

Cell Tracer Violet and CellTracker Red CMTPIX Staining of Purified Mature Plasmodium-infected Red Blood Cells

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[Abstract] Efficient staining methods to identify *Plasmodium*-infected red blood cells (iRBCs) are crucial to discriminate precisely the immune cells responsible for their elimination from circulation. Here, we describe the protocol for the purification of iRBCs and their subsequent staining with the vital dyes Cell Tracer Violet (CTV) or CellTracker Red CMTPIX (CMTPIX), both of which readily diffuse into cells and bind covalently to intracellular amines. The iRBCs stained by using this protocol were used in *ex vivo* phagocytosis assays, to determine the ability of splenic dendritic cells of phagocytizing these parasites (Borges da Silva *et al.*, 2015).

Materials and Reagents

1. 15 ml tubes (TPP, catalog number: 91015)
2. 1 ml syringes (BD Biosciences, catalog number: 309659)
3. B6.Rag2^{-/-} (RAGKO) mice (Jackson Laboratories, C57BL/6)
4. *Plasmodium chabaudi* (AS strain)
5. RPMI 1640 (Thermo Fisher Scientific, catalog number: 11875093)
6. Fetal bovine serum heat inactivated (Thermo Fisher Scientific, catalog number: 10437028)
7. Penicillin-Streptomycin (Thermo Fisher Scientific, catalog number: 15140122)
8. L-glutamine (Thermo Fisher Scientific, catalog number: 25030081)
9. Sodium pyruvate (Thermo Fisher Scientific, catalog number: 11360070)
10. 2-mercaptoethanol (Thermo Fisher Scientific, catalog number: 21985023)
11. Cell Tracer Violet (CTV) (Thermo Fisher Scientific, catalog number: C34557)
12. CellTracker Red CMTPIX (CMTPIX) (Thermo Fisher Scientific, catalog number: C34552)
13. Halothane (Sigma-Aldrich, catalog number: H0150000)
14. NaCl
15. KCl
16. Na₂HPO₄
17. KH₂PO₄
18. Percoll (GE Healthcare Dharmacon, catalog number: 17089101)
19. 1x phosphate buffered saline (PBS) (see Recipes)
20. 74% Percoll (see Recipes)

Equipment

1. Centrifuge (Eppendorf, model: 5804)
2. 1-ml micropipettes
3. 25 gauge needle (BD Biosciences, catalog number: 305122)
4. Laminar flow hood (AirClean Systems, catalog number: AC8000HLF)
5. Epi-Fluorescent microscope (Leica Microsystems, model: DM6000B)

Procedure

1. 6-8 weeks old RAG2KO mice (used here because we observed higher proportion of mature parasite forms in circulation instead bound to endothelial cells-personal observation) are infected *i.p.* with 10^6 *Pc*-infected iRBCs. The infection in RAGKO allows the posterior collection of a greater proportion of mature iRBCs (Borges da Silva, personal observation).
2. Seven days after infection, mice are euthanized with halothane by inhalation and bled by cardiac puncture with a 1 ml syringe with a 25 gauge needle, in the presence of approximately 50 μ l of heparin. A volume of approximately 500 μ l of blood is enough to collect up to 5×10^8 mature iRBCs.

Note: Verify the institution approved ethical regulations on animal welfare if other euthanization methods are in place.

3. The blood collected is diluted in approximately 1 ml of PBS 1x (1:1), and placed on a 74% Percoll solution in 15 ml tubes (5 ml). The tubes are centrifuged for 30 min at $2,500 \times g$, at room temperature with acceleration/break of 5/0, respectively.
4. After the centrifugation, the mature iRBCs are located as a layer above the 74% Percoll (Figure 1), and are collected by using 1-ml micropipettes into new 15 ml tubes. The mature iRBCs are then washed twice with 1x PBS.

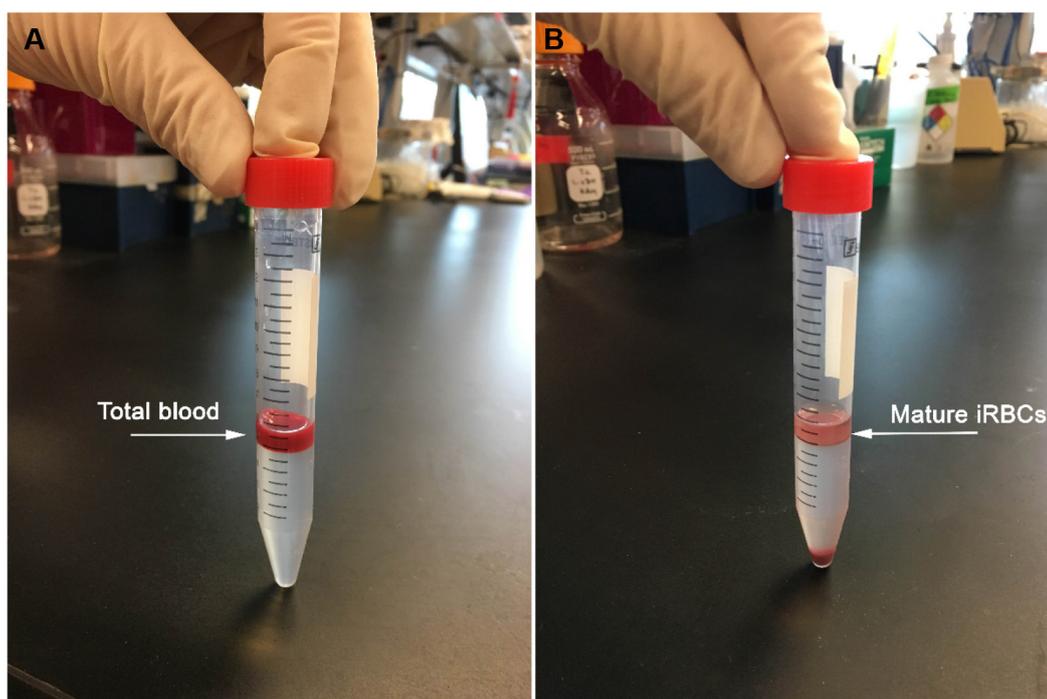


Figure 1. Percoll-based purification of mature iRBCs. A. A representative picture of 15-ml conical tubes containing RBCs over Percoll 74% before centrifugation. B. A representative picture of 15-ml conical tubes containing the layer with mature iRBCs after Percoll 74% centrifugation.

5. After washing, a sample of the mature iRBCs (approximately 5 μ l) is collected for a blood smear, to confirm the purity for mature iRBCs versus non-mature iRBCs (Figure 2) by counting the percentage of mature iRBCs versus non-mature iRBCs in the blood smear (at this point, it is expected up to 97% of mature iRBCs in the blood smear). Then, 1×10^8 mature iRBCs (resuspended in 2 ml of PBS) are incubated with 5 μ M of CTV or with 5 μ M of CMTPX (both dyes previously diluted in DMSO and with final suspension volume of 2 ml), at 37 $^{\circ}$ C, for 30 min.

Notes:

- a. *The expected purity for the samples is between 90-99% of mature iRBCs.*
- b. *During the incubation, mix the cells gently for homogenization.*

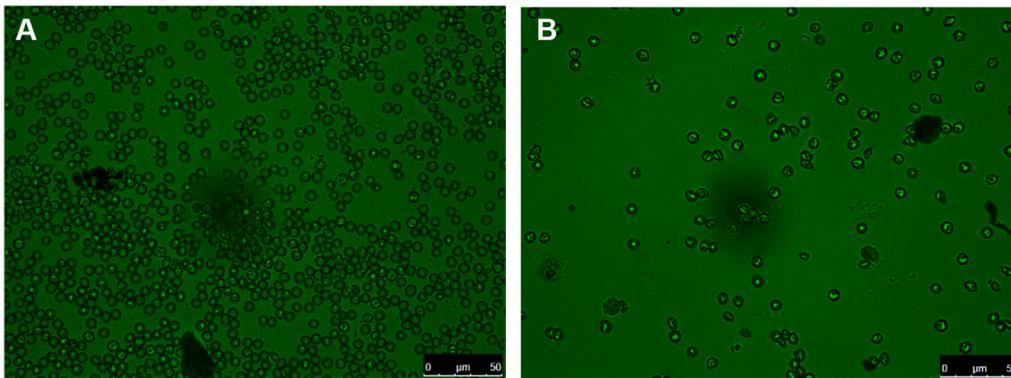


Figure 2. Mature iRBCs purification assessment. A. A representative picture of a blood smear of a *Plasmodium*-infected mouse, pre-Percoll enrichment. B. A representative picture of a blood smear of post-Percoll enrichment blood of the same mice. Obs: The infected RBCs can be detected by the bright green spots (these images were acquired in Bright Field, in a Leica DM6000B Epi-Fluorescent Microscope).

6. After incubation, the mature iRBCs are washed twice with 1x PBS for removal of excess dye. The stained mature iRBCs are, then, ready for use in phagocytosis assays as described in (Borges da Silva *et al.*, 2015).

Notes

1. This procedure usually yields between 95-97% of mature iRBCs purity. However, when done with mice with lower parasitemia levels, there is a drop in these numbers up to 50% purity. Thus, it is preferential to collect blood from RAG2KO mice with > 50% parasitemia (verified upon a blood smear previously done to select mice).

Recipes

1. 1x phosphate buffered saline (PBS)
 Dissolve the following in 800 ml distilled H₂O
 8 g NaCl
 0.2 g KCl
 1.44g Na₂HPO₄
 0.24 g KH₂PO₄
 Adjust pH to 7.4
 Adjust volume to 1 L with additional distilled H₂O
 Sterilize the solution
Note: Adjust the pH using HCl and NaOH.
2. 74% Percoll

Percoll is commercially available at a 10x concentration. To make a 1x concentrated Percoll solution (which is equivalent to a 100% Percoll solution), it is necessary to dilute 9 parts of Percoll (9 ml GE Healthcare) with 1 part of 10x PBS (1 ml).

To make 74% Percoll, it is necessary to dilute 3.7 ml of 100% Percoll with 1.3 ml of 1x PBS.

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