Chapter 8

Fate-Mapping of Yolk Sac-Derived Macrophages

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Abstract

To better understand the distinct functions of yolk-sac-derived tissue-resident macrophages (TRMs) and bone-marrow-derived macrophages in homeostasis and disease, it is important to trace the ontogeny of these cells. The majority of TRMs originate from erythro-myeloid progenitors (EMPs). EMPs develop into pre-macrophages (pMacs), which can be detected starting at embryonic developmental day (E)9.0, and which give rise to all TRM during early development. pMacs start expressing the gene Cx3cr1, allowing us to genetically target the early yolk-sac wave of pMacs and their progeny. Here, we describe the protocol for the identification of yolk sac-derived TRMs utilizing in utero labelling of the inducible fate mapping *Cx3cr1*^{CreERT}*; Rosa26*$^{LSL\text{-}eYFP}$ mouse model.

Key words Yolk sac, Embryo, Tissue-resident macrophages, Fate-mapping, EMPs, pMacs, Tamoxifen

1 Introduction

The origin of macrophages can be divided into three waves based on the appearance of progenitors with different hematopoietic potential. The first and second waves originate from the yolk sac (YS) and give rise mainly to erythrocytes and macrophages, but also to granulocytes, megakaryocytes, and mast cells [1, 2]. Many fetal-derived macrophages originating from the YS persist until adulthood via proliferation and do not rely on replacement from bone-marrow-derived monocytes. The third wave originates from the embryo proper and gives rise to hematopoitic stem cells (HSCs), which later seed the fetal liver and subsequently the bone marrow producing monocyte-derived macrophages, which can contribute to the TRM-pool in some adult tissues [3, 4].

Macrophages play a fundamental role in fetal development. They are involved in organogenesis via phagocytosis of dead cells during footplate remodeling [5], modulation of neuron outgrowth and positioning during the development of the brain [6], tissue vascularization [7], or bone remodeling [8]. Distinguishing YS-derived TRMs from monocyte-derived cells during

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embryogenesis, but also in adulthood, can serve us for a better understanding of how these cells are involved in establishment of homeostasis and progression of diseases. An inducible Cre recombination system can be used to genetically target YS-derived TRMs, which is achieved by fusing the Cre protein with estrogen receptor variants [9]. In this case, the Cre is in a cytoplasmic inactive form until activated by the administration of an estrogen-analog hydroxy-tamoxifen (OH-TAM) causing the translocation of the Cre into the nucleus where it can excise loxP sites. Activation of Cre was achieved by tamoxifen in the past, but recent studies demonstrated the indispensability of using OH-TAM, the active metabolite of tamoxifen, in in utero fate-mapping studies due to its faster mode of action, which will result in more accurate time-controlled labeling. The translocation of Cre to the nucleus is a transient process and is inactive when the OH-TAM is washed out from the system.

Here, we show an example of how to fate-map YS-derived macrophages utilizing an inducible mouse model targeting pMacs. To this end, *Cx3cr1-Cre*^{ERT} males are mated with *Rosa26*$^{LSL\text{-}eYFP}$ females, which are subjected to a single dose of OH-TAM to obtain embryos in which eYFP expression is induced at E9.0, labeling the first wave of EMP-derived macrophages. Additionally, the preparation of fetal tissues (brain and liver) for flow cytometry analysis is described.

2 Materials

2.1 Mouse Model

Here we use *Cx3cr1*^{CreERT} (JAX Strain #:020940) and *Rosa26*$^{LSL\text{-}eYFP}$ (JAX Strain #:006148) mice to generate *Cx3cr1*^{CreERT}; *Rosa26*$^{LSL\text{-}eYFP}$ embryos where Cre activation is induced at day E9.0 by OH-TAM. Also time points up until E9.5 can be fate-mapped with the same protocol to target the first EMP/pMac wave. Please refer to Chapters 2 and 9 in this book for further fate-mapping models of distinct hematopoietic waves.

2.2 Consumables

1. Scissors (standard, blunt-end, and spring scissors angled to side ball tip).
2. Forceps (standard and fine).
3. Embryo spoon.
4. 15 mL tubes.
5. 1.5 mL microcentrifuge tubes.
6. 48-well plate.
7. 96-well plate, U-shaped.
8. 10 cm tissue culture dish.

9. 35 mm petri dish.
10. FACS tubes.
11. Cell strainers (70 and 100 μm).
12. 1 mL syringe with 25G needle.

2.3 Buffers and Solutions

1. 4-hydroxytamoxifen stock solution (OH-TAM): 10 mg/mL OH-TAM (Sigma, H7904) in 10% (v/v) molecular grade Ethanol and 90% (v/v) sunflower seed oil (Sigma, 88921). Prepare by decanting the OH-TAM powder into a 15 mL tube. Add 250 μL Ethanol (100%) to the remaining powder in the glass bottle, pipette up and down and transfer the solution into the 15 mL tube. Vortex and sonicate in a sonication bath for 30 min (*see* **Note 1**). Add 2.25 mL of sunflower seed oil to obtain a final volume of 2.5 mL OH-TAM solution. Vortex for approximately 5 min at maximum speed and sonicate for 30 min in a sonication bath (*see* **Note 2**). Store at 4 °C, protected from light for a maximum of 1 month (*see* **Note 3**).
2. Progesterone stock solution: 10 mg/mL Progesterone (Sigma, P3972) in sunflower seed oil (Sigma, 88921). Weight approximately 10 mg of powder into a 15 mL falcon tube. Add sunflower seed oil to obtain a 10 mg/mL stock solution. Vortex until the progesterone is dissolved. Store at 4 °C (*see* **Note 4**).
3. 1x Phosphate-buffered saline (PBS).
4. FACS buffer: 0.5% bovine serum albumin (BSA) and 2 mM Ethylenediaminetetraacetic acid (EDTA) in 1x PBS. Filter buffer through a 0.2 μm filter, e.g., using a bottle top vacuum filter. Store at 4 °C (*see* **Note 5**).
5. 2x digestion mix: 0.4 mg/mL DNase, 1 mg/mL Collagenase D, 6% fetal calve serum (FCS) in 1x PBS. Calculate the desired volume of the digestion mix (150 μL/sample). Keep on ice until used for tissue digestion.
6. Blocking solution: 1% (v/v) anti-mouse CD16/32 (e.g. clone 93 from Biolegend), 2% (v/v) of rat serum in FACS buffer. Calculate the desired volume of blocking solution (50 μL/sample).
7. *Antibody mix:* Calculate antibody mixes based on the number of your samples (*see* **Note 6 + 7**). Fill a 1.5 mL tube with the calculated amount of FACS buffer. Add respective antibodies into microcentrifuge tube and mix by pipetting.

   For the brain: CD45-BUV805, clone 30-F11, 0.25 μg/mL; CD11b-BUV737, clone M1/70, 1 μg/mL; Cx3cr1-PE-

CF594, clone SA11F11, 0.5 μg/mL; CD206-BV711; clone C068C2; 1 μg/mL.

For the liver: CD45-BUV805, clone 30-F11, 0.25 μg/mL; CD11b-BUV737, clone M1/70, 1 μg/mL; F4/80, clone BM8, 1 μg/mL; Cx3cr1-PE-CF594, clone SA11F11, 0.5 μg/mL; Tim4-BV786; clone 21H13; 1 μg/mL.

8. Live/dead staining: 5 μg/mL Hoechst33258 in FACS buffer.

2.4 Equipment

1. Ultrasonic unit (*see* **Note 8**).
2. Flow cytometer.
3. Centrifuge for FACS tubes and plates.
4. Optional: Epifluorescence microscope (or similar) with GFP detection.

3 Methods

3.1 In Utero Pulse Labeling by OH-TAM

1. On the morning of injection, sonicate the OH-TAM solution in a ultrasonic bath for 10–30 min until it is completely dissolved. Vortex well.
2. Pre-warm progesterone to room temperature.
3. Weigh the pregnant dam and prepare 75 mg/kg OH-TAM and 37.5 mg/kg progesterone (*see* **Note 9**) solution from stock-solutions in a 1.5 tube under the fume hood (*see* **Notes 10** and **11**).
4. Load the solution into a 1 mL syringe with a 25G needle under the fume hood (*see* **Note 12**).
5. In the animal facility, perform intraperitoneal injection by slowly injecting OH-TAM/progesterone solution into the pregnant dam on the day of interest (*see* **Notes 13** and **14**).
6. Keep the needle for approx. 10 s in the peritoneum to allow the oil to exit the syringe.
7. After withdrawing the needle, gently massage the abdomen to distribute the solution (*see* **Note 15**).

3.2 Preparation of Cell Suspension from Embryonic Tissue for Flow Cytometry Analysis

The organs of the embryo/fetus are very soft and the protocol of cell suspension preparation can be applied to the embryonic or fetal organs such as the skin, brain, liver, or spleen. Here we show an example of how to prepare cell suspension from E14.5 liver and brain suitable for flow cytometry analysis.

3.2.1 Embryo and Organ Isolation

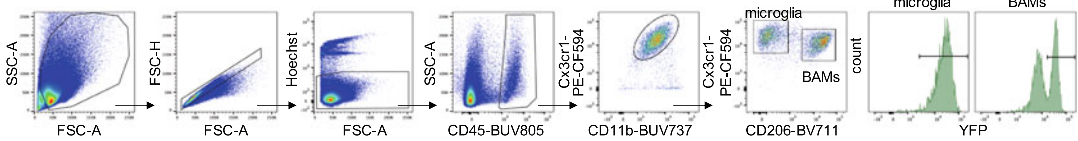
1. Sacrifice the pregnant mouse by cervical dislocation.
2. Perform a V-formed section on the abdomen and take out the uterus containing the embryos into a 10-cm tissue culture dish filled with cold PBS on ice (*see* **Notes 16** and **17**).
3. Catch the open side of one uterine horne using forceps and cut the uterus tissue using spring scissors until you reach the uterine junction. Repeat the same procedure for the other side of the uterus (*see* **Note 18**).
4. Collect single embryos in their YS into a new 10-cm tissue culture dish filled with cold PBS on ice using an embryo spoon.
5. Use fine foreceps to open the YS, so that embryos remain connected to the placenta via their umbillical vein.
6. Move each embryo into a separate 35 mm tissue culture dish filled with cold PBS on ice and cut off their heads to sacrifice them (*see* **Note 19**).
7. Isolate livers and brains using forceps and clean them from surrounding tissue (*see* **Note 20**).
8. Transfer isolated tissues into 48 well-plate filled with 150 μL of cold PBS per well. Keep on ice until all tissues have been collected (*see* **Note 21**).

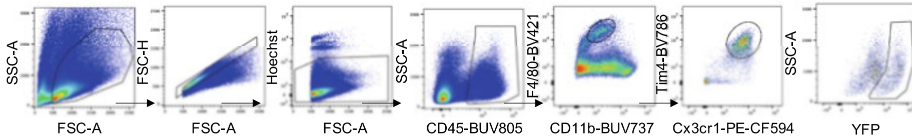
3.2.2 Cell Suspension Preparation

1. Prepare sufficient amount of the 2× concentrated digestion mix once you know the number of embryos being analyzed.
2. Fill each well of the 48 well-plate containing tissue with 150 μL of 2× digestion mix.
3. Cut tissues within the well with blunt-end scissors to obtain small pieces (*see* **Note 22**).
4. Incubate the tissues in the digestion mix at 37 °C for 30 min.
5. Place the 48 well-plate with samples on ice.
6. Add 500 μL of FACS buffer to each well to stop digestion.
7. Gently pipet the sample with a 1 mL pipet up and down to obtain a single-cell suspension.
8. Filter the samples through a 100 μm filter into the FACS tubes prefilled with 2 mL of FACS buffer.
9. Spin the FACS tubes with samples for 5 min, 400 g, 4 °C.
10. Remove the supernatant.

3.2.3 Staining of Cell Suspension with Antibodies

1. Add 50 μL of blocking solution into each FACS tube and mix by pipetting or careful vortexing.
2. Incubate for 15 min on ice.
3. Transfer 25 μL of the cell suspension into a 96-well plate and add 200 μL FACS buffer (*see* **Note 23**).





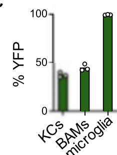


Fig. 1 Analysis of *Cx3cr1*^{CreERT}; *Rosa26*$^{LSL\text{-}eYFP}$ embryos at E14.5, with OH-TAM induction at E9.0. (**a**) Representative gating strategy for microglia and border-associated macrophages (BAMs) and the eYFP expression in both populations. (**b**) Representative gating strategy for Kupffer cells (KCs) and their eYFP expression. (**c**) Labeling efficiency. $n = 3$

4. Spin down the 96-well plate for 5 min, 400 g, 4 °C.
5. Remove supernatant.
6. Add 20 μL of the antibody mix and resuspend the pellet by pipetting.
7. Incubate on ice for 30 min in the dark (*see* **Note 24**).
8. Add 200 μL FACS buffer (*see* **Note 25**).
9. Spin down the 96-well plate for 5 min, 400 g, 4 °C.
10. Remove the supernatant.
11. Resuspend cells in 100 μL of FACS buffer.

3.2.4 Flow Cytometry Analysis

1. Filter the sample into the FACS tube through a 70 μm strainer.
2. Add 100 μL Hoechst33258 into the FACS tube containing the sample 1 min before the measurement.
3. Vortex sample and measure using a flow cytometer. An example showing the gating strategy for liver and brain, as well as the labeling efficiency of OH-TAM induction of YFP expression is shown in Fig. 1.

4 Notes

1. Make sure that the water bath does not heat up, otherwise add ice. The OH-TAM suspension must turn into a white homogenous suspension. If not, prolong the time of sonication.
2. The final OH-TAM solution must be a white homogenous suspension. If not, prolong the time of sonication.
3. If you will not use up 2.5 mL OH-TAM within 1 month, you can store the OH-TAM diluted in EtOH and aliquoted at –

20 °C. Make sure that you sonicate the OH-TAM well after thawing, do not do thaw/freeze cycles, and follow labeling efficiency results carefully as the efficiency of OH-TAM may decrease with time.

4. Note, that the solution is clear when stored.
5. Mix BSA well, best is to prepare fresh buffer the day before the experiment.
6. For embryonic tissue the 20 μL of antibody mix is sufficient, you may need to scale this volume up if you are staining in FACS tubes or more cells.
7. The majority of the flow cytometry antibodies contain sodium azide as protection against microbial contamination. Despite this fact, we recommend preparing antibody mixes in sterile conditions.
8. Use floating stands for 15 mL tubes to keep tubes upright. If your water bath is small and heats up quickly, add ice during sonication. The OH-TAM should be warmer than 42 °C as it will lose its efficiency after being exposed to higher temperatures.
9. It is crucial to supplement OH-TAM with progesterone to reduce abortion after tamoxifen administration to the pregnant dam. The OH-TAM treatment often leads to problems during birth, we suggest scarifying the mother and performing a cesarean section on E20, removing the pups, and using foster mothers if the study needs to be performed on the offspring at postnatal ages.
10. Depending on the mouse weight, you will inject about 100–150 μL in total, but a 25G syringe has about 50 μL dead-volume, thus, make sure you prepare enough OH-TAM/progesterone mixture.
11. 75 mg/kg OH-TAM is a high concentration, which will lead to high labelling efficiency, but also a higher abortion rate or increased mortility during labor. To circumvent these problems, you can decrease the OH-TAM concentration by half, especially when performing initial experiments where high labelling efficiency is not required.
12. Despite sonication, OH-TAM stays an emulsion. Thus, to make sure that small pieces are not getting stuck in the syringe during the injection, take up the mixture through the needle. Be very careful when placing the cap back on the needle if you have to transport the syringe to the animal facility.
13. Inject at the middle line of the belly so that you do not accidentlly inject into one of the embryos. Enter the perito-

neum with the needle at a low angle (approx. 15–20°, instead of the typical 45° angle for interperitoneal injecitons). Make sure by pushing the needly slightly up against the peritoneal wall that you are actually in the peritoneum and not injecting subcutaniously.

14. The day of interest is in this case the day of embryonic development (E). Embryonic development is estimated considering the day of vaginal plug formation as E0.5. Recombination is induced by a single injection of OH-TAM/progesterone solution. Example: The vaginal plug was observed in the morning, and the embryo's development is considered E0.5. If the induction should take a place at E9.0, the injection will be performed at 8:00 am 9 days after the vaginal plug discovery. If the induction should take place at E9.5, the injection will be performed at 1:00 pm, 9 days after vaginal plug discovery.
15. If you see a big oil spot after syringe withdrawal, make a note for this mouse and its offspring. It is likely that your labelling efficiency will be lower than expected.
16. It is important to work with cold PBS and on ice. If not, embryos will be degraded very fast.
17. If both sides of the uterus have embryos, it is easiest to grab the uterine junction near the cervix by standard foreceps and pull both uterine hornes out of the body. Then cut the uterus on both sides, remove any access of adipose tissue during the procedure.
18. The ball tip helps preventing piercing of the embryos. If you are familiar with dissections you may also use other fine sciccors to carfully cut open the uterus.
19. It is better if the embryo is floating in the dish because dissection of the embryo is easier.
20. Even other organs can be isolated. E12.5 embryos have already visible organs, so it is possible to work without a microscope. In the case, of smaller embryos, we recommend using a microscope or table magnifier.
21. The YFP signal can be detected when using the GFP channel of an Epifluorescence microscope. Like that, all Cre-positive and 1–2 Cre- embryos can be genotyped before running the samples.
22. It is important to use blunt scissors to avoid scratching the bottom of the well. The released plastic particles can kill the cells.
23. You can use the rest of the cell suspension to calculate cell numbers or run an additional antibody panel.

24. You can place the plate into the fridge. If you use aluminum foil and keep the plate on ice, you may need to increase the staining time since the ice/ice-water has a lower temperature than the fridge.
25. If you analyze many tissues and/or many embryos a multichannel pipette should be used.

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