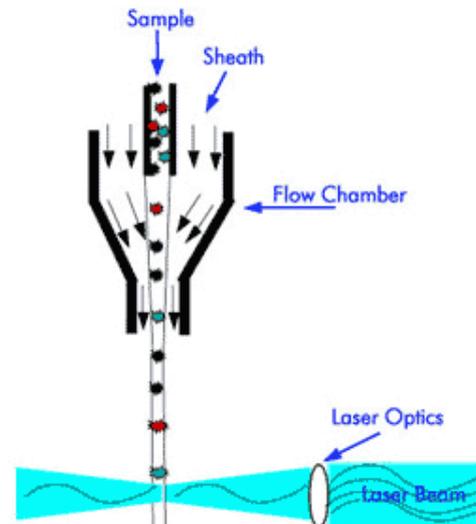


FLOW CYTOMETRY (FC)

Introduction

Flow cytometry is a technique for counting, examining and sorting microscopic particles suspended in a stream of fluid. A beam of light of a single wavelength is directed onto a hydro-dynamically focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam; one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC)) and one or more fluorescent detectors. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). The combination of scattered and fluorescent light is picked up by the detectors, and by analyzing fluctuations in brightness at each detector (one for each fluorescent emission peak) it is then possible to extrapolate various types of information about the physical and chemical structure of each individual particle.

Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. A vibrating mechanism causes the stream of cells to break into individual droplets. The scatter and fluorescence signal is compared to the sort set criteria on the instrument. If the particle matches the selection criteria, the droplet is charged as it exits the nozzle of the fluidics system. The droplets eventually pass through a strong electrostatic field, and are deflected left or right based on their charge.



Surface staining can be done by direct staining and indirect staining.

In direct immunofluorescence staining, cells are incubated with an antibody conjugated with a fluorochrome (e.g. FITC). This has the advantage of requiring only one antibody incubation step and eliminates the possibility of non-specific binding from a secondary antibody.

Indirect labeling requires two incubation steps; first with a primary antibody followed by a compatible secondary antibody. The secondary (and not the primary) antibodies have the fluorescent dye (FITC, PE, Cy5, etc.) conjugated.

A successful staining procedure in all cases is dependent on optimization of experimental conditions through titrating of antibodies, use of appropriate controls to set up the flow cytometer correctly, and optimized fixation and permeabilization methods if necessary.

Materials / reagents

- **Sample: Cells expressing protein of interest**
- **Phosphate Buffered Saline (PBS)**
- **Fluorochrome conjugated antibody (direct staining)**
- **Primary antibody (indirect staining)**
- **Fluorochrome conjugated secondary antibody (indirect staining)**
- **Propidium Iodide (PI) solution**
Mix 10 µg/ml in PBS
- **Fixation buffer**
 - Mix 100 ml PBS, pH 7.4 with 0.1 ml 1 M Azide
 - Add 2 gram paraformaldehyde to 100 ml PBS with azide
 - Heat to 70 degrees Celsius in a fume hood, or in a 56 degree Celsius water bath, just until the paraformaldehyde goes into solution
 - Allow to cool to room temperature, then adjust to pH 7.4 using 0.1 M NaOH or 0.1 M HCl, as needed
 - This stock solution can be stored at 4 °C for at most one year
 - Add 10 ml of this stock solution to 30 ml PBS with azide
 - Store at 4°C, this solution is stable for up to 1 week
- **Permeabilization buffer:**
Mix 5 ml 10% Saponin in PBS (A) + 95 ml PBS/BSA/Azide buffer (B)

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A: 10% Saponin

- Mix 5 g Saponin with 50 ml PBS, pH 7.4
- Place at 37°C until the Saponin has dissolved completely
- Sterile filter the mixture (0.22 µM)
- Store at 4°C

B: PBS/BSA/Azide buffer

- Mix 500 ml PBS, pH 7.4 with 0.5 ml 1 M azide
- Layer 2.5 g of Bovine Serum Albumine (BSA) on top of liquid mixture
- Allow BSA to dissolve at room temperature
- Sterile filter the mixture
- Store at 4°C for at most one year

Procedure

A: Surface staining

Direct Staining

1. Prepare the cells and adjust cell suspension to a concentration of $1-5 \times 10^6$ cells/ml in PBS.
2. Cells can be stained in any container for which you have an appropriate centrifuge e.g. test tubes, eppendorf tubes, and 96-well round bottomed microtiter plates. In general, cells should be spun down hard enough that the supernatant fluid can be removed with little loss of cells, but not so hard that the cells are difficult to resuspend.
3. It is always useful to check the viability of the cells which should be around 95% not less than 90%.
4. Resuspend the cells to approximately $1-5 \times 10^6$ cells/ml in ice cold PBS.
5. Aliquot 100 µl of cell suspension into as many test tubes as required.
6. Add the fluorochrome conjugated antibody (see for recommended dilution the specific datasheets).
7. For live/dead discrimination, add 10 µl [propidium iodide \(PI\) solution](#) at this point.
8. Mix well and incubate for at least 30 min at room temperature or 4°C. This step will require optimization and must be done in the dark.
9. Wash the cells 3 times with PBS and centrifugate each time at 400 g for 5 minutes and discard the resulting supernatant. You may need to adjust the conditions of the centrifugation (the force and the time) for the cell types used.
10. Resuspend them in 500 µl to 1 ml of ice cold PBS.
11. Keep the cells in the dark on ice or at 4°C in a fridge until acquisition of the flow cytometer, preferably within 24 hours.

Indirect staining

1. Prepare the cells and adjust cell suspension to a concentration of $1-5 \times 10^6$ cells/ml in PBS.
2. Cells can be stained in any container for which you have an appropriate centrifuge e.g. test tubes, eppendorf tubes, and 96-well round bottomed microtiter plates. In general, cells should be spun down hard enough that the supernatant fluid can be removed with little loss of cells, but not so hard that the cells are difficult to resuspend.
3. It is always useful to check the viability of the cells which should be around 95% not less than 90%.
4. Resuspend the cells to approximately $1-5 \times 10^6$ cells/ml in ice cold PBS.
5. Aliquot 100 µl of cell suspension into as many test tubes as required.
6. Add the primary antibody (see for recommended dilution the specific datasheets).
7. Mix well and incubate for at least 30 min at room temperature or 4°C, this step will require optimization.
8. Wash the cells 3 times with PBS and centrifugate each time at 400 g for 5 minutes and discard the resulting supernatant. You may need to adjust the conditions of the centrifugation (the force and the time) for the cell types used.
9. Dilute the appropriate fluorochrome conjugated secondary antibody at the recommended dilution (see specific datasheets) and then resuspend the cells in this solution.
10. For live/dead discrimination, add 10 µl [propidium iodide \(PI\) solution](#) at this point.
11. Fix well and incubate at room temperature for 30 minutes. This step will require optimization and must be done in the dark.
12. Wash the cells 3 times with PBS and centrifugate each time at 400 g for 5 minutes and discard the resulting supernatant.
12. Resuspend them in 500 µl to 1 ml of ice cold PBS.
13. Keep the cells in the dark on ice or at 4°C in a fridge until acquisition of the flow cytometer, preferably within 24 hours.

B: Intracellular staining

1. Prepare the cells and adjust cell suspension to a concentration of $1-5 \times 10^6$ cells/ml in PBS.
2. Cells can be stained in any container for which you have an appropriate centrifuge e.g. test tubes,

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- eppendorf tubes, and 96-well round bottomed microtiter plates. In general, cells should be spun down hard enough that the supernatant fluid can be removed with little loss of cells, but not so hard that the cells are difficult to resuspend.
3. Wash cells twice in PBS.
 4. If required, perform staining of cell-surface antigens using appropriate directly conjugated monoclonal antibodies at this stage. Following staining, wash cells once in PBS and discard the supernatant.
 5. Fixate the cells in [fixation buffer](#) using 100 μ l per 1×10^6 cells for 8 minutes at 4°C.
 6. Wash twice in PBS (Note that one wash may be sufficient, but more washes may decrease the background).
 7. Permeabilize the cells in [permeabilization buffer](#) using 50 μ l per 1×10^6 cells for 8 minutes at 4°C.
 8. Incubate cells with fluorochrome-conjugated antibody diluted in permeabilization buffer for 1 hour at 4°C.
 9. For live/dead discrimination, add 10 μ l [propidium iodide \(PI\) solution](#) at this point.
If fixing cells before analysis, do not add PI.
 10. Wash cells twice in permeabilization buffer
 11. Keep the cells in the dark on ice or at 4°C in a fridge until acquisition of the flow cytometer, preferably within 24 hours.
- Note: PI can not be used on permeabilized cells.*

Safety

- Under no circumstances shall Hbt be liable for any damage arising from the use of this protocol. User should be trained and be familiar with the test procedure.
- Samples of tissue, serum or blood origin should be handled to guidelines for prevention of transmission of blood borne diseases.
- Some enzyme substrates are considered hazardous, due to potential carcinogenicity. Handle with care and refer to Material Safety Data Sheets for proper handling precautions.
- Wear appropriate protective clothing, gloves, and eyewear to avoid any accidental contact with reagents.
- Paraformaldehyde is highly toxic and aerates easily, avoid breathing in the powder. Use a fume hood.
- Reagents which contain preservatives may be toxic if ingested, inhaled, or in contact with skin.
- Propidium iodide (PI) is known to be toxic and carcinogenic.

Notes

- Please note that this is a general protocol. Optimal dilutions for the primary and secondary antibodies, cells preparation, controls, as well as incubation times will need to be determined empirically and may require extensive titration. Ideally, one would use the primary antibody as recommended in the product data sheet.
- Protocol is to be used for research purposes only.
- Appropriate standards should always be included e.g. an isotype-matched control sample
- Analysis on the same day is recommended. For extended storage (16 hr) as well as for greater flexibility in planning time on the cytometer, resuspend cells in 1% paraformaldehyde to prevent deterioration.
- Staining with ice cold reagents/solutions at 4°C is recommended.
- Presence of sodium azide prevents the modulation and internalization of surface antigens which can produce a loss of fluorescence intensity.
- When making buffer(s) fresh every time, there is no need to add sodium azide to the buffer.
- The fixation method is also good for cells labeled by fluorochrome-conjugated antibodies to membrane antigens. It will stabilize the light scatter and labeling for up to a week in most instances, allowing you to be more flexible in scheduling cytometer time. Furthermore, it inactivates most biohazardous agents, so it is important from a safety standpoint as well.

Technical assistance

For technical assistance and more information please contact Hycult Biotech directly at support@hycultbiotech.com.