Chapter 5

Isolation of Tissue Macrophages in Adult Zebrafish

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Abstract

Tissue macrophages are essential components of the immune system that also play key roles in vertebrate development and homeostasis, including in zebrafish, which has gained popularity over the years as a translational model for human disease. Commonly, zebrafish macrophages are identified based on expression of fluorescent transgenic reporters, allowing for real-time imaging in living animals. Several of these lines have also proven instrumental to isolate pure populations of macrophages in the developing embryo and larvae using fluorescence-activated cell sorting (FACS). However, the identification of tissue macrophages in adult fish is not as clear, and robust protocols are needed that would take into account changes in reporter specificity as well as the heterogeneity of mononuclear phagocytes as fish reach adulthood. In this chapter, we describe the methodology for analyzing macrophages in various tissues in the adult zebrafish by flow cytometry. Coupled with FACS, these protocols further allow for the prospective isolation of enriched populations of tissue-specific mononuclear phagocytes that can be used in downstream transcriptomic and/or epigenomic analyses. Overall, we aim at providing a guide for the zebrafish community based on our expertise investigating the adult mononuclear phagocyte system.

Key words Zebrafish, Macrophages, Microglia, Flow cytometry, *mpeg1.1*, *p2ry12*

1 Introduction

For years, the teleost fish zebrafish (*Danio rerio*) has been extensively used as a vertebrate model by developmental biologists due to the transparency and external development of the embryo, and its genetic similarity and amenability, among other features [1]. More recently, zebrafish has also emerged as a powerful tool for the study of hematopoiesis and immunity [2, 3], contributing notably to new insights into the developmental and immune functions of leukocytes [4–8]. Initially conducted on embryos and larvae, immunological investigations in zebrafish have progressively extended to the adult, which is now increasingly used to study the contribution of leukocytes, with a particular interest on macrophages, in a range of physiological processes and diseases, such as cancer, infection, as well as organ regeneration [9–14]. Due to the

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paucity of fish-specific antibodies and the poor antibody cross-reactivity between fish and mammals, these studies mostly rely on the use of transgenic animals engineered to express a fluorophore under the control of lineage-specific regulatory elements [15], and allowing to identify zebrafish leukocytes in vivo, on tissue sections or by flow cytometry [3]. While an increasing number of stable lines with fluorescently labeled macrophages have been generated and validated for use in transparent embryos and larvae, one caveat is that their reliability in the adult is not systematic. One striking example is seen with the widely used collection of reporter lines generated using the *mpeg1.1* promoter [16]. Indeed, although Tg (*mpeg1.1*:EGFP) or Tg(*mpeg1.1*:mCherry) transgenics can reliably serve as pan-macrophage reporters in the embryo and larvae, the situation changes in the adult due to the *mpeg1.1* promoter being active in other cell types, including B lymphocytes and non-leukocytic metaphocytes [17–19]. As B cells and metaphocytes both appear at around the juvenile stage [19, 20], this precludes the sole use of *mpeg1.1*-driven reporters for the isolation of tissue macrophages in the adult.

To overcome these limitations and facilitate future studies, we have established optimized protocols to analyze and isolate enriched populations of mononuclear phagocytes from the main zebrafish hematolymphoid organs. One protocol takes advantage of the unique combined expression patterns of the *mpeg1.1*:GFP [16] and the *cd45*:DsRed [21] fluorescent reporters, which are both readily available to the zebrafish community. Indeed, in double transgenic animals, GFP is expressed in mononuclear phagocytes, B lymphocytes and nonleukocytic metaphocytes [17], while DsRed labels all leukocytes with the exception of B cells [21]. This combination thus makes it possible to discriminate mononuclear phagocytes from B cells and/or metaphocytes in the brain, liver, skin, and the whole kidney marrow (WKM), the site of adult hematopoiesis in teleosts [22]. Finally, another approach makes use of the Tg(*p2ry12*:p2ry12-GFP) transgenic line [23] which, in combination with the *cd45*:DsRed reporter, offers additional means for the specific isolation of microglia, the macrophage population of the central nervous system. We detail the whole procedure step-by-step for each organ, from the dissociation and generation of a single-cell suspension to the creation of the flow cytometry gates allowing for the successful isolation of the populations of interest (Fig. 1).

2 Materials

2.1 Zebrafish Lines (See Notes 1 and 2)

1. *Wild-type* strain (AB*).
2. Tg(*mpeg1.1*:EGFP)^{gl22}, here referred to as *mpeg1.1*:GFP.
3. Tg(*ptprc*:DsRed)^{sd3}, here referred to as *cd45*:DsRed.

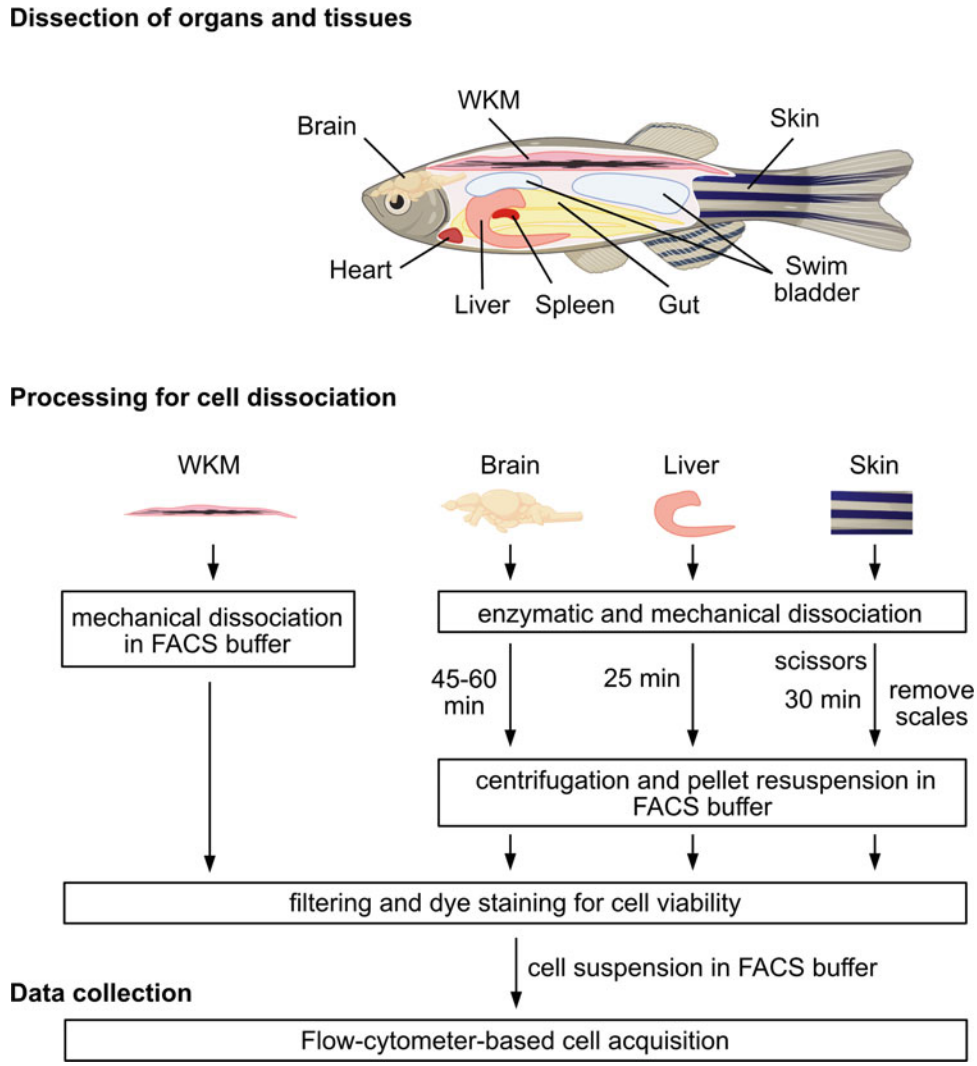

Fig. 1 Workflow of analysis of macrophages from the Whole Kidney Marrow (WKM), brain, liver and skin. Schematic representation of an adult zebrafish with the anatomical structures of interest labelled. Protocols to obtain single cell suspensions are optimized for every organ. The WKM is the only tissue that does not require enzymatic digestion and is mechanically homogenized in solution

4. TgBAC(*p2ry12*:p2ry12-GFP)^{hdb3}, here referred to as *p2ry12:* p2ry12-GFP.
5. Tg(*mpeg1.1*:GFP; *cd45*:DsRed).
6. Tg(*p2ry12*:p2ry12-GFP; *cd45*:DsRed).

For clarity, throughout the text, transgenic animals are referred to without allele designations.

2.2 Plasticware and Equipment

1. Scalpels.
2. Microdissection forceps.
3. Microdissection scissors.
4. Dissection scissors.
5. Petri dishes.
6. FACS tubes: 12 × 75 mm polystyrene round bottom tubes (5 mL).
7. Syringes, microfine insulin model (1 mL).
8. 20 G and 26 G needles.
9. 40-μM cell strainers.
10. Pipettes (5, 10, 25 mL).
11. Micropipettes and autoclaved pipette tips (P10, P200, P1000 μL).
12. Sterile unfiltered tips (P10, P200, P1000 μL).
13. Incubator, to be set at 33 °C.
14. Refrigerated swing-bucket centrifuge suitable for FACS tubes (10 min, 290 g at 4 °C).
15. Vortex mixer.
16. Aluminum foil.
17. Cell sorter, e.g. BD FACS ARIA III.

2.3 Media and Reagents

1. Sterile Dulbecco's Phosphate Buffered Saline (DPBS) 1×.
2. DPBS 0.9×: 500 mL 1×(DPBS) + 55 mL deionized water. Keep on ice.
3. Liberase stock solution (Thermolysin Medium): prepare a stock solution of 5 mg/mL in DEPC water: reconstitute by injecting 1 mL of DEPC water in the 5 mg vial. Place the vial on ice to rehydrate and gently agitate the vial at 2–8 °C until the enzyme is completely dissolved (30 min maximum). Store in single use aliquots at −20 °C. Avoid repeated freezing and thawing.
4. Liberase solution: Prepare a 1/100 dilution of the liberase stock in 0.9× DPBS (calculate a final volume of 1 mL per sample). Keep on ice.
5. FACS Buffer: 2% inactivated fetal calf serum in 0.9× DPBS. Keep on ice.
6. Live dead dye (e.g., SYTOX™ Red).

3 Methods

3.1 Tubes to Prepare Before to Start

1. Label 1.5 mL sterile microcentrifuge tubes and 2 sets of corresponding FACS tubes in serial numbers (*see* **Note 3**).
2. Dispense 1 mL of Liberase solution into microcentrifuge tubes, except for WKM samples. Keep the tubes on ice.
3. For WKM samples, dispense 200 μL of ice-cold FACS Buffer into microcentrifuge tubes (*see* **Note 4**).
4. In one set of FACS tubes, add 3 mL of FACS Buffer, but leave the tubes for the WKM samples empty. Keep all tubes on ice.

3.2 Dissection of Adult Zebrafish Organs or Tissues

1. Euthanize the fish according to the permissions and ethical rules of local authorities.
2. Dissect the brain: Under a dissection microscope, place the fish on the side, cut the head with a scalpel, remove the eyes using forceps, and transfer the head in a Petri dish with ice-cold 0.9× DPBS. Hold the head with the forceps in one hand, and with the other, perform a small cut with the microdissection scissors in the posterior part of the skull. Be careful not to damage the brain. Break open the skull with the forceps and remove the right and left flank of the skull carefully keeping the head still with a forceps. Collect the brain with one forceps while keeping the head still with the other forceps. Take great care not to dissociate the olfactory bulbs, as they can easily detach from the telencephalon. Place the brain into the corresponding microcentrifuge tube containing 1 mL of ice-cold Liberase solution.
3. Dissect the liver: Make a ventral, midline incision from the anal fin towards the head using microdissection scissors under the microscope. Be careful not to damage the internal organs. Open the lateral muscles and look for the swim bladder, located in the upper cavity. Remove and discard it. If the fish is a female, remove the eggs present in the cavity to visualize the intestine, pancreas, liver, and spleen. Take the posterior intestine located near the anal fin with the forceps and pull towards the middle intestine, removing along all viscera of the body cavity as a full pluck. Carefully transfer into a Petri dish with ice cold 0.9× DPBS. Clean from fat and the gonads using the forceps. Stretch the intestine and locate the liver, which can be identified by its large size with lobed morphology, yellowish/pink color, and extensive vascularization. Place it directly into the corresponding microcentrifuge tube containing 1 mL of ice-cold Liberase solution.
4. Dissect the skin: With the fish placed on the side, make a vertical incision posterior to the operculum. With one forceps, pull the skin along the side of the fish from anterior to posterior while holding the rest of the body with the other forceps. If

necessary, carefully pull free any underlying fat that remain attached. Transfer the skin including the scales into the corresponding microcentrifuge tube containing 1 mL of ice-cold Liberase solution.

5. Dissect the WKM: Once all internal organs are removed, the kidney is exposed. Lay the fish on its back and locate the thin, translucent, and pigmented structure that runs along the axis of the vertebral column. If possible, discard the dorsal vein with forceps. Keep the forceps as parallel as possible to the spine to avoid contaminating the sample with bone or muscle debris. Carefully pull out the entire WKM away from the dorsal body wall, from the head (anterior) to the tail (posterior) kidney with the forceps. Place it directly into the corresponding microcentrifuge tube prefilled with 200 µL of ice-cold FACS Buffer (*see* **Note 5**).

3.3 Processing the Organs

3.3.1 WKM

1. Predissociate the WKM by gently pipetting up and down with a P200 unfiltered tip. Be careful as the sample can be sticky.
2. Add 300 µL of FACS Buffer and complete dissociation by passing the sample up and down multiple times through a 1 mL syringe with 26 G needle (*see* **Note 6**).
3. Filter the suspension through a 40-µm cell strainer directly into an ice-cold and empty FACS tube. Store on ice and protect from light by covering with aluminum foil.

3.3.2 Brain and Liver

1. Incubate in Liberase solution at 33 °C for 45–60 min (brain samples) or 25 min (liver samples) (*see* **Note 7**).
2. After 5 min, gently triturate with a P1000 to help physical cell dissociation. Make sure to use unfiltered tips to avoid the samples to stick to the inside of the tip.
3. Every 15 min, gently pipet the sample up and down several times with a P1000 for additional mechanical disruption.
4. Repeat until the tissue is fully dissociated. Do not let the sample digest longer than needed as it can damage cells.
5. At the end of the incubation, gently pass the sample through a 1-mL syringe with a 26 G needle, up and down multiple times.
6. Using the syringe, transfer the cell suspension to the corresponding FACS tube containing 3 mL of ice-cold FACS Buffer (*see* **Note 8**).

3.3.3 Skin

1. Using fine scissors, carefully cut the skin sample into small pieces in the microcentrifuge tube containing 1 mL Liberase solution and transfer at 33 °C.
2. After 10 min incubation, triturate the sample and pipet up and down with a P1000. Repeat every 10 min until the skin starts to disaggregate (*see* **Note 9**).

3. Once the skin is dissociated, which usually takes around 30 min, the suspension will appear greyish.
4. Keep the tube still until the scales sediment to the bottom. Transfer the cell suspension into a new empty microcentrifuge tube using a P1000 pipette. Take care not to transfer the scales.
5. Homogenize by gently pipetting up and down with a P1000 and a syringe with a 26 G needle.
6. Using the syringe, transfer the solution to the corresponding FACS tube containing 3 mL of ice-cold FACS Buffer (*see* **Note 8**).

3.4 Pelleting the Cells

1. Centrifuge the FACS tubes (except the WKM) containing the dissociated tissues (Subheading 3.3) at 290 g at 4 °C for 10 min (*see* **Note 10**).
2. Taking great care not to disturb the cell pellet, discard the supernatant using a P1000, leaving about 100 μL above the pellet.
3. To resuspend the cells, add 300 μL of ice-cold FACS Buffer. Disperse the pellet by gently pipetting up and down twice using a P1000.

3.5 Filtering and Staining for Dead Cell Discrimination

1. Using a P1000, pass the suspension through a 40-μm cell strainer into a new and empty FACS tube (second set).
2. Add live/dead dye (in our case 1/1000 Sytox Red dye with a final concentration 5 nM) to each tube.
3. Mix by flicking, and then keep the tubes on ice and in the dark until the scheduled time for analysis (*see* **Note 11**).

3.6 Data Collection

1. Collect data on a BD FACSAria III cytometer using the following gating strategy:
2. Start by drawing the first gate (P1) on cells displayed based on side (SSC-A, y axis bi-exponential scale) and forward (FSC-A, x axis linear scale) scattering characteristics. Exclude events with low FSC or high SSC (dead cells, debris and aggregates). This gate needs to be adjusted depending on the tissue (Fig. 2a).
3. To filter cell doublets out, display the P1 events based on the width of the side scatter (SSC-W, y axis linear scale) and the height of the side scatter (SSC-H, x axis bi-exponential scale). Draw the gate for the singlet population (P2) (Fig. 2b).
4. As a second step to ensure doublet exclusion, plot the P2 gate based on forward scatter-width (FSC-W, y axis linear scale) and forward scatter-height (FSC-H, x axis linear scale). Draw the gate for the singlet population (P3) (Fig. 2c).
5. Next, exclude Sytox Red positive dying cells by visualizing P3 on a plot of far red (x axis bi-exponential scale) versus side

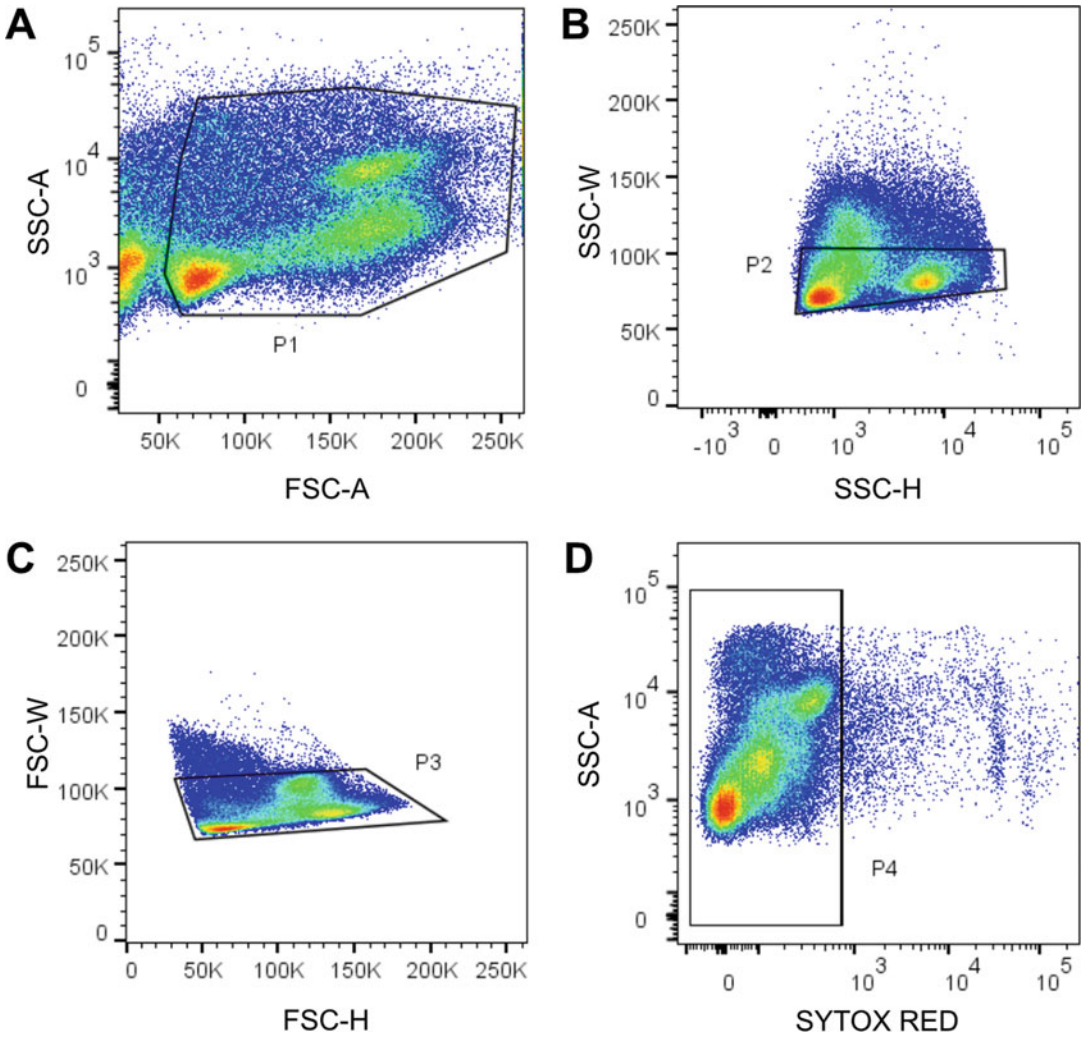

Fig. 2 General gating strategy to identify single and live cells in adult zebrafish organs using flow cytometry. The WKM is shown as an example. (**a**) The P1 gate depicts events based on cell size and granularity. (**b**, **c**) Exclusion of cell doublets using the combination of pulse width and height side scatter or forward scatter parameters. (**d**) Selection of live cells (P4, or Sytox red negative fraction). Note that cells are displayed based on SSC-A (y-axis) and Sytox Red fluorescence (x-axis). As an alternative, cells can be plotted based on Sytox red fluorescence and GFP or DsRed (instead of SSC-A), allowing to visualize cell viability directly within the population of interest (not shown). Figures 3, 4, 5, and 6 will show the gating strategy for every organ to follow

scatter-area (SSC-A, y axis bi-exponential scale). Draw P4, which consists of single, live cells (Fig. 2d).

6. Plot the live P4 population based on side (SSC-A, y axis bi-exponential scale) and forward (FSC-A, x axis linear scale) scatter properties. In a tissue-specific manner, draw a new gate (P5) that excludes the lymphoid fraction and is enriched in myeloid cells (*see* **Note 12**) (Fig. 3 for WKM, Fig. 4 for brain, Fig. 5 for liver and Fig. 6 for skin).

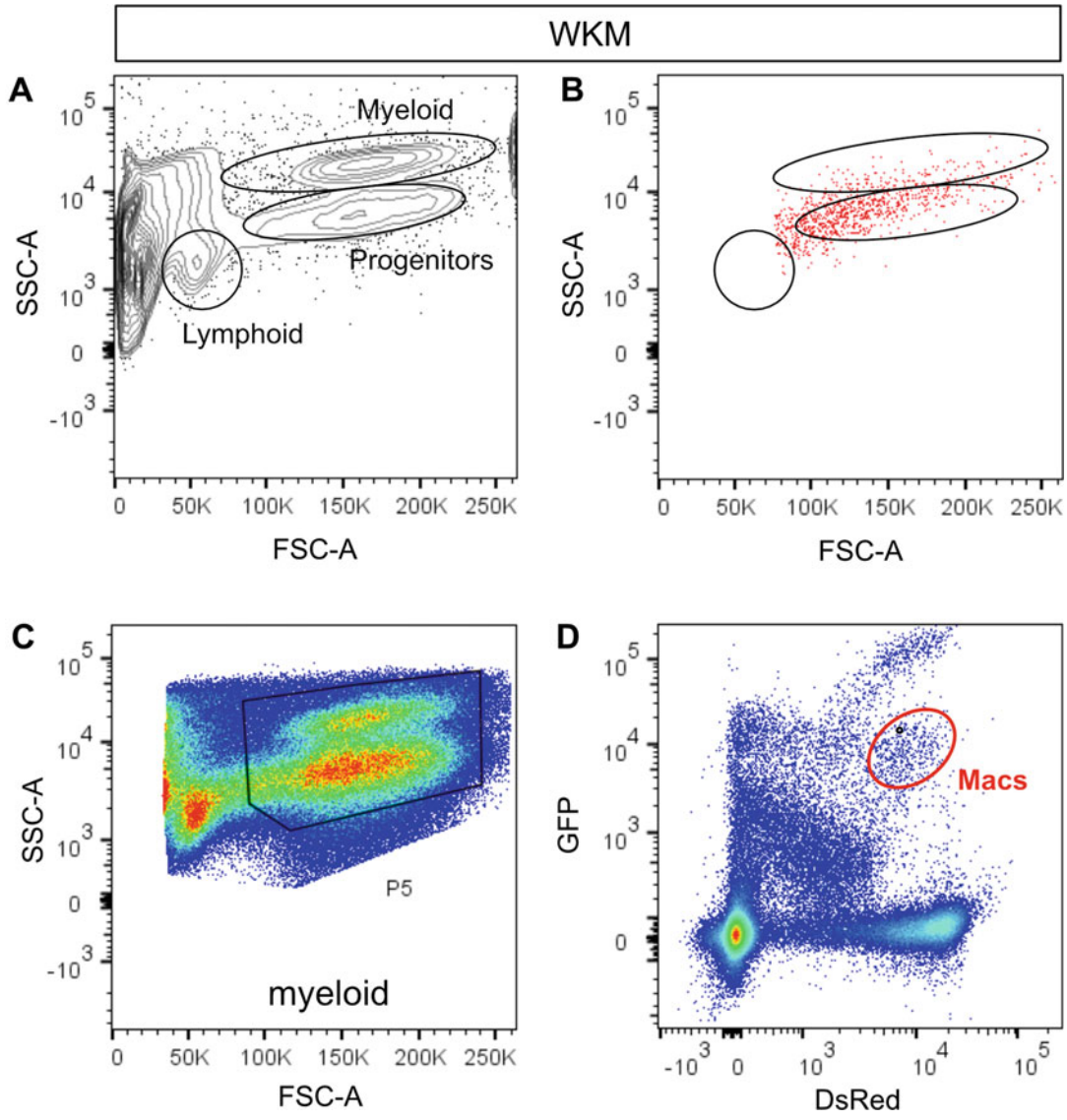

Fig. 3 Representative dot plots and gating strategy for macrophage isolation in the WKM using Tg(*mpeg1.1*:GFP;*cd45*:DsRed). (**a**) Gating strategy to isolate lymphoid, progenitor and myeloid lineages using light-scattering characteristics. (**b**) Mature macrophages (red dots), reanalyzed by forward and side scatter, overlap the myeloid and progenitor fractions in the WKM. (**c**) The P5 gate visualizes the P4 population in a more restrictive manner, with most of the lymphoid population excluded. (**d**) Macrophages are identified as GFP^{int}; $DsRed^{high}$ cells (red circle). Macs, macrophages

7. Once you have discriminated events by size, granularity, and viability, and delineated P5, gate on fluorescently labeled transgenic cells. Plot P5 based on GFP (y axis bi-exponential scale) and DsRed (x axis bi-exponential scale) fluorescence (Fig. 3 for WKM, Fig. 4 for brain, Fig. 5 for liver and Fig. 6 for skin).

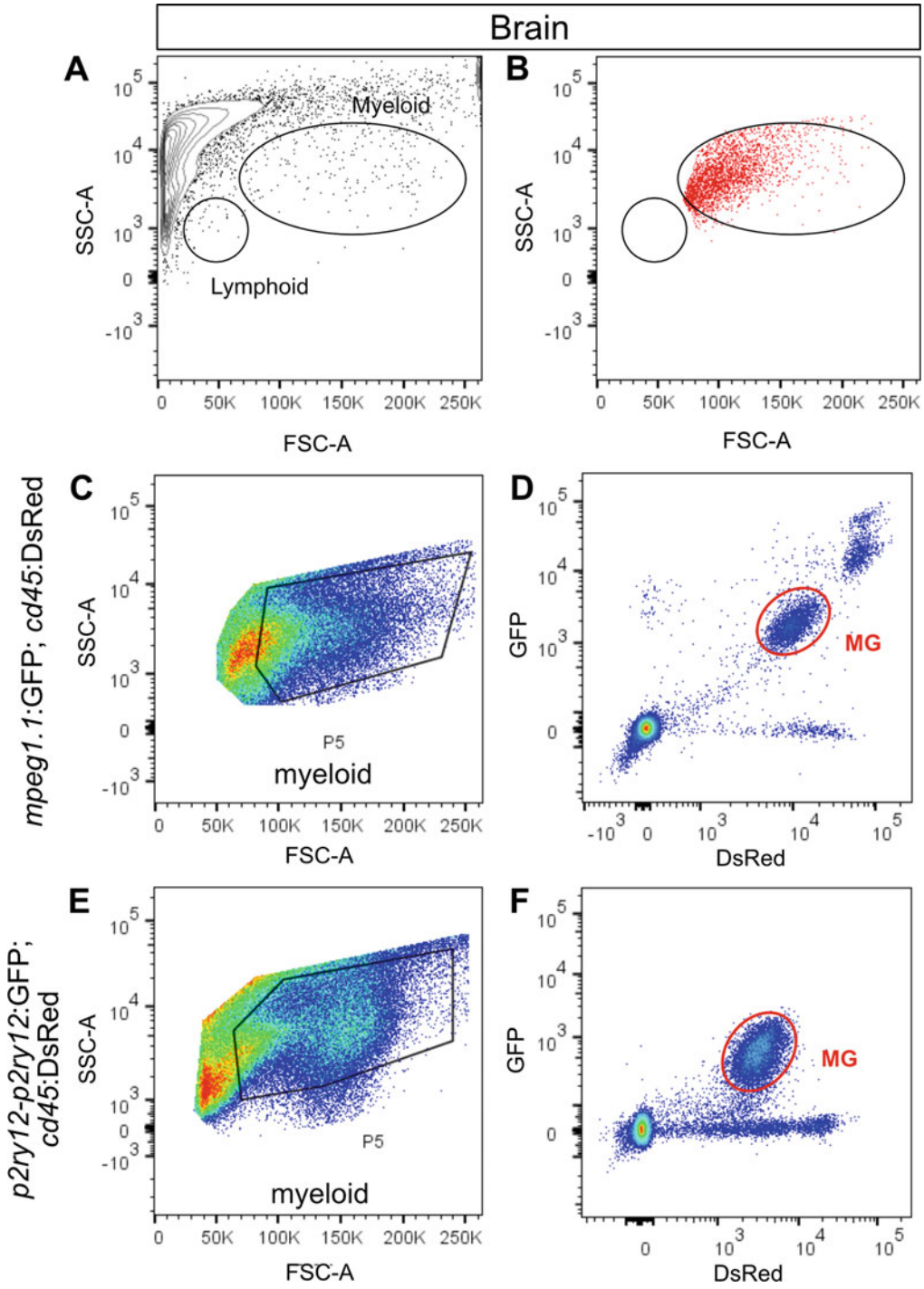

Fig. 4 Representative dot plots and gating strategy for isolation of brain microglia using Tg(*mpeg1.1*:GFP; *cd45*:DsRed) or TgBAC(*p2ry12*:p2ry12-GFP;*cd45*:DsRed). (**a**) Gating strategy to analyze lymphoid and myeloid cells using light-scattering characteristics. (**b**) Microglial cells (red dots) reanalyzed by forward and side scatter, localize within the SSC^{int}; FSC^{int} myeloid gate. (**c, e**) The P5 gate displays the P4 population in Tg (*mpeg1.1*:GFP;*cd45*:DsRed) (**c**) and TgBAC(*p2ry12*:p2ry12-GFP;*cd45*:DsRed) (**e**) in a more restrictive manner. (**d, f**) The GFP^{low}; $DsRed^{low}$ population (red circle) represents microglia in Tg(*mpeg1.1*:GFP;*cd45*:DsRed) fish (**d**), while in the brain of TgBAC(*p2ry12*:p2ry12-GFP;*cd45*:DsRed) animals, microglia are the GFP^{+}; $DsRed^{+}$ double positive population (red circle) (**f**). MG microglia

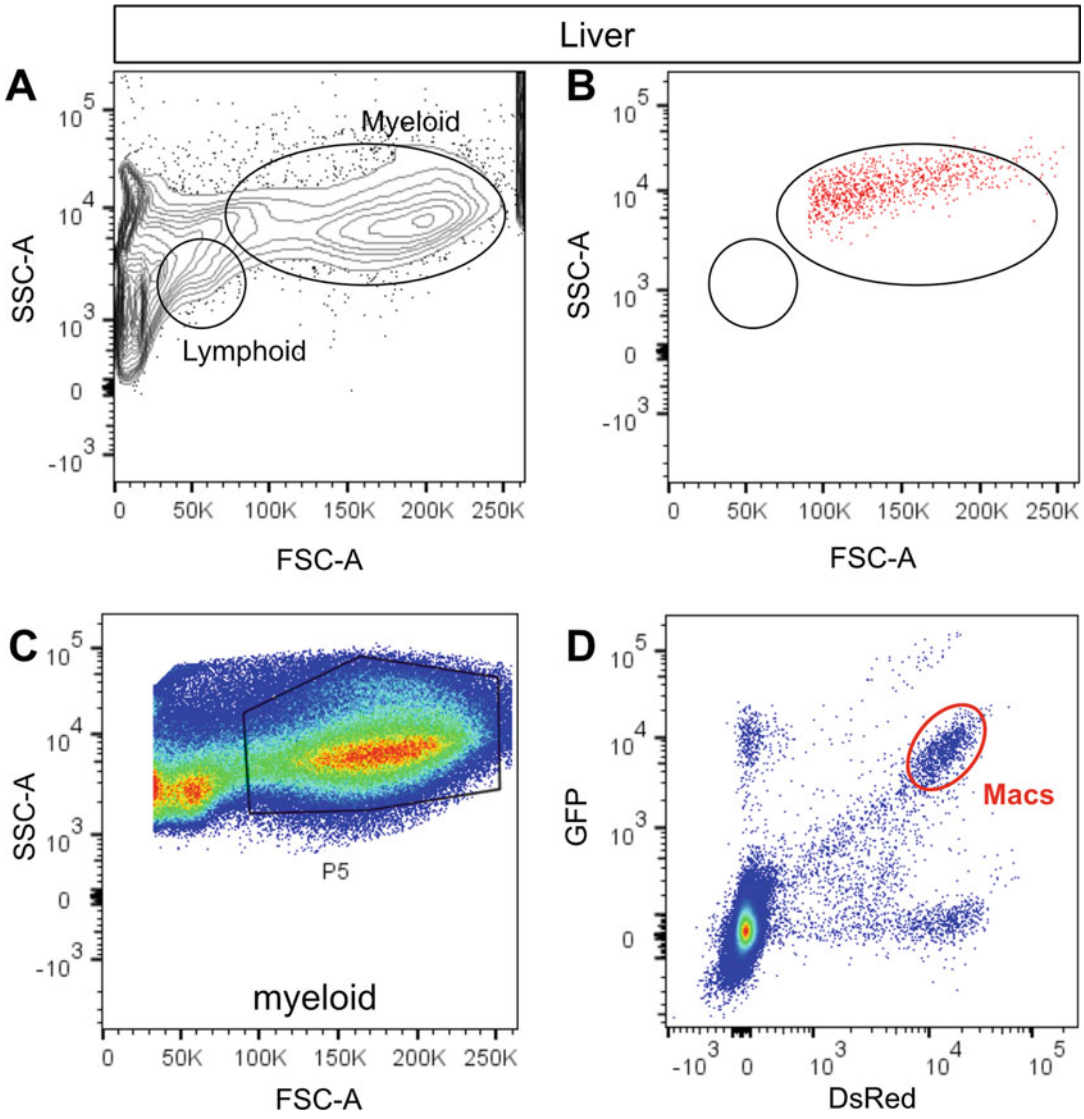

Fig. 5 Representative dot plots and gating strategy for isolation of liver macrophages using Tg(*mpeg1.1*:GFP; *cd45*:DsRed). (**a**) Gating strategy to isolate lymphoid and myeloid lineages using light-scattering characteristics. (**b**) Macrophages (red dots) reanalyzed by forward and side scatter, are contained within the myeloid fraction. (**c**) After setting up the P4 gate, P5 is a more restrictive gate into the myeloid fraction. (**d**) Liver macrophages represent a well-defined population of GFP^{+}; $DsRed^{+}$ double positive cells (red circle). Macs macrophages

8. Make sure to use unlabeled samples (from nontransgenic fish) and single-fluorescent samples (from single GFP and DsRed reporter carriers) to set appropriate detector gains (PMT voltages) and thresholds for fluorescence channels.

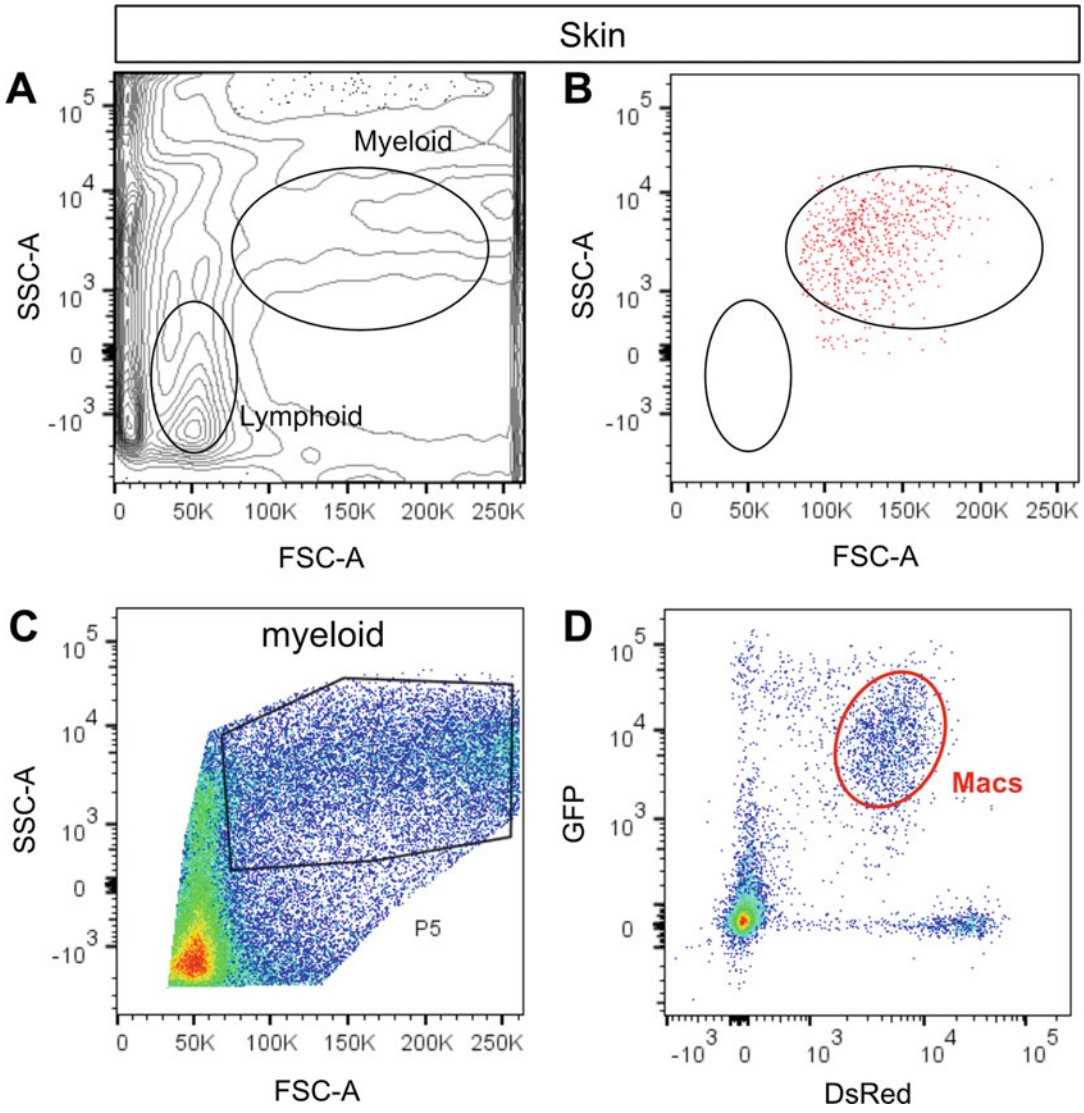

Fig. 6 Representative dot plots and gating strategy for isolation of skin macrophages using Tg(*mpeg1.1*:GFP; *cd45*:DsRed). (**a**) Gating strategy to isolate lymphoid and myeloid lineages using light-scattering characteristics. Note that this tissue is very different from the other examples given. (**b**) Mononuclear phagocytes (red dots) reanalyzed by forward and side scatter, localize within the myeloid fraction. (**c**) The P5 gate visualizes the P4 population in a more restrictive manner, with the lymphoid population excluded. **(d)** In this setting, skin macrophages are identified as GFP^+; $DsRed^+$ double positive (red circle). Macs macrophages

3.7 Expected Outcomes for Each Tissue

1. *WKM, using the Tg(mpeg1.1:GFP, cd45:DsRed) line*: In this tissue, differentiated mononuclear phagocytes represent a discrete *mpeg1.1*$^+$ cell population with a specific scatter profile overlapping the myeloid and progenitor fractions (Fig. 3a, b). The majority of the cells shows the characteristics of macrophages, with kidney-shaped nuclei and vacuoles [21]. As the

site of adult hematopoiesis, the WKM also contains large amounts of B lymphocytes that share expression of the *mpeg1.1*:GFP reporter with macrophages [17], which can thus affect their analysis. Therefore, exclusion of unwanted GFP^+ B lymphocytes is firstly achieved by gating on the combined myeloid and scatter fractions (Fig. 3c) and, secondly, using Tg(*mpeg1.1*:GFP; *cd45*:DsRed) double transgenic animals, where GFP^+; $DsRed^+$ mononuclear phagocytes are discerned from GFP^+; $DsRed^-$ B cells based on differential DsRed expression. Importantly, WKM mononuclear phagocytes can be further divided into two subpopulations, identified as macrophages (GFP^{int}; $DsRed^{high}$) and putative DC-like cells (GFP^{high}; $DsRed^{high}$), respectively (Fig. 3d) (*see* **Note 14**). GFP^- $DsRed^+$ cells comprise a mix of hematopoietic progenitors, T cells, eosinophils, and neutrophils. The latter is abundant in the WKM [24].

2. *Brain, using the Tg(mpeg1.1:GFP, cd45:DsRed) line*: Brain samples contain two main populations of GFP^+; $DsRed^+$ cells that can be discriminated based on differential levels of fluorophore expression: microglia, the brain macrophages, which are GFP^{low}; $DsRed^{low}$, and a less abundant GFP^{high}; $DsRed^{high}$ population (Fig. 4d), which mostly comprises DC-like cells and monocytes (Rovira, Ferrero and Wittamer, unpublished) (*see* **Note 14**). In this setting, GFP^+; $DsRed^-$ cells are B lymphocytes while GFP^- $DsRed^+$ cells consist of a mixed population of T lymphocytes, NK cells, and neutrophils, among others.
3. *Brain, using the Tg(p2ry12:p2ry12-GFP; cd45:DsRed) line*: Microglia can also be easily identified based on expression of the *p2ry12*:p2ry12-GFP transgene, the purinergic receptor *p2ry12* being widely accepted as a canonical microglia marker [25], including in zebrafish [26, 27]. Importantly, expression of the *p2ry12* transgene is coupled to CFP expression in the lens (serving as a transgenesis marker), which can make analysis of microglia challenging. Using animals carrying both the *p2ry12*:p2ry12-GFP and the *cd45*:DsRed reporters allows to overcome this limitation (Fig. 4f), as in this setting microglia are GFP^+; $DsRed^+$ and can be discriminated from unwanted CFP^+; $DsRed^-$ lens cells (*see* **Note 13**). Importantly, due to low *p2ry12*:p2ry12-GFP transgene expression in microglia (especially in comparison to the *mpeg1.1*:GFP reporter), fine adjustment of the GFP voltage is required to achieve efficient separation between microglia (GFP^{low}; $DsRed^+$) and the remaining immune cells (identified as GFP^- $DsRed^+$).
4. *Liver, using the Tg(mpeg1.1:GFP, cd45:DsRed) line*: The liver contains a well-defined population of *mpeg1.1* positive cells, which mostly comprises macrophages [28]. These cells are GFP^+; $DsRed^+$ and are easily separated from GFP^+; $DsRed^-$ B

lymphocytes (Fig. 5d). The GFP$^-$ DsRed$^+$ population contains the remaining leukocytes.

5. *Skin, using the Tg(mpeg1.1:GFP, cd45:DsRed) line*: The skin contains a heterogenous population of *mpeg1.1*$^+$ cells, of which B lymphocytes and metaphocytes account for a high proportion [19]. These cells are discerned from skin macrophages based on differential DsRed expression. Accordingly, skin macrophages are GFP$^+$DsRed$^+$, and B cells and metaphocytes are GFP$^+$DsRed$^-$ (*see* **Note 14**). Drawing a gate on the myeloid fraction (P5) will ensure enrichment for GFP$^+$DsRed$^+$ macrophages (Fig. 6c).

4 Notes

1. Analyses are conducted on fish between 4 and 9 months of age. Animal experiments must conform to national and institutional regulations.
2. Sample preparation for flow cytometry analyses does not require antibody staining since the protocol relies on detection of the endogenous fluorescence in the cells of interest. However, not fluorescent (obtained from nontransgenic fish) and single-fluorescent (obtained from single EGFP and DsRed reporters) control samples are needed to set appropriate laser voltages that identify clear negative and positive populations.
3. The sample preparation time before analysis at the flow cytometer is around 3 h. In practice, this timing will depend on the sample size. If large numbers of samples have to be analyzed, we recommend that two people participate: one in charge of collecting the organs while the second processes the samples. The protocol has no stopping step until the end and the experimenter must proceed quickly. Transgenic fish should be screened in advance and selected the day prior the experiment. If necessary, validation of the reporter lines can be conducted on anesthetized adult fish placed under a fluorescence binocular. Transgenic GFP$^+$ and DsRed$^+$ cells are easily visualized in the skin and gills of Tg(*mpeg1.1*:GFP) and Tg(*cd45*:DsRed) animals, respectively. The Tg(*p2ry12*:p2ry12-GFP) line is identified by a lens-specific expression of cerulean fluorescent protein (CFP) that serves as a transgenesis marker and is easily visible in adult animals. The fish to sample the next morning can be isolated in breeding tanks overnight.
4. Mechanical disruption of soft tissues like the kidney is sufficient to release cells into a single-cell suspension.
5. The order of the dissection of the tissues or organs will depend on your interests. However, some tissues are more sensitive to degradation such as the WKM and the brain. If several organs

are collected from the same animal, we recommend proceeding as followed: cut the head first, place it into a Petri dish with 0.9× DPBS, and keep on ice until processing. Remove the viscera as a full pluck and keep it in cold 0.9× DPBS until processing. Collect the WKM, and then dissect the brain in cold 0.9× DPBS. Quickly peel off the skin. Finally, dissect the liver in cold 0.9× DPBS.

6. To fully dissociate the WKM, always use a P200 and a syringe sequentially. First pipet up and down with the P200 until no clumps are visible. This will avoid clogging the syringe in the subsequent step. The use of 26 GA needles is necessary for complete tissue homogenization. The mixing up and down must be gentle to not harm the cells. The inside diameter of the needle must not be smaller than the diameter of a cell, as this will result in cell lysis.
7. The time required for optimal dissociation using enzymatic digestion will differ between tissues and will depend on the tissue size. Typically, for an adult brain it takes 40–50 min, and 20–30 min for the liver. During incubation, check your samples regularly to prevent overdigestion.
8. Dilution of the homogenized tissue into 3 mL of ice-cold FACS Buffer will ensure enzymatic inactivation of the liberase.
9. To speed up the dissociation process, pass the sample through a syringe with a 20 GA needle.
10. When processing large numbers of samples, we recommend centrifuging maximum 10 tubes at a time as proceeding quickly will prevent detachment of the cell pellets.
11. Always keep the samples on ice to stop cell death and protected from light to prevent loss of fluorescence. As the cells are not fixed, the samples cannot be stored more than a few hours so proceed with the flow cytometry analysis without delay.
12. Using flow cytometry, each of the major hematopoietic lineages can be resolved by light-scatter characteristics. In the zebrafish WKM, the main distinct scatter populations are termed “lymphoid,” “progenitor,” and “myeloid”. Mature mononuclear phagocytes are identified as a distinct forward scatter $(FSC)^{high}$ side scatter $(SSC)^{int}$ population, overlapping the conventional myeloid and precursor fractions. The precursor population is not present in other zebrafish organs, and mononuclear phagocytes largely localize in the myeloid gate.
13. Because eyes are separated from the brain during dissection, contamination of *p2ry12*:p2ry12-GFP microglia by CFP lens cells in the single cell suspension should be minimal (unlike in the case of cell suspensions produced from embryos and/or larvae).

14. Limitations: While tissue macrophages will undoubtedly represent the major population analyzed with this protocol, it is important to keep in mind that other mononuclear phagocyte subsets, such as monocytes and dendritic cells, will likely also be comprised in the isolated population of *mpeg1.1*$^{+}$; *cd45*$^{+}$ myeloid cells. Although our approach also permits to identify putative dendritic cell-like populations, notably in the brain and in the WKM, whether these cells represent the zebrafish counterparts of mammalian DCs remains to be determined. Likewise, zebrafish monocytes have not been characterized yet. Therefore, more markers and new transgenic lines are needed to discriminate the different subsets of the mononuclear phagocyte compartment in the zebrafish model. Finally, it is also possible that the *mpeg1.1* promoter fails to label all macrophages in transgenic fish, although this appears unlikely based on available transcriptomic data.

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