[Protocol 4.06] FACS analysis of markers in undifferentiated iPSCs

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Media and Reagents:
Anti-SSEA4-VioBlue (Miltenyi Biotec, 130-098-366)
Anti-Oct3/4-APC (Miltenyi Biotec, 130-123-318)
Anti-Tra1-60-Vio488 (Miltenyi Biotec, 130-106-872)
CD15 (Anti-SSEA1)PE-Vio770 (Miltenyi Biotec, 130-114-012)
Anti-Nanog-PE (Cell Signaling, 14955S)
PBS without Ca2+ and Mg2+ (Thermo Fisher, 14190-094)
BSA stock solution (Miltenyi Biotec, 130-091-376)
UltraPure™ 0.5 M EDTA (Thermo Fisher, 15575-020)
FoxP3 Staining Buffer Set (Miltenyi Biotec, 130-093-142)
Instead of Fox P3 Staining Buffer: (BD Perm/Wash buffer BD, 554723)
Cell Culture Grade H2O (Corning, 25-055-CM)
MACSQuant Running Buffer (Miltenyi Biotec,130-092-747)

Materials and Equipment:
MACS Quant VYB
5ml FACS tubes
100 µm Cell strainer
Aspiration pump
5ml/10ml pipettes
15ml conical tubes
Cell counting equipment

Note: Prepare different buffers (Fixation, Permeabilisation and Wash Buffer) for FACS staining always freshly and before starting the procedure. Therefore follow instructions in the appendix!

1. Preparation of cell suspension
1. Use 2 to 4 wells of a 6-well plate with cells (about 60-80% dense) to prepare a single cell suspension with TryplE. Refer to Protocol 1.03.0 Passaging of iPSC into single cells using TrypLE/Accutase - FACS tube with strainer (blue cap)
2. Perform cell count (cells are in E8+rock) and fill a volume containing $2x10^5$ cells in FACS tubes. (The cell number is not really important, but need to document how many they were, really important to have single cell suspension) Prepare two falcon tubes with cells for each cell line to be tested, one non-stained sample (sample 1) as reference and one sample (sample 2) for the staining.
3. As a right negative control you should add a sample stained with all the antibodies but not with Anti-SSEA1-PE-Vio770 and as a control you can add an FMO (fluorescence minus one control = sample stained with all the antibodies but not Anti-Nanog-PE). (For staining use 96 well with v form, it's easy & quick to pipette).
4. If you have enough cells, it is also recommended for at least once a month, or if you change something from the protocol to do single antibody staining (5 more samples)
5. Centrifuge cells at 300 g for 5 min, tilt the supernatant over washbasin, resuspend the pellet in 500µl buffer.
6. Centrifuge cells and re-suspend the pellet of sample 1 in 100 µl buffer and the pellet of sample 2 in 90 µl buffer (see appendix). It is recommended to make one dilution of Anti-Tra1-60-Vio488 before (for example 3 samples with Anti-Tra1-60-Vio488: 0,49µl Anti-Tra1-60-Vio488 + 315µl buffer -> add 90µl per FACS tube)

Table 1: Samples with different experimental conditions

Staining	Antibodies	Samples	neg. ctrl	isotype ctrl	fmo ctrl	ctrl without any ABs
surface staining	Anti-SSEA4-VioBlue	+	+	+	+	-
	Anti-SSEA1-PE-Vio770	+	-	+	+	-
	Anti-Tra1-60-Vio488	+	+	+	+	-
intracellular staining	Anti-Nanog-PE	+	+	+	-	-
	Anti-Oct3/4-APC	+	+	+	+	-

*in case too less cells < 1 M = no neg. ctrl & < 800 K = no fmo ctrl

2. Surface marker staining

1. Add antibodies as indicated in table 2 to sample 2. Add nothing to sample 1.

Table 2: Volumes of surface marker antibodies per sample *(Can vary depending on the reaction size of antibodies) they are in the box FACS antibodies.*

Antibodies	Volume [µl]
Anti-SSEA4-**VioBlue**	5
Anti-SSEA1-PE-Vio770 (if the signal is more than 10 %, it's not pluripotent anymore = CD45)	1
Anti-Tra1-60-Vio488	0,2

2. Mix well and incubate all samples for 10 minutes in the dark at 4°C (refrigerator).

Note: Higher temperature or longer incubation time may lead to non-specific cell labeling.

3. Wash cells by adding 100 µl of FACS buffer and centrifuge at 300xg for 4 minutes.
4. Remove the supernatant (just shake the plate down and then check if pallets are still there) and re-suspend with 180 µl of FACS buffer again and centrifuge at 300xg for 5 minutes

3. Inside marker staining

1. Re-suspend pellets of the samples in 100 µl of cold, freshly prepared Fixation/Permeabilisation Solution (for preparation see appendix).
2. Mix well and incubate for 30 minutes in the dark at 4°C (refrigerator) (can be 45 min but not longer than 2 hours) (1. 30 hrs until this step in case of Sandra)
3. Add 100 µl of cold FACS buffer and centrifuge at 300xg for 4 minutes. Aspirate supernatant completely.
4. Wash cells by adding 100 µl of cold 1X Permeabilisation Buffer (for preparation see appendix) and centrifuge at 300xg for 4 minutes. Aspirate supernatant completely.
5. Re-suspend pellet of sample 1 in 100 µl and pellet of sample 2 in 94 µl of cold 1X Permeabilisation Buffer (if you have a lot of samples it is recommended to make one mix of the antibodies in Permeabilisation Buffer and add 100µl to all the samples you want to stain)
6. Add 100 µ of Permeabilisation Buffer in case of unstained samples.Add antibodies as indicated in table 3 to sample 2.

Table3: Volumes of inside marker antibodies per sample (Can vary depending on the reaction size of antibodies) + 97 µl of perm buffer to be 100 µl. (FMO no oct, isotype each 2 µl, isotype will be prepared at this step not before) Isotype needs just 1 ul for each AB

Antibodies	Volume [µl]
Anti-Nanog-PE	1
Anti-Oct3/4-APC	2

7. Mix well and incubate for 30 minutes in the dark at 4°C (refrigerator).
8. Wash cells by adding 180 ul of cold 1X Permeabilisation Buffer and centrifuge at 300xg for 5 minutes. Aspirate supernatant completely.Re-suspend pellets in 200 µl MACS running buffer (=FACS buffer).
9. If you see any cell clumps you can pipet the cell suspensions through a 100 µm cell strainer into 5 ml FACS tubes. Perform FACS analysis immediately.
10. For the analysis you can use the Pluripanel template.

Appendix

Buffer preparations

Buffer

Prepare a buffer containing PBS (pH 7.5), 0.5% bovine serum albumin (BSA) and 2 mM EDTA.

Ingredient	Volume [ml]
PBS without Ca^{2+} and Mg^{2+}	95
BSA stock solution 10%	5
UltraPure™ 0.5 M EDTA	0,4

Keep buffer at 4°C for storage.

FoxP3 staining buffer Set

Note: Always prepare buffers freshly!

Fixation and Permeabilisation Buffer

The Fix/ Perm Solution 1 has to be diluted 1:4 with Fix/ Perm Solution 2.
Prepare 0.5 ml mixture per sample.

Sample number	Volume to be prepared [ml]	Fix/Perm Solution 1 [ml]	Fix/Perm Solution 2 [ml]
1	0.5	0,125	0,375
6	3	0,75	2,25
12	6	1,5	4,5

2. Permeabilisation Buffer

The 10X Permeabilisation buffer has to be diluted 1:10 with distilled water (MilliQ).
Prepare 1.20 ml solution per sample. (Need to wash x3)

Sample number	Volume to be prepared [ml]	Permeabilisation buffer [ml]	Distilled water [ml]
1	1,2	0,12	1,08
6	7,2	0,72	6,48
12	14,4	1,44	12,96