FACS Protocol

Flow cytometry is a useful tool for simultaneously measuring multiple physical properties of individual particles (such as cells). As each cell passes through the laser beam, the cytometer records how the cell or particle scatters incident laser light and emits fluorescence.

1. Reagents

Methanol Buffer

Formaldehyde Buffer

1 g	Paraformaldehyde powder
100 ml	PBS Buffer

Store at 4 °C

Blocking Buffer

3 g	BSA
100 ml	PBS Buffer

Store at 4 °C

Washing Buffer

0.5 ml	Tween 20
1000 ml	PBS Buffer

Store at 4 °C

PBS Buffer

8.5 g	NaCl
1.4 g	Na_2HPO_4
0.2 g	NaH_2PO_4
1000 ml	ddH_2O

Adjust to pH 7.4
Store at 4 °C

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2. Procedure

2.1 Cell preparation

a. For suspension cells

1. Collect media in a conical tube and spin down cells to a pellet at 1000 rpm for 10 minutes at room temperature then discard supernatant.
2. Resuspend the cell with cold *PBS Buffer* and spin down at 1000rpm for 10 minutes at room temperature then discard supernatant.

b. For adherent cells

1. Trypsinize cells for 10 minutes and collect media in a conical tube and spin down cells to a pellet at 1000 rpm for 10 minutes at room temperature then discard supernatant.
2. To wash cells, resuspend the cell with cold *PBS Buffer* and spin down at 1000rpm for 10 minutes at room temperature then discard supernatant.

c. For peripheral blood mononuclear cells

1. Dilute blood sample 1:1 with *PBS Buffer* in a conical tube.
2. Underlay the diluted sample with a volume of Ficoll® that is equal to the original sample volume.
3. Spin down cells at 1000rpm for 20 minutes and harvest PBMC located at the interface of the *PBS Buffer* and Ficoll® layers into a fresh tube.
4. Wash cells with *PBS Buffer* and spin down the cells at 400 rpm for 5 minutes at 4 °C, discard supernatant.

2.2 Fixation

Note: this step is not necessary for cell surface antigens staining.

1. Resuspend cells with *Formaldehyde Buffer* and fix cells for 10 minutes at 37 °C and place tubes on ice for 5 minutes.
2. Spin down to a pellet at 1000 rpm for 10 minutes and then decant supernatant.
3. Resuspend cells in 1 ml *PBS Buffer* and spin down to a pellet at 1000 rpm for 10 minutes then decant supernatant.
4. Tap the conical tube gently to loosen the pellet.

2.3 Permeabilization

a. For cell surface antigens staining

Skip the permeabilization step and turn to immnunostaining directly.

b. For intracellular antigens staining

1. Incubate cells with ice-cold *methanol* for 30 minutes on ice to permeabilize the cell membrane.
2. Spin down cells to a pellet at 1000 rpm for 10 minutes then decant supernatant.

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3. Resuspend cells in *PBS Buffer* and spin down to a pellet at 1000 rpm for 10 minutes and decant supernatant.

2.4 Immunostaining

1. Resuspend cells with *Blocking Buffer* to approximately 1-5x10^6 cells/ml and incubate the cells for 30 minutes at 37 °C.
2. Aliquot 0.5-1x10^6 cells into each tube (by volume).

a. For a unconjugated primary antibody

3. Dilute the unconjugated primary antibody and the unconjugated isotype control antibody separately in *Blocking Buffer*.

   Note: The primary antibody should be diluted at the optimal dilution according to the manufacturer's instructions

4. Resuspend the cells with the diluted primary antibody or diluted isotype control antibody to each tubes and incubate the cells for 1 hour at 37 °C.
5. Spin down the cells to pellet at 1000 rpm for 10 minutes and discard the supernatant.
6. Resuspend the cells with *Washing Buffer* and spin down the cells to pellet at 1000 rpm for 10 minutes and then discard the supernatant. Repeat twice.
7. Dilute the corresponding fluorochrome conjugated secondary antibody in *Blocking Buffer*.
8. Resuspend the cells with the diluted secondary antibody to each tube and incubate the cells for 30 minutes at 37 °C.

   Note: the secondary antibody should be diluted at the optimal dilution according to the manufacturer's instructions and this incubation must be done in the dark.

9. Resuspend the cells with *Washing Buffer* and spin down the cells to pellet at 1000 rpm for 10 minutes and then discard the supernatant. Repeat twice.
10. Resuspend cells in 0.5 ml *PBS Buffer* and analyze on flow cytometer.

b. For a fluorochrome conjugated primary antibody

3. Dilute the fluorochrome conjugated primary antibody and the corresponding fluorochrome conjugated isotype control antibody separately in *Blocking Buffer*.

   Note: the primary antibody should be diluted at the optimal dilution according to the manufacturer's instructions

4. Resuspend the cells with the conjugated primary antibody or conjugated isotype control antibody to each tube and incubate the cells for 1 hour at 37 °C.

   Note: this incubation must be done in the dark.

6. Spin down the cells to pellet at 1000 rpm for 10 minutes and discard the supernatant.

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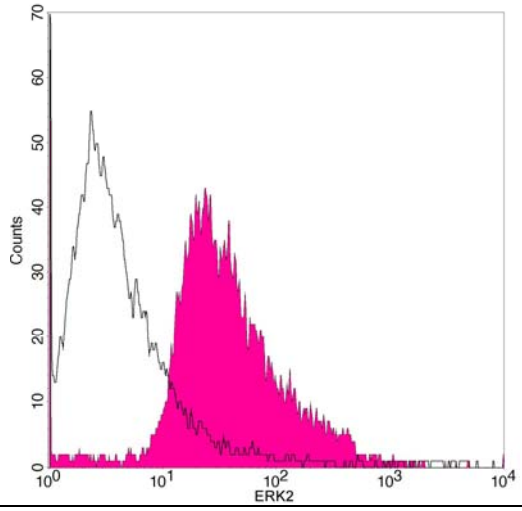
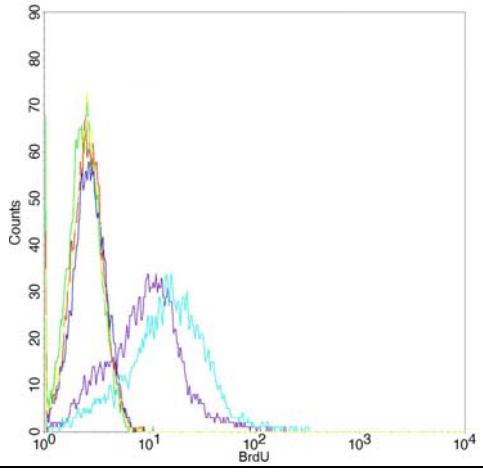
7. Resuspend the cells with *Washing Buffer* and spin down the cells to pellet at 1000 rpm for 10 minutes and then discard the supernatant. Repeat twice.
8. Resuspend cells in 0.5 ml *PBS Buffer* and analyze on flow cytometer.

3. FACS Examples



Flow cytometric analysis of HeLa cells using **ERK2 Antibody, pAb, Rabbit** (GenScript, A01194; shaded histogram) or with an isotype control antibody (GenScript, A01008; open histogram), followed by R-PE conjugated anti-rabbit IgG.



Flow cytometric analysis of untreated or BrdU incorporated Hela cells using GenScript **THE™ BrdU Antibody, mAb, Mouse** (yellow and cyan, respectively), Competitor A Mouse Anti-BrdU mAb (blue and purple, respectively), or mouse IgG1 isotype control (green and red, respectively).

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4. Recommended Products

Name	Cat. No.
Human IgG Control(Whole Molecule), Purified	A01006
Mouse IgG control (Whole Molecule), Purified	A01007
Rabbit IgG Control (Whole Molecule), Purified	A01008
Goat IgG Control (Whole Molecule), Purified	A01009
Chicken IgY Control (Whole Molecule), Purified	A01010

5. Troubleshooting

Problem	Possible Cause	Solution
Weak or no staining	Antibody concentration is too low	Increase the concentration of primary and/or secondary antibodies.
		Use secondary antibody that will interact with primary antibody.
		Replace with a new batch of antibody
High background	Non-specific binding of primary antibodies to tissue or antibody concentration is too high	Non-specific binding may be reduced by using high dilution of primary antibodies
	Non-specific binding of secondary antibodies to tissue	Use pre-adsorbed 2nd antibody, i.e. use rabbit anti-rat IgG, mouse adsorbed, on mouse tissue, or use rabbit anti-mouse IgG, rat adsorbed, on rat tissue.
	Inadequate washing of sections	Wash at least 3 times between steps

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