold	4°C	forever	

94. Prepare PCR mix for cDNA amplification.

cDNA Amplification PCR Mix		
Reagent	Final concentration	Volume per well (μL)
KAPA HiFi HotStart ReadyMix (2×)	1×	12.50
IS PCR primers (100 μM)	0.1 μM	0.25
Nuclease-free water	N/A	2.25
Total	N/A	15

95. Add 15 μL of PCR mix to each well.
96. Cover the plate with an adhesive seal, vortex briefly and spin down.
97. Run PCR to amplify the cDNA.

cDNA Amplification Program			
Steps	Temperature	Time	Cycles
Initial denaturation	98°C	3 min	1
Denaturation	98°C	20 s	20
Annealing	67°C	15 s	
Extension	72°C	6 min	
Final extension	72°C	5 min	1
Hold	4°C	forever	

▮▮ **Pause point:** The cDNA can be stored at –20°C for 6 months or longer.

98. Before starting the purification of cDNA, equilibrate Ampure XP beads at room temperature for 15 min, and then vortex thoroughly to ensure that the beads are properly suspended in the buffer.

99. Add 16.25 μL of Ampure XP beads (0.65 \times ratio) to each well of the 96-well plate.
100. Cover the plate with an adhesive seal, vortex briefly and incubate for 5 min at room temperature.
101. Place the 96-well plate on the magnetic stand for 5 min.
102. Wash the beads with 200 μL of 80% (vol/vol) ethanol solution twice.
103. After removing the washing solution, leave the plate at room temperature for approximately 5 min until the beads have dried completely.
104. Add 22 μL of EB solution to each well and mix 10 times with the pipet to resuspend the beads.
105. Incubate the plate off the magnet for 2 min at room temperature.
106. Place the plate back on the magnet for 2 min or longer.
107. Transfer 20 μL of supernatant containing clean cDNA into a new 96-well plate.

Pause point: The clean cDNA can be stored at -20°C for 6 months or longer.

108. Evaluate the quality of the cDNA. Following [the manufacturer's instruction](#), analyze the size distribution of the cDNA using a HS NGS Fragment Kit on an Agilent Fragment Analyzer Instrument.

Note: A good quality cDNA preparation should be almost free of small fragments (<500 bp) and should peak at $\sim 1.5\text{--}2$ kb.

109. Measure the concentration of cDNA generated from each sample using PicoGreen.
110. Normalize cDNA to a concentration of 0.1–0.15 ng/ μL in nuclease-free water.
111. Prepare DNA libraries using Nextera XT DNA Library Preparation Kit (Illumina). 0.75 μL of normalized cDNA is used in a miniaturized 15% volume (3 μL total) Nextera tagmentation reaction. Miniaturized reactions are set up in a 384-well plate. Volumes of the following steps are adjusted accordingly. For the detailed information on the Nextera XT kit, refer to [the manufacturer's manual](#). A Mosquito HV liquid-handling robot is used for pipetting.

Pause point: The final libraries can be stored at -20°C .

112. Prepare a library pool containing 96 libraries by combining 1.2 μL of each final library in one tube.
113. Purify the library pool with 0.8 \times Vol Ampure XP beads and elute in 30 μL resuspension buffer.
114. Evaluate the quality of the library pool with an HS NGS Fragment Kit on an Agilent Fragment Analyzer instrument.
115. Dilute the library pool to the loading concentration specific for the sequencing system.
116. Sequence the libraries with e.g., the Illumina NovaSeq 6000 system using a 100 cycles reagent kit and PE 2 \times 51 sequencing.

EXPECTED OUTCOMES

The aim of this protocol is to visualize and characterize cancer cells undergoing partial and full EMT in mammary tumors of MMTV-PyMT transgenic mice. We generated a Tamoxifen-inducible dual recombinase-specific fluorescent color-switching system to label breast cancer cells by the expression of mCherry (red) and, upon Tamoxifen administration, trace the cells that have undergone either a partial or full EMT by irreversible switching to the expression of GFP (green). Moreover, the dual recombinase strategy excludes the labeling of any non-tumoral cells, such as fibroblasts, even though these cells express mesenchymal markers.

Using the composite mouse models, we describe a protocol for tracking the morphology and behavior of partial and full EMT cells in mammary tumors. With this protocol, around 10 precision slices with 350 μm thickness would be expected to obtain from a piece of tumor that is around

5 × 5 × 5 mm in size using a vibratome. Once slices are in culture, these tissues are expected to survive for up to 3 days at least and maintain their architecture during this time of culture. Besides tracking the migration of cells of interest, these slice cultures can be applied for further analyses such as investigating their interactions with non-tumoral cells including endothelial cells and immune cells all within the tumor microenvironment ([Methods videos 1 and 2](#)).

Finally, this protocol describes the preparation procedure of 5-cell cDNA libraries ready for sequencing. Using 5-cell pools allows to obtain a higher depth of sequencing compared to single-cell RNA sequencing, yet still explore the heterogeneity present within each population of choice. After sequencing, bioinformatic analysis using a single cell analysis pipeline, for example Bioconductor Single Cell Analysis workflow ([Amezquita et al., 2020](#)), can be used to reveal the transcriptional content of individual 5-cell pools. In combination with EMT score analysis using a database published by Tan et al. ([Tan et al., 2014](#)), the degree of EMT between full epithelial, intermediate, and full mesenchymal states can be assessed in every sample. RNA velocity analysis using the package *velociraptor* (1.0.0) further infers the transition kinetics between different EMT stages in tumors as reported in our study ([Luond et al., 2021](#)).

LIMITATIONS

The Tamoxifen-inducible dual recombinase system presented here allows not only the labeling of cancer cells undergoing an early partial EMT or a late full EMT in tumors at chosen time points and time frames of choice, but also the investigation of the reversibility of cells upon EMT after initial recombination induced by CreER^{T2}. The treatment of the mice with Tamoxifen twice a week at 2 mg/mouse had only a minor and transient suppressive effect on primary tumor growth. However, due to the transient nature of an EMT, our lineage tracing system that relies on the continuous administration of Tamoxifen may not trace all cancer cells undergoing an EMT throughout breast cancer progression. The use of different genetic tracing tools, for examples the seamless EMT lineage tracing system developed by Li et al. ([Li et al., 2020](#)), could have the potential for more sensitive mapping of the cells of interest during mammary gland tumorigenesis. Yet there are discrepancies regarding the contributions of different states of the EMT spectrum to metastatic tumor progression between distinct EMT lineage-tracing models. The selective labeling of the examined cancer cells, the selection of specific markers for EMT, and the different kinetics of tumor progression associated with different genetic background of mice should be considered in order to design the specific lineage tracing models of interest in various (sub)types of cancers.

In the tumor slice culture, blood and lymphatic vessels are not correctly perfused. In our hands, a gradual deterioration of tissue architecture was observed in mammary tumor tissue slices cultured beyond 3 days. Furthermore, the signal from fluorophore-conjugated antibodies that visualize the stromal cells was gradually diminished during the culture period due to degradation and/or dissociation of the antibodies. Introducing circulatory devices such as microfluidics into the slice tissue culture system could potentially allow to maintain tissue structure and functions for up to several weeks or months ([Komeya et al., 2016](#)). Despite these limitations, our tumor slice culture system is well suited for short-term tracing of particular cells within regions of interest in the tumor microenvironment for up to 3 days and could be applied for experiments aimed at revealing the acute local reactions upon treatments using anti-tumor drugs, antibodies, and immune cells. Furthermore, by manipulation of the culture conditions, for example by applying hypoxia or therapeutic treatments, the changes in the morphology and behavior of cells can be monitored within their niche.

In the MMTV-PyMT transgenic mouse model of breast cancer, the numbers of partial and full EMT cells in tumors are small ([Luond et al., 2021](#)). Due to this limitation, we recommend to use most of the total tumor volume for the sorting of partial and full EMT subpopulations. Flow cytometry analysis prior to sorting of cells for RNA sequencing is recommended to predict the frequency of specific populations in tumors and to estimate the time required for sorting. To obtain sufficient biological

replicates for 5-cell RNA sequencing, at least 5 mice should be used for each EMT lineage tracing model. Moreover, due to the limiting number of GFP⁺ cells – particularly in the full EMT tracing model – there may not be enough starting material to process the same mouse for tumor slice culture and 5-cell RNA sequencing.

TROUBLESHOOTING

Problem 1

Tamoxifen is not dissolving ([before you begin](#), step 5a).

Potential solution

Vortex the solution intermittently while shaking at 37°C. Prepare a low volume, e.g., 1 mL in a 2 mL Falcon tube to allow thorough mixing.

Problem 2

No mCherry⁺ and/or GFP⁺ cells detected by microscopy and flow cytometry. ([part 2](#), step 34 and [part 3](#), step 68).

Potential solution

Wrong genotype due to false-positive PCR or mix-up of mice. Repeat genotyping using a biopsy collected from the animal at sacrifice for clarification.

Problem 3

The agarose blocks do not tightly adhere to the specimen disc. ([part 2](#), step 10).

Potential solution

Before fix to the stage, place the agarose block on kimwipes to remove the extra liquid from the surface of the agarose gel.

Problem 4

Difficulty in tissue slicing ([part 2](#), step 18).

Potential solution

The integrity and stiffness of the tissue affects obtaining of precision slices. Ensure that adipose tissue is removed from mammary tumor pieces using a scalpel. If the tissue is too soft due to tissue necrosis, embed it in higher concentration agarose gel (> 3%) to provide stability, and slice with higher amplitude and lower speed. Setting a larger section thickness up to 400 μm may also help.

Problem 5

Focus drift during live-cell imaging ([part 2](#), step 34).

Potential solution

Due to the stage movement, uneven heating of imaging chamber, and vibration produced by the instruments, axial fluctuations manifested by slow changes to specimen focus during live-cell imaging creating a focus drift. Specimen movement in the culture insert can also cause a shift completely out of the original field of view. Before starting the session, heat the imaging chamber and incubate the plate on the stage for at least 30 min to equilibrate the system. Verify that the actual temperature inside the imaging chamber and on the stage is stable at 37°C. Ensure that the imaging plate is positioned correctly and secured in the stage aperture. Verify that the PFS settings are set correctly as described in step 29e to ensure that the focus position is automatically adjusted during image acquisition. Any excess medium in the culture insert should be removed to avoid tissue floating.

Problem 6

Signal is reduced during live-cell imaging ([part 2](#), step 34).

Potential solution

Ensure that the system equipment including imaging chamber, temperature, gas mixture for CO₂ and air, flow rate, and humidifying module are set-up and operating correctly. Optimize laser power and scanning time to keep minimum levels of photo-toxicity and photo-bleaching during imaging.

Problem 7

Poor cell viability during tissue digestion ([part 3](#), step 45).

Potential solution

Ensure the concentrations of the components in collagenase/trypsin solution and incubator settings, e.g., temperature and shaking speed. Do not incubate longer than 1.5 h.

Problem 8

Clogging of the 40 µm filter when filtering cells ([part 3](#), step 60).

Potential solution

If the cell suspension is highly concentrated, the 40 µm filter may be obstructed during filtration. To avoid the obstruction, dilute the sample with FACS buffer before filtration.

Problem 9

Nozzle clogs during sorting ([part 3](#), step 72).

Potential solution

Dilute the sample and filtrate using a 40 µm filter. The nozzle should be sonicated.

Problem 10

Poor cell viability during sorting ([part 3](#), steps 72 and 82).

Potential solution

Reduce the event rate on the sorter by decreasing the speed of the sorting.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Gerhard Christofori (gerhard.christofori@unibas.ch).

Materials availability

Mouse lines and cell lines reported in the paper ([Luond et al., 2021](#)) are listed in the [key resources table](#) and will be shared by the [lead contact](#) upon request under a completed MTA with University of Basel.

Data and code availability

- All five-cell RNA sequencing data are deposited at GEO database and are publicly available. The accession number is GSE171940 (<https://www.ncbi.nlm.nih.gov/geo/browse/>).
- The original code for drift correction is deposited as a DOI and is publicly available. The accession number is DOI: [10.5281/zenodo.6103102](https://doi.org/10.5281/zenodo.6103102).
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101438>.

ACKNOWLEDGMENTS

This work was financially supported by the SystemsX.ch MTD project MetastasiX, Switzerland; the Swiss National Science Foundation, Switzerland; the Swiss Cancer League, Switzerland; and the Krebsliga Beider Basel, Switzerland.

We are grateful to S. Dymecki (Department of Genetics, Harvard Medical School, Boston, Massachusetts) and N. Hynes (FMI, Basel, Switzerland) for sharing RC::FrePe and MMTV-PyMT (FVB/N) mice, respectively. We thank E. Panoussis, I. Galm, and U. Schmieder (DBM, University of Basel) for technical support, P. Lorentz (DBM microscopy core facility) for expert support with microscopy, M. Abbate (arivis AG) for image analysis with Arivis software, and T. Lopes and G. Auray (DBM, University of Basel) for support with flow cytometry operation and data analysis. We also thank A. Offinger, L. Schneider, and the DBM animal facility for animal husbandry.

AUTHOR CONTRIBUTIONS

N.S. and G.C. conceived the idea and supervised the project. N.S. designed and established the slice tissue culture and FACS/5-cell RNA sequencing protocols and performed and analyzed most of the experiments. F.L. established mouse lines and designed, performed, and analyzed FACS experiments. C.B. designed and performed the RNA sequencing experiment. L.G. wrote the scripts for image analysis. T.R.B. performed 5-cell sorting by FACS. N.S., G.C., and F.L. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Amezquita, R.A., Lun, A.T.L., Becht, E., Carey, V.J., Carp, L.N., Geistlinger, L., Marini, F., Rue-Albrecht, K., Risso, D., Sonesson, C., et al. (2020). Orchestrating single-cell analysis with Bioconductor. *Nat. Methods* *17*, 137–145. <https://doi.org/10.1038/s41592-019-0654-x>.
- Attalla, S., Taifour, T., Bui, T., and Muller, W. (2021). Insights from transgenic mouse models of PyMT-induced breast cancer: recapitulating human breast cancer progression in vivo. *Oncogene* *40*, 475–491. <https://doi.org/10.1038/s41388-020-01560-0>.
- Bang, S.J., Jensen, P., Dymecki, S.M., and Commons, K.G. (2012). Projections and interconnections of genetically defined serotonin neurons in mice. *Eur. J. Neurosci.* *35*, 85–96. <https://doi.org/10.1111/j.1460-9568.2011.07936.x>.
- Engleka, K.A., Manderfield, L.J., Brust, R.D., Li, L., Cohen, A., Dymecki, S.M., and Epstein, J.A. (2012). Islet1 derivatives in the heart are of both neural crest and second heart field origin. *Circ. Res.* *110*, 922–926. <https://doi.org/10.1161/circresaha.112.266510>.
- Guy, C.T., Cardiff, R.D., and Muller, W.J. (1992). Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol. Cell Biol.* *12*, 954–961. <https://doi.org/10.1128/mcb.12.3.954-961.1992>.
- Komeya, M., Kimura, H., Nakamura, H., Yokonishi, T., Sato, T., Kojima, K., Hayashi, K., Katagiri, K., Yamanaka, H., Sanjo, H., et al. (2016). Long-term ex vivo maintenance of testis tissues producing fertile sperm in a microfluidic device. *Sci. Rep.* *6*, 21472. <https://doi.org/10.1038/srep21472>.
- Li, Y., Lv, Z., Zhang, S., Wang, Z., He, L., Tang, M., Pu, W., Zhao, H., Zhang, Z., Shi, Q., et al. (2020). Genetic fate mapping of transient cell fate reveals N-cadherin activity and function in tumor metastasis. *Dev. Cell* *54*, 593–607.e5. <https://doi.org/10.1016/j.devcel.2020.06.021>.
- Luond, F., Bill, R., Vettiger, A., Oller, H., Pelczar, P., and Christofori, G. (2019). A transgenic MMTV-flippase mouse line for molecular engineering in mammary gland and breast cancer mouse models. *J. Mammary Gland Biol. Neoplasia* *24*, 39–45. <https://doi.org/10.1007/s10911-018-9412-4>.
- Luond, F., Sugiyama, N., Bill, R., Bornes, L., Hager, C., Tang, F., Santacroce, N., Beisel, C., Ivanek, R., Burglin, T., et al. (2021). Distinct contributions of partial and full EMT to breast cancer malignancy. *Dev. Cell* *56*, 3203–3221.e11. <https://doi.org/10.1016/j.devcel.2021.11.006>.
- Picelli, S., Faridani, O.R., Bjorklund, A.K., Winberg, G., Sagasser, S., and Sandberg, R. (2014). Full-length RNA-seq from single cells using Smart-seq2. *Nat. Protoc.* *9*, 171–181. <https://doi.org/10.1038/nprot.2014.006>.
- Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* *9*, 671–675. <https://doi.org/10.1038/nmeth.2089>.
- Tan, T.Z., Miow, Q.H., Miki, Y., Noda, T., Mori, S., Huang, R.Y., and Thiery, J.P. (2014). Epithelial-mesenchymal transition spectrum quantification and its efficacy in deciphering survival and drug responses of cancer patients. *EMBO Mol. Med.* *6*, 1279–1293. <https://doi.org/10.15252/emmm.201404208>.