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Chapter 11

Lineage Tracing in the Mammary Gland Using Cre/lox Technology and Fluorescent Reporter Alleles

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Abstract

Lineage tracing using Cre/lox technology has become a well-established technique to study the contribution of different (stem) cell populations to organ development and function. When used in the mammary gland, it forms a valuable addition to the already existing experimental toolbox and an important alternative to other readouts measuring stem cell potential, such as the fat pad transplantation assay.

Here I describe how to set up and analyze an in vivo lineage tracing experiment using tamoxifen-inducible Cre/lox technology, highlighting the specific challenges that the investigator faces when employing this method and interpreting the results in the mammary gland.

Key words Lineage tracing, Mammary gland stem cells, Tamoxifen, Whole mount, Confocal microscopy

1 Introduction

Owing to its unique developmental properties, the mouse mammary gland is an excellent model system to study various biological processes associated with tissue growth and regeneration. Located immediately below the skin and well outside the body cavity, it offers easy access. Moreover, it is a non-essential organ, thus allowing relatively straightforward experimental manipulation. More important than these practical considerations, however, are its morphological and functional characteristics. Biologically speaking, the mammary gland is a very dynamic tissue that undergoes dramatic changes in cell proliferation and differentiation during the life span of an organism [1].

The adult mouse mammary gland is composed of a bilayered, ductal epithelium that lies embedded in the stromal tissue of the fat pad (Fig. 1). This elaborate ductal network forms during puberty, when rapid branching morphogenesis causes the rudimentary mammary epithelium to grow out and invade the entire length of the fat pad. During pregnancy, a second wave of tissue expansion

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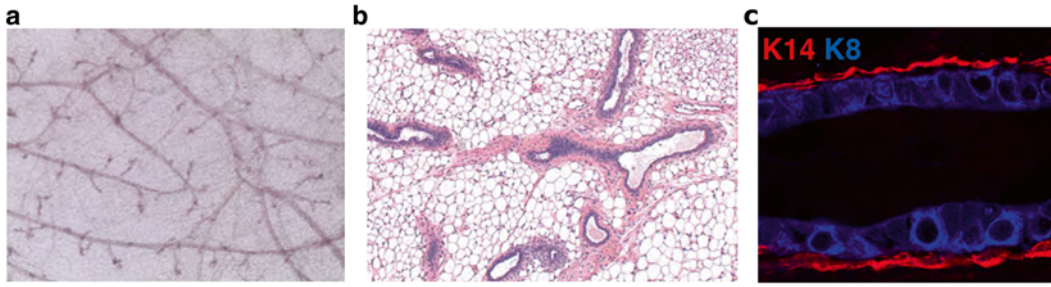

Fig. 1 Macroscopic and microscopic appearance of the mouse mammary gland. (**a**) Whole-mount picture of a carmine stained mouse mammary gland reveals a branched ductal network. (**b**) Tissue section of a hematoxylin/eosin stained mouse mammary gland reveals how the ductal epithelium lies embedded in the stromal tissue of the fat pad, which consists mostly of adipocytes. (**c**) Cross section of the mouse mammary epithelium revealing the bilayered appearance of the epithelium following immunofluorescent detection of basal, K14-positive (*red*) and luminal, K8-positive (*blue*) cells

occurs, this time associated with the formation and terminal differentiation of milk-producing alveoli. After lactation, these alveolar structures regress in a process called involution, when massive tissue remodeling takes place and the gland is essentially restored to a pre-pregnancy state.

Both developmental and cancer biologists have long been fascinated by these dynamic growth properties and in particular by the identity of the underlying stem and progenitor cell populations. As a result, a wide variety of experimental techniques exist to probe mammary cell behavior and function [2]. These include the prospective isolation of distinct cell populations based on the combinatorial expression of defined cell-surface markers by fluorescence-activated cell sorting (FACS) [3, 4] and their subsequent transplantation into the cleared fat pad [5]. First devised in the late 1950s, this transplantation assay remains a robust and powerful method to test the regenerative capacity of putative stem cell populations. However, it is important to realize that it requires cells to be taken out of their natural environment. In contrast, dedicated in vivo lineage tracing studies are designed to track cell fate in situ. Importantly, a direct comparison of the two experimental approaches has revealed important differences between the normal developmental potential tested by lineage tracing and the regenerative potential displayed by the same cell population upon transplantation [6, 7].

In this chapter I provide stepwise instructions for setting up and analyzing an in vivo lineage tracing experiment using genetically engineered mouse models and Cre/lox technology. This includes breeding of the required compound genotypes, initiation and duration of the trace, processing of the mammary gland for histological analysis, and 3D reconstruction using the Fiji software program. I specifically focus on the practical considerations related to tamoxifen-inducible Cre driver lines and (multicolor) fluorescent reporter alleles.

2 Materials

2.1 Compound Mutant Mice

Given the amount of time and money involved with long-term, in vivo lineage tracing analyses, selection of the genetically engineered mouse strains with which to perform the experiment is the most crucial step of the entire experiment. Ample time should be dedicated to making this decision. Ultimately the choice is up to the investigator, as it will depend on the specific research question. Although alternatives exist (*see* **Note 1**), the most straightforward and flexible approach is to make use of the tried and tested Cre/lox technology [8, 9].

In this scenario, the first mouse strain (hereafter referred to as the "Cre driver") should express the Cre recombinase protein in the cell population of interest (i.e., the cell population of which the investigator wishes to study the developmental fate). Spatial control is provided by the promoter and enhancer sequences used to drive Cre expression (*see* **Note 2**). Modified versions of the Cre protein (Cre^{ER}, Cre^{ERT}, or Cre^{ERT2}, *see* **Note 3**) in addition offer temporal control, allowing the investigator to decide the onset of Cre recombinase activity and thus the developmental time point at which the tracing experiment is initiated. In most cases, this is recommended (*see* **Note 4**). The Jackson mouse repository offers a large selection of (inducible) Cre strains (http://cre.jax.org/index.html). Practical considerations and pitfalls associated with selecting the appropriate Cre driver are discussed elsewhere [10, 11]. Ultimately, however, investigators may feel the need to generate novel Cre driver lines, as these will be best suited to address their specific research question. This falls out of the scope of the current chapter.

The second mouse strain (hereafter referred to as the "reporter") needs to meet two criteria. First, it should contain a conditional, Cre-inducible reporter allele that will "switch" from state A to state B in the presence of Cre-recombinase activity. This marks the cell population of interest (*see* **Note 5**). Second, after Cre-mediated recombination, expression of the marker gene itself should be driven by a constitutively active promoter, such that all cells in the lineage can be visualized at any given time point. Multiple Cre/lox technology based reporter lines are available from the Jackson mouse repository (http://jaxmice.jax.org/list/xprs_creRT1805.html). The simplest versions are binary OFF/ON switches, such as the trustworthy *Rosa26-lacZ* reporter [12]. More sophisticated reporters, such as the *Rosa26-mTmG* line [13], allow cells to be visualized both before and after recombination by switching on the expression of a different fluorescent protein. Yet other reporters, such as the *Rosa26-Confetti* line [14] are specifically suited for studying the clonal outgrowth of individual stem cells. Again, the choice of reporter will depend on the specific

research question to be addressed (*see* **Note 6**). Some characteristics of the three reporter lines mentioned above, pertaining to their use for lineage tracing in the mammary gland, are compared in Table 1.

As an example, the remainder of this protocol will assume the presence of the following mouse strains:

1. *Axin2-Cre^{ERT2}* (available from Jackson labs, stock #018867): a tamoxifen-inducible Axin2-Cre^{ERT2} allele [6] that marks Wnt/β-catenin responsive cells based on their expression of the negative feedback target gene *Axin2*.
2. *Rosa26-mTmG* (available from Jackson labs, stock #007676): a Cre-inducible reporter allele that marks all cells in the animal with a membrane-bound dTomato (mT) fluorescent protein prior to recombination. Cells that recombine the reporter allele lose mT expression and gain expression of a membrane-bound eGFP (mG) fluorescent protein.
3. *Axin2-Cre^{ERT2}*;*Rosa26-mTmG* double-heterozygous mice (*see* **Note 7**) in which the actual lineage tracing experiment is performed (Fig. 2).

2.2 Inducing Tamoxifen-Mediated Recombination

1. Gloves.
2. Tamoxifen solution: Dissolve tamoxifen (Sigma, *see* **Note 8**) at 5–20 mg/ml in 90 % corn oil (Sigma) and 10 % ethanol. A standard stock of 10 mg/ml tamoxifen consists of 10 mg tamoxifen, 900 μl corn oil, and 100 μl ethanol (*see* **Note 9**).
3. Corn oil control solution: Mix 900 μl corn oil and 100 μl ethanol.
4. Nutator mixer or tube roller.
5. Two 3 ml syringes.
6. Two 22 μm syringe filters.
7. Two 1.5 ml or 2 ml eppendorf tubes.
8. Two 1 ml BD™ slip-tip syringes with 26 G × 5/8 in. subQ needles.

2.3 Harvesting the Mammary Glands

1. Euthanasia chamber.
2. Dissection pad.
3. Spray bottle with 70 % ethanol.
4. 6–8 Pushpins.
5. One pair of surgical scissors.
6. One eppendorf tube.
7. One pair of Dumont No. 5 forceps.
8. Two pairs of fine (Iris or Graefe) forceps.
9. Four pieces of 3 mm Whatman paper, 4 cm × 1 cm each.

Table 1
Comparison of the *Rosa26-lacZ* (generated by the lab of Phil Soriano [12]), *Rosa26-mTmG* (generated by the lab of Liqun Luo [13]) and *Rosa26-Confetti* (generated by the lab of Hans Clevers [14]) reporter lines

Strain	Official name	Jackson strain	Before Cre	After Cre
Rosa26-lacZ	B6.129S4-Gt*(ROSA)26Sortm1Sor*/J	003474	Unlabeled	lacZ
Rosa26-mTmG	B6.129(Cg)-*Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo*/J	007676	dTomato	EGFP
Rosa26-Confetti	B6.129P2-Gt*(ROSA)26Sortm1(CAG-Brainbow2.1)Cle*/J	017492	Unlabeled	CFP, GFP, YFP, or RFP
Strain	Detection	Advantages	Disadvantages	
Rosa26-lacZ	– Enzymatic detection of β-galactosidase activity by X-gal staining	– Sensitive and robust – Allows early detection (24 h after Cre) – Allows co-staining with structural markers by IHC – Permanent record of X-gal stained tissue slides	– No direct detection of recombined cells in unfixed material – Not suited for confocal analysis – Not well suited for live-cell sorting by FACS	
Rosa26-mTmG	– Direct fluorescence – anti-GFP staining (IHC or IF)	– Both dTomato en EGFP are membrane bound, which nicely visualizes the cell outlines and provides free structural information – Well suited for whole-mount confocal analysis –Well suited for FACS analysis	– Earliest detection in the mammary gland at 48 h after Cre – No permanent record (unless stained with anti-GFP by IHC)	
Rosa26-Confetti	– Direct fluorescence	– Allows individual cell clones to be discriminated with more certainty – Allows “re-switching” of already recombined cells – Suited for whole-mount confocal analysis	– Recombination in the mammary gland is very inefficient – No permanent record	

Each line has its own advantages and disadvantages. Abbreviations: *IF* immunofluorescence; *IHC* immunohistochemistry; *FACS* fluorescence-activated cell sorting

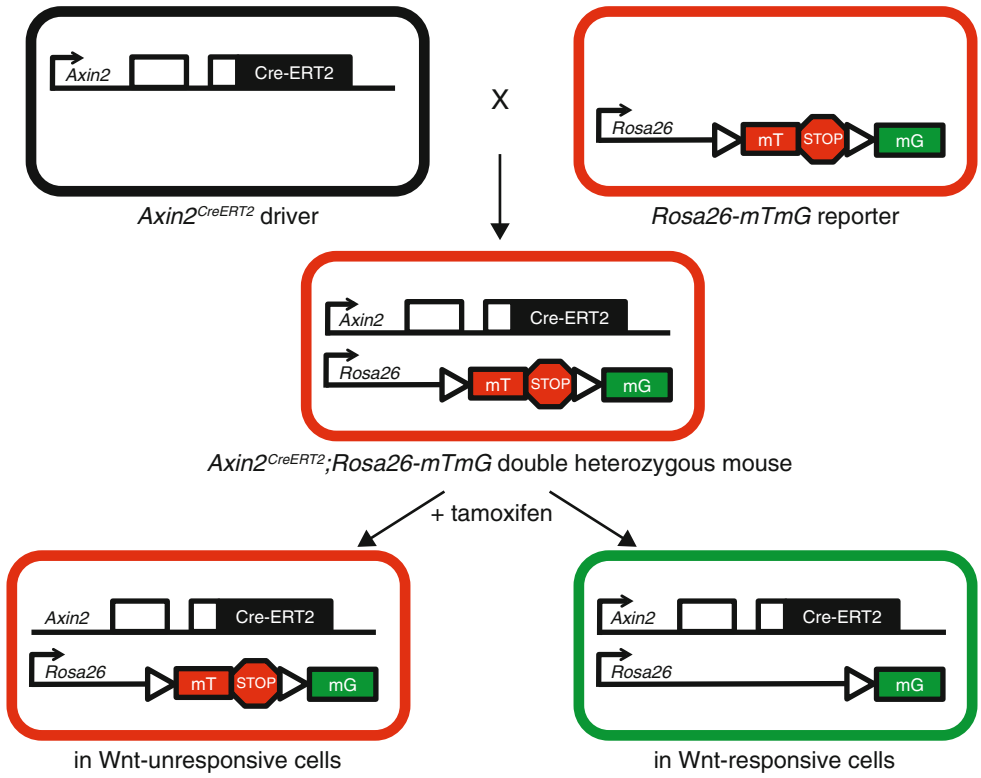

Fig. 2 Lineage tracing with the *Axin2-Cre* ^{ERT2} driver and the *Rosa26-mTmG* reporter. Schematic depicting the genetic principle behind Cre/lox-mediated lineage tracing. After crossing *Axin2-CreERT2* and *Rosa26-mTmG* mice (*top*), double-heterozygous offspring will express the Cre recombinase in all Axin2-positive, Wnt/β-catenin responsive cells (*middle*). However, owing to the presence of an ERT2 moiety, the Cre recombinase will be inactive. Therefore, prior to tamoxifen administration, all cells in the animal will be *red*, due to expression of a membrane-bound dTomato, which is expressed from the *Rosa26* reporter allele. The administration of tamoxifen will result in a pulse of Cre-activity in Axin2-positive, Wnt/β-catenin responsive cells, which in turn will recombine the *Rosa26-mTmG* reporter allele. This results in a switch from membrane-bound dTomato to membrane-bound GFP expression in Wnt-responsive cells, but not in Wnt-unresponsive cells (*bottom*). Because the switch is genetic, it is permanent and will remain present for the remainder of the life span of the recombined cell. In addition, it will be passed on to the cell's offspring, allowing its lineage to be traced

10. Glass vial with 10 % neutral buffered formalin (4 % paraformaldehyde).
11. Aluminum foil.

2.4 Whole-Mount Confocal Analysis of Fluorescent Reporter Alleles

1. Nutator mixer or tube roller.
2. Phosphate buffered saline (PBS) solution.
3. 30 % Ethanol: 30 ml ethanol in 70 ml PBS.
4. 50 % Ethanol: 50 ml ethanol in 50 ml PBS.
5. 70 % Ethanol: 70 ml ethanol in 30 ml deionized H_2O.
6. 100 % Ethanol.

7. Glass vial with methylsalicylate (*see* **Note 10**).
8. Single concave microscope slide (14 mm diameter).
9. #1.5 Coverslip.
10. Nail polish.
11. Confocal microscope with 488 nm and 561 nm laser lines for *Rosa26-mTmG* or with 458 nm, 488 nm, 514 nm, and 561 nm laser lines for *Rosa26-Confetti* (*see* **Note 11**).
12. Acetone.
13. Kleenex tissues.
14. Glass vial with paraffin.
15. Paraffin for refreshing.
16. Hybridization oven.
17. Embedding cassette.
18. Computer with Fiji software (downloadable from http://fiji.sc/Fiji).

3 Methods

3.1 Inducing Cre-Mediated Recombination

1. Design the appropriate lineage-tracing experiment (Fig. 3).
2. Breed *Axin2-Cre^{ERT2};Rosa26-mTmG* double-heterozygous mice, which carry both the Cre-driver and the reporter allele, by intercrossing the *Axin2-Cre^{ERT2}* and the *Rosa26-mTmG* strains. I usually cross a heterozygous *Axin2-Cre^{ERT2}* male to a homozygous *Rosa26-mTmG* female. Half of the offspring will be double-heterozygous (*see* **Note 12**).
3. Genotype the mice by PCR (*see* **Notes 13–15**).
4. Divide the mice into experimental cohorts (*see* **Notes 16–19**).
5. Prepare the tamoxifen and the corn oil control solution. It is best to always prepare these fresh. Remember to wear gloves when handling tamoxifen!
6. Incubate the solution on a nutator or tube roller. Tamoxifen will take some time to dissolve.
7. Filter the solutions through a 22 μm syringe filter into an eppendorf tube (*see* **Note 20**).
8. When you are ready to inject the mice, fill a 1 ml syringe attached to a subQ needle with each of the solutions (*see* **Note 21**).
9. Remove all air bubbles by holding the syringe vertically, pulling down on the plunger and flicking the syringe with your fingers to force the air to the top.
10. Insert the plunger to let all air escape.
11. Put down the syringe and open the mouse cage.

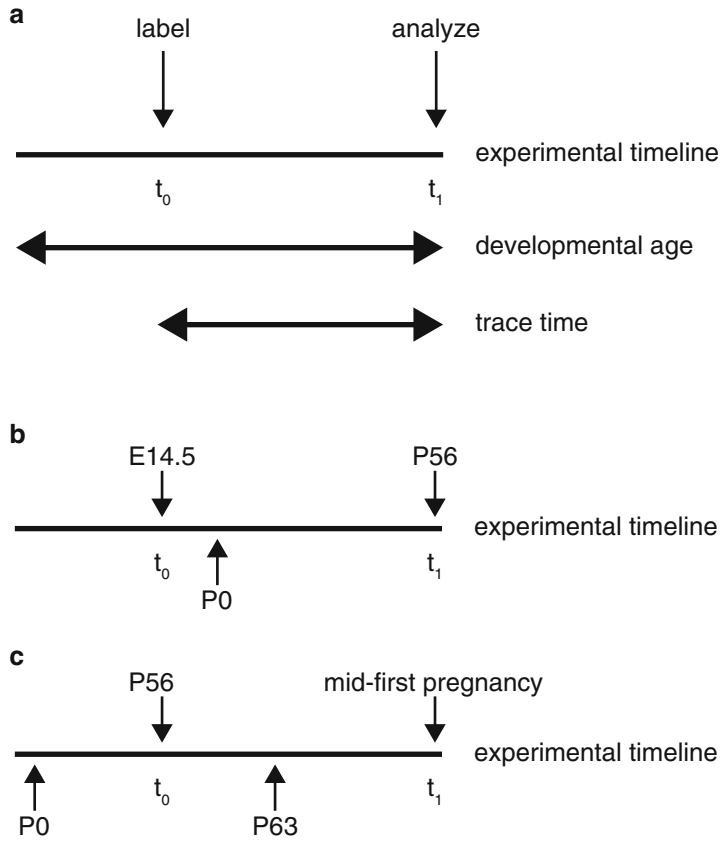

Fig. 3 Experimental setup of an in vivo lineage tracing experiment. (**a**) Timeline depicting the two critical time points of a lineage tracing experiment. Using an inducible system, Cre-mediated recombination can occur at any time of choice. The time point at which the cells are labeled following tamoxifen administration (t_0) represents the onset of the actual tracing experiment. The trace can be stopped for analysis at any time point greater than 24 h (the minimum amount of time for reporter activity to be detectable) after tamoxifen administration (t_1). (**b**) The trace can be started prior to birth by administering tamoxifen to pregnant mothers. As an example, recombination can be induced in E14.5 embryos, after which the mice are traced through puberty and analyzed as adults at 8 weeks (56 days, P56) of age. (**c**) Alternatively, the trace can start at any time after birth. As an example, recombination can be induced at P56, after which the cells are analyzed at mid-pregnancy

12. Holding it by the tail, lift the mouse that is to be injected out of the cage.
13. Lower the mouse onto the wire rack of a cage lid and let it grab hold of the bars.
14. With the thumb and index finger of your other hand, firmly grab the loose skin at the back of its neck and pull back to secure its head.

15. Turn the mouse over and secure its tail between your fourth finger or pinky and the palm of your hand. Slightly tilt your hand, such that the head of the mouse is lower that its abdomen. Your other hand should now be free again (*see* **Note 22**).
16. Pick up the syringe and gently insert it into the lower right quadrant of the abdomen at an approximately 30-degree angle (*see* **Note 23**).
17. Pushing down on the plunger, inject the required amount of tamoxifen (*see* **Notes 24–27**).
18. Pull back the syringe. Dispose of it immediately if you are done injecting and a sharps container is within reach. Alternatively, put down the syringe and dispose of it in a sharps container after you have finished injecting all mice and the animals have been returned to the cage (*see* **Note 28**).

3.2 Tracing

1. In the first few days after administering tamoxifen, check on the mice regularly to ensure there are no ill side effects of tamoxifen administration.
2. Wait for the desired amount of trace time to pass (*see* **Note 29**). If required, set up timed-matings to trace the contribution of your cell population of interest to alveoli formation during pregnancy.

3.3 Analyzing the Trace Experiment by Whole-Mount Confocal Microscopy

1. Transfer the mouse to a euthanasia chamber.
2. Start the flow of CO_2 to euthanize the animal (*see* **Note 30**).
3. Confirm that the animal is dead.
4. Spray the mouse with 70 % ethanol and pin it to a dissection pad.
5. Cut off a piece of tail and store it in an Eppendorf tube at –20 °C to confirm the genotype of the animal by PCR at a later time point.
6. Remove the mammary glands as shown in Fig. 4, working as described in **steps 7–18** (*see* **Note 31**).
7. Using a pair of forceps to hold the skin, make an incision along the ventral midline with a pair of sharp scissors. The mammary glands are located outside the peritoneum, so try to leave it intact when you cut open the skin.
8. Moving from the midline towards the hind limb, make an incision between the fourth and the fifth mammary gland. This should result in an incision that is at an approximate 45-degree angle to the incision along the ventral midline made in **step 7**. Be careful not to cut any blood vessels.
9. Moving from the midline towards the front limb, make another incision at a 45-degree angle to the incision along the ventral midline. Be careful not to cut any blood vessels (*see* **Note 32**).

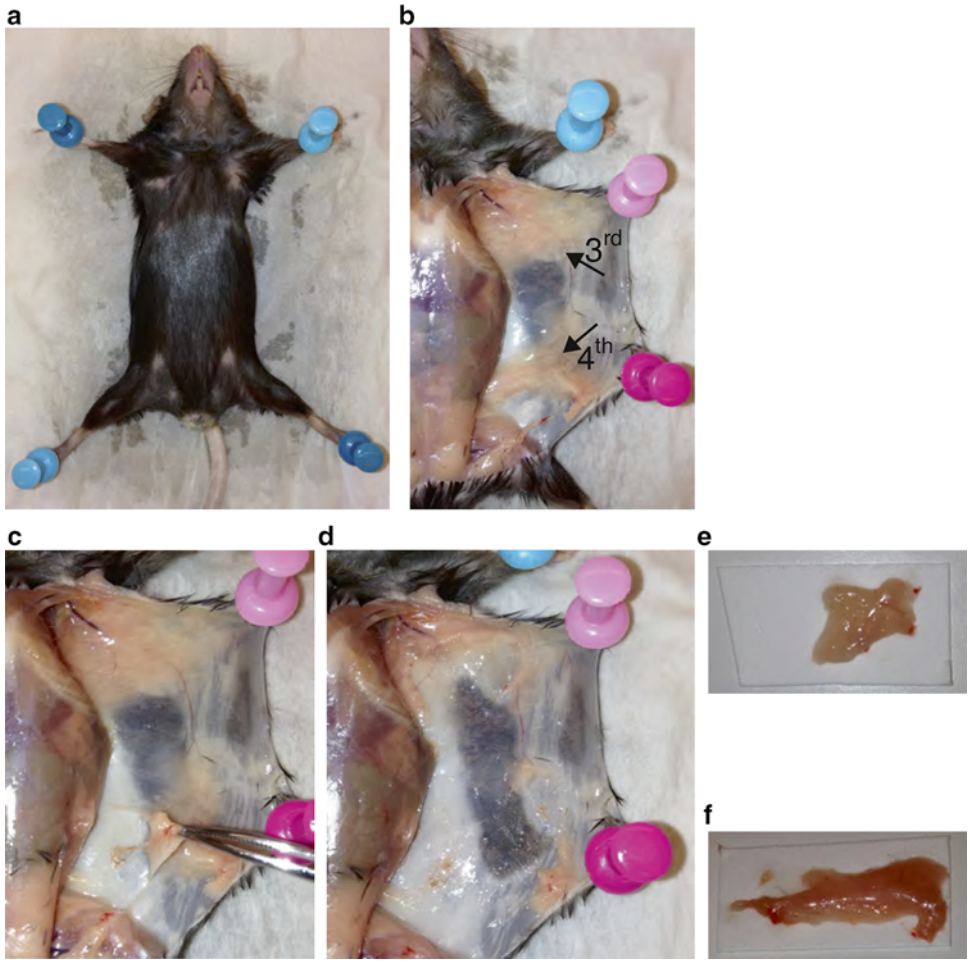

Fig. 4 Isolation of the third and fourth mammary gland as described in Subheading 3.3, **steps 1–18**. (**a**) Image showing the secured animal on a dissecting pad, as described in **step 4**. (**b**) Image showing that both the third and the fourth mammary gland (*arrows*) can be easily accessed after following **steps 7–10**. (**c**) Image showing how to remove the fourth mammary gland by following **steps 12–14**. (**d**) Image showing the skin after both the third and the fourth mammary gland have been removed. (**e–f**) Image showing the third (**e**) and the fourth (**f**) mammary gland after isolation and transfer to a piece of 3 mm Whatman paper

10. Using one pair of fine forceps to hold the peritoneum and a second pair of fine forceps to grab the skin, peel the skin flap in between the upper and lower incisions away from the peritoneum and pin it down to the dissection pad. The third and the fourth mammary glands should now be exposed.
11. Repeat **steps 7–10** on the contralateral side.
12. Using one hand to grab the most distal tip (i.e., closest to the spine) of the fourth mammary gland with a pair of fine (or Dumont no. 5) forceps.

13. Holding a pair of scissors in your opposite hand, gently cut away the fine connective tissue that attaches the mammary gland to the skin, starting at the distal tip and progressing towards the nipple.
14. As you cut away the connective tissue, gently pull the mammary gland upwards.
15. Cut off the mammary gland close to the nipple.
16. Position the mammary gland on a piece of 3 mm Whatman paper and use a pair of Dumont no. 5 forceps to stretch it out as well as possible (*see* **Note 33**).
17. Repeat **steps 12–16** for the fourth mammary glad on the contralateral side.
18. Remove the third pair of mammary glands in a similar fashion (*see* **Note 34**).
19. Process the mammary glands for whole-mount confocal microscopy as shown in Fig. 5, working as described in **steps 20–34**.
20. Transfer the pieces of Whatman paper with the mammary glands to a vial with 10 % neutral buffered formalin (*see* **Note 35**).
21. Wrap the vial in aluminum foil and fix the mammary glands by incubating them on a nutator or tube roller for up to 1 h at room temperature (*see* **Note 36**).
22. Peel away the pieces of Whatman paper and replace the formalin solution with PBS.
23. Incubate on a nutator or tube roller for 15 min at room temperature (*see* **Note 37**).
24. Replace the PBS solution with 30 % ethanol and incubate on a nutator or tube roller for 15 min at room temperature.
25. Replace the 30 % ethanol solution with 50 % ethanol and incubate on a nutator or tube roller for 15 min at room temperature.
26. Replace the 50 % ethanol solution with 70 % ethanol and incubate on a nutator or tube roller for 15 min at room temperature.
27. Replace the 70 % ethanol solution with 100 % ethanol and incubate on a nutator or tube roller for 15 min at room temperature.
28. Replace with fresh 100 % ethanol and incubate on a nutator or tube roller for another hour at room temperature (*see* **Note 38**).
29. Transfer the mammary glands to a fresh vial with methylsalicylate, cover it in aluminum foil and incubate on a nutator or tube roller for up to 1 h, or until the mammary glands are sufficiently cleared (*see* **Note 39**).
30. Use a pair of fine forceps to lift the cleared mammary glands from the vial.

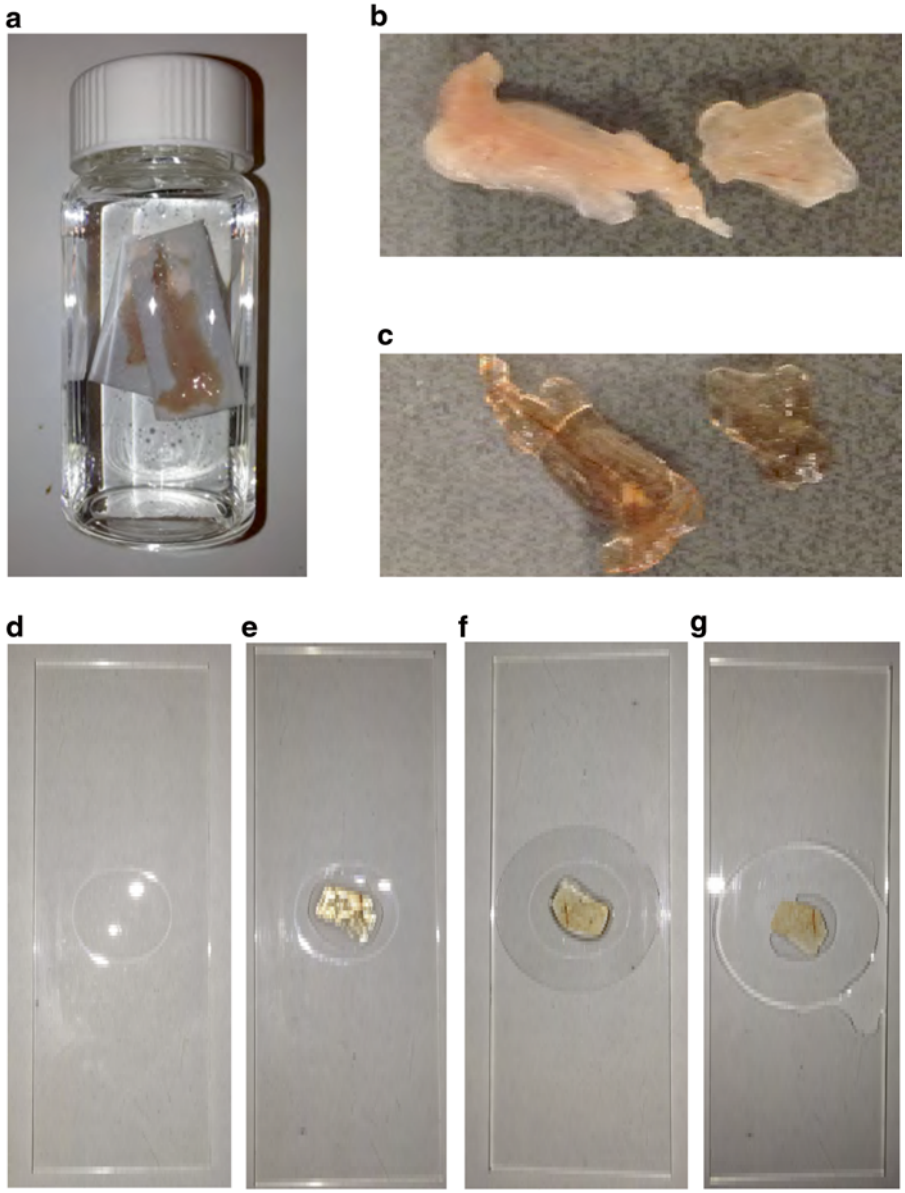

Fig. 5 Processing the mammary glands for confocal microscopy as described in Subheading 3.3, **steps 19–33**. (**a**) Image showing the isolated mammary glands in a glass vial with formalin fixation solution. (**b**) Image showing the mammary glands after fixation, prior to clearing. (**c**) Image showing cleared mammary glands following fixation, dehydration, and incubation in methylsalicylate. (**d**) Image showing a 14 mm concave microscope slide. (**e**) Same as in (**d**) with a trimmed piece of cleared mammary gland tissue. (**f**) Same as in (**e**) with a coverslip positioned onto the specimen. (**g**) Same as in (**f**) after carefully exerting pressure on the coverslip to flatten the tissue, while simultaneously removing excess methylsalycilate

31. Using a pair of scissors, cut a piece that will fit in the opening of a concave microscope slide (*see* **Note 40**).
32. Position the piece in the middle of the 14 mm diameter circle and add a drop of methylsalicylate from the incubation vial.
33. Cover the mammary gland with a coverslip and (wearing gloves) gently press down on the coverslip to flatten the tissue and remove excess methylsalicylate.
34. Wipe away the excess methylsalicylate with a Kleenex tissue (*see* **Note 41**).
35. Seal the coverslip with nail polish.
36. Let the nail polish dry completely before taking your samples to the confocal microscope (*see* **Note 42**).
37. Mount the slide on the stage of the confocal microscope.
38. Use a 488 nm laser line to excite the membrane-bound GFP and a 568 laser line to excite the membrane-bound dTomato (*see* **Note 43**).
39. Image the endogenous fluorescence signal using a 20x/0.7NA objective. If applicable, record a Z-stack (*see* **Note 44**).
40. When you are done, remove your slide from the microscope stage and switch off the microscope (the following **steps 41–46** are optional).
41. To process the cleared glands for paraffin embedding, remove the coverslip from the microscope slide using a bit of acetone and a Kleenex tissue.
42. Transfer the mammary gland to a glass vial with molten paraffin.
43. Incubate in a hybridization oven at 60 °C for 1 h.
44. Refresh the paraffin.
45. Incubate overnight in a hybridization oven at 60 °C.
46. Embed the mammary gland in a tissue cassette for future paraffin sectioning (*see* **Note 45**).

3.4 Constructing a 3D Picture of Labeled Alveoli Using Fiji

1. Download Fiji software from http://fiji.sc/Fiji and install the software on your computer.
2. Import the source files from your confocal microscopy experiment and visualize your Z-stack with Image 5D (*see* **Notes 46** and **47**).
3. Select 'Color' and assign colors to each of your channels.
4. If required, select Image → Adjust → Brightness/Contrast and adjust the sliders to improve the signal to noise ratio (*see* **Note 48**).
5. Select Plugin → Image 5D → Image 5D stack to RGB.

6. To make a three-dimensional reconstruction, follow **steps 8–11**.
7. To make a maximum projection, follow **steps 12–14**.
8. Make sure the RGB stack window is selected.
9. From the menu select Plugins → 3D viewer and press 'OK'.
10. Make sure the ImageJ 3D viewer window is selected.
11. From the menu, select View → Record 360 degrees rotation.
12. Make sure the RGB stack window is selected.
13. From the menu select Image → Stacks → Z-project.
14. Choose the appropriate projection (usual Maximum Intensity or Average Intensity will be the right option) and press 'OK'.

4 Notes

1. Another approach would be to use FLP/FRT technology. However, the number of available mouse strains based on Cre/lox technology far exceeds the collection of existing FLP/FRT lines.
2. Most available Cre drivers are conventional transgenic lines, meaning that the transgene cassette (comprising the Cre recombinase gene and the selected promoter and enhancer sequences driving its expression) has randomly integrated into the genome. As a result, Cre expression is unlikely to completely recapitulate the expression pattern of the original gene from which the promoter and enhancer sequences are derived. In addition, it is important to realize that not all strains with the same name carry an identical transgene insertion as individual founder lines or independent transgenic constructs may have been shared within the scientific community. Cre knock-in alleles, in which the Cre recombinase is targeted to the locus of interest, are more likely to faithfully recapitulate the endogenous gene expression pattern. Regardless, it is recommended that investigators do not solely rely on the published literature describing the Cre expression pattern. Few if any lines have been exhaustively characterized and ideally, Cre activity in each line should be carefully assessed in both spatial and temporal terms prior to commencing the actual lineage tracing experiment.
3. When using a straight (i.e., non-inducible) Cre allele, the first cells to express the recombinase will be labeled. For instance, K14-Cre first becomes active in the developing embryo. This results in labeling of $K14^+$ cells in the embryonic mammary bud and precludes the specific interrogation of $K14^+$ cells in the postnatal mammary gland. Modified versions make use of a Cre-ER fusion gene, in which the Cre recombinase has been

fused to a moiety of the estrogen receptor (ER), thereby rendering the Cre recombinase inactive in the absence of estrogen analogues [15]. Later generations contain specific point mutations in the ER portion of the fusion gene (Cre^{ERT} [16] or Cre^{ERT2} [17, 18]), which greatly reduce sensitivity to circulating endogenous estrogens. This tightens the system by eliminating leakiness, while simultaneously allowing the induction of Cre recombinase activity by the administration of the synthetic estrogen analog tamoxifen.

4. Alternatives to this system already exist and as the experimental methodologies continue to be refined, more will undoubtedly become available in the future. For instance, lineage tracing can also be performed using a three-component system comprising a tissue-specific rtTA driver, a tetO-Cre recombinase and a floxed reporter allele. Each system has its own advantages and disadvantages, but the use of more than two alleles greatly complicates mouse breeding.
5. Cre-inducible reporter alleles usually contain a so-called stop cassette, which is flanked by loxP sites and which prevents transcription of the downstream marker gene. Upon recombination of the reporter allele, the stop cassette is excised and the marker gene is expressed. Genetic recombination is irreversible. Therefore, the mark not only remains present for the remainder of the cell's life span but it is also passed on to all of the cell's offspring. It is this property that allows lineage tracing.
6. A more sophisticated reporter does not necessarily represent a better choice. For instance, compared to *Rosa26-mTmG*, the *Rosa26-Confetti* reporter recombines less efficiently in the mammary gland. Thus, whereas the *Rosa26-mTmG* reporter can be used to label cells at all stages of embryonic and postnatal mammary gland development in combination with the *Axin2CreERT2* driver, in my hands use of the *Rosa26-Confetti* reporter is effectively limited to puberty.
7. It is recommended to backcross the Cre driver and reporter lines to the same genetic background (usually C57/B6 or FVB), owing to differences in mammary gland development between individual mouse strains. The reporter lines listed in Table 1 can be maintained as a homozygous stock on a C57/B6 background without any breeding problems. Whether the same holds true for the Cre driver will depend on the specific strain. I prefer to keep the $Axin2^{CreERT2}$ mice as a heterozygous stock on a C57/B6 background. Even though the $Axin2^{CreERT2}$ allele is not leaky, I always maintain separate stocks of the driver and reporter lines, intercrossing them only as required. The presence of a single copy of the reporter allele is sufficiently sensitive to perform lineage-tracing experiments in the mammary gland.

8. Tamoxifen is a structural estrogen analog and is classified as a carcinogen. Gloves should be worn when handling and weighing the product.
9. Tamoxifen is notoriously difficult to dissolve. Including 10 % ethanol helps to get the tamoxifen into solution. Instead of corn oil, it is also possible to dissolve the tamoxifen in oil that is fit for human consumption (e.g., sunflower oil bought in the supermarket).
10. Methylsalicylate (also known as oil of wintergreen) is toxic upon ingestion. Inhalation and contact with the skin should be prevented. It has a very penetrant smell and even when the stock bottle is opened in the fume hood it is impossible to prevent the smell from entering the lab. It has, however, long been used as a clearing agent and in my hands, after testing many different methods, it gives the best results for whole-mount confocal microscopy. The commercially available clearing agent Focus Clear (Cedarlane Labs) gives good results as well, but it is very expensive.
11. Given the cost associated with purchasing a confocal microscope, the average investigator will most likely have little choice with respect to the equipment that is available for use. He/She is advised to talk to a local expert, who can help with the appropriate settings. In the past we used a Leica SP5 equipped with 3 HyD detectors and 2 PMTs, which is user friendly and offers the benefit of having a slider rather than fixed-width bandpass filters. The latter is particularly useful for multi-color reporter (e.g., *Rosa26-Confetti*) imaging. We are currently using a Nikon A1 to detect endogenous fluorescence of the *Rosa26-mTmG* and the *Rosa26-Confetti* reporter alleles and we have also observed endogenous fluorescence on a Carl Zeiss LSM510.
12. As an investigator, you are both ethically and scientifically responsible. Make sure that all animal experiments are approved by your local animal welfare committee prior to starting and always adhere to the local guidelines.
13. Always genotype your mice, even if you "know" what the genotype is supposed to be! It is almost inevitable that at some point or another animals will be switched (either because they are put in the wrong cage, or because their mark is misread), especially when you are breeding large numbers of mice and/or multiple strains. It is better to catch a mistake early on than after many months (or years) of crossing. I genotype the *Axin2-Cre*^{ERT2} mice using the forward primer RVA283 (5′CGATGCAACGAGTGATGAGGTTC3′) and the reverse primer RVA282 (5′GCACGTTCACCGGCATCAAC3′), with

an annealing temperature of 58 °C and a total of 35 cycles. This results in a Cre-transgene specific product of approximately 350 base pairs. I genotype the *Rosa26-mTmG* mice using the three-primer PCR posted on the Jackson labs website for this strain. Primers RVA284 (or oIMR7318, 5′CTC TGCTGCCTCCTGGCTTCT3′), RVA285 (or oIMR7319, 5′CGAGGCGGATCACAAGCAATA3′), and RVA286 (or oIMR7320, 5′TCAATGGGCGGGGGTCGTT3′) give a band of 330 base pairs for the wild-type *Rosa26* locus and a band of 250 base pairs for the *Rosa26-mTmG* allele. This PCR can also be run with an annealing temperature of 58 °C and a total of 35 cycles. Primers RVA284, RVA285, and RVA286 can also be used to genotype the *Rosa26-Confetti* mice.

14. It is easiest to genotype the mice around the time they are weaned (approximately postnatal day 21, P21) and to also give each of the animals a unique identifier at this point (e.g., earclip). Collect a piece of tail, toe or ear tissue (according to local guidelines) and lyse the tissue. The easiest way to get DNA out, is to lyse your samples in Viagen tail lysis buffer (use approximately 100–200 μl per sample) supplemented with 100 μg/ml proteinase K. Following overnight incubation at 55 °C, inactivate the proteinase K by heating the samples to 85 °C for 15–45 min. You can now directly use 1–10 μl the lysate as input for your genotyping PCR. Alternatively, you can follow a DNA isolation protocol that uses homemade lysis buffer [19]. Using the commercial lysis buffer is faster, but more expensive. There is no difference in the quality of the DNA in terms of performance in the PCR.
15. This only holds for traces that are started in postnatal animals. If you start an embryonic trace (e.g., as outlined in Fig. 3b) you can only genotype the experimental animals after they are born. When you initiate a trace in early postnatal animals (e.g., P14, but in reality anything prior to weaning), it is often unpractical to genotype the mice beforehand as well. In these cases it is advisable to set up your breeding schemes in such a way that your litters have a high chance of containing double-heterozygous mice, but remember that not all strains perform well as breeders when they are homozygous.
16. Like any experiment, a lineage-tracing analysis should be well controlled. A technical positive control will usually be difficult (if not impossible) to obtain, since it would require an independent Cre-driver that is known to work in your tissue of interest. If possible, use a different tissue from the double-heterozygous mice in which you initiate the trace as a positive control at the time of analysis. For instance, *Axin2-Cre*^{ERT2} also marks intestinal stem cells. Because Cre-mediated recombination in the

intestine is far more efficient than in the mammary gland, I often check if my experiment has worked by isolating a piece of intestine from tamoxifen-injected double-heterozygous *Axin2-Cre^{ERT2};Rosa26-mTmG* mice and quickly score that under a fluorescent microscope.

17. As a negative control, inject double-heterozygous *Axin2-Cre^{ERT2};Rosa26-mTmG* mice with corn-oil control solution. This will allow you to ascertain if your Cre allele is leaky. Because animal numbers are often limiting, it is virtually impossible to take this control along in every experiment. However, leakiness should ideally be tested for every Cre strain for each experimental protocol (i.e., breeding scheme and age of analysis) at least once at the outset of the experiment.
18. To be sure that the fluorescence signal you are scoring in your analysis is real, you can take along a littermate that does not carry the Cre-allele, but which is heterozygous for the *Rosa26-mTmG* allele. This is especially important when you are just getting started and still need to become familiar with the pattern of clonal outgrowth that you will see and/or if you are analyzing the mammary gland by FACS analysis. If you are truly performing clonal tracing analyses (i.e., each mammary gland will only contain a few positive clones), the majority of the tissue will in fact be an intrinsic negative control and individual (or patches of) labeled cells will be easy to identify in an otherwise unrecombined epithelium.
19. For the correct interpretation of your tracing experiments, proper biological controls are essential. For instance, when you are studying the contribution of mammary stem cells to turnover of the mammary epithelium during multiple rounds of pregnancy, lactation and involution, be sure to trace nulliparous and primiparous mice as well. Given the biological variation between individual mice, it is best to always use littermates of the multiparous mice you are tracing and, when the mice are not in a mating scheme, to house these females in the same cage as much as possible.
20. Filter sterilizing may not always be required and most likely depends on the quality of the oil you use to dissolve the tamoxifen. When using corn oil, I always do it as a precaution, after experiencing problems with injecting unfiltered corn oil at some point. Be careful when exerting pressure on the syringe, as the solutions will be very viscous.
21. This protocol will assume that tamoxifen is administered by means of an intraperitoneal injection. Alternatively, oral gavage may be used. This is a matter of personal preference.
22. Find out what is most comfortable. Either inject with your dominant hand and hold the mouse with the other, or vice versa.

23. Injecting at a shallow angle in this quadrant minimizes the chance of damaging internal organs, such as the liver, small intestine, caecum, and bladder. If you want to make sure you are in the right spot, you can always aspirate a small volume prior to injecting. If the aspirate is green/brown, yellow, or red, you have penetrated the intestine, bladder, or blood vessels and you should not inject. However, as long as you do not insert the needle too deep (i.e., up until about 0.5 cm) this should not be a problem.
24. A tamoxifen inducible system offers experimental control over the onset, but also the extent of recombination. This is particularly important when the lineage-tracing experiment is designed to label stem cells. To be able to track clonal outgrowth, Cre-mediated recombination should be sporadic. This ensures that labeled cell clusters really are the clonal offspring of a single recombination event in an individual stem cell. Theoretically, each mammary gland should thus contain a single cell clone. In practice however, it means that there should be sufficient space (i.e., stretches of unlabeled tissue) between labeled patches of tissue. This can be achieved by lowering the amount of tamoxifen that is injected. I often get asked what the half-life of tamoxifen is following injection as it will circulate in the animals for some time following injection. In practice, however, it is very well possible to label even fast-dividing cells (e.g., in the intestinal crypt [6, 20] or the developing embryo [21]) by essentially causing a pulse of Cre-activity.
25. In my experience (at least with the *Axin2-Cre^{ERT2}* mice), tamoxifen-mediated recombination in the mammary gland is far less efficient than in other tissues. For instance, in the skin [22] and the intestine [6], which also contain Wnt-responsive stem cells, we can inject as little as 0.1–0.2 mg tamoxifen per 25 g of body weight into adult mice and still score a sufficient number of clones. By comparison, recombination in the mammary gland requires 4 mg tamoxifen per 25 g of body weight in adult mice. This translates to administering 200 μl of a 20 mg/ml tamoxifen solution into an 8–10-week-old mouse. The amount of tamoxifen that is to be injected will vary depending on the experiment (e.g., if clonal labeling is not required, more tamoxifen can be injected), the reporter that is used (e.g., using the same amount of tamoxifen, recombination is far less efficient in *Axin2-Cre^{ERT2};Rosa26-mTmG* mice than in *Axin2-Cre^{ERT2};Rosa26-Confetti* animals) and the Cre driver.
26. The absolute amount of tamoxifen (as well as the total injection volume) should always be adjusted for the age and weight of the mice. For instance, when inducing sporadic recombination in P14 animals, I try to inject at most 1–2 mg tamoxifen

in a total volume of 100 µl. Injecting tamoxifen at this age can result in a growth deficit. The animals will eventually catch up, but it may require them to stay with the mother for a bit longer. Extra care should be taken when inducing recombination in the developing embryo by injecting tamoxifen into pregnant animals. I prefer to use a low stock concentration (i.e., a tamoxifen solution of 2–5 mg/ml) and try to inject no more than 0.5 mg tamoxifen in total. Even so, the injection of tamoxifen into pregnant females can induce termination of the pregnancy and/or delivery problems. This can be avoided by co-injecting progesterone [21].

27. Please be aware that tamoxifen is a mixed estrogen agonist/antagonist, meaning that it can either mimic or counteract the effects of estrogen in different tissues. Since the mammary gland is particularly sensitive to estrogen, it is important to be aware of potential side effects. For instance, we have observed that even a single injection of tamoxifen prior to or during puberty (at concentrations below those reported in the literature) can delay mammary gland outgrowth [23]. Although the tissue eventually catches up, this is something to keep in mind. This is a downside of using tamoxifen-mediated Cre/lox recombination for lineage-tracing experiments in the mammary gland, but at present this remains the most flexible, informative and widely used system of choice.
28. Never recap a used needle.
29. To interpret the outcome of a long-term tracing experiment, it is advisable to collect samples at (i.e., set up dedicated tracing experiments for) various time points along the way. First, you will always want to determine which cells first recombine the reporter allele (i.e., your starting population) by performing a short term tracing experiment. The *Rosa26-lacZ* reporter will allow you to analyze the mammary gland as early as 24 h after tamoxifen administration, whereas the GFP signal in the *Rosa26-mTmG* cannot be detected robustly until 48 h after tamoxifen administration. Second, if you are interested in tracing stem cells, it is important to note that (even when you are using a driver that is known to label stem cells, such as *Axin2-Cre*^{ERT2}) not all labeled cells are indeed stem cells. To qualify as a stem cell, a cell needs to meet two criteria: It has to give rise to differentiated offspring and it has to survive turnover of the tissue. The only way to be sure that the mammary epithelium has completely turned over is by tracing cells through a complete round of pregnancy, lactation and involution. Here too, it will be helpful to have some analytical time points along the way (e.g., prior to pregnancy, mid-pregnancy, mid-lactation, mid-involution, post-involution).

30. Please follow the local guidelines for euthanizing animals.
31. There are many ways in which a lineage tracing experiment can be analyzed. This includes FACS analysis, but also various histological analyses. The remainder of the protocol will describe analysis by whole-mount confocal analysis, which allows 3D reconstruction of labeled structures.
32. Accidentally hitting a blood vessel is more likely to occur near the front limbs than near the hind limbs. Try to prevent it by not cutting too far away from the midline.
33. The idea is to fix the mammary gland in such a way that it stays flat. Another way to achieve this is by stretching the mammary gland on a glass slide, covering it with a second glass slide and fixing this "sandwich" in a 50 ml Falcon tube. This works well, but uses a lot more formalin.
34. The boundaries of the third mammary gland are not as well defined as the fourth mammary gland, so isolating it may feel a bit intuitive at first. It is closely associated with the first and second mammary gland. In addition, there is a high chance of collecting some muscle tissue and of hitting blood vessels, both of which may obscure a whole-mount preparation. On the up side, the third mammary gland is a lot flatter than the fourth, which helps for whole-mount confocal microscopy.
35. You can use glass scintillation counter vials that hold up to 25 ml for processing multiple glands together, or smaller snap cap glass vials that hold up to 4 ml for processing an individual gland.
36. As little as 15 min may be enough. In fact, if you are interested in analyzing the *Rosa26-mTmG* reporter by whole-mount confocal microscopy, you can omit the formalin fixation step altogether and transfer the isolated mammary glands directly to 100 % ethanol. Because the GFP and dTomato are membrane bound, their signal will be preserved. Importantly, this will not work for the *Rosa26-Confetti* reporter, which does require formalin fixation. Therefore, it is safest to include this step for all fluorescent reporters.
37. Formalin should be disposed of as toxic waste. Please follow local guidelines.
38. It is important that the tissue is completely dehydrated prior to transfer into methylsalicylate, otherwise the clearing will not work.
39. Use approximately 5 ml per mammary gland. Provided that the mammary glands are properly dehydrated, clearing will become apparent within 15 min.
40. For the third mammary gland it is often sufficient to trim the edges. For the fourth mammary gland, I usually cut straight through the lymph node. This separates the proximal from the

distal portion of the gland and still allows the lymph node to be used for orientation. After this, trim the edges of the fat pad and remove excess pieces of tissue from the top or bottom.

41. At this point, there should be a continuous ring of methylsalicylate surrounding the concave opening. If you see any air pockets, this means the specimen either needs to be flattened further or is too thick. In the latter case, you can remove the tissue and use pair of scissors to trim it until it does fit. The entire dimple holding the tissue does not need to be filled with methylsalicylate, but to prevent the tissue from drying out, it is best to only mount the specimen immediately prior to microscopic analysis.
42. At this point, I usually dry the slides in the dark (i.e., in a drawer). There is no need to switch off the overhead lights during any of the previous steps. The fluorescent signal will not be quenched.
43. For the *Rosa26-Confetti*, use 458 nm (for CFP), 488 nm (GFP), 514 nm (YFP), and 561 nm (RFP) laser lines.
44. This is the most difficult part of the procedure. It takes time and patience to get a feel for the appearance of the whole-mount tissue under the microscope. I usually scan the tissue through the eyepiece, using a UV lamp and a GFP filter. With the *Rosa26-mTmG* reporter, this gives you just enough cell outlines to see where you are in the tissue. Using the focus knob to move up and down, you will be able to discern epithelial branches and blood vessels surrounded by adipocytes. After a while, you will be able to identify GFP-positive cell clusters based on their increased intensity. Finding labeled cells with the *Rosa26-Confetti* reporter takes more effort, because you no longer have the information of the cell outlines provided by the membrane-bound dTomato, but can be done as well. Using a 20x/0.7 NA objective should give you enough working distance to make a Z-stack through an entire alveolar cluster as shown in Fig. 6.
45. The described protocol is compatible with paraffin embedding of the cleared mammary glands. The same labeled cell clusters can thus be imaged as whole mounts and on tissue sections in combination with other markers. During the processing for paraffin embedding, the dTomato signal is always lost. This frees up a channel for co-staining with antibodies directed against structural markers. Sometimes, the GFP signal will be preserved, but it is advisable to detect recombined cells with an anti-GFP antibody (I like the Abcam chicken-anti-GFP, ab13790). The downside of using methylsalicylate cleared samples for paraffin embedding is that it precludes heat-induced epitope retrieval (HIER) as the tissue tends to come off the slide. However, on paraffin sections from *Rosa26-mTmG*

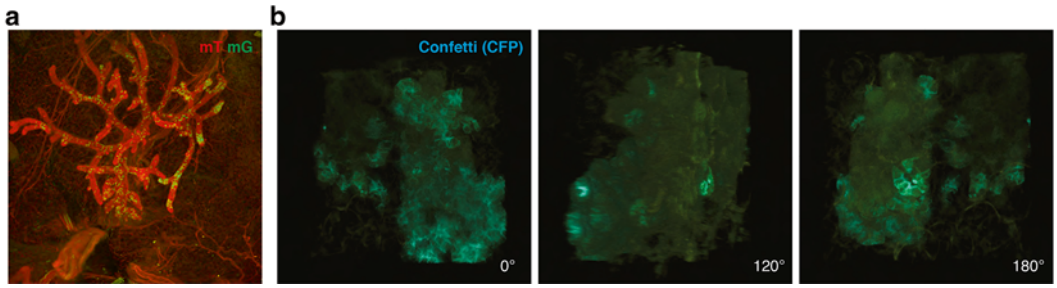

Fig. 6 Whole-mount confocal microscopy images after processing in Image J. (**a**) Maximum Z-projection of a whole-mount *Axin2-CreERT2;Rosa26-mTmG* mammary gland showing recombined (mG, *green*) and unrecombined (mT, *red*) cells (168 slices, imaged on a Leica SP5 confocal microscope using a 20x/0.7NA objective). For this particular experiment, the trace was initiated at E13.5 and the mammary glands were isolated at P8. Prior to puberty, the ductal network is small and an entire epithelial tree fits into the frame of view when using a 20× objective. (**b**) Snapshots from a movie showing the 3D rotation of a reconstructed alveolar cluster of a whole-mount *Axin2-CreERT2;Rosa26-Confetti* mammary gland demonstrating the clonal outgrowth of recombined (CFP, *blue*) cells (107 slices, 1024 × 1024 pixels, imaged on a Leica SP5 confocal microscope using a 20x/0.7NA objective and a 4× zoom). For this particular experiment, the trace was initiated at P42 and the mammary glands were isolated at E14.5 of the first pregnancy

mammary gland samples that are processed for whole-mount confocal microscopy with methylsalicylate but without including the formalin fixation step (*see* **Note 36**), you can often use antibodies that would normally require HIER on regular formalin-fixed paraffin-embedded sections (including the anti-GFP antibody mentioned above). You should test which conditions are optimal for your antibodies. Alternatively, you can always process one mammary gland for whole-mount confocal microscopy and the contralateral gland for formalin fixation and paraffin embedding, thus allowing HIER and colocalization studies by confocal microscopy.

46. When using a Leica SP5 confocal microscope, you can simply drag the *.lif file into the Fiji toolbar. You will then be presented with the option to 'View as Image 5D'. Choose this option. For other microscopes, the images may open as a stack. In that case, select Plugin → Image 5D → Stack to Image 5D and proceed as described.
47. If you happen to only be able to import individual *.tif files, wait for all the files in your stack to import. Then select Image → Stacks → Images to Stacks. Make sure the resulting stack is selected and choose Image → Hyperstacks → Stacks to Hyperstacks. Assign the correct number of channels (c) and sections (z). For Display Mode select 'Color'. Make sure that the resulting hyperstack is selected and choose Plugins → Image 5D → Stack to Image 5D and proceed as described.
48. Be careful when adjusting the sliders and try to prevent image adjustments as much as possible.

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