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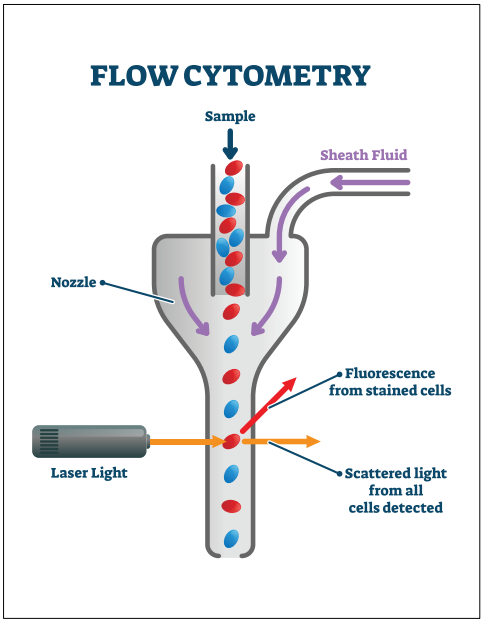
Flow Cytometry Protocols for Extracellular & Intracellular Targets



Flow cytometry is a high throughput quantitative application that employs fluorescent-labeled molecules for multi-parametric analysis of millions of individual cells or particles. In a typical flow cytometry assay of cells, the sample in suspension format is guided through a fluidics system and the hydrodynamic focusing aligns various cells one by one as a single cell stream. Once the cells pass through a laser beam, the light scatters and this scatter data provides information about the size/granularity of the cell. Additionally, the laser excites the fluorophores on conjugated antibodies causing them to emit different wavelengths of light which are then filtered/collected by fluorescent detectors and finally converted to a digital signal. This technique can detect the presence of different fluorophores and can also quantify the amount of each fluorophore present.

Flow cytometry is useful for a variety of research areas and applications including but not limited to:

- Immunophenotyping in a heterogeneous cell population (i.e. cell population analysis)
- Quantitative analysis of protein expression
- Live and dead cells analysis
- Quantitative analysis of cell cycle progression
- Detection of surface markers on cells
- Cell signaling pathway analysis
- Detection of post-translational modifications
- Characterization of organelles, e.g. exosomes

Considerations for Protocol Design

Extracellular & Intracellular Staining

While flow cytometry applications are becoming more complex with the additions of sorting, mass spectrometry, and imaging, the basic premise of protocol design remains the same: is the target being investigated on the outside surface of the cell (extracellular) or inside the cell, whether in the cytoplasm or nucleus? Addressing this question is key to the experimental design and being able to examine the factors of interest. To detect and quantify intracellular targets, the cells are fixed to maintain cellular integrity and then permeabilized so that the antibodies or dyes being used can cross the cell membrane and bind their targets. Fixing cells preserves as many of the cell features in their native state as possible prior to permeabilization and keeps the cells available for analysis for a longer period of time. For extracellular targets, cells can be stained without fixation, and following flow cytometry analysis the live cells can be collected for downstream assays.

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Flow Cytometry Protocols for Extracellular & Intracellular Targets

	Pros	Cons
Live Cells	• Targets are not degraded from fixative. • Cells can be used in downstream assays (e.g. *in vitro* culture).	• Samples need to be analyzed quickly.
Fixed Cells	• Samples can be stored for extended periods of time prior to and after staining.	• Targets can be degraded from fixative. • Since cells are dead, they cannot be used for downstream assays.

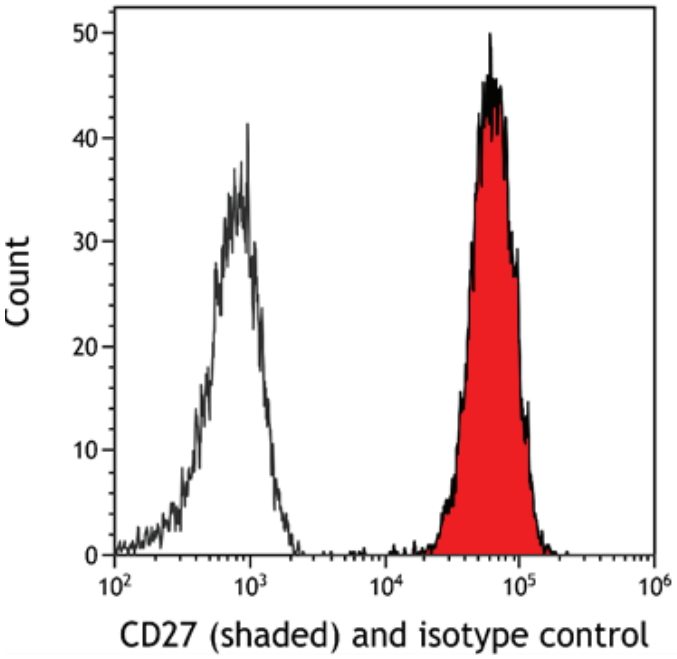
Representative Data

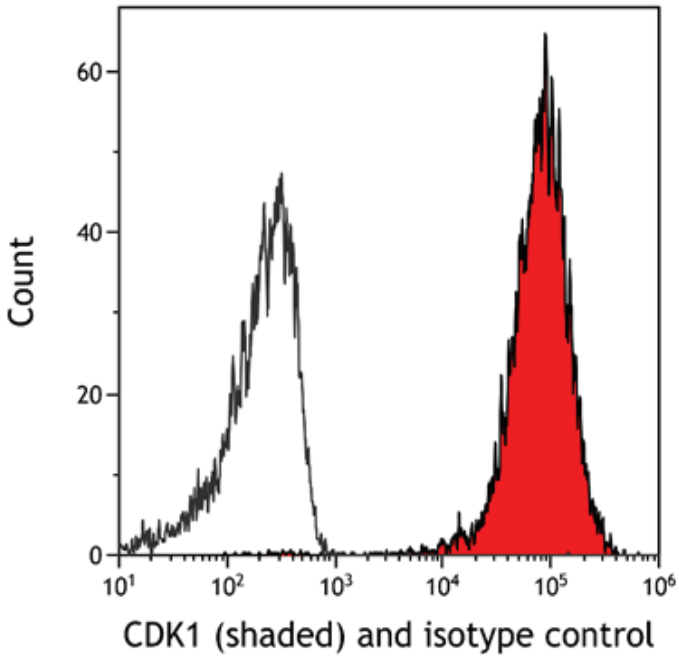
Extracellular domain target: CD27

CD27 is a member of the tumor necrosis factor receptor superfamily. It is a costimulatory receptor primarily located on T cells. When bound to its ligand, CD70, the complex promotes survival and differentiation of T cells.

Intracellular domain target: CDK1 (cyclin-dependent kinase 1)

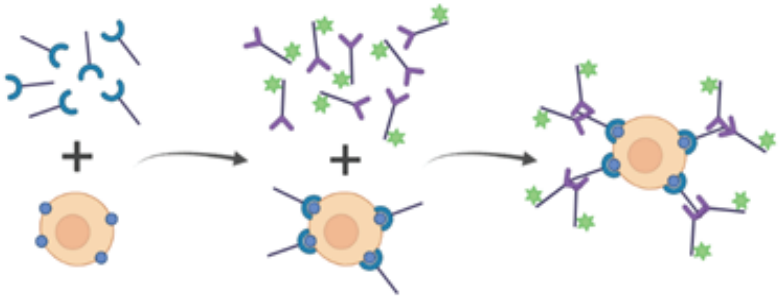
CDK1 is an important player in cell cycle regulation. In complex with cyclins, CDK1 phosphorylates proteins which results in cell cycle progression.

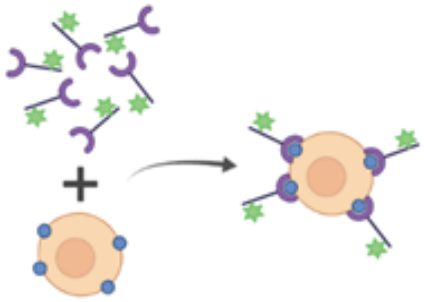




Direct vs Indirect Staining

Method	Direct	Indirect
Description	Primary antibodies are directly conjugated to a fluorophore. Therefore, mixing the antibody and cell sample together results in a detectable signal.	Primary antibodies are unconjugated, meaning they are not bound to a fluorophore. Thus, a secondary antibody conjugated to a fluorophore must be used to detect the signal.
Advantages	• Quick, one-step staining. • Can use multiple antibodies from the same host. • More easily used in multicolor assays	• Amplification of signal by secondary antibodies. • A single secondary antibody can be used for multiple primary antibodies
Disadvantages	• No signal amplification. • Each antibody must be conjugated to a fluorophore.	• Two-step staining process. • Requires primary antibodies produced in different hosts. • More limited when used for multicolor assays.





Flow Cytometry Protocols

Surface Staining Protocol

This protocol describes a fixation and permeabilization method for staining cell surface antigens by flow cytometry.

1. Collect cells and wash with PBS.
2. Count cells.
3. Pellet cells so that 50µL will contain the desired number of cells for staining.
   a. Typically, $5x10^5$ – $1x10^6$ cells are used per sample.
4. Wash cells in flow staining buffer.
5. Resuspend cells at $1x10^7$ cells/mL in flow staining buffer containing blocking agent.
   a. Option 1: Fc block: Block cells using IgG Fc Fragment (P80-104).
      i. Add 1ug (1µL) for each 10^6 cells.
      ii. Incubate at room temperature for 10 minutes.
   b. Option 2: Normal Serum: If performing indirect staining, use serum from the animal the secondary antibody is derived. (Often goat serum or calf serum is used.)
      i. Dilute serum to a final concentration of 5% normal serum.
      ii. Incubate on ice for 30 minutes.
6. Dilute primary antibody in 50µL of flow staining buffer.
7. Add 50µL of antibody solution with the 50µL of cell suspension for a final volume of 100µL.
8. Incubate on ice for 30 minutes. If the primary antibody is conjugated to a fluorophore, make sure the incubation is performed in the dark.
9. Wash cells with flow staining buffer.
10. Repeat wash.
11. If using an unconjugated primary antibody:
    a. Dilute your secondary antibody in 100uL/sample.
    b. Add 100µL of secondary antibody to each sample.
    c. Incubate on ice for 30 minutes.
    d. Wash cells twice with flow staining buffer.
12. Resuspend cells in at least 200µL flow staining buffer.
13. Analyze cells on the flow cytometer as soon as possible.

Notes:

1. Flow staining buffer: PBS with 1% BSA and 0.1% sodium azide
2. All centrifugations were performed at 300xg for 5 minutes.
3. If it will be an hour or more before you can analyze your cells, you may need to fix the cells.

Intracellular & Nuclear Staining Protocol

This protocol describes a fixation and permeabilization method for staining intracellular and nuclear antigens by flow cytometry.

1. Collect cells and wash with PBS.
2. Count cells.
3. Resuspend cells to be $5x10^6$ cells/mL.
4. **Fix cells:** fixing cells crosslinks proteins so that the native conformation of the cell is maintained for extended periods of time.
   a. Resuspend cells with paraformaldehyde to a final concentration of 2% by combining equal volumes of cell suspension and 4% PFA.
   b. Incubate cells on ice for 15 minutes.
   c. Pellet cells at 300xg for 5 minutes.
   d. Discard supernatant in appropriate waste container.
5. **Permeabilize cells:**
   a. Resuspend cells in 90% methanol at $1x10^7$ cells/mL.
   b. Incubate at -20°C for at least 30 minutes.
   c. Note: Cells can be stored in 90% methanol for an extended period of time.
6. Aliquot the appropriate amount of cell suspension for your experiment.
   a. Typically, $5x10^5$ – $1x10^6$ cells are used per sample.
7. Pellet cells.
   a. Discard supernatant in appropriate waste container.
8. Wash cells twice in flow staining buffer.
9. Resuspend cells in flow staining buffer containing blocking agent.
   a. Option 1: Fc block: Block cells using IgG Fc Fragment (P80-104).
      i. Add 1 µg (1µL) per 10^6 cells.
      ii. Incubate at room temperature for 10 minutes.
   b. Option 2: Normal Serum: If performing indirect staining, use serum from the animal the secondary antibody is derived. (Often goat serum or calf serum is used.)
      i. Dilute serum to a final concentration of 5% normal serum.
      ii. Incubate on ice for 30 minutes.
10. Dilute primary antibody in 50µL of flow staining buffer.
11. Add 50µL of antibody solution with the 50µL of cell suspension for a final volume of 100µL.
12. Incubate on ice for 30 minutes. (If the primary antibody is conjugated to a fluorophore, make sure the incubation is performed in the dark.)
13. Wash cells with flow staining buffer.
14. Repeat wash.
15. If using an unconjugated primary antibody:
    a. Dilute your secondary antibody in 100µL/sample.
    b. Add 100µL of secondary antibody to each sample.
    c. Incubate on ice for 30 minutes.
    d. Wash cells twice with flow staining buffer.
16. Resuspend cells in at least 200µL flow staining buffer.
17. Analyze cells on the flow cytometer as soon as possible.

Notes:

1. Flow staining buffer: PBS with 1% BSA and 0.1% sodium azide.
2. All centrifugations were performed at 300xg for 5 minutes.