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Processing PBMCs for multiplexed scRNA-seq

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Human Cell Atlas Method Development Community

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ABSTRACT

Purpose: to prepare viable single cells from frozen PBMCs for scRNA-seq, multiplexing strategy is applied to reduce batch effect and cost.

ATTACHMENTS

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MATERIALS TEXT

Supplies/Equipment

●Ultra-Low Attachment Multiple Well Plate, 24 well (Corning Costar #3473)
●1.5 mL microcentrifuge tube, low binding Eppendorf tube
●70 um cell strainers
●Hood (Biological Safety Cabinet - BSC)
●Incubator for cells at 37°C
●Water bath at 37°C
●Inverted microscope
●Centrifuge for 50 mL, RT
●Centrifuge for 5 mL, 4°C
●Centrifuge for 1.5 mL, 4°C
●Timer
●Pipettes and sterile filter-tips (all sizes)
●1 mL and 200 uL wide-bore tip
●Pipetboy and sterile serological pipettes (all sizes)
●Regular hemacytometer
●Cell counter
●50 mL sterile falcon tubes
●5 mL FACS tube
●White column racks for FACS tube
●MS columns (Miltenyi Biotec #130-042-201)
●Flowmi Cell Strainers (Sigma # BAH136800040-50EA)

Reagents

●Complete medium (for PBMC culture)
RPMI-1640 + 10% FBS + 100 U/mL penicillin + 100 U/mL streptomycin + 1 mM sodium pyruvate + 2 mM L-glutamine
●Washing medium
50% Complete medium + 50% X-vivo 15 medium + 25 U/mL benzonase (3.1 uL of 400 U/mL benzonzase into 50 mL Washing medium)
●Benzonase (Millipore #71205-3)
●Lipopolysaccharide (LPS). Ultrapure lipopolysaccharide from E. coli K12 (InvivoGen #tlrl-peklps)
●Interferon β (IFN β). Recombinant Human IFN-beta Protein (R&D systems #8499-IF-010/CF)
●1X D-PBS
●Trypan blue 0.4% (filtered)
●Staining Buffer. Cell staining buffer (BioLegend #420201)
●Dead cell removal kit (Miltenyi Biotec # 130-090-101)
●Distilled water. UltraPure distilled water (Invitrogen #10977-015)
●4% BSA. Prepared from BSA powder in PBS, aliquots stored in -20C.
●Bleach

SAFETY WARNINGS

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Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

BEFORE STARTING

Carefully determine the PBMCs will be used in one experiment based on individual information. Cells from unrelated individuals can be pooled together.

Thawing PBMCs

1 Prepare Complete medium and Washing medium, and warm to 37°C in a water bath. Prepare 50 mL Falcon tubes with 25 mL Washing medium each.

> Note: Add benzonase into the Washing medium immediately before use.

2 Remove cryovials from liquid nitrogen storage and place them on dry ice.

> *Note: Carefully determine the PBMCs will be used in one experiment based on individual information. Cells from unrelated individuals can be pooled together.*

3 Thaw frozen vials in the water bath at 37°C for 1 min (set timer), only one vial each time. Remove from the water bath when a tiny ice crystal remains. Wipe the vials with 70% ethanol and bring them to the hood.

4 Pour the thawed cells gently into a 50 mL conical tube containing 25 mL pre-warmed Washing medium. Use one 50 mL conical tube for each vial.

> *Note: Avoid pipetting cells in the "Thawing PBMCs" steps, do tapping or vortexing instead.*
> *Note: The washing medium contains benzonase, which is a gentle DNase. Using benzonase to get rid of potential DNA released from dead cells, thus avoiding clustering of cells.*

5 Rinse the cryovial with 1 ml pre-warmed Washing medium and add the rinse to the 50 mL tube.

6 Centrifuge at 350 xg for 5 min at room temperature. Aspirate the supernatant.

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7 Resuspend cell pellet in 1 mL of pre-warmed Washing medium (dropped slowly along the side of the tube) by gently tapping. Add 4 mL of pre-warmed Washing medium. Invert gently the tube several times to homogenize the cell suspension. Take 10 uL for cell counting (step 8). Add another 5 mL of pre-warmed Washing medium. Centrifuge at 350 xg for 5 min at room temperature.

8 Count cells and determine viability. Count the cells by adding 10 uL of cells to 10 uL of trypan blue and pipetting up and down to mix. Apply 10 uL of the mixture to the hemacytometer or cell counter. Calculate the cell number.

9 Resuspend cell pellet in 1 mL of pre-warmed Complete medium by gently tapping. Add another 9 mL of pre-warmed Complete medium. Briefly vortex (optional). Centrifuge at 350 xg for 5 min at room temperature. Note: Using Complete medium is to wash away benzonase.

10 Resuspend cells in Complete medium and adjust to 1 x 10^6 cells per mL. Gently tapping.

11 Seed cells into 24-well plate (low attachment plate), at 1 x 10^6 cells per well in 1 mL Complete medium, using a wide-bore 1 mL pipet tip.

12 Transfer the cell culture plate to the incubator (37°C, 5% CO2) for 16-24 hrs, and leave the cells for recovery.

Stimulation of the PBMCs with ligands

13 Prepare LPS (200ng/mL, 20x), IFNβ (2000U/mL, 20x) in Complete medium.

Note: prepare these 20x solutions in Complete medium and can be stored in 4°C overnight. To prepare 1000 uL of 200 ng/mL LPS, add 10 uL of 20 ug/mL LPS (-80°C stock) into 990 uL Complete medium. To prepare 1000 uL of 2000 U/mL IFNβ, add 2 uL of 1 x 10^6 U/mL IFNβ (-80°C stock) into 998 uL Complete medium.

14 Add 50 uL of LPS (200 ng/mL, 20x) in one well (with 1 mL medium) at a final concentration of 10 ng/mL. Or add 50 uL of IFNβ (2000U/mL, 20x) in one well (with 1 mL medium) at a final concentration of 100 U/mL. For control, add 50 uL Complete medium.

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Note: We usually add LPS or IFNβ at 7AM, thus harvesting cells at 1PM.

15 Tap plate 10 times (at each side of the plate) to resuspend cells and mix medium. Transfer the cell culture plate to the incubator (37°C, 5% CO2) and incubate for 6 hrs.

Harvest PBMCs and pool samples

16

Note: Before harvesting cells, set centrifuge for 5 mL tubes at 4°C. Prepare/label FACS tubes. Bring Dead Cell Removal beads and buffer to room temperature. Bring 4% BSA to room temperature.

At the end of the incubation (6 hr after adding LPS or IFNβ), remove the cell culture plate from the incubator.
Check cells under the microscope if any cell clusters.
Transfer cells with medium into 5 mL FACS tube, using 1 mLwide-bore pipette.
Rinse the well with 1 mL PBS and add the rinse to the FACS tube.
Check cell plate under a microscope if any cell leftover. If cells are attached to the well, add 200 uL Tryple E, incubate at 37°C for 5 min, rinse with another 1 mL PBS and transfer to the FACS tube.

17 Centrifuge at 350 xg for 8 min at 4°C.
Pour off the supernatant into sink (usually will have 50 uL liquid leftover).
Invert the tube and dry the tube top with a Kim wipe.

18 Wash cells with Staining Buffer.
Add 0.5 mL Staining Buffer (from BioLegend) to each sample.
Tap on the white columns several times to resuspend cells.
Add another 1.5 mL Staining Buffer to each sample.
Centrifuge at 350 xg for 5 min at 4°C.
Pour off the supernatant and dry the tube top.

19 Wash cells again with Staining Buffer.
Add 500 uL Staining Buffer (now total volume around 550 uL).
Tap on the white columns to resuspend cells.
Take 10 uL for Trypan Blue staining and count cells using cell counter Countess.
Calculate the cell number and volumes (will use in step 21).

20 Add 2 mL Staining Buffer.
Centrifuge at 350 xg for 5 min at 4°C. Pour off the supernatant. Dry the tube top.

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Note: Now set centrifugate for 1.5 mL EP tube at 4°C and fast cool down.

21 Resuspend cells in the Staining Buffer and adjust to 5 x 10^6 cells per mL.
Tap on the white columns to resuspend cells. Place cell tubes on ice.
Take out (using a wide-bore tip) the same number of cells from indicated samples and pool cells into low binding 1.5 mL Eppendorf tubes.

For example, we thawed PBMCs from 8 individuals and each received 3 treatments (Ctrl, LPS, IFNβ), thus we had 24 cell samples; we pool 8 samples (from 8 individuals) into one tube, thus 24 samples were pooled into 3 tubes. For each cell sample, we take 0.3 million cells (60 uL, 5 x 10^6 cells per mL), thus every pool had 2.4 million cells (480 uL, 5 x 10^6 cells per mL).

Note: Total cell number after pooling should be better higher than 2 million, at least higher than 1 million; if less, cell number after dead cell removal and filtration will be too low due to cell loss in the following step. Use low-binding Eppendorf tubes for all steps below.

Dead cell removal

22

Note: Before step 22, prepare Binding buffer (from Dead cell removal kit, 4°C), add 0.5 mL uL Binding buffer into 9.5 mL distilled water. Bring to RT before use. Prepare 10 mL 0.4% BSA in PBS, add 1 mL 4% BSA (-20°C aliquots) into 9 mL PBS (no Ca2+, no Mg2+). Keep on ice. Prepare 10 mL 0.04% BSA in PBS, add 1 mL 0.4% BSA into 9 mL PBS (no Ca2+, no Mg2+). Keep on ice

Filter cell with 70 um cell strainer using normal 1 mL tip. Centrifuge at 350 xg for 5 min at 4°C. Remove the supernatant.

23 Add 100 uL dead cell removal microbeads (well-vortexed). Resuspend by pipetting using P100 pipet. Leave at room temperature for 15 min.

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Note: During the 15 min incubation, bring 10x Genomics Gel beads to room temperature, and bring 10x Genomics reagents on ice, bring 50% Glycerol and TSO (template switch oligo) to room temperature.

24 During the last 1 min, rinse the MS column with 500 uL binding buffer (room temperature). Keep MS column wet all the time. Add 400 uL binding buffer to the pooled cells. Transfer the cell suspension onto the column and leave them to pass through. Collect the flowthrough in a low binding 1.5 mL Eppendorf tube.

25 Rinse the column with another 500 uL binding buffer. Collect the flowthrough in the same tube from the last step. Centrifuge at 350 xg for 5 min at 4°C.

Optional: Rinse the column with 500 uL binding buffer twice. Save the flowthrough in another 1.5 mL tube and place on ice (in case more cells can be collected).

Prepare single-cell suspension

26 Resuspend the cell pellet with 1 mL 0. 4% BSA in PBS, slowly pipet using a wide-bore tip. Centrifuge at 350 xg for 5 min at 4°C. Use a pipet to remove SPN.

27 Resuspend the cell pellet with 200 uL-400 uL 0.04% BSA in PBS (depends on how big the cell pellet, if tiny pellet, do 200 uL). Pass through the 40 um Flowmi Cell Strainer using P1000. Do not spin down cells after filtration. Take 10 uL for Trypan Blue staining and count cells using cell counter Countess, record cell viability.

28 Adjust the cell number to 1500 cells/uL (this is 10X Genomics suggestion) and place cell on ice. We will use 40 uL of 1500 cells/uL cell suspension for each reaction using 10X Genomics single cell 3' kit, i.e. 60K cells.

29 Move forward to 10X Genomics protocol of "Chromium Next GEM Single Cell 3' Reagent Kits v3.1" immediately.

Note: Immediately load cells into the 10X Genomics instrument for generating GEM. Determine the number of reactions for each pool based on your target singlet number per sample.

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