MONOCYTE CELL SORTING FOR RNASEQ

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EQUIPMENT/MATERIALS

- Antibodies (See page 4)
- FACs Wash (0.5% (w/v) BSA, 5mM EDTA (pH 7.4-7.6))
- eBioscience RBC Lysis Buffer (10X) (420301)
- RPMI Media
- PBS
- Leucosep tubes filled with ficoll (VWR: 89048-934)
- 50 mL conical tubes
- 5mL polystyrene round bottom tubes (FACS Tubes)
- 5mL polystyrene round bottom tubes with cell-strainer cap (352235)
- 5mL polypropylene round bottom tubes (FACS Tubes)
- BD Pharmingen 7 AAD (559925)
- eBioscience OneComp eBeads (01-1111-41)
- Qiagen QIAzol Lysis Reagent (79306)

PROTOCOL

Cell Sorting for Human Monocytes
Comparing PBMCs to Fresh Blood for RNA seq.

*** from 100uL of whole blood average of 8000 classical, 230 Intermediate, 300 Non-Classical Monocytes*

*** from 30 million PBMCs average 6.29x10^5 classical, 0.24x10^5 intermediate and 0.22x10^5 non classical*

**** aiming for 50 000 x all three classifications.*

WHOLE BLOOD MONOCYTES

1) Collect 3x10 mL of whole blood in a heparin collection tube. Allow to sit at room temperature for at least 30 minutes before processing.
2) Spin at 1500 rpm for 10 mins and remove plasma.
3) Combine the volume of blood 1:1 with PBS in a 50mL conical tube.
4) Divide the volume into two Leucosep tubes and centrifuge without a break at 2300 rpm for 10 mins.
5) Remove plasma layer from the Leucosep tube and combine buffy layers from both tubes into one 50mL conical tube.
6) Wash cells by adding cold PBS up to 50mL. Centrifuge at 1500rpm for 5 minutes and remove supernatant.
7) Suspend cell pellet in 20mL of PBS and count PBMCs with a hemocytometer.
8) Determine what volume of the 20mL of cells to divide into a second conical tube for an unstained control. At this point one 50mL tube should have volume with approximately 20-35x10^6 cells for full staining and sorting. The second tube should have a smaller volume with approximately 5-10x10^6 cells to be used as an unstained control.
9) Spin at 1500 rpm for 5 mins and remove supernatant.
10) Resuspend each tube of cells in 200uL of cold FACS Wash and transfer into FACS tubes for staining.
    a. Unstained tube – 5-10 million cells
    b. Full stain – 20-35 million cells

ISOLATED PBMC HUMAN MONOCYTES

**All reagents, centrifuges and cells must be kept at 4C*

1) Add thawed cells to 15mL of cold RPMI Media.
2) Spin down at 1500rpm for 5 minutes and remove supernatant.
3) Suspend cell pellet in 2.5 mL of PBS and count PBMCs with a hemocytometer.
4) Spin down at 1500rpm for 5 minutes and remove supernatant.

5) Wash by adding PBS up to 2.5mL, count cells, then spin down and remove supernatant.
6) Resuspend each tube of cells in 200uL of cold FACS Wash and transfer into FACS tubes for staining. (Leave out a small volume (~20 µL) in a separate FACS Tube as an unstained control)

STAINING MONOCYTES

1) Add 100uL of 3X surface stain to cells in FACS tube and incubate in the dark at 4°C for 30 minutes. (Stain is optimized for 10-35 million cells (ideally 20 million)/100uL of stain).

Antigen	**Fluorophore**	**Antibody Concentration in 3x stain**	**One Sample Full Stain**
CD45	BV510	1/50	2 µL
CD16	PECy7	1/50	2 µL
CD14	BV421	1/50	2 µL
CD11b	APC	1/25	4 µL
HLA-DR	PerCpCy5.5	1/25	4 µL
CD15	AF700	1/50	2 µL
CD3	AF700	1/25	4 µL
CD56	AF700	1/25	4 µL
CD19	AF700	1/25	4 µL
	Total Antibody		**28.0 µL**
	FACS Wash		**72.0 µL**

2) Cool centrifuge to 4°C.
3) To wash, top up tube with 4mL with FACS Wash, spin down and remove supernatant.
4) Top up tube with 4mL of RBC Lysis Buffer and incubate in the dark at 4°C for 5 minutes.
5) Spin at 1500 rpm for 5 mins and remove supernatant.
6) Add 2mL of FACS Wash and count cells.
7) Spin at 1500 rpm for 5 mins and remove supernatant.

PREPARTION FOR CELL SORT

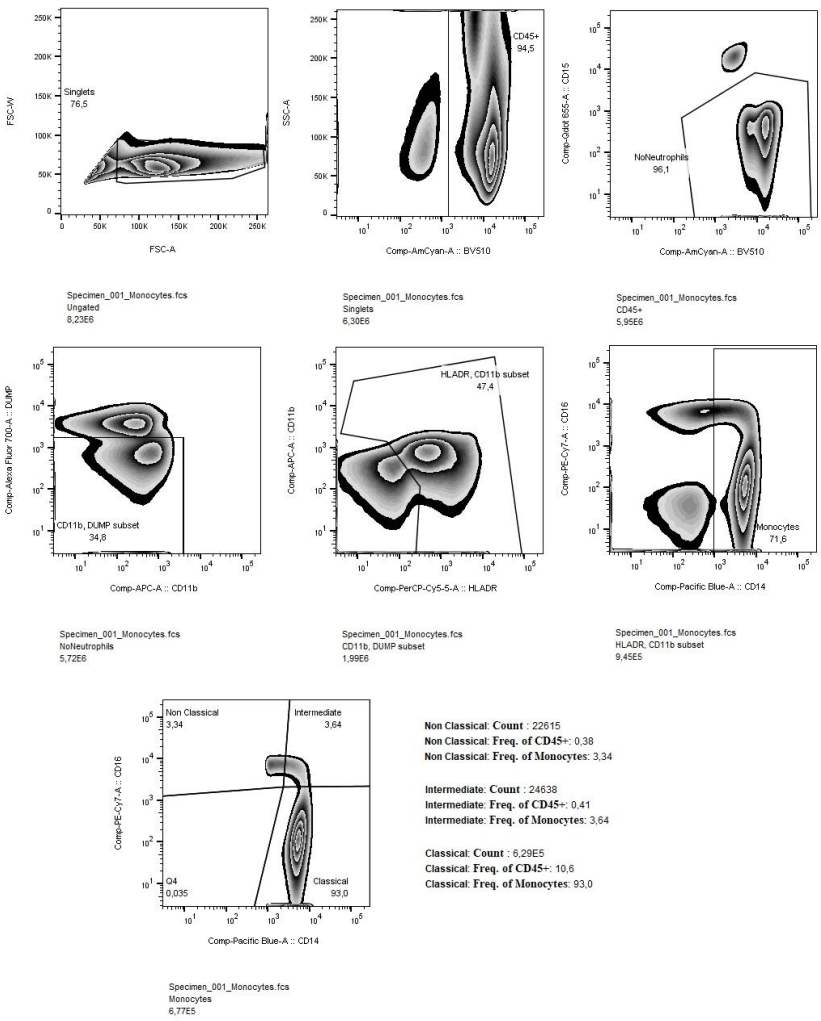
1) Prepare for cell sorting by resuspending in 1.0 mL of FACS Wash.
2) Prepare one tube with 20uL of fully stained cells as an 7AAD FMO control (add 220uL of FACS Wash)
3) Add 7AA-D at 1/250 (V/V) (4 µL) to each sample. Mix with pipette.

4) Prepare one tube for cytometer set up (20ul of fully stained cells (with 7AAD) and 220 FACS Wash)
5) Prepare compensation controls. Note: 7AAD compensation is made with unstained cells rather than beads.
6) Set up voltages and compensation on sorter.
7) Filter cells through 0.45um mesh just prior to sorting.
8) Coat 2mL polypropylene FACS Tubes with FACS Wash (Cells will bust if hitting dry tubes).
9) Empty tubes before the cell sort.
10) Sort ~50 000 cells directly into 700uL QIAzol. Note that every 2000 cells are sorted in ~6uL of sorting buffer.
11) Verify viability and purity of cells post-sorting.
    **Plan 1 hour of set up time on the cytometer
    **Plan 1.5-2hours/sample of sort time.

ANTIBODY LIST

Antibody	Fluorophore	Company	Clone	Catalog #
CD45	BV510	BioLegend	HI30	304036
CD16	PE-Cy7	eBioscience	eBioCB16	25-0168-42
CD14	BV421	BioLegend	M5E2	301830
CD11b	APC	BD Pharmingen	ICRF44	550019
HLA-DR	PerCPCy5.5	eBioScience	LN3	45-9956-42
CD15	AF700	BioLegend	W6D3	323026
CD3	AF700	BD Pharmingen	UCHT1	557943
CD19	AF700	invitrogen	HIB19	56-0199-42
CD56	AF700	BioLegend	HCD56	318316

SORTING STRATEGY



Non Classical: **Count** : 22615
Non Classical: **Freq. of CD45+**: 0,38
Non Classical: **Freq. of Monocytes**: 3,34

Intermediate: **Count** : 24638
Intermediate: **Freq. of CD45+**: 0,41
Intermediate: **Freq. of Monocytes**: 3,64

Classical: **Count** : 6,29E5
Classical: **Freq. of CD45+**: 10,6
Classical: **Freq. of Monocytes**: 93,0