Multidimensional fluorescence imaging of embryonic and postnatal mammary gland development

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Abstract

Multidimensional fluorescence imaging represents a powerful approach for studying the dynamic cellular processes underpinning the development, function and maintenance of the mammary gland. Here, we describe key multidimensional imaging strategies that enable visualisation of mammary branching morphogenesis and epithelial cell fate dynamics during postnatal and embryonic mammary gland development. These include 4-dimensional intravital microscopy and *ex vivo* imaging of embryonic mammary cultures, in addition to methods that facilitate 3-dimensional imaging of the ductal epithelium at single cell resolution within its native stroma. Collectively, these approaches provide a window into mammary developmental dynamics, and the perturbations underlying tissue dysfunction and disease.

Running title: Multidimensional imaging in the mammary gland

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1. Introduction

Biological imaging is a fundamental and universal tool in the life sciences. For over 80 years, researchers have harnessed microscopy to reveal the inner workings of the mammary gland, a secretory organ essential for the survival of over 5000 mammalian species [1]. Light and electron microscopy of fixed tissue sections and cells are mainstay tools in a mammary gland biologist's armoury. While immensely useful, two-dimensional (2D) static analyses are limited in their ability to provide detailed topological and/or dynamic functional information. Thus, to fully comprehend the intricate organisation of the branched mammary epithelium, including the dazzling architecture of the lactating mammary gland, three-dimensional (3D; x-, y-, z-) imaging is essential. Moreover, interrogating the inherently dynamic cellular processes underpinning this complex tissue demands the ability to visualise mammary epithelial and stromal cells by live, four-dimensional (4D; x-,y-, z-, t-) imaging [2].

Due to its widespread accessibility and flexible multicolour acquisition capabilities, confocal laser scanning microscopy (CLSM) is a commonly used optical sectioning technique for volumetric fluorescence imaging [3]. However, conventional confocal modalities rely on single-photon excitation wavelengths in the visible range for imaging, which suffer from tissue light scattering and absorption. This limits confocal microscopy to depths of ~100 μm in most tissues [3, 4]. As such, microscopes equipped with pulsed, infrared multiphoton lasers are favoured for deep tissue imaging. Alongside decreased photo-toxicity, multiphoton infrared excitation is less prone to light scattering and absorption, enabling deeper penetration and tissue imaging depths of up to 1 mm [5, 6]. These features make multiphoton systems particularly useful for *in vivo* fluorescence imaging by intravital microscopy (IVM).

In this chapter, we describe strategies for high-resolution, multidimensional imaging of live and fixed mammary tissues using CLSM and multiphoton microscopy. These include step-by-step protocols for 4D imaging of the adult and embryonic mammary gland using IVM and *ex vivo* imaging of embryonic cultures respectively. We also describe approaches that facilitate high-resolution volumetric imaging of the mammary epithelium within its native stroma. These methodologies are particularly useful in contexts that demand the ability to visualise large regions of the mammary epithelium at high spatiotemporal resolutions, such as in genetic-fate mapping studies focused on delineating the differentiation potential and capacity of distinct populations of mammary progenitor cells [2, 7, 8]. Importantly, the approaches outlined enable *in situ* visualisation of the dynamic interplay between mammary epithelial cells and their stromal microenvironment. While not discussed herein, many of the described protocols are also compatible with mammary tumour imaging, providing important insights into breast cancer cell biology [9–12].

IVM is a powerful technique that facilitates high-resolution, real-time fluorescence imaging of cells deep inside live animals [2, 13]. For short-term, acute IVM under non-recovery anaesthesia, mammary gland tissues and tumours can be made accessible to the microscope's objective via a surgical 'skin-flap' incision [14, 15]. Longitudinal IVM studies, however, require the surgical implantation of an imaging window to protect and provide optical access to tissues during repeated imaging sessions [9, 16–18]. Here, we describe the surgical procedure for implanting mammary imaging windows (MIW), based on protocols developed for their similarly-designed abdominal counterparts [17, 19, 20]. These imaging windows consist of a glass cover-slipped titanium frame that can be fixed above tissues using a purse-string suture. Alongside, we suggest methods for re-tracing regions of interest in consecutive imaging sessions, facilitating *in situ* visualisation of specific mammary epithelial cells and tissue structures for extended periods of time. For studies focused on the embryonic mammary gland, we describe methods for high-resolution, time-resolved CLSM and multiphoton imaging of embryonic mammary buds established in *ex vivo* culture [21–23]. This represents a highly tractable system for interrogating epithelial cell fate dynamics and behaviours during embryonic mammogenesis, a fundamental phase in mammary gland development that remains inaccessible to IVM.

Finally, we outline strategies for high-resolution, wholemount immunostaining and 3D visualisation of fixed mammary tissues, aimed at circumventing issues associated with antibody penetration and depth of imaging in this optically-opaque organ. This includes an enzymatic digestion-based procedure that facilitates improved deep tissue immunostaining and whole gland imaging of slide-mounted tissues [24–27]. This approach, however, risks proteolytic-mediated damage to mammary epithelial and stromal cells, limiting its utility in some contexts. Consequently, we also provide two optical tissue clearing methods that enable high-resolution, deep tissue mammary gland imaging in the absence of enzymatic digestion or mechanical dissection; namely 'see deep brain' (SeeDB) [28] and 'clear unobstructed brain imaging cocktails' (CUBIC) [29]. By mitigating tissue light scattering caused by cellular and extracellular components with different refractive indices (RIs), these methods allow for the visualisation of expansive regions of the mammary epithelial tree at single-cell resolution within its native stroma [11, 30–35]. These fixed tissue methods are particularly valuable when applied downstream of *in vivo/ex vivo* live-imaging, allowing mammary cells visualised in 4D to be further characterised by immunostaining for biomarkers of interest. The refinement of the imaging approaches described herein - and their application to novel experimental models and methods for visualising distinct cells and cellular processes – will continue to provide important insights into mammary gland and breast cancer biology.

2. Materials

2.1 Materials for mammary imaging window (MIW) preparation

1. Custom-made titanium MIW (*See* **Note 1**).
2. Glass coverslips (12 mm).
3. 100% Ethanol - prepare 70% (v/v) for use.
4. 100% acetone.
5. Cyanoacrylate-based glass glue.
6. Cotton swabs.
7. Sterile 0.9% saline.
8. *Optional step:* Sterile 1 ng/ml Poly(L-amino acid)-polyethylene glycol/poly(L-lysine)-polyethylene glycol (PLL-g-PEG) solution prepared in 10 mM HEPES buffer, pH 7.4.

2.2 Surgical instruments and equipment for intravital imaging

1. Extra fine Graefe forceps.
2. Straight Bonn surgical scissors.
3. *Optional:* Needle holder.
4. Autoclave pouches.
5. Bead steriliser.
6. Sterile surgical gloves, gown, hair net and face mask.
7. Sterile surgical drapes.
8. Sterile cotton gauze swabs.
9. Isoflurane inhalation anaesthetic.
10. Isoflurane vaporiser (Vet Tech Solutions) with oxygen supply.
11. Anaesthetic scavenger unit (Vet Tech Solutions).
12. Anaesthesia induction box/cage.
13. Heat pad / heated operating stage.
14. Analgesics e.g. 0.3 mg/ml buprenorphine hydrocholoride (Temgesic).
15. Physiological saline solution.
16. 1 ml Syringe and 25-guage needle.
17. Ophthalmic ointment.
18. Tape.
19. Pet hair clipper.
20. *Optional:* depilatory cream.
21. Antiseptic surgical scrub – e.g. Betadine.
22. Sterile non-absorbable polypropylene surgical suture (4-0) (*See* **Note 2**).

2.3 Intravital microscopy (IVM)

1. Multiphoton confocal microscope surrounded by a heated dark box (e.g. Nikon A1R MP, equipped with a Spectra-Physics Insight Deepsee laser, conventional and resonant scanners and GaAsP non-descanned detectors).
2. Long-working distance objectives (e.g. Nikon, 25×/NA1.1 2.0 mm WD water immersion objective).
3. Custom-made imaging window stabilisers e.g. microscope stage insert or imaging box.
4. Physiological saline solution.
5. Syringe and butterfly wing needle (for long-term (>3 h) imaging).
6. Mouse vital sign monitor (e.g. MouseOx Plus, Starr Life Sciences).
7. *Optional:* Injectable fluorescent probes to label mammary cells or structures of interest.
8. Image analysis software and processing tools: e.g. ImageJ (https://imagej.nih.gov/ij/), Imaris (http://www.bitplane.com/).

2.4 Materials for embryonic mammary gland dissection

1. Timed-pregnant mice e.g. fluorescent reporter mouse models (*Lgr5-GFP* [36] or *Rosa26-mTmG* reporter strains [37]).
2. 1 ml syringe and 25-gauge needles.
3. Dissection tools: micro-dissecting scissors, spring scissors, Dumont #5 forceps and curved micro-dissecting forceps.
4. 100 mm diameter Petri dish.
5. 24 well plates.
6. Stereoscopic dissecting microscope with transmitted illumination.
7. Sterile phosphate-buffered saline solution (PBS).
8. Embryonic culture medium: DMEM/F-12, 2 mM GlutaMAX™, 10% Fetal Bovine Serum (FBS) (v/v) and 20 U/ml Penicillin-Streptomycin (PS).
9. *Optional (for Cre-inducible reporter models):* Tamoxifen free base (MP Biomedicals), dissolved in sunflower oil.

2.5 Ex vivo embryonic mammary gland culture and 4D live-cell imaging

1. Tissue Culture Dish with Cover Glass Bottom (35 mm).
2. Cell culture inserts (0.4 µm, 30 mm diameter; Millicell).
3. 10x Pancreatin solution pH 7.0: 2.5 g of pancreatin from porcine pancreas (Cat. # P3292-25G, Sigma) and 0.85 g NaCl dissolved in 100 ml of cold MilliQ water and filter-sterilised (for preparation guidelines *See* **Note 3**).
4. Porcine Trypsin, 1:250 (Cat. # 85450C-25G, SAFC).

5. Thyrode's solution (pH 7.4): 8 g/l NaCl, 0.2 g/l KCl, 0.05 g/l NaH_2PO_4 + H_2O, 1 g/l glucose, 1 g/l $NaHCO_3$ dissolved in 1 litre of distilled water and filter sterilised.
6. Pancreatin-trypsin working solution pH 7.4: 0.225 g porcine trypsin made up in a final volume of 10 ml Thyrode's solution containing 1 ml 10x pancreatin stock solution and 20 µl of PS. Filter sterilise before use (for preparation guidelines *See* **Note 4**).
7. Ascorbic acid: 10 mg/ml in distilled water and filter sterilised (Cat. # A4544-25G, Sigma).
8. Horizontal laminar flow hood.
9. Inverted CLSM microscope (e.g. Zeiss LSM780/880) or two-photon confocal microscope (e.g. Leica SP8 microscope with a Chameleon Vision II laser), equipped with a heated environmental chamber.
10. Long-working distance objective (e.g. Zeiss 40x/1.0NA water immersion objective, Leica 25x/0.95NA water immersion objective).
11. Image analysis software and processing tools: e.g. ImageJ (https://imagej.nih.gov/ij/), Imaris (http://www.bitplane.com/).

2.6 Enzymatic digestion and wholemount immunostaining of mammary tissues

1. Enzyme digestion (ED) mix: HBSS containing 300 U/ml collagenase (Cat. # C0130, Sigma) and 300 µg/ml hyaluronidase (Cat. # 4272, Sigma).
2. HBSS.
3. PBS.
4. 4% Paraformaldehyde in PBS.
5. NH_4Cl, 0.5 M.
6. ED Blocking buffer: 1% (w/v) bovine serum albumin (BSA), 5% (v/v) horse serum and 0.8% (w/v) Triton-X100 in PBS.
7. PBST: PBS + 0.2% (w/v) Tween20.
8. 4',6-diamidino-2-phenylindole (DAPI) dilactate.
9. Primary and fluorescent secondary antibodies (as required).
10. Microscope slides and coverslips (No. 1.5).
11. Aqua Poly/Mount.

2.7 CUBIC-based optical tissue clearing

1. 10% Neutral Buffered Formalin (NBF).
2. Card or glass microscope slides.
3. PBS.

4. CUBIC Reagent 1A: 10% (w/w) urea, 5% (w/w) N,N,N',N'-tetrakis(2-hydroxypropyl)ethylenediamine, 10% (w/w) Triton-X100 and NaCl (25 mM) in distilled water (*See* **Notes 5 and 6**).
5. CUBIC Reagent 2: 44% (w/w) sucrose, 22% (w/w) urea, 9% (w/w) 2,2',2"-nitrilotriethanol and 0.1% (w/w) Triton-X100 in distilled water.
6. CUBIC Blocking buffer: 10% (v/v) goat serum, 0.5% (w/v) Triton-X100 in PBS.
7. 4',6-diamidino-2-phenylindole (DAPI) dilactate.
8. *Optional:* Primary and fluorescent secondary antibodies as necessary.
9. Glass bottomed imaging dishes (e.g. Cat # 81158, Ibidi).

2.8 SeeDB-based optical tissue clearing

1. 10% Neutral Buffered formalin (NBF).
2. Card or glass microscope slides.
3. PBS.
4. α-thioglycerol.
5. Serial fructose solutions made up in distilled water – 20%, 40%, 60%, 80%, 100% (w/v), SeeDB (115% (w/v)) (*see* **Note 7**).
6. SeeDB Blocking buffer: 10% (w/v) bovine serum albumin (BSA), 1% (w/v) Triton-X100 in PBS.
7. 4',6-diamidino-2-phenylindole (DAPI) dilactate.
8. *Optional:* Primary and fluorescent secondary antibodies as necessary.
9. Glass bottomed imaging dishes (e.g. Cat # 81158, Ibidi).

3. Methods

3.1 Mammary imaging window implantation for 4D-IVM

All animal studies must be approved by institutional ethical committees and national authorities as required. Perform surgeries according to the LASA Guiding Principles of Preparing and Undertaking Aseptic Surgery to minimise infection risk (*See* **Note 8**). Due to its fast induction and recovery, alongside the ability to precisely control anaesthesia depth and length, inhalation anaesthetics should be used for window implantation and intravital imaging [14, 17]. While acute intravital imaging of the mammary gland under non-recovery anaesthesia (i.e. where the animal is euthanised immediately at the end of imaging) can be performed via a skin-flap incision [14, 38, 39], this approach poses challenges for maintaining optimal mouse hydration and physiology. Thus, implanting a MIW is also beneficial when performing non-recovery imaging studies spanning several hours. When applied to fluorescent reporter mouse models, IVM is a powerful approach for tracing the fate, migration, proliferation, geometry and

re-arrangements of individual cells in real-time during mammary gland postnatal development and tissue homeostasis.

3.1.1 Mammary imaging window (MIW) preparation

1. Apply a thin layer of cyanoacrylate-based glass glue on the etched inset of the MIW that will contact the coverslip.
2. Using forceps, place the coverslip on the glue-covered inset and apply gentle pressure using a cotton swab for 1 min. If necessary, apply glue at the interface of the coverslip and the window.
3. Allow the glue to dry completely by placing it on its side in a flow cabinet for a minimum of 2 h.
4. If required, use a cotton swab soaked in 100% acetone to remove excessive and condensed glue from the coverslip. Use a cotton swab soaked in 70% (v/v) ethanol to remove the acetone.
5. Place the window glass-side down on a dry tissue and fill with physiological saline to assess for leaks. If the window is watertight, the tissue will remain dry (*See* **Note 9**).
6. Sterilise the MIW by steam sterilisation, or by placing it in 70% (v/v) ethanol for a minimum of 30 min. Do not autoclave cover-slipped windows as it will degrade the glue.
7. *Optional*: In a sterile flow cabinet, coat the interior side of the coverslip with PLL-g-PEG solution. Incubate for 1 h at room temperature. Subsequently, wash the window in sterile PBS (*See* **Note 10**).

3.1.2 Surgery preparation

1. Sterilise surgical tools by autoclaving or dry sterilization (*See* **Note 11**). Thoroughly disinfect the entire operating station (including heat-pads, anaesthesia nose cones and adjustment controls) using 70% ethanol.
2. Anaesthetise the mouse in an induction chamber using 3-4% (v/v) gaseous isoflurane (*See* **Note 12**).
3. Once anaesthetised, transfer the mouse onto a heat-pad situated away from the sterile operating area, and continue to supply anaesthesia via a nose cone. Reduce the isoflurane concentration to 1.5-2% (v/v) (*See* **Note 13**).
4. Apply ophthalmic ointment to both eyes to prevent corneal drying.
5. Administer Buprenorphine at a dose of 0.1 mg/kg mouse body weight by sub-cutaneous injection (*See* **Note 14**).
6. Shave the skin over the 4^{th} mammary gland using electronic pet clippers. As stray hairs under the window pose an infection risk and can obstruct imaging, it is important to

remove as much residual hairs as possible from the surgical field. Brief exposures to depilatory cream can be used if necessary.

7. Disinfect the shaved skin using Betadine solution and a cotton gauze. Begin at the centre of the surgical site and move out towards the periphery using an ever-widening circular motion. Repeat 3 times using a clean gauze each time.
8. Gently transfer the mouse to the disinfected heated operating stage. Continue to supply the mouse with 1.5-2% (v/v) isoflurane via a nose cone to maintain anaesthesia.
9. Loosely immobilise the hind legs using tape. Cover the mouse with a sterile surgical drape equipped with an opening to allow access to the prepared surgical field.

3.1.3 Surgical implantation of the MIW

1. Verify that the mouse is sufficiently anaesthetised by pinching a hind paw through the overlying sterile drape. If the mouse is unresponsive, proceed to the next step. Otherwise, adjust the isoflurane dose and wait until reflex behaviours are absent.
2. Use sterile forceps to gently lift the skin away from the abdominal wall and make a ~12 mm long incision in the flank area above the 4th abdominal mammary gland using sterile scissors (Fig. 1a, panel 1. *See* **Notes 15 and 16)**.
3. Use sterile forceps to blunt dissect the skin away from the underlying mammary gland, taking care not to damage the tissue.
4. Place a circular purse-string suture around the incision, approximately 4 mm from skin edges. Keep the external sections of the suture loose at this stage, leaving 4 butterfly-wing shaped outer loops (Fig. 1a, panel 2).
5. Using forceps, carefully insert the window on the exposed mammary gland and gently place the sutured skin into the window groove (Fig. 1a, panel 3).
6. Carefully pull the loops of the purse-string to tighten the skin in the window groove, securing it in place (Fig. 1a, panel 3). Place a double knot to fix the purse-string suture, making sure to hide the knot underneath the upper ring of the window to prevent the mice from biting it open (Fig. 1b).
7. Proceed immediately to intravital imaging (Subheading 3.2), or allow the mouse to recover from the anaesthesia in a cage placed on a heating pad at 37 °C. Once recovered, the mouse may be group-housed with other window-bearing mice.
8. Post-operative care: Closely monitor mice for signs of pain and discomfort after surgery. If required, provide post-operative analgesics in accordance with local veterinary guidance (*See* **Note 17**). Inspect the surrounding skin and the tissue underneath the MIW daily for signs of inflammation and necrosis.

3.2 Longitudinal 4D-IVM by multiphoton microscopy

1. If not proceeding directly from MIW surgery (Subheading 3.1), anaesthetise the mouse in an induction chamber using 3-4% (v/v) gaseous isoflurane.
2. *Optional*: administer fluorescent probes by tail vein injection to label mammary cells and structures as necessary (*see* **Note 18**).
3. To maintain mouse hydration during short-term experiments (< 3 h), administer a maximum of 500 µl saline by sub-cutaneous injection prior to imaging. In experiments exceeding 3 h, saline should be provided periodically (~ 50–100 µl/hour) using an indwelling intraperitoneal line [14, 17] (*See* **Note** 19).
4. Transfer the mouse onto the stage insert of a multiphoton microscope for imaging, continuing to supply 1.5-2% (v/v) isoflurane via a nose cone to maintain anaesthesia.
5. Position and immobilise the MIW for imaging. On an inverted set-up, a microscope insert or box customised with a hole that precisely fits the window can be used for stabilisation [19]. To image in the upright configuration, the window can be fixed using custom-made holders or microstage devices [38] (*See* **Note 20**). Maintain the mouse's body temperature at 37 °C during imaging, ideally using a dark heated chamber that surrounds the microscope stage.
6. Once the mouse is secure and stabilised, reduce the isoflurane concentration to between 0.8%-1.2% (*See* **Note 21**).
7. Closely monitor the mouse's vital parameters during imaging:

   a) Regularly check the breathing rate and adjust the isoflurane supply accordingly if irregular or abnormal breathing is observed.

   b) Measure the mouse's temperature during imaging using a rectal probe. Adjust the temperature of the heated imaging chamber as necessary.

   c) Monitor capillary blood flow to ensure optimal conditions for imaging.

   d) Continuous assessment of vital parameters can be performed using a non-invasive pulse oximetry monitoring system (e.g. MouseOx system) that measures the mouse's temperature, arterial oxygen saturation, breathing distension, and heart and respiratory rates during imaging.
8. When anaesthesia levels are tightly controlled and animal vitals well-maintained, 4D-IVM can be performed continuously for up to ~40 h under non-recovery anaesthesia [39]. Alternatively, longitudinal IVM can be performed whereby mice undergo repeated, shorter imaging sessions over extended periods of time (*See* **Note 22**).
9. For longitudinal imaging of specific cells and tissue structures, a number of approaches can be used to help retrace regions of interest in consecutive imaging sessions, including:

a) Using a motorized stage: In configurations where the relative position of the imaging window is fixed in repeated imaging sessions, the coordinates of individual regions can be stored and used to relocate imaging areas.

b) Morphological landmarks: the branched mammary epithelial network is structurally heterogeneous, providing unique patterns of ductal structures and adjacent blood vessels that can be readily recognised in repeat imaging sessions though the eyepiece or by manual scanning (*See* **Note 23**). Intravenous injection of fluorescent agents to label vasculature (e.g. fluorescently-labelled dextrans), in addition to second harmonic generation (SHG) imaging of tissue collagen organisation, can also assist with retracing regions of interest in consecutive imaging sessions (Fig. 1c).

c) Fluorescent reporter mouse models: Heterogeneous labelling of mammary epithelial cells gives rise to unique and identifiable colour patterns for serial imaging e.g. using fluorescent reporter mouse models such as *R26R-Confetti* [9, 40] or *Rosa26-mTmG* [37] mouse strains (Fig. 1c).

10. *Optional:* To aid the re-identification of intravitally imaged mammary tissue regions in downstream 3D or 2D histological analyses, at the end of the final imaging session use focused high laser power to generate distinct auto-fluorescent “photo-tattoos” in nearby tissue areas, taking care not to damage cells and tissue structures of interest [41].
11. At experimental endpoints, euthanise the mouse according to institutional/national guidelines.
12. Taking note of the orientation of the tissue relative to the coverslip, harvest and fix the intravitally imaged mammary gland for downstream histological analysis (e.g. by 3D wholemount immunostaining as described in Subheading 3.5). Harvest the contra-lateral, non-window mammary gland as a control.
13. To reuse the MIW, clean the titanium ring using soap and hot water. Incubate overnight in 100% acetone to remove the glue and release the used coverslip. Prepare the MIW for future implantation following the instructions detailed in Subheading 3.1.1.
14. Process and analyse intravital images using ImageJ (https://imagej.nih.gov/ij/) and/or commercial software such as Imaris (http://www.bitplane.com/), depending on availability.

3.3 Establishing mammary embryonic buds in culture for 4D ex vivo imaging

The mammary epithelium is first specified as placodes at approximately embryonic (E) day 11, which invaginate into the underlying mesenchyme to form mammary buds by E12. After

E15.5, buds undergo sprouting and branching morphogenesis to give rise to a rudimentary epithelial tree by E18.5 [42]. To visualise this process in real-time, mammary embryonic buds must be established in *ex vivo* culture [21–23]. In this section, we describe methods for high-resolution longitudinal or time-lapse fluorescence imaging of *ex vivo* cultured embryonic mammary buds by CLSM and multiphoton microscopy. Similarly to IVM, when applied to fluorescent reporter mouse models this approach allows for the dynamic behaviour and fate of individual embryonic mammary cells to be traced in real-time during embryonic mammary morphogenesis.

3.3.1 Dissection of the embryonic mammary gland

1. Set up timed mouse matings to obtain pregnant females bearing embryos of the desired genotype and age. Embryo stage is determined by the detection of a vaginal plug the following day (detection at mid-day defined as 0.5 days-post-coitus i.e. E0.5).
2. *Optional*: If using Cre-inducible fluorescent reporter mice, administer a low dose of tamoxifen to pregnant females 24 h before tissue dissection to induce fluorescent labelling in embryos. While a tamoxifen dose of 0.1 mg/g of mouse body weight is commonly used, precise doses will vary depending on specific study requirements [26].
3. At the desired embryonic stage, sacrifice the pregnant female and harvest the embryos from the uterus in a 100 mm diameter Petri dish filled with cold PBS.
4. Remove the yolk sac and separate each embryo from its placenta, taking care not to damage the tissue (Fig. 2, panel 1).
5. Sacrifice each embryo by decapitation and place in separate wells of a 24 well plate filled with cold PBS. Keep tissues to confirm genotypes and sex by PCR (*see* **Note 24**).
6. Place one embryo in a 35 mm culture dish filled with set silicon. Secure the embryo in place by pinning the neck and tail joint using needles or dissection pins (Fig. 2, panel 2).
7. Remove the limbs. This makes the 1st and 5th mammary buds accessible for dissection (Fig. 2, panel 3).
8. Perform a small incision above the tail joint of the embryo. Using spring scissors, cut along the dorsal-lateral line from the hind limb to the forelimb in the right flank of the embryo (Fig. 2, panel 4).
9. Detach the flank of the embryo from the incision along the dorsal-lateral line to the midline (Fig. 2, panel 5). Hold the tissue using Dumont #5 forceps, and use the spring scissors to trim the right flank (*see* **Note 25**).

10. Repeat step 8 and 9 with the left flank of the embryo, but this time cutting along the dorsal-lateral line from the forelimb to the hind limb.
11. Transfer both flanks to a new 24 well plate with PBS (Fig. 2, panel 6).

3.3.2 Separating the embryonic skin epithelium and mesenchyme

This procedure entails proteolytic digestion of dissected embryonic flanks, based on a protocol developed by the laboratory of M. Mikkola [23].

1. Replace the PBS with pancreatic-trypsin working solution (*see* Materials 2.5) and incubate for 4-5 min. Optimal incubation times are heavily dependent on embryo stage, in addition to the particular enzyme batches used [23]. Thus, closely monitor the tissue under the stereomicroscope during enzyme treatment.
2. When the skin epithelium starts to detach from the edges of the mammary mesenchyme, inactivate the enzymes by replacing the pancreatic-trypsin solution with DMEM/F-12 embryonic culture medium (*see* Materials 2.4 and **Note 26**).
3. Incubate the mammary tissue on ice for 30-45 min.
4. Place the mammary tissue in a 35 mm culture dish. Using two needles, gently peel the skin epidermis away from the mesenchyme containing the embryonic mammary buds. The mesenchyme is required for embryonic mammary epithelial development [21] and should be maintained as intact as possible around mammary buds (Fig. 2, panel 7).
5. Once isolated, use a plastic pipette to transfer the intact mesenchyme containing the embryonic mammary buds to fresh DMEM/F-12 embryonic culture medium in a 24 well plate. Repeat this process for all harvested tissues.

3.3.3. Establishing mammary buds in ex vivo culture

1. Per embryo, prepare a 35 mm cover glass-bottomed tissue culture dish containing 1 ml of DMEM/F-12 embryonic culture medium (see Materials 2.4) freshly supplemented with 75 µg/ml ascorbic acid.
2. Place the embryonic mammary tissue on a cell culture insert (containing 0.4 µm pores) using a 1000 ml pipette.
3. Using curved micro-dissecting forceps, carefully and slowly place the cell culture insert into the prepared glass-bottomed tissue culture dish to avoid bubble formation. This ensures mammary embryonic buds are cultured on an air-liquid interface, whereby the tissue remains exposed to air while maintaining contact with the embryonic cell culture medium through the pores of the cell culture insert (Fig. 2, panel 8).
4. Maintain mammary cultures in a tissue culture incubator at 37 °C and 5% CO_2 atmosphere.

5. Exchange the culture media with fresh media every second day for the duration of the experiment. Cultures can be maintained *ex vivo* for up to 2 weeks.

3.4 4D time-lapse and longitudinal imaging of ex vivo embryonic mammary cultures

Once established in *ex vivo* culture, embryonic mammary branching development can be recorded daily by 3D fluorescence imaging for up to 2 weeks. Use an inverted CLSM or multiphoton microscope (e.g. Zeiss LSM780/880 or Leica SP8) equipped with long-working distance objectives to acquire high-resolution 3D image stacks at each time-point (Fig. 3a). Alternatively, time-lapse imaging can be performed for up to 24 hours (Fig. 3b). Below, we provide an overview of the experimental conditions that facilitate high-resolution, *ex vivo* imaging of fluorescently labelled embryonic mammary epithelial cells over time.

1. Isolate embryonic mammary buds from a fluorescent reporter mouse model and establish in *ex vivo* culture as described in sub-heading 3.3. Fig. 3a and 3b show a cultured embryonic mammary bud established from a *Lgr5-GFP* [36] or *Rosa26-mTmG* [37] reporter mouse embryo respectively. In the absence of Cre-mediated recombination in the *Rosa26-mTmG* mammary bud (Fig. 3b), all embryonic mammary epithelial and stromal cells are labelled with a membrane-bound Tomato fluorescent protein.
2. Due to superior depth of imaging and low photo-toxicity, a multiphoton confocal microscope is preferred for time-lapse imaging of *ex vivo* embryonic mammary cultures e.g. an inverted Leica SP8 microscope equipped with a femtosecond Chameleon Vision II multiphoton laser (680–1350 nm; Coherent, Inc.) and long-working distance objectives (e.g. Leica 25×/0.95NA water immersion objective).
3. The microscope should be equipped with an incubation chamber that maintains tissues at 37 °C, 5% CO_2 atmosphere and 95% humidity (*see* **Note 27**). To avoid sample drift in the Z-axis, allow environmental conditions of the microscope chamber to stabilise for 30-60 min before commencing image acquisition.
4. Acquire image sequences as Z-stacks of the desired volume. Image acquisition time may vary between 30-60 min, depending on tissue thickness and area imaged, as well as the number of fluorophores to detect.
5. To image the whole embryonic mammary epithelium, overview tile scans can be acquired. Ensure at least a 10% overlap between tiled images, which can be stitched into larger mosaics using the microscope's acquisition software, or ImageJ Plugins (e.g. the Grid Collection/Stitching plugin) (https://imagej.nih.gov/ij/).

6. Time-lapse processing and analysis can be performed using ImageJ and/or commercial software such as Imaris (http://www.bitplane.com/), depending on availability.

3.5 3D fluorescence imaging of fixed mammary gland tissues

In this section, we provide three protocols for wholemount immunostaining and 3D visualisation of fixed mammary gland tissues, encompassing proteolytic digestion and two optical tissue clearing methods, namely CUBIC [29] and SeeDB [28]. These can be applied to freshly harvested tissues, or after completing 4D *in vivo/ex vivo* imaging to further characterise live-imaged tissue regions by immunostaining. While proteolytic-digestion methods are particularly useful for whole-gland imaging of immunostained mammary tissues [24–27], CUBIC and SeeDB optical tissue clearing methods facilitate visualisation of the mammary epithelial tree at single-cell resolutions within its intact stroma [11, 30–34] (*see* **Note 28**). For an overview of the optical tissue clearing methods previously applied to the mammary gland, in addition to their advantages and disadvantages, see [2, 11].

3.5.1 Proteolytic digestion-based immunostaining of mammary gland tissues

1. Euthanise mice according to institutional/national guidelines.
2. Excise mammary glands and immerse in Enzyme Digestion solution (see Materials 2.6) for 30-60 min (depending on tissue size) at 37 °C with gentle agitation. Due to its small size, high quality wholemount immunostaining can be performed in embryonic mammary gland tissues and explants without the need for enzymatic digestion [43] (Fig. 4).
3. Wash tissues 3 times in HBSS (5 min each time).
4. Fix tissues for 2 h at room temperature in 4% PFA.
5. Wash samples twice in 0.5 M NH_4Cl (10 min each time).
6. Wash tissues 3 times in PBS (10 min each time).
7. Incubate tissues in ED blocking buffer (see Materials 2.6) for 3 h at room temperature, or overnight at 4 °C with gentle agitation.
8. Incubate samples in primary antibodies diluted in blocking buffer overnight at room temperature.
9. Wash samples three times in PBST (10 min each time).
10. Incubate samples with secondary antibodies diluted in blocking buffer for 5 h at room temperature, or overnight at 4 °C with gentle agitation.
11. Wash samples three times in PBST (10 min each time).

12. Incubate tissues with DAPI (10 µM) for 30 min to 1 h at room temperature with gentle agitation to stain nuclei.
13. Mount tissues on microscope slides using Aqua Poly/Mount, taking care to avoid bubbles.
14. Acquire images using CLSM (e.g. Zeiss LSM780/880 or Leica SP8) with a long-working distance objective to facilitate deep tissue imaging (e.g. Zeiss 25x/0.8 oil immersion objective or Leica 25x/0.95NA water immersion objective).
15. Imaging considerations: Adjust laser power and gain manually to give optimal fluorescence intensity for each fluorophore with minimal photobleaching. Acquire Z-stacks using step-sizes and line averaging appropriate for the desired resolution. If performing tile-scans, at least a 10% overlap is recommended for optimal stitching of tiled images into larger mosaics (Fig. 4). Stitching can be performed using the microscope's acquisition software, or using ImageJ Plugins (e.g. the Grid Collection/Stitching plugin) (https://imagej.nih.gov/ij/).
16. Process and analyse image stacks using ImageJ (https://imagej.nih.gov/ij/) and/or commercial software such as Imaris (http://www.bitplane.com/) depending on availability.

3.5.2 Modified CUBIC tissue clearing and immunostaining of mammary gland tissues

1. Euthanise mice according to institutional/national guidelines.
2. Excise mammary glands and spread immediately on card (Tetra Pak) or glass microscope slides (*see* **Note 29**).
3. Fix tissues by immersing in 10% NBF for 6-9 h (according to tissue size/thickness) at room temperature (*see* **Note 30**).
4. Wash tissues briefly in PBS with gentle agitation to remove residual NBF. Cut tissues into large (~15×15×2 mm) pieces if necessary (*see* **Note 31**).
5. Immerse tissues in modified CUBIC Reagent 1A (see Materials 2.7) at 37 °C for 2-3 days, depending on the size of the tissue, exchanging the solution with fresh R1A each day. For timeline, see Fig. 5a.
6. Wash samples 3 times in PBS (10 min each time) to remove excess R1A solution. If tissues are to be imaged for genetically-encoded fluorescent proteins and do not require immunostaining, proceed to step 12.
7. *Optional immunostaining:* Immerse tissues in CUBIC blocking buffer (see Materials 2.7) and incubate overnight at 4 °C with gentle agitation.
8. Incubate tissues with primary antibodies diluted in CUBIC blocking buffer at 4°C for 4 days with gentle agitation. Incubation times may be optimised for specific antibodies.

9. Wash tissues 3 times in PBS (1 h each time) with gentle agitation at room temperature.
10. Incubate samples with Alexa Fluor-conjugated secondary antibodies made up in PBS for 2 days at 4°C with gentle agitation (*see* **Note 32**).
11. Wash tissues 3 times in PBS (1 h each time) with gentle agitation at room temperature.
12. Incubate with DAPI (10 μM) for 2-3 h at room temperature with gentle agitation.
13. Transfer samples to CUBIC Reagent 2 (see Materials 2.7) and incubate at 37°C for at least 24 h for refractive index matching before imaging (Fig. 5b).
14. Image samples in CUBIC Reagent 2 by CLSM or multiphoton microscopy within 1 week (e.g. Zeiss LSM780/880 or Leica SP8 equipped with long-working distance objectives to facilitate deep tissue imaging). Samples may be imaged on inverted microscopes using glass-bottomed iBidi dishes, or mounted in CUBIC Reagent 2 for imaging (Fig. 5c) (*see* **Note 33**).
15. Imaging considerations: adjust laser power and gain manually to give optimal fluorescence intensity for each fluorophore with minimal photobleaching. Acquire Z-stacks using step-sizes and line averaging appropriate for the desired resolution. If performing tile-scans, at least a 10% overlap is recommended for optimal stitching of tiled images into larger mosaics. Stitching can be performed using the microscope's acquisition software, or using ImageJ Plugins (e.g. the Grid Collection/Stitching plugin) (https://imagej.nih.gov/ij/).
16. If tissues were previously imaging by 4D intravital imaging, place cleared samples in the same orientation for imaging. To re-trace regions of interest, some of the strategies suggested for longitudinal IVM may be used (see Subheading 3.2, Step 9) e.g. using morphological landmarks or laser-induced autofluorescent photo-tattoos.
17. Process and analyse image stacks using ImageJ (https://imagej.nih.gov/ij/) and/or commercial software such as Imaris (http://www.bitplane.com/) depending on availability.

3.5.3 SeeDB tissue clearing and immunostaining of mammary gland tissues

1. Euthanise mice according to institutional/national guidelines.
2. Excise mammary glands and spread immediately on card (Tetra Pak) or glass slides (*see* **Note 29**).
3. Fix tissues by immersing in 10% neutral buffered formalin (NBF) for 6-9 h (according to tissue size/thickness) at room temperature (*see* **Note 30**).
4. Subsequently, wash tissues briefly in PBS with gentle agitation to remove residual NBF. Cut tissues into large (~15×15×2 mm) pieces if necessary (*see* **Note 31**).
5. If tissues are to be imaged for genetically-encoded fluorescent proteins and do not require immunostaining, proceed to step 11. For timeline, see Fig. 6a.

6. *Optional immunostaining*: Immerse tissues in SeeDB blocking buffer (see Materials 2.8) overnight at 4°C with gentle agitation.
7. Incubate tissues with primary antibodies diluted in SeeDB blocking buffer at 4°C for 4 days with gentle agitation. Incubation times may be optimised for specific antibodies.
8. Wash tissues 3 times in PBS (1 h each time) with gentle agitation at room temperature.
9. Incubate samples with Alexa Fluor-conjugated secondary antibodies made up in PBS for 2 days at 4°C with gentle agitation.
10. Wash tissues 3 times in PBS (1 h each time) with gentle agitation at room temperature.
11. Incubate with DAPI (10 μM) for 2-3 h at room temperature with gentle agitation.
12. Subsequently, serially incubate samples for 8-16 h (twice daily changes i.e. at beginning and end of the day) at room temperature with gentle agitation in increasing fructose solutions: 20%, 40%, 60% and 80% (w/v) fructose solutions containing freshly added 0.5% (v/v) α-thioglycerol to inhibit the Maillard reaction [28].
13. Incubate samples in 100% (w/v) fructose solution containing freshly added 0.5% (v/v) α-thioglycerol for 24 h at room temperature.
14. Incubate samples in SeeDB (115% (w/v)) fructose solution containing freshly added 0.5% (v/v) α-thioglycerol for 24 h at room temperature before imaging (Fig. 6b).
15. Image samples in SeeDB reagent by CLSM or multiphoton microscopy within 2 weeks (e.g. Zeiss LSM780/880 or Leica SP8 equipped with long-working distance objectives to ensure deep tissue imaging). Samples may be imaged on inverted microscopes using glass-bottomed iBidi dishes, or mounted in SeeDB reagent for imaging (Fig. 6c) (*see* **Note 33**).
16. Imaging considerations: adjust laser power and gain manually to give optimal fluorescence intensity for each fluorophore with minimal photobleaching. Acquire Z-stacks using step-sizes and line averaging appropriate for the desired resolution. If performing tile-scans, at least a 10% overlap is recommended for optimal stitching of tiled images into larger mosaics. Stitching can be performed using the microscope's acquisition software, or using ImageJ Plugins (e.g. the Grid Collection/Stitching plugin) (https://imagej.nih.gov/ij/).
17. If tissues were previously imaging by 4D intravital imaging, place cleared samples in the same orientation for imaging. To re-trace regions of interest, some of the strategies suggested for serial IVM may be used (see Subheading 3.2, Step 9) e.g. using morphological landmarks or laser-induced autofluorescent photo-tattoos.
18. Process and analyse Z-stack images using ImageJ (https://imagej.nih.gov/ij/) and/or commercial software such as Imaris (http://www.bitplane.com/) depending on availability.

4. Notes

1. This protocol describes a re-usable titanium MIW that is based on the design of the abdominal imaging window [17, 19, 20]. This consists of an upper and a lower titanium ring (outer diameter 14 mm), separated by a 0.9 mm width groove. Titanium possesses superior biocompatibility compared to other stainless alloys.
2. Suture material may depend on user preference. Non-absorbable 5-0 nylon and 6-0 silk sutures is also used for imaging window implantation.
3. Dissolve pancreatin from porcine pancreas and NaCl in MilliQ water using a magnetic stirrer on ice for 3-4 h (or at 4 °C overnight). Centrifuge 5000 rpm for 10 min and filter using suction prior to use.
4. Dissolve 0.225 g porcine trypsin in 6 ml of ice-cold Thyrode's solution using a magnetic stirrer on ice. Once in solution add 1 ml of 10x pancreatin stock solution, in addition to 20 µl of PS. Using NaOH, adjust the pH of the solution to 7.4. Make up the solution to a final volume of 10 ml using additional ice-cold Thyrode's solution. Filter and prepare single-use aliquots.
5. CUBIC Reagent 1A is a modified, unpublished version of CUBIC Reagent 1 [29] available at http://cubic.riken.jp/. CUBIC reagents require heat (60 °C) and agitation to dissolve.
6. N,N,N',N'-tetrakis(2-hydroxypropyl)ethylenediamine is highly viscous. Dispense this reagent first and adjust other reagent amounts accordingly. In the original protocol [29], the authors state that a specific brand of Triton X-100 (Nacalai Tesque Inc., 25987-85, Japan) is required to avoid quenching endogenous EGFP fluorescence. However, in our hands, Triton X-100 purchased from VWR International is compatible with EGFP fluorescent protein in 10% NBF-fixed mammary gland tissues. If quenching is suspected, consider immunostaining tissues with a GFP antibody, or use an alternative optical tissue clearing method [2, 44].
7. Fructose solutions greater than 60% are difficult to get into solution. Use heat (60 °C) and agitation to dissolve.
8. To avoid contaminating the surgical station, prepare the mouse for surgery in a separate area before transferring to the sterilised operating station. Autoclaved tools should only come into contact with sterile gloves, surfaces and the disinfected skin area undergoing surgery. To safeguard sterility throughout the procedure, the surgeon should only contact sterile tools and the prepared surgical field. Mouse preparation and handling, adjustment of anaesthesia flow rates and similar tasks should be performed by an assistant.
9. For optimal results, it is essential that the seal between the titanium ring and coverslip is air and watertight.

10. PEG-based coating of the glass coverslip can be performed to prevent cell attachment and to improve its biocompatibility, limiting the risk of an inflammatory or immune reaction in response to implantation.
11. Ideally, a new set of sterilised tools should be used per mouse. If this is unfeasible, a bead sterilizer may be used to sterilise tools between limited numbers of mice.
12. Isoflurane - which requires oxygen as a carrier gas - is a well-tolerated inhalation anaesthetic with minimal side-effects and a short recovery time. Isoflurane induction and maintenance doses may require optimisation depending on animal parameters (including strain, age and condition) and the available set up. It is advisable to use relatively high doses of isoflurane for rapid induction (up to 4%), reducing doses to maintenance levels as quickly as possible to minimise the time exposed to high concentrations [14]. Isoflurane should be handled in well ventilated areas using systems equipped with a gas scavenger to minimise user exposure to exiting gases.
13. Maintaining the physiological body temperature of the mouse is critical for long-term survival under anaesthesia. This can be achieved using adjustable heated anaesthesia posts and induction boxes, heat-pads or environmental chambers during intravital imaging. Conversely, hyperthermia may amplify the effect of the inhalation anaesthetic, depressing respiration rates. Monitor the mouse's body temperature throughout surgery and intravital imaging using a rectal probe.
14. Buprenorphine may cause mild respiratory depression. Multi-modal regimes consisting of Buprenorphine and a NSAID (e.g. Carprofen at a 5 mg/kg) may be recommended by your local veterinarian. However, depending on the nature of the study, suppression of the immune system by NSAIDs may impact important experimental parameters.
15. Gently handle the skin at incision edges when using forceps to avoid compression-associated damage. Consider using non-serrated forceps to minimise this risk. For optimal implantation, the incision size should closely match the size of the window.
16. While the MIW can be implanted over inguinal (3^{rd}) mammary glands, the 4^{th} is less impacted by respiratory movements during imaging.
17. Post-operative buprenorphine (0.1 mg/kg for up to 3 days) may be administered to provide additional pain relief. NSAIDs (e.g. Carprofen at a 5 mg/kg dose and/or ibuprofen administered in drinking water) may also be used if appropriate (*see* Note 14). Local application of a topical analgesic (e.g. 1% Xylocaine) at the surgical site can be used to provide additional pain relief [17].
18. Endogenous and injectable fluorescent probes may be used to study aspects of mammary gland/tumour cell biology and tissue morphology [10, 45]. For example, blood vessels can be labelled by injecting fluorescently-conjugated dextrans into the circulation to investigate vascular flow and permeability, in addition to the invasion of

mammary tumourigenic cells into nearby vessels. Vessel labelling can also aid identification of imaging regions in repeated IVM sessions, in addition to the registration of serially acquired image stacks.

19. A winged infusion set attached to a syringe can be used to administer saline during imaging. This can be performed manually, or by using a programmable syringe pump for controlled, continuous administration.
20. An inverted microscope is preferable as it provides better stabilisation, reducing image distortions arising from respiration-induced tissue movement. It is important not to compress the underlying tissue and impair blood flow when immobilising the MIW.
21. These anaesthesia levels are optimal for long-term maintenance of mice in a nonresponsive state with a constant and non-forced breathing pattern. Irregular and abnormal breathing patterns are associated with persistent anaesthesia greater than 1.5%, which can decrease survival times [14, 39].
22. Due to the limited tolerance of mice to repeated anaesthesia, the duration and frequency of imaging sessions in longitudinal experiments should be adapted according to the study parameters under investigation, and in line with institutional/national ethical rules. For instance, if mice are to be anaesthetised daily for imaging, this should be restricted to shorter study time periods, with imaging sessions kept as brief as possible to aid recovery.
23. Generating a tile scan of the imaging field using a low-magnification objective also aids retracing of mammary regions of interest.
24. Before E14, there are no obvious morphological differences between female and male mammary embryonic buds. To distinguish the embryos' sex, perform a PCR using the following primer sequences and cycling conditions:
    5'-TGGATGGTGTGGCCAATG-3', 3'-CACCTGCACGTTGCCCTT-5'
    94 °C for 2 min; then 35 cycles of: 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec; finally 72 °C for 5 min.
    Run PCR products on a 2% agarose gel. One band is observed for female embryos and two bands for male embryos.
25. Avoid cutting the tissue into small pieces as this will make the next steps challenging.
26. To exchange the medium hereafter, use pipette tips with narrow orifices (e.g. gel loading tips) to carefully remove the medium without contacting mammary tissues.
27. Humidified conditions are required to minimise evaporation of the culture medium during imaging.
28. Of a number of tissue clearing techniques tested in the mammary gland, SeeDB and CUBIC protocols provided optimal results [11]. These protocols have subsequently been further developed [46, 47], although they have yet to be tested in mammary

tissues. In the event of issues with penetration and staining performance of some antibodies, other tissue clearing protocols are available [44], including FUnGI, a method recently developed for the mammary gland [48].

29. Well-spread tissues are thinner and easier to render transparent. Fibres from card/paper can transfer to tissues after fixation, hampering imaging from that side. Using Tetra Pack card (e.g. milk carton card) overcomes this issue. Alternatively, foam biopsy pads may be used.
30. Fixation time depends on the thickness and size of tissues, and may require antibody-specific optimisation. Thinner or smaller tissue pieces and embryonic tissues may be fixed after only 2-3 h at room temperature. A ~10:1 ratio v/v of NBF to tissue is required for adequate fixation. Alternatively, fresh solutions of 4% PFA (for 2-4 h at room temperature) may be used. In our hands, 10% NBF is compatible with several genetically-encoded fluorescent proteins, including TdTomato, EGFP, YFP, RFP and CFP in *Rosa26-mTmG*, *R26R-Confetti* and *Rosa26-tdTomato* reporter mouse strains [11, 30, 31]. If quenching of fluorescent proteins is suspected, consider using methanol-free Formaldehyde solutions.
31. While best to process samples immediately after harvesting, fixed tissues can be stored at 4°C in PBS containing 0.05% (w/v) sodium azide for up to 8 weeks. Cutting samples into large pieces may improve antibody penetration and immunostaining, in addition to allowing more immunostainings to be performed in tissues harvested from the same mouse.
32. CUBIC clearing is also compatible with wholemount immunohistochemistry using HRP-conjugated secondary antibodies and horseradish peroxidase-3,3-diaminobenzidine detection, in addition to the detection of β-glucosidase expression (a magenta histochemical stain) (see [11, 30]).
33. Tissues may be mounted between coverslips using iSpacer chambers or concave glass microscope slides. However, in these contexts, samples can be difficult to adjust or repositioned for optimal illumination, making working distance a limiting factor in image acquisition [11]. To mitigate this, sample thickness must be closely matched to the thickness of the iSpacer or concave chamber, and/or specialised imaging objectives with long working distances must be used. Alternative refractive index matching solutions may also be considered.

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Ethics Statement

All animal experimentation were carried out in accordance with the Animal (Scientific Procedures) Act 1986/609 and with local ethics committee approval.

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Figure Legends

Figure 1. Intravital imaging through the mammary imaging window (MIW). (a) Schematic representation of the surgical procedure. 1. Make an incision above the 4th mammary gland. 2. Place a purse-string suture around the incision. Dotted line denotes internal suture position. 3. Insert the MIW above the mammary gland and place the sutured skin within the groove. Secure the skin around the MIW by tightening the purse-string suture. Protocol based on [17, 19]. **(b)** Photograph of the MIW implanted over the 4th abdominal mammary gland. LN; Lymph Node. **(c)** Longitudinal imaging of mammary epithelial cell dynamics by multiphoton IVM. Images show a mammary epithelial duct in a 5 week old *K5-Cre^{ERT2};Rosa26-mTmG* female mouse. In this model, all cells are labelled with a membrane-bound Tomato fluorescent protein (mT, red). Tamoxifen administration induces Cre-mediated recombination of the reporter allele in sporadic Keratin (K)5-expressing mammary myoepithelial cells, resulting in membrane-bound EGFP fluorescent protein expression (mG, green). Images show the migratory behaviour of four EGFP^{+ve} mammary myoepithelial cells over time. Second harmonic generation (SHG) microscopy reveal the collagen organisation surrounding the ductal structure. Scale bars: 100 μm (left panels), 25 μm (right panels).

Figure 2. Embryonic mammary gland dissection protocol. (1) Collect all embryos from the uterus of a pregnant female mouse. (2) Attach the decapitated embryo to a dish filled with set silicon. (3) Remove the limbs. (4) Cut along the dorsal-lateral line of the embryo (dashed line). (5) Detach the flank (see arrowheads) to the midline. (6) Collect both flanks of the embryo containing the embryonic mammary buds (marked with dashed circles). (7) Remove skin epithelium after enzymatic digestion with pancreatic-trypsin working solution. Dashed circles denote the location of embryonic mammary buds within the mesenchyme. (8) Culture the isolated tissues on an air-liquid interface. Protocol based on [23].

Figure 3. 4D *ex vivo* imaging of mammary embryonic bud cultures (a) Longitudinal fluorescence imaging of embryonic mammary branching morphogenesis. Schematic representation of the microscope configuration for live-cell imaging of embryonic mammary

bud cultures. The tissue (i) is deposited on a cell culture insert (ii) placed in contact with the culture medium (iii) in a glass bottom dish. Use an inverted confocal or multiphoton microscope equipped with a long-working distance objective for imaging. Images show the growth of an E15.5 embryonic mammary bud isolated from a *Lgr5-GFP* [36] mouse embryo over 5 days. Maximum intensity projections of image sequences are displayed, and the rendered surface of the mammary epithelium is outlined in white. Scale bars: 100 μm. **(b)** Time-lapse imaging of embryonic mammary cultures**.** Close-up of one embryonic mammary bud dissected from a *Rosa26-mTmG* [37] mouse embryo at day E13.5, and cultured *ex vivo* for 5 days prior to time-lapse imaging. Images show the growth of an epithelial branch over 10 h (white box). In this reporter mouse strain, all cells are labelled with a membrane-bound Tomato fluorescent protein (red). Inset images show a thin optical slice. Individual mammary epithelial cells can be clearly visualised in *ex vivo* cultures by high-resolution multiphoton microscopy. Branching morphogenesis can be monitored for up to 24 h by time-lapse confocal or multiphoton imaging. The rendered surface of the mammary epithelium is outlined in white. Scale bars: 100 μm.

Figure 4: Wholemount immunostaining of embryonic mammary cultures. Immunofluorescence staining of an embryonic mammary gland dissected at day E13.5 and grown in *ex vivo* culture for 8 days. Left panel: Maximum intensity projection (MIP) showing the expression of the luminal epithelial marker protein Keratin 8 (K8, red), and the basal epithelial marker protein p63 (green) in embryonic mammary cells. Nuclei are stained with DAPI (blue). Centre panel: A single optical section (z). White boxes mark regions displayed in the right panels. Scale bar: 100 μm. Right panel: Close-up images of K8 (red) and p63 (green) expressing cells in a tip (upper panel) and branch (lower panel) region of the embryonic mammary epithelium. Scale bar: 25 μm.

Figure 5: Clear unobstructed brain imaging cocktails (CUBIC) optical clearing and 3D imaging of mouse mammary tissues. **(a)** CUBIC optical tissue clearing and immunostaining protocol and timeline. Black arrow shows the stage at which (optional) immunostaining may be performed. The experimental timeline may be adapted depending on the desired degree of transparency, and the size and nature of the tissue. **(b)** Transmission images of whole abdominal (4^{th}) virgin and lactating mammary glands, before and after CUBIC clearing. Adapted from [11] with permission from Springer under http://creativecommons.org/licenses/by/4.0/. **(c)** Example three-dimensional confocal images of cleared mammary tissues immunostained with basal mammary epithelial cell markers (K5 or smooth muscle actin (SMA)) showing compatibility of CUBIC clearing with high-resolution imaging of genetically-encoded fluorescent proteins. Top panels show a close up image of a mammary duct in a virgin *SMA-Cre^{ERT2}; Rosa26-mTmG* reporter mouse. Membrane labelling in recombined (mG, green) mammary basal cells can be observed at high resolutions by

CUBIC clearing. Non-recombined cells express membrane-bound Tomato fluorescent protein (mT, red). Middle and bottom panels show 3D images of cleared mammary tissues from involuting and lactating *R26R-Confetti* fluorescent reporter mice respectively. Reporter expression (nuclear GFP, cytosolic YFP and cytosolic RFP) is induced at low, sporadic levels. Scale bars: 50 μm (top two panels), 100 μm (middle and bottom panels). MIP, maximum intensity projection; z, single optical section.

Figure 6: See deep brain (*SeeDB*)-clearing and 3D imaging of mouse mammary tissues **(a)** SeeDB tissue clearing and immunostaining protocol and timeline. Black arrow shows the stage at which (optional) immunostaining may be performed. The experimental timeline may be adapted depending on the desired degree of transparency, and the size and nature of the tissue. **(b)** Transmission images of whole abdominal (4^{th}) virgin and lactating mammary glands, before and after SeeDB clearing. Adapted from [11] with permission from Springer under http://creativecommons.org/licenses/by/4.0. **(c)** Example three-dimensional confocal images of SeeDB-cleared mammary tissues immunostained with a basal cell marker (SMA) showing compatibility of SeeDB clearing with high-resolution imaging of genetically-encoded fluorescent proteins. Top and middle panels show images of the mammary epithelium in virgin and pregnant *N1-Cre^{ERT2}; Rosa26-mTmG* reporter mice respectively. Membrane labelling in recombined (mG, green) and unrecombined (mT, red) mammary luminal cells can be observed at high resolutions. Bottom panels show images of SeeDB-cleared mammary tissues from a lactating *Rosa26-YFP* mouse model immunostained with a GFP antibody. MIP, maximum intensity projection; z, single optical section. Scale bars: 50 μm.