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Ashley M. Vaughan *Editor*

# Malaria Vaccines

## Methods and Protocols

# Methods in Molecular Biology

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# Malaria Vaccines

## Methods and Protocols

Edited by

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# Preface

The most effective way to control and ultimately eliminate an infectious disease is through vaccination. Man has successfully eliminated small pox with this ingenious strategy but other diseases are proving harder to eradicate, even when highly effective vaccines do exist. Malaria is caused by the eukaryotic pathogen parasite *Plasmodium*, and to date no efficacious vaccine against any eukaryotic pathogen is widely available. Nevertheless seminal studies in the 1960s showed the power of immunity in controlling malaria disease. In 1961 Sydney Cohen and colleagues showed that the passive transfer of gamma immunoglobulin from adults living in areas of high malaria endemicity to young children with severe malaria disease could help eliminate parasites from the blood. This study clearly demonstrated the ability of humoral immunity to control severe disease. In 1967, Ruth Nussensweig and colleagues demonstrated that the immunization of mice with irradiated *Plasmodium berghei* sporozoites led to the generation of an immune response that completely protected the immunized mice from a sporozoite challenge. Subsequently, in 1973, David Clyde and colleagues repeated these studies in man using irradiated *Plasmodium falciparum* parasites and again showed that complete protection could be achieved. These pivotal breakthroughs have fueled decades of research into malaria vaccine efforts focusing on both blood stage vaccines and preerythrocytic vaccines. It is now known that both humoral and cellular immunity are important partners in effective vaccine design, and large bodies of work have shown that antibodies can prevent both merozoite and sporozoite invasion while $CD4^+$ T cells and $CD8^+$ T cells play critical roles in the destruction of infected erythrocytes and hepatocytes respectively.

The goal of this volume, which focuses exclusively on malaria vaccinology, is to introduce researchers to a subset of the many methods regularly being used in this field. This volume complements a recent "Methods in Molecular Biology" volume that is devoted exclusively to malaria and provides a complete overview of the protocols and tools used by the molecular and cellular malariologist. Working with the human malaria parasite both in vitro and in vivo is challenging due to its unique tissue tropism, and research efforts on malaria vaccine design have required the creation of novel methodologies for determining vaccination efficacy as well as pinpointing correlates of protection. These methodologies have been fine-tuned over the years, and this volume brings together a large number of nuanced chapters from leading experts in the field that will help any aspiring malaria vaccinologist determine the effectiveness of vaccine regimens. Thus, the volume provides a unique resource and exquisitely detailed methodologies that are not typically found in published literature.

The chapters contained within talk to interventions concerning all aspects of life cycle progression—measuring antibody responses to blood stage parasite survival, the T cell responses engendered by attenuated sporozoite vaccination, and the unique effect on transmission of antibodies that target the mosquito stage of the life cycle. Additionally, methods concerning the ability to generate targeted gene deletions and replacements in the genome of *Plasmodium* parasites convey how *Plasmodium* parasite phenotypes can be created to

precise specifications. More recently, the potential power of humanized mouse models of disease progression has been demonstrated and these are discussed herein.

We thank all authors for their dedication in creating step-by-step methodologies that will undoubtedly lead to further discoveries and further improvements. Hopefully these findings will ultimately lead to the creation of an effective vaccine regimen for the elimination and ultimately the eradication of malaria.

***Seattle, WA, USA*** ***Ashley M. Vaughan***

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# Part I

## Pre-erythrocytic Stages

# Chapter 1

# Isolation of Non-parenchymal Cells from the Mouse Liver

Isaac Mohar, Katherine J. Brempelis, Sara A. Murray, Mohammad R. Ebrahimkhani, and I. Nicholas Crispe

## Abstract

Hepatocytes comprise the majority of liver mass and cell number. However, in order to understand liver biology, the non-parenchymal cells (NPCs) must be considered. Herein, a relatively rapid and efficient method for isolating liver NPCs from a mouse is described. Using this method, liver sinusoidal endothelial cells, Kupffer cells, natural killer (NK) and NK-T cells, dendritic cells, CD4+ and CD8+ T cells, and quiescent hepatic stellate cells can be purified. This protocol permits the collection of peripheral blood, intact liver tissue, and hepatocytes, in addition to NPCs. In situ perfusion via the portal vein leads to efficient liver digestion. NPCs are enriched from the resulting single-cell suspension by differential and gradient centrifugation. The NPCs can by analyzed or sorted into highly enriched populations using flow cytometry. The isolated cells are suitable for flow cytometry, protein, and mRNA analyses as well as primary culture.

**Key words** Liver, Perfusion, Cell isolation, Sinusoidal endothelial cells, Kupffer cells, Hepatic stellate cells

## 1 Introduction

The principle cell types in a healthy liver are hepatocytes, liver sinusoidal endothelial cells (LSEC), Kupffer cells, and hepatic stellate cells (HSC) [1–3]. Fewer in number are bile duct cells, venous and arterial endothelial cell, hepatic progenitor cells, and dendritic cells. Furthermore, the number and proportion of leukocytes can increase tremendously in an infected or damaged liver [4, 5]. As a result, granulocytes, monocytes, natural killer (NK) and NK-T cells, dendritic cells, CD4+ and CD8+ lymphocytes, and B cells are important determinants of the liver biology. Thus, the dissected dynamics of each cell type can provide powerful information to understand the pathology and immunology of the tissue. This information, in combination with serological, histological and tissue-level observations, allows for a comprehensive assessment of each experimental mouse, thus reducing the number of experimental mice while increasing the likelihood of discovery.

Isaac Mohar and Katherine J. Brempelis are co-first authors of this chapter.

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The purpose of this protocol is to provide a detailed description of materials and methods by which liver cell populations can be isolated from the mouse liver and studied, while also permitting the collection of blood and intact liver tissue. The liver dissociation protocol is derived from the method published by Seglen [6] for isolating rat liver cells. Dr. Seglen provides an extensive description of the theory behind rat liver dissociation that extends to the mouse. We have evolved the method of Seglen to allow rapid, yet effective, isolation of mouse liver cells, permitting the dissociation of up to five livers per hour by two skilled technicians—one conducting perfusions and dissections, the other processing cell suspensions.

The basic protocol relies upon in situ perfusion of the liver via the portal vein. Peripheral blood and cells are flushed from the liver in a $Ca^{2+}$-free buffer, prior to perfusion with the collagenase digestion solution. Following liver digestion, the liver is removed and mechanically dissociated. Hepatocytes are separated by low-speed centrifugation, and then non-parenchymal cells (NPCs) are enriched by gradient separation. The enriched NPCs allow for relatively efficient cell type-specific analysis and/or further purification by flow cytometry [7]. For purification, magnetic bead-based methods can be applied and in certain circumstances are preferred [8], however, cell sorting allows for multi-way separation from each preparation.

Although liver NPCs are the focus of this protocol, hepatocytes are readily purified and cultured with good success. In addition, it is not yet clear if this protocol is able to isolate the population of sessile Kupffer cells, which are radioresistant and appear somewhat distinct in function from their non-sessile counterparts [2]. This caveat in mind, this protocol establishes a reproducible method to isolate and enable the study of many cell types from the mouse liver. Indeed, a parallel understanding of cell-specific responses associated with tissue immune and pathological responses offers promise of new insights into treatment and prevention of infection and disease.

## 2 Materials

All solutions and consumables should be purchased as "tissue culture tested" from a trusted commercial source in order to assure minimal contamination with endotoxin and sterility. All surgical instruments should be thoroughly washed, rinsed and autoclaved for sterility, especially if primary culture is the end goal. As with any protocol involving animals, institutional guidelines for handling, anesthesia, and waste disposal should be followed.

### 2.1 Anesthesia

1. Anesthesia approved for terminal procedures such as Avertin; 1.25 % (w/v) 2,2,2-tribromomethanol, 2.5 % (v/v) 2-methyl-2-butanol, sterile water. Filter-sterilize and then store at 4 °C protected from light (*see* **Note 1**).

2. 28G ½ inch needle, suitable for intraperitoneal injections.
3. 1-cc syringe.

### *2.2 Perfusion/Liver Dissociation Hardware Components*

1. Peristaltic pump; such as Gilson MINIPULS 3 with medium flow-rate pump head.
2. Pump tubing and connectors; such as F1825113 and F1179951.
3. Tubing extension with slip-tip end; such as Hospira 1265528.
4. Catheter; 24G, IV, such as BD 381412 (*see* **Note 2**).
5. Scissors, straight fine-tipped dissection.
6. Forceps, 2 blunt tip.
7. 50-ml conical tubes.
8. 15-ml conical tubes.
9. 5-cm sterile petri dish (optional).
10. 10-cm sterile petri dish.
11. Stainless steel mesh "tea strainer."
12. 10-cc syringe.
13. 100-μm filter.
14. 70-μm filter (optional).
15. Gauze pads, large-size.
16. Surgical tape, such as 3 M Transpore.
17. Disposable absorbent underpads.
18. 37 °C water bath with 50-ml conical rack.

### *2.3 Perfusion/Liver Dissociation Solution Components*

1. Hank's Balance Salt Solution (HBSS); no $Ca^{2+}$, no $Mg^{2+}$, no phenol red.
2. HBSS with phenol red.
3. Phosphate buffered saline (PBS), pH 7.4.
4. Distilled water, TC-grade.
5. PBS, 10×.
6. HEPES; 1 M (Stock).
7. EDTA; 0.5 M (Stock).
8. $CaCl_2$; 0.5 M (Stock).
9. Fetal bovine serum (FBS).
10. Collagenase; *Clostridium histolyticum*, Sigma-Aldrich C5138 (*see* **Note 3**).
11. OptiPrep; 60 % iodixanol solution in water.
12. Tissue fixative; 4 % formaldehyde in PBS.
13. 70 % ethanol.

**Table 1**
**Antibodies for FACS-based purification of some of the major liver NPC and leukocytes**

| Epitope | Fluorophore | Clone | Dilution |
|---|---|---|---|
| CD8a | Pacific Blue | 53-6-7 | 1:250 |
| CD4 | PerCP-Cy5.5 | RM4-5 | 1:250 |
| CD11b | FITC | M1/70 | 1:200 |
| NK1.1 | Per-Cy7 | PK136 | 1:200 |
| Tie2 | PE | TEK4 | 1:250 |
| F4/80 | APC | BM8 | 1:200 |
| GR1 | APC-Cy7 | RB6-8C5 | 1:200 |
| N/A | Live/Dead Violet | N/A | 1:1000 |

The antibodies listed here will allow for selection or analysis of some of the most numerous liver NPC as well as some leukocytes

These solutions can be prepared in advance and stored at 4 °C.

1. Perfusion Buffer, 5–10 ml per mouse; HBSS, 5 mM HEPES, 0.5 mM EDTA.
2. Wash Buffer, 50 ml per mouse; PBS, 4 % FBS, 0.5 mM EDTA.
3. PBS Flow Buffer (PFB), 20 ml per mouse; PBS, 1 mM EDTA, 2 % FBS.

These solutions should be prepared on the day of isolation.

1. Collagenase solution, 5–10 ml per mouse; HBSS (w/phenol red), 5 mM HEPES, 0.5 mM $CaCl_2$, 0.5 mg/ml collagenase.
2. 40 % iodixanol in PBS, 2.5 ml per mouse; 1.67 ml OptiPrep + 0.25 ml 10× PBS + 0.58 ml TC-grade water.

### 2.4 Cell Analysis and Purification Components

1. Flow cytometer; such as BD Biosciences, LSRII or Aria.
2. Flow cytometry tubes (*see* **Note 4**).
3. Antibodies for sorting cell type and/or analysis (Table 1) (*see* **Note 5**).

## 3 Methods

### 3.1 Prepare for Perfusion(s)

1. Warm perfusion and collagenase solutions to 37 °C for approximately 15 min prior to beginning the perfusion.
2. Prepare tubing for perfusion (*see* **Note 6**).
3. Prepare perfusion area with absorbent pad, dissection tools, gauze, 10-cm petri dish, tea strainer, and 10-cc syringe (Fig. 1).
4. Fill perfusion line with perfusion solution.

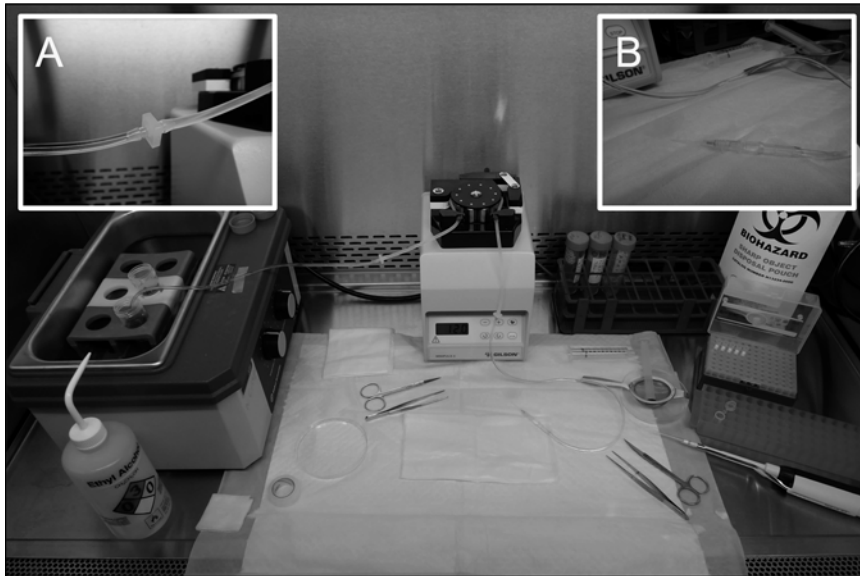


**Fig. 1** Suggested workspace set-up. Position the water bath and pump to allow the perfusion tubing to reach the bottom of the 50-ml conical tubes. The water bath should be to the *left*, in order to allow switching of the perfusion line while holding the catheter with the *right hand*. Place absorbent pad on the work surface; this pad will both absorb perfusion solutions and act as the foundation to adhere the mouse. Place large gauze pad in the center of the work area; this small pad will absorb most of the perfusion solutions as well as blood and should be changed after every other if not every mouse. Place tea strainer in a 10-cm petri dish. Place the lid of the dish to the left of the smaller gauze pad. Place one pair of sharp scissors and forceps above the gauze. Place the other scissors and forceps to the right of the gauze. Position the surgical tape, small gauze pads, and 70 % ethanol within easy reach. *Inset* (**a**) illustrates the connection between extension tubing and silicon peristaltic pump tubing. *Inset* (**b**) illustrates the catheter connected to the male end of the extension tubing

### 3.2 Anesthetize Mouse

1. Inject mouse with appropriate amount of anesthesia.
2. Once adequate level anesthesia is obtained, proceed to Subheading 3.3 (*see* **Note** 7).

### 3.3 Surgical Preparation

1. Place mouse belly-up on large gauze pad.
2. Secure mouse by footpads using surgical tape in an X orientation (Fig. 2a).
3. Disinfect and wet mouse fur using 70 % ethanol. Wipe off excess.
4. Open skin to expose the peritoneal membrane (Fig. 2b).
5. Open peritoneal membrane (Fig. 2c), gently move intestines and stomach to the right and very gently “stick” the liver to the diaphragm. This should expose the portal vein and descending vena cava (*see* **Note 8**) (Fig. 2d).
6. Use sharp scissors to nick the portal vein; blood will flow (*see* **Note 9**).

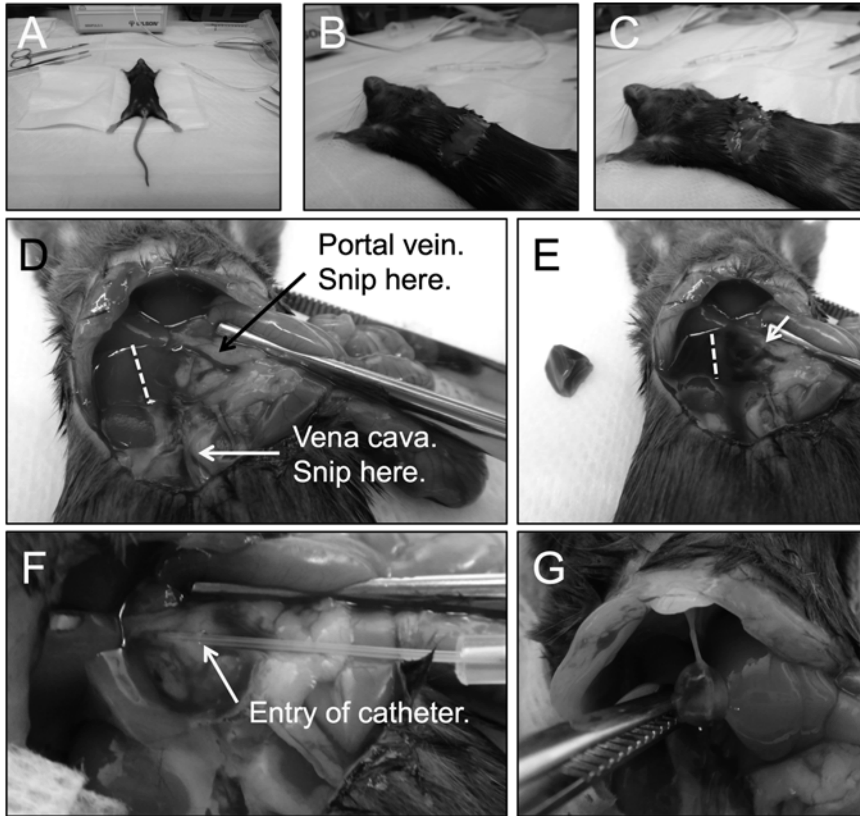


**Fig. 2** General perfusion anatomy and procedure. (**a**) Adhere anesthetized mouse overtop of the gauze in an X-configuration. (**b**) Make a crosswise incision through the mouse skin to reveal the peritoneum. (**c**) Being careful to avoid cutting internal organs, make a crosswise incision through the peritoneum. (**d**) Move the gastrointestinal organs to the *left*, revealing the portal vein. Place forceps to hold tissue off of the vein. (**e**) Snip the portal vein (collect blood if desired), then remove a portion of the intact *right* posterior lobe. Catheterize the portal vein, then immediately cut the descending vena cava. (**f**) The liver will blanch once the portal vein is catheterized, and will fully perfuse once the vena cava is cut. Avoid pushing the catheter too far into the vein. The tip of the catheter should be easily observed within the vein. (**g**) Once digested, remove the liver by the falciform ligament, along the top of the medial lobe. The gall bladder is a good landmark for identifying the ligament

### *3.4 Blood and Tissue Collection (Optional)*

1. Collect 0.2–0.5 ml of blood as it pools near the portal vein. Transfer to proper collection tube.
2. Locate and remove ~2/3 of the right posterior liver lobe (Fig. 2d, e). Transfer to 4 % formaldehyde for fixation or further divide for other assessments.

### *3.5 In Situ Liver Dissociation*

1. Turn on pump to flow of ~2 ml/min.
2. Drip perfusion buffer onto the cut portal vein.
3. Use gauze sponge to draw perfusion solution to the left.
4. Identify the opening in the vein (*see* **Note 10**).

5. Gently catheterize the vein; the liver should blanch (*see* **Note 11**) (Fig. 2f).
6. Cut the descending vena cava; blood and buffer should visibly flow from the vena cava.
7. Relax your hand (*see* **Note 12**).
8. Perfuse liver with 5–10 ml of perfusion buffer. Most perfusion tubing setups hold about 5 ml of solution, thus once the descending vena cava is cut, proceed to **step 9**.
9. Stop pump.
10. Switch line to collagenase, using the left hand.
11. Resume pump flow (*see* **Note 13**).
12. Swell the liver using forceps to occlude buffer flow from the vena cava, every 45–60 s for 5–10 s. If part of the right posterior lobe was removed, use the forceps to occlude flow into this lobe (*see* **Note 14**).
13. Perfuse liver with 5–10 ml of collagenase buffer. After 3–4 min, the liver should soften and the left lobe will begin to fall over the portal vein. When this happens, use forceps to lift up the lobe to periodically check that the catheter is properly positioned. After 5 min, the internal structure of the liver cracks. This indicates a good digestion, and is most evident in the right anterior lobe.
14. Stop the pump.
15. Remove catheter from vein.
16. Reverse pump to return unused collagenase solution to the 50-ml conical tube.
17. Switch line back to perfusion solution and refill the line in preparation for the next mouse.

### 3.6 Single Cell Suspension

1. Using wide-tipped forceps, grasp the liver just to the left of the gall bladder along the falciform ligament (Fig. 2g).
2. Use scissors to separate the liver from the diaphragm and all other points of connection. Care should be taken to avoid cutting the gastrointestinal tract.
3. Transfer the digested liver into the tea strainer within a 10-cm petri dish.
4. Remove the gall bladder (*see* **Note 15**).
5. Add 30 ml of cold wash buffer to the dish.
6. Use the rubber plunger of 10-cc syringe to gently massage the liver through the tea strainer, shake the strainer to disperse the cells. The liver should easily disperse with only the capsule and ligament remaining in the strainer.

7. Use 10-cc syringe (or 10-ml pipet) to gently disperse any clumps.
8. Filter (100 μm) the cell suspension into a 50-ml conical tube.
9. Store on ice or at 4 °C for no longer than 15 min before proceeding to Subheading 3.8.

### 3.7 Isolate Splenocytes (Optional, See Note 16)

1. Locate and remove spleen.
2. Place spleen into 5-ml petri dish filled with 10 ml of PFB.
3. Place the spleen on the rough surface of a glass slide.
4. Use the rough surface of a second glass slide to dissociate the spleen by gentle pressure applied in a circular motion. Continue this gentle mashing until the tissue is clearly dispersed.
5. Scrape the cells into the buffer using the edge of the slide.
6. Disperse the cells by pipetting.
7. Filter (70 μm) into 50-ml conical tube.
8. Store on ice until the NPC isolation reaches Subheading 3.10, **step** 7, then process as NPC.

### 3.8 Crude Liver Cell Fractionation

1. Centrifuge the cell suspension at 50 × *g* for 3 min at room temperature. At this speed and duration, hepatocytes and debris will pellet while most NPCs will remain in suspension.
2. Transfer the supernatant, which contains the hepatocyte-depleted NPCs, to a new 50-ml conical tube.

### 3.9 Hepatocyte Enrichment (Optional)

1. Wash the hepatocyte pellet in 40 ml of wash buffer.
2. Pellet at 50 × *g* for 3 min.
3. Resuspend in 10 ml of media.
4. The resulting hepatocytes can be further enriched by magnetic bead depletion of contaminating cells and/or plated on collagen-coated tissue culture dishes. For the mouse, anti-CD45 and anti-CD146 microbeads will deplete most immune cells and endothelial cells, respectively.

### 3.10 Non-parenchymal Cell Enrichment

1. Pellet the NPC suspension at 500 × *g* for 5–7 min at 4 °C.
2. Gently resuspend in 2.5 ml of PFB.
3. Mix cell suspension with 2.5 ml of 30–40 % iodixanol solution in 15-ml conical. A final concentration of 20 % iodixanol will enrich for most if not all intact NPCs.
4. Gently overlay with 2 ml of PFB.
5. Centrifuge at 1500 × *g* for 25 min at room temperature. If available, turn the brake OFF on the centrifuge to minimize disturbance to the cell interface.

6. During the centrifugation add 10 ml of cold PFB to a 15 ml conical tube.
7. After centrifugation a well-defined interface of cells should be visible. Carefully transfer this cell layer from the iodixanol gradient to the 10 ml of PFB in order to wash away excess iodixanol.
8. Centrifuge at 500 × *g* for 5 min at 4 °C.
9. Resuspend the enriched NPC pellet in 0.5 ml of cold PFB or appropriate buffer for desired applications.

### 3.11 Staining NPCs for Flow Cytometry

1. Prepare the necessary number of flow cytometry tubes.
2. Add anti-CD16/anti-CD36 (Fc receptor blocking) antibody to each sample to a final concentration of 1:250 (*see* **Note 17**).
3. Incubate for 5 min at room temperature.
4. Add antibody cocktail (*see* Table 1).
5. Vortex briefly and gently.
6. Incubate for 20 min at 4 °C.
7. Wash the cells by adding 1 ml of PFB to each sample.
8. Centrifuge at 500 × *g* for 5 min at 4 °C.
9. Aspirate supernatant.
10. Resuspend cell pellet in 0.5 ml of PFB.
11. In order to minimize clogs during cell sorting, filter the cell suspension.

### 3.12 Identifying and Sorting Liver NPC by Flow Cytometry

Liver NPCs have yet to become absolute in their defining characteristics. However, many distinct cell populations can be sorted from a mouse liver. Those identified here represent a cross-section of major cell types, including endothelial cells, macrophage, quiescent hepatic stellate cells, lymphocytes, and natural killer cells. If a population of cells appears diffuse in characteristics, separation by an additional dimension may reveal multiple cell populations. The successful isolation of pure and viable cells is as much art as science and will be aided by the direction and advice of a skilled flow cytometrist with an appreciation for the complexity of sorting from dissociated tissue. The gating strategy depicted in Fig. 3 is one approach to sorting liver NPCs.

### 3.13 Quality Control Analysis of Enriched Liver Cell Populations

Quality control analysis of enriched and sorted liver cell populations can be conducted by in vitro culture of the cells to confirm morphology and/or function [7]. In addition, enriched cells can be analyzed for expression of genes known to be relatively specific to cell types. The basic protocol and representative results are presented below.

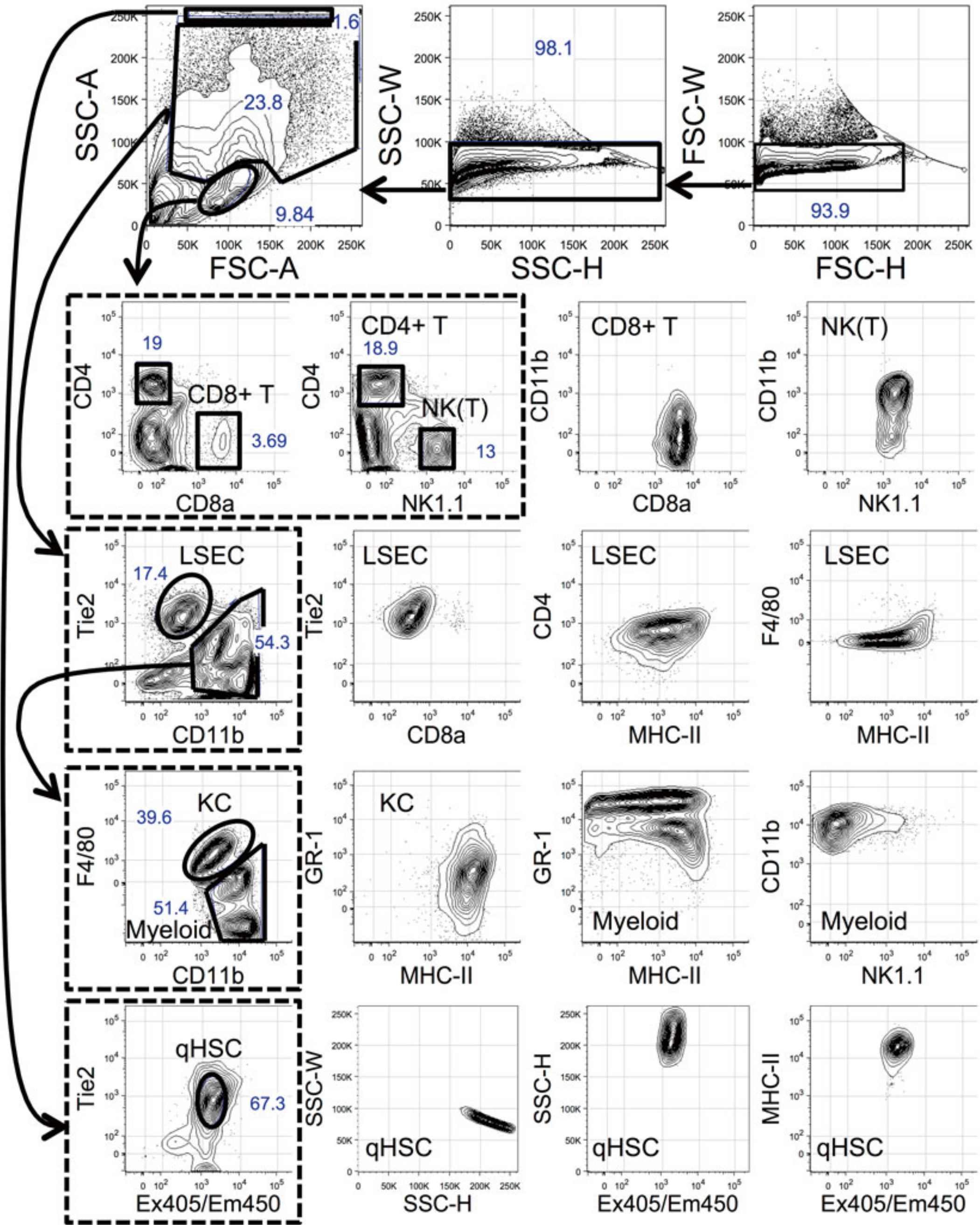


**Fig. 3** NPC sort strategy. Representative NPC sorting strategy from a C57BL/6J mouse 68 h following injection of 50,000 *Plasmodium yoelii* sporozoites. Labeled gates are sorted populations. Exclude doublets by FSC-H vs. FSC-W and SSC-H vs. SSC-W, but if quiescent hepatic stellate (qHSC) are desired, be sure to include the SSC-H events. From a standard FSC-A vs. SSC-A scatter plot, separate lymphocyte-sized cells from cells with high granularity (SSC) and larger size (FSC). Hepatic stellate cells contain highly refractive retinol droplets and are autofluorescent when excited with 405 nm and emitting at 450 nm. Lymphocytes can be separated into many populations. Here, CD8+ T cells are collected against CD8a vs. CD4. CD4+ T cells and NK(T) cells are collected against NK1.1 vs. CD4. A significant population of NK-T cells are CD4+ in the mouse. The best identifier of NK-T cells is CD1d (stained by tetramer, not conducted here). NK(T) cells induce CD11b expression when activated. From the larger cells, LSEC, Kupffer cells (KC), and infiltrating myeloid cells (including monocytes and granulocytes) can be collected. LSEC are selected against CD11b vs. Tie2. From the CD11b$^{int/hi}$ Tie2$^{int/lo}$