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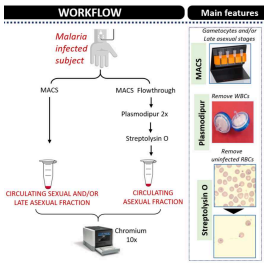
Isolating Plasmodium sexual and asexual parasites from malaria infected individuals for single cell RNA sequencing



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We use this protocol and it's working

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Abstract

This protocol isolates circulating sexual and asexual *Plasmodium* parasites from an infected individual's blood such that the resulting parasites are viable for single cell RNA sequencing, as well as bulk RNA sequencing and genome sequencing.
The important features of the protocol include (i) preventing the activation of gametocytes by re-suspending the blood in Suspended Animation (SA) buffer, (ii) using MACS columns to concentrate capture of circulating gametocytes and late stages from blood, (iii) using Plasmodipur filters to eliminate human contamination from white blood cells, and (iv) performing selective erythrocyte lysis using Streptolysin O (SLO) to remove as many uninfected erythrocytes as possible and improve the percentage of infected erythrocytes for further analysis.

We have successfully used this protocol on parasites captured from the blood of naturally infected carriers in Mali to generate single cell RNA sequencing data on both asexual and sexual parasites of *P. falciparum*, *P. ovale* and *P. malariae* using the 10X 3' protocol. Because both the asexual stage parasites and gametocytes of *P. malariae* and *P. ovale* spp. circulate in the peripheral blood, we recover asexual stages in both the MACS and non-MACS fractions for these two species.

Human contamination from high quantity transcripts present in erythrocytes remains a problem as SLO is not always successful at removing the majority of uninfected erythrocytes. This contributes to a significant proportion of sequencing output, and decision to sequence at required depth should be taken upon inspection of the RBC-specific transcript peak (~700 nt) post-cDNA amplification QC. In our experience, when there is a significant RBC-specific peak, we recommend not proceeding with single cell RNA sequencing unless at least 3000 cells can be targeted for recovery.

Materials

Reagents and Consumables

- D-PBS (#D8537-500ML, from Sigma Aldrich)
- Nuclease-free water (not DEPC-treated) (#AM9937, Invitrogen™)
- MACS LS Columns (#130-042-401, from Miltenyi Biotec Ltd).
  Pre-cooled, store in fridge the day before
- Suspended Animation (SA) buffer –
  10 mM Tris-HCL (#H5125, from Promega)
  166 mM NaCl (#27810.364, AnalaR NORMAPUR® - VWR)
  10 mM Glucose (#G7021, from Sigma-Aldrich)
  pH 7.37 (pH is very important, make sure the meter is calibrated)
- Streptolysin O from Streptococcus Pyrogenes (#S5265-25KU, from Sigma Aldrich)
- 1M DTT (#P2325, from Life Technologies Ltd)
- Plasmodipur Filter Units (#8011Filter25U, from Euro Proxima)
- CPDA Vacuette blood collection tube, 9ml, CPDA (#455056, from Greiner Bio-One Ltd)
- C-Chip Hemocytometer Chips (#DHC-N01, from NanoEntek)
- Microscope slides (E.g: double-frosted microslide 45° 20mm glass, #630-2012, from VWR)
- Gloves (material and size as needed)
- Surface cleaning paper wipes
- Any bin/container to dispose of used plasticware
- Bin with appropriate lysis solution to discard bio-waste and inactivate consumables in contact with blood.

Equipment

- MACS MultiStand (#130-042-303, from Miltenyi Biotec Ltd)
- QuadroMACS Separation Unit (#130-090-976, from Miltenyi Biotec Ltd)
- Single channel pipettes (100-1,000 µL, 20-200 µL, 2-20 µL)
- Pipette Controller, such as Pipetboy
- Falcon tube racks
- Tabletop centrifuge for 1.5mL Eppendorf tubes
- Tabletop centrifuge with temperature settings for 15mL and 50mL falcons
- Spray bottle (70-80% ethanol for cleaning surfaces)

Other Plasticware

- DNA LoBind Tubes (#EP0030108051, Eppendorf)
- 15/50 mL falcon tubes
- 5/10/25 mL Stripette
- 20/200/1000 µL tips

Troubleshooting

Before start

Preparation

1. Pre-cool QuadroMACS Separation Unit and MACS columns (keep them in the fridge the day before the experiment)
2. Ice bucket
3. Keep required amount of SA buffer in 50 mL falcons (filled to the top) on ice
4. Keep centrifuge for 15 ml/50 ml falcons at 4°C

Activate SLO

1. Resuspend Streptolysin O from Streptococcus Pyrogenes (#S5265-25KU, from Sigma Aldrich) in 5mL of D-PBS and make 150 µl aliquots of 5 units/µl each.
2. Prepare 50 µl aliquots of 20mM DTT solution from 1M DTT stock solution (with RNAse free water)
3. Activate SLO by mixing 150 µl SLO solution (5 units/µl) with 50 µl 20mM DTT solution and incubate at 37°C for 2 hours (to activate the toxin). Activated aliquots may be stored at -20C

Blood Draw

1 Draw fresh blood into a CPDA vacutainer, mix by inverting 4-5 times and immediately keep on ice (Heparin might inhibit PCR/RT. EDTA affects parasites if left for a few hours). 5m

Note

1. Draw at least 4 mL of blood to proceed with the rest of the protocol.
2. Take care to mix the blood with the CPDA solution properly. Improper mixing can lead to coagulation.
2. Proceed with the protocol immediately after blood draw. If transportation is needed, make sure the sample is maintained on ice. Samples held on ice for 9 hours have yielded good quality single cell transcriptomes in our experience.

2 Following fresh blood draw or transportation as above, add 5 mL of cold SA buffer to 3 mL of subject blood sample in a 15mL falcon and keep it on ice 1m

Purification of circulating gametocytes and late stage parasites.

3 Take a cooled MACS LS column and insert it into the precooled magnet. Place a labeled 50 mL falcon beneath it for flow-through. 5m

Note

Leave the MACS LS columns and the Quadromacs™ separator in the fridge the day before the experiment. The pre-cooling helps prevent activation of gametocytes.

4 Pre-wet the MACS LS column with 800 µL of cold SA buffer onto the MACS LS column. 1m

5 Decant the sample from **Step 2** on the MACS LS column and let it elute. 10m

6 Washing the MACS column to elute uninfected erythrocytes and rings (e.g. cells not bound magnetically):

6.1 Wash with 4 mL of SA buffer 5m

Note

Repeat wash if the eluate solution is not clear.

6.2 Once the eluate solution is clear, wash with an extra 6 mL of SA buffer. 5m

6.3 Optional: Wash again with an extra 6 mL of SA buffer. 5m

Note

Additional washes help in getting rid of uninfected RBCs and WBCs that get trapped in the column, and thereby obtaining a higher parasitemia of late stages and gametocytes in the MACS fraction.

7 Spin the flow through at 500 x g, 4°C, 00:03:00 and keep on ice after the spin is complete. This is the fraction that contains early asexuals. (This fraction is processed according to section "Purification of circulating early asexual stages" from **Step 12**) 3m

8 Elute the MACS fraction:

8.1 During the spin above (Step 7), remove the column from the magnet and place into a labeled 15 mL falcon tube. Add 4 mL of SA buffer to the column to elute the cells trapped in the MACS column by gravity. 5m

8.2 Add another 6 mL of SA buffer to the MACS column and insert plunger contained in the MACS column packet and elute by applying gentle (important) pressure. 2m

8.3 Spin down the MACS fraction at 5m
800 x g, 4°C, 00:05:00 , (slow acceleration and slow decceleration setting)

Note

You might see a faint or very faint pellet or nothing at all. Proceed to the next step anyway.

8.4 Remove all but ~ 120 µL of SA buffer and resuspend the pellets gently. 3m

Note

Pre-mark the falcon with a line indicating 120 µl remaining volume to assist with supernatant removal steps. Remove supernatant until 1 mL mark with a stripette and remove the rest using a 200 µL pipette. The number of cells per µL can be concentrated by leaving 60 µl of SA buffer instead of 120 µl. Extra caution should be taken in this case to not to aspirate the cells.

9 Resuspend and take a sample of 100 µL and transfer to an 1.5 mL Eppendorf and keep on ice. 3m

Note

This fraction will be used for the 10X run, and contains gametocytes and late stage asexuals, if any.

10 Take 10 µL from the rest of the 20 ul and use it to count on a hemocytometer. 5m

11 Spin the rest of the 10 µL at 15m
800 x g, 4°C, 00:05:00 , (slow acceleration and slow decceleration setting) ,
remove most of the supernatant, leaving enough to place 2 x ~2 µl drops on a glass slide, let it dry for 00:10:00 and
stain with Giemsa to estimate parasitemia.

Purification of circulating early asexual stages.

12 From the flowthrough fraction (from **Step 7**), remove the supernatant and any visible buffy coat (WBCs). Wash by resuspending the pellet with 10 mL of D-PBS and spin at 500 x g, 00:03:00 . 3m

13 Remove supernatant and resuspend in 10 mL of D-PBS for an additional wash. Spin again at 500 x g, 00:03:00 3m

14 Remove supernatant and resuspend pellet in 10 mL of D-PBS 3m

15 During the above two spins, remove the plunger from a 20 mL syringe, mount it onto a Plasmodipur filter and load 2 mL of sterile PBS and pre-rinse it with gentle pressure. Do the same for a second Plasmodipur. (We are going to pass each sample through 2 Plasmodipur filters to get rid of WBCs) 3m

16 Now, load the sample from **Step 14** into the syringe as above and filter it through the Plasmodipur into a 50 mL falcon. 3m

17 Reload the filtrate through the second pre-rinsed Plasmodipur and filter it into a 50 mL falcon. 3m

18 Spin down the Plasmodipur flowthrough at 500 x g, 00:03:00 and remove the supernatant. 3m

19 Take 50 µL from the blood pellet above in a 1.5mL Eppendorf tube and resuspend in 950 µL PBS + 100 µL activated SLO. 3m

20 Incubate for 00:10:00 at 37 °C for SLO lysis. 10m

Note

SLO binds to cholesterol-containing membranes and permeabilizes host cell membrane. Differences in cholesterol content of infected and uninfected RBCs allows us to use SLO to preferentially lyse uninfected RBCs. Lysis of uninfected RBCs appears to vary between subjects. Time of incubation of lysis and concentration of SLO can be varied to obtain desired extent of lysis. In cases when after first SLO treatment, lysis appears to be minimal or not optimal, additional lysis can be performed after washes (**Step 23**).

21 Following SLO lysis, spin 1500 x g, Room temperature, 00:02:00 and remove the supernatant (should show heavy lysis) 2m

22 Add 1 mL PBS to the tube (even if a clear pellet is not discernible), spin at 1500 x g, Room temperature, 00:02:00 and discard supernatant (a proteinaceous translucent red layer sits above the actual pellet) 2m

23 Repeat washing until the supernatant is clear. A clear pellet might not be discernible after the washed. Proceed anyway. 10m

24 Resuspend in ~ 60 µL of D-PBS and keep on ice. 2m

> Note
>
> This fraction will be used for the 10X run, and contains cells not bound to the MACS column, namely, the early stage asexuals.

25 Take an aliquot of 10 µL to count under a hemocytometer. 5m

26 Take an aliquot of 10 µL to spin and smear (either a drop smear or a normal smear depending on the size of the pellet) and stain with Giemsa to estimate parasitemia. 20m

Example Application: Sample Loading on Chromium 10x

27 Obtain the number of cells per µL in the isolated fractions using a hemocytometer. 5m

28 Estimate parasitemia of isolated fractions after staining with Giemsa. 5m

29 Calculate the number of infected RBCs per µL by using the cell number and parasitemia estimate. 2m

Note

For loading, we estimate only the number of parasites per µL. Though the presence of RBCs does not affect the single cell loading per se, when the uninfected RBCs have not been completely lysed or washed, we observe this in the form of RBC-specific transcript peak at ~700 nt post-cDNA amplification QC (https://kb.10xgenomics.com/hc/en-us/articles/217233386-What-are-the-additional-peaks-in-my-post-cDNA-amplification-QC-). This contributes to a significant proportion of sequencing data. Hence, the number of uninfected RBCs in the final fraction should be minimal.

30 Follow the guidelines from **Chromium 10x User Guide** to target required recovery of cells.