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Flow Cytometry Protocol

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We use this protocol in our group and it is working.

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Abstract

Fluorescent activated cell sorting (FACS) is a specialized type of flow cytometry used for sorting and analyzing a heterogeneous mixture of cells into different subpopulations based on the specific light scattering and fluorescent characteristics (from the specific labels) of each cell. The number of measurable parameters that can be used by this technology to separate cell populations is immense – starting from simple surface immunophenotyping to metabolic functions, cell cycle status, redox state, and DNA content analysis to name a few.

Since its inception, FACS has been used extensively in biomedical research and clinical diagnostics and therapeutics. The most common usage of FACS is seen in:
- Analysis of whole human blood for diagnosing diseases, immunophenotyping
- Sorting different blood cell fractions for ex-vivo manipulations and/or transplantations
- Immuno-phenotypic analysis of murine blood to identify transgenic/knockout animals
- Sorting and analysis of a slew of cell lines for various biological assays
- Characterization and isolation of rare cells types like adult stem cells and cancer initiating cells

Each human cell expresses hundreds of thousands of cell surface antigens that specify their cell type, biological function, development stage, and much more. Cells residing in different organs have characteristic cell surface antigens, and determination of these cells using the specific fluorophore-conjugated antibodies can be analyzed by flow cytometry. The following general protocols are recommended for various common FACS staining procedures. Staining with unconjugated purified antibody needs an additional step of staining with a fluorescent conjugated secondary antibody (indirect immunostaining).

Guidelines

This information is to serve as a guide as individual investigators may need to optimize protocols for their particular cell type.

Troubleshooting

Preparation of cells for staining

1 Cells for flow cytometry analysis are usually derived from 4 main sources:
- Adherent or suspension cultures
- Cryopreserved samples
- Whole blood or buffy coats
- Solid tissues e.g. bone marrow, spleen, intestine etc.

2 Regardless of the source, the final cell preparation should be:
- A homogenous single-cell suspension free of clumps and dead cell debris
- At a density of 10^6-10^7 cell per ml
- Suspended in a suitable staining buffer

3 PBMCs isolated from whole blood through Ficoll gradient centrifugation or RBC lysed whole blood or non-adherent cultured cells are readily available for flow cytometric analysis. Adherent cultured cells or cells present in the solid organs should be first made into a single cell suspension before flow analysis by using enzymatic digestion or mechanical dissociation of the tissue, respectively. Mechanical filtration should be followed to avoid unwanted instrument clogs or lower quality flow data.

4 Use the following sample preparation protocols based on your appropriate starting materials:
- **Preparation of suspension culture cells**
- **Preparation of adherent culture cells**
- **Preparation of cryopreserved cells**
- **Preparation of cells from blood or buffy coat**
- **Preparation of cells from murine bone marrow**

Preparation of suspension culture cells

5 **Key Reagents – PBS, staining buffer**
1. Decant cells from tissue culture vessel into centrifuge tube(s).
2. Centrifuge at 300-400 x g for 5-10 minutes at room temperature.
3. Discard supernatant and re-suspend pellet in PBS and repeat the previous step.
4. Discard supernatant and re-suspend in a suitable volume of cold staining buffer.

Preparation of adherent culture cells

6 **Key Reagents – PBS, staining buffer, 0.25% trypsin**
1. Discard culture medium and rinse the cell monolayer with sterile PBS.
2. Add just enough warmed trypsin to cover the monolayer and incubate at 37°C for 5-10 minutes (depending on the cell type).

3. Neutralize the reaction with culture medium (serum added) and detach the cells by gently shaking the vessel.
4. Continue as with suspension culture cells preparation protocol.

go to step #5 Suspension Culture Cells Preparation

Preparation of cryopreserved cells

7 **Key reagents – PBS, staining buffer, culture medium with 10% FBS**

1. Thaw the cryo-tubes rapidly in a water bath set at 37°C.
2. Transfer to a chilled centrifuge tube and add ice cold culture medium drop by drop until the cells are diluted 10X. Perform on ice!
3. Centrifuge at 300-400 x g for 5 minutes at 4°C.
4. Discard supernatant and wash once with cold staining buffer.
5. Re-suspend cells in a suitable volume of cold staining buffer.

Preparation of cells from blood or buffy coat

8 **Key reagents – PBS, staining buffer, suitable gradient medium like Ficoll or Histopaque**

1. Dilute whole blood or buffy coat with an equal volume of room temperature PBS.
2. Carefully layer the diluted blood over an equal volume of the gradient medium.
3. Centrifuge at 400-500 x g for 30-40 minutes at room temperature. The centrifuge brakes should be turned off!
4. Aspirate the PBMCs from the thin interface between the upper plasma layer and the lower medium layer.
5. To remove granulocytes, aspirate the whitish colored layer just above the RBC sediment.*
6. Re-suspend the cells in PBS and centrifuge at 300-400 x g for 10 minutes at room temperature.
7. Wash with PBS once or twice more.
8. Re-suspend the cells in a suitable volume of staining buffer.

Note

*Specialized gradient media have been formulated to enrich for different granulocyte populations.

Preparation of cells from murine bone marrow

9 **Key reagents – PBS, staining buffer (see appendix), RBC lysis buffer [check Appendix for recipe or use a commercially available buffer]**

1. Dissect out the tibia and femurs and remove all extraneous fat, muscle, and connective tissue. Put the bone in cold PBS and store on ice – perform all following steps on ice.
2. Fill up a syringe with cold culture medium and fit an 18-gauge needle to it. Drill the ends of the bones with the needle and flush out the contents onto a non-tissue culture-treated plate.
3. Break up the cell masses into a single cell suspension with the help of a 22-gauge needle.
4. Pellet down the cells at 300-400 x g and wash once more with cold PBS.
5. Re-suspend the cells in a suitable volume of staining buffer and perform a cell count before pelleting the cells again.
6. Re-suspend the cells in RBC lysis buffer at 10^6 cells/ml and incubate at room temperature for 5 minutes.
7. Pellet down the cells, discard the lysis buffer, and wash once with PBS.
8. Re-suspend the cells in a suitable volume of staining buffer.

Common FACS Staining Protocols

10 Each human cell expresses hundreds of thousands of cell surface antigens that specify their cell type, biological function, development stage, and much more. Cells residing in different organs have characteristic cell surface antigens. Determination of these cells using the specific fluorochrome-conjugated antibodies can be analyzed by flow cytometry. Staining with unconjugated purified antibody needs an additional step of staining with a fluorescent conjugated secondary antibody (indirect immunostaining).

Note

If the cells are stained in a 96 well U- or V-bottom plate, washing procedure should be set up first for maximum removal of unbound primary antibodies.

11 Common FACS staining protocols include:

1. **Direct immunostaining of surface antigens**
2. **Indirect immunostaining of surface antigens**
3. **General immuno-staining procedure for intracellular antigens**
4. **Intracellular cytokine/phospho-immunostaining**
5. **In Vitro Cell Stimulation Reference Table**
6. **Dye efflux staining**
7. **DNA content or Cell cycle analysis**

Common FACS Staining Protocols - Direct immunostaining of surface antigens

12 **Key Reagents – PBS, staining buffer, FACS buffer, PFA fixing buffer**

1. Prepare a single cell suspension using the appropriate protocol and adjust the cell concentration to 10^7 cells/ml in staining buffer.
2. Dispense 100µl of cell suspension into as many staining tubes as needed (unstained control, compensation controls, optional isotype and FMO controls, and test sample).
3. Add the optimized dilution of antibodies to the respective tubes and incubate at 4°C (on ice) for 30 minutes in the dark.
4. Wash the cells once with ice-cold PBS at 300-400 x g and re-suspend in 100-200µl FACS buffer/PFA fixing buffer.
5. Store at 4°C in darkness and acquire preferably within 24 hours.

Common FACS Staining Protocols - Indirect immunostaining of surface antigens

13 **Key Reagents – PBS, staining buffer, FACS buffer, PFA fixing buffer**

1. Prepare a single cell suspension using the appropriate protocol and adjust the cell concentration to 10^7 cells/ml in staining buffer.
2. Dispense 100µl of cell suspension into as many staining tubes as needed (unstained control, compensation controls, optional isotype and FMO controls, and test sample).
3. Add the optimized dilution of primary antibodies to the respective tubes and incubate at 4°C (on ice) for 30 minutes.
4. Wash the cells once with ice-cold PBS at 300-400 x g and re-suspend in 100µl staining buffer.
5. Add the specific secondary antibodies at the proper dilution and incubate the cells at 4°C (on ice) for 30 minutes in the dark.
6. Wash the cells once with cold PBS at 300-400 x g and re-suspend in 100-200µl FACS buffer/PFA fixing buffer.
7. Store at 4°C in darkness and acquire preferably within 24 hours.

Common FACS Staining Protocols - General immuno-staining procedure for intracellular antigens - Permeabilization with methanol

14 **Key Reagents – PBS, staining buffer, FACS buffer, 0.5-4% PFA in PBS (exact concentration of PFA has to be standardized for every antibody panel), 100% methanol**

1. Perform surface staining as per protocols 1 or 2 along with the suitable controls.
2. Aliquot the stained cells in 0.5-4% PFA at 10^7 cells/ml. Prepare unstained aliquots for the intracellular staining controls.
3. Fix the cells on ice for 10-30 minutes away from light.
4. Wash out the fixative at 300-400 x g and slowly add ice-cold 100% methanol with gentle vortexing.
5. Incubate the cells on ice for 30 minutes away from light.
6. Wash out the methanol at 400-500 x g and re-suspend the cells in 100µl staining buffer.

7. Dispense 100µl of cell suspension into as many staining tubes as needed (unstained control, compensation controls, optional isotype and FMO controls, and test sample).
8. Add the optimized dilution of primary antibodies to the respective tubes and incubate at 4°C (on ice) for 30 minutes.
9. Wash the cells once with ice-cold PBS at 400-500 x g and re-suspend in 100µl staining buffer.
10. Add the specific secondary antibodies at the proper dilution and incubate the cells at 4°C (on ice) for 30 minutes in the dark.
11. Wash the cells once with cold PBS at 400-500 x g and re-suspend in 100-200µl FACS buffer/PFA fixing buffer.
12. Store at 4°C in darkness and acquire preferably within 24 hours.

Common FACS Staining Protocols - General immuno-staining procedure for intracellular antigens - Permeabilization with saponin

15 **Key Reagents – PBS, staining buffer, FACS buffer, 0.5-4% PFA in PBS [exact concentration of PFA has to be standardized for every antibody panel], 0.1% saponin**

1. Perform surface staining as per protocols 1 or 2 along with the suitable controls.
2. Aliquot the stained cells in 0.5-4% PFA at 10^7 cells/ml. Prepare unstained aliquots for the intracellular staining controls.
3. Fix the cells on ice for 10-30 minutes away from light.
4. Wash out the fixative at 300-400 x g and add 0.1% saponin.
5. Incubate the cells at room temperature for 15 minutes.
6. Wash out saponin at 300-400 x g and re-suspend the cells in 100µl staining buffer.
7. Dispense 100µl of cell suspension into as many staining tubes as needed (unstained control, compensation controls, optional isotype and FMO controls, and test sample).
8. Add the optimized dilution of primary antibodies to the respective tubes and incubate at 4°C (on ice) for 30 minutes.
9. Wash the cells once with ice-cold PBS at 400-500 x g and re-suspend in 100µl staining buffer.
10. Add the specific secondary antibodies at the proper dilution and incubate the cells at 4°C (on ice) for 30 minutes in the dark.
11. Wash the cells once with cold PBS at 400-500 x g and re-suspend in 100-200µl FACS buffer/PFA fixing buffer.
12. Store at 4°C in darkness and acquire preferably within 24 hours.

Common FACS Staining Protocols - Intracellular cytokine/phospho-immunostaining

16 The intracellular staining procedure allows for direct measurement of antigens (cytokines or transcription factors) present inside the cytoplasm or the nucleus of a cell in addition to surface antigen determination simultaneously. In this procedure, the fixation and permeabilization of cells are required after staining with fluorescent conjugated surface

antigens. This modified staining procedure allows direct measurement of functional activity of any cell of interest present in blood or other tissues without further separation. To achieve better results, additional *in vitro* cell stimulation with some common mitogen (e.g., PMA, Ca++ or peptide epitopes and protein transport inhibitor, Brefeldin A, etc.) may be required, which allows increased production of cytokines inside cells. Refer to the table in Step 18 as a guideline for common cell stimulation procedures.

17 **Key Reagents – PBS, staining buffer, FACS buffer, 0.5% PFA in PBS, 0.1% saponin or 100% methanol, suitable cell stimulant, brefeldin A or monensin**

1. Harvest cells using the suitable protocol and aliquot them in tubes at the pre-determined concentration (depending on cell type and stimulant).
2. Add the specific stimulant and incubate the cells at 37°C for the requisite time. The table in Step 18 is a handy reference of different stimulants and incubation time vis-à-vis the target proteins.*
3. Stop the stimulation by fixing the cells with the final concentration of 0.5% PFA.
4. Vortex gently and keep cells on ice for 15 minutes.
5. Wash off the fixative and proceed with surface staining as per protocol 1 or 2.
6. Re-suspend cells in the preferred permeabilizing reagent and proceed with the permeabilization and intracellular staining accordingly as per protocol 3a or 3b (100% methanol or 0.1% saponin).

Note

If the cells are stained in a 96 well U- or V-bottom plate, washing procedure should be set up first for maximum removal of unbound primary or secondary antibodies.

Note

*In the case of staining for secreted cytokines, add brefeldin A or monensin during the incubation period at the concentration recommended by the manufacturer.
*Set aside some unstimulated aliquots for the unstained control and stained baseline controls.

go to step #12 Protocol 1
go to step #13 Protocol 2
go to step #14 Protocol 3a
go to step #15 Protocol 3b

18 **In Vitro Cell Stimulation Reference Table**

	Target cytokine/phosphoprotein	Target cells	Stimulant	Duration	Surface marker
	IL-2	PBMCs	PMA (50ng/ml)	4-6 hours	CD3
	IL-3	T-cells	PMA(50ng/ml) + ionomycin (1µg(ml)	4-6 hours	CD4
	IL-4	PBMCs	PMA(50ng/ml) + ionomycin (1µg(ml)	4-6 hours	CD4
	IL-6	PBMCs	LPS (100ng/ml)	4-6 hours	CD14
	IL-10	PBMCs	LPS (100ng/ml)	18-24 hours	CD14
	GM-CSF /IFNγ/TNFα/TNFβ	PBMCs	PMA(50ng/ml) + ionomycin (1µg(ml)	4-6 hours	CD3
	pStat5	PBMCs	GM-CSF (20ng/ml) + IL3 (20ng/ml)	15 min.	CD123, CD116
	pStat3	PBMCs	G-CSF (20ng/ml) + IL6 (20ng/ml)	15 min.	CD126, CD114
	pERK	PBMCs	IL3 (20ng/ml) + IL6 (20ng/ml) + FLT3L (20ng/ml)	15 min.	CD123, CD126, CD135

Common FACS Staining Protocols - Dye efflux staining

19 Dye exclusion staining is performed to separate live and dead cells, as well as to isolate the rare stem cell "side populations". If viability staining is included in your regular immunostaining, it should be performed before any other staining.

Common FACS Staining Protocols - Dye efflux staining - Propidium iodide (PI) staining (viability)

20 **Key reagents – PBS, staining buffer, PI solution (10µg/ml in PBS)**

1. Harvest the cells and wash once with PBS.
2. Re-suspend cells in staining buffer at 10^7 cells/ml.
3. Add 5µl of PI stain per 100µl of cell suspension, mix gently, and let it stay in the dark for 1 minute.
4. Wash out the dye and re-suspend cells in a suitable volume of staining buffer.

Common FACS Staining Protocols - Dye efflux staining - 7-Amino actinomycin D (7-AAD) staining (viability)

21 **Key reagents – PBS, staining buffer, 7-AAD solution (100µg/ml in PBS)**

1. Harvest the cells and wash once with PBS.
2. Re-suspend cells in staining buffer at 10^7 cells/ml.
3. Add 2µl of 7-AAD stain per 100µl cell suspension, mix gently, and incubate the cells on ice for 30 minutes.
4. Wash out the dye and re-suspend cells in a suitable volume of staining buffer.

Common FACS Staining Protocols - Dye efflux staining - Rhodamine 123 or Hoechst 33342 staining (side population)

22 **Key reagents – PBS, 5% FBS in PBS, staining buffer, Hoechst 33342 solution (1mM in PBS) or Rho123 solution (10µg/ml in PBS)**

1. Harvest the cells and wash once in PBS.
2. Re-suspend cells in 5% FBS at 10^7 cells/ml.
3. Add 1µl of the dye per 100µl of cell suspension and incubate in a water bath at 37°C for 30 minutes.
4. Wash out the dye, re-suspend the cells in pre-warmed, dye-free 5% FBS, and repeat the previous incubation step.
5. Wash the cells again and re-suspend in a suitable volume of staining buffer.

Common FACS Staining Protocols - DNA content or Cell cycle analysis

23 **Key reagents – PBS, staining buffer, PI solution (50µg/ml in PBS), RNase A (10µg/ml), 70% ethanol**

1. Harvest cells and wash once in PBS.
2. Re-suspend cells in staining buffer at 10^7 cells/ml.
3. Aliquot 500µl of cells into separate tubes (pre-chilled) and add ice cold 70% ethanol dropwise with gentle vortexing.
4. Keep cells on ice for 1 hour.
5. Wash the cells twice in PBS at 400-500 x g for 10 minutes.
6. Add 1ml of PI solution to the cell pellet and mix well. Add 50µl of RNase to a final concentration of 0.5µg/ml.
7. Incubate the cells at 4°C overnight.
8. Wash once in PBS and re-suspend in a suitable volume of staining buffer.