CYTOFLUORIMETRY PROTOCOL

Protocol for surface antigens staining

Because membrane proteins are readily accessible to the antibody, no permeabilization steps are required. However, experimental conditions, such as antibody concentration, incubation time, and temperature, should be optimized for each experiment.

1. Centrifuge cells at 500 xg for 5 minutes and wash three times in PBS with 0.5% BSA to remove any residual of growth factors that may be present in the culture medium. Adherent cell lines may require pre-treatment with 0.5 mM EDTA or trypsin to facilitate removal from their substrates.

Note: Titration experiments must be performed to determine the optimal antibody concentration to be used.

2. Add the conjugated antibody (10 µl/10^6 cells, or a previously titrated amount) and vortex. Incubate the cells for 45 minutes on ice in the dark.
3. Wash cells three times in PBS with 0.5% BSA

Note: If an unconjugated primary antibody has been used, proceed with incubation with an appropriate secondary antibody. Dilute the secondary antibody as the datasheet. suggested Incubate for 20-30 minutes in the dark and wash

4. Resuspend the cells in 200-500 µl of PBS ready for the analysis

Note: For a negative control, a separate group of cells should be stained with an antibody as isotype control using the procedure described above.

Definition of IMC: control antibody that has no specificity for the cell of our interest but which maintains all the non-specific characteristics of the antibodies used in an experiment. The purpose of the use of such control is to:

- Confirm the binding specificity of the primary antibody.
- Exclude non-specific cellular protein interactions or binding to other Fc receptors.

The isotype control should ideally correspond to the host species and to the isotype of each primary antibody. When an isotype control is use, it is important to use the same antibody concentration (in µg) for both the isotype and the primary antibody. Use of an isotype control is recommended for surface staining.

Autor: T. Apuzzo **1/02/2015** **Approved: Giorgio Stassi**

CYTOFLUORIMETRY PROTOCOL

Summary

Direct staining:

1. Label the Eppendorf tubes for IMC, unstainted cells and for specific markers of interest
2. Wash 3X times the cells in PBS0.5% BSA and divide them into each tube
3. Add the conjugated antibody for 45 min on ice
4. Wash 2X times the cells in PBS 0.5% BSA
5. Resuspend the cells in 0.2 -0.5 ml of PBS and analysed to cytofluorimeter

Indirect staining:

1. Label the Eppendorf tubes for IMC, unstainted cells and for specific markers of interest
2. Wash 3X times the cells in PBS 0.5% BSA and divide them into each tube
3. Add the non- conjugated antibody for 45 min on ice
4. Wash 2X times the cells in PBS 0.5% BSA
5. Add the appropriate secondary antibody (1: 200) 20-30 min on ice
6. Wash 2X times the cells in PBS 0.5% BSA
7. Resuspend the cells in 0.2 -0.5 ml of PBS and analysed to cytofluorimeter

Protocol for the intracellular staining

1. Centrifuge cells at 500 xg for 5 minutes and wash three times in PBS with 0.5% BSA to remove any residual of growth factors that may be present in the culture medium. Adherent cell lines may require pre-treatment with 0.5 mM EDTA or trypsin to facilitate removal from their substrates.

Note: staining of surface antigens can be done at this point.

2. Add 0.250 ml of Fixation and permeabilizzation cold buffer and vortex. Incubate at room temperature or ice for 10 minutes. Shake cells to maintain a suspension of single cells.

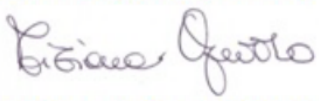
3. Wash the cells twice with Permeabilization / Wash Buffer. Resuspend the cell pellet in 100-200 µl of Permeabilization / Wash Buffer.

4. Add the conjugated antibody (10 µl/10^6 cells, or a previously titrated amount) and vortex. Incubate the cells for 45 minutes on ice in the dark.

Note: Because cellular permeabilization with saponin is a reversible process, it is important to maintain cells in the presence of Permeabilization Buffer during the intracellular staining.

5. Wash the cells twice with Permeabilization / Wash Buffer.

Autor: T. Apuzzo **1/02/2015** **Approved: Giorgio Stassi**



Note: If an unconjugated primary antibody has been used, proceed with incubation with an appropriate secondary antibody. Dilute the secondary antibody as the datasheet. suggested Incubate for 20-30 minutes in the dark and wash

6. Resuspend the cells in 200-500 µl of PBS ready for the analysis

Note: For a negative control, a separate group of cells should be stained with an antibody as isotype control using the procedure described above.

Summary

Direct staining:

1. Label the Eppendorf tubes for IMC, unstainted cells and for specific markers of interest.

2. Wash 3X times the cells in PBS 0.5% BSA and divide them into each tube.

3. Add 0.250 ml of Fixation and permeabilizzation cold buffer and vortex. Incubate at room temperature or ice for 10 minutes.

4. Wash the cells twice with Permeabilization / Wash Buffer.

5. Resuspend the cells in 0.2 -0.5 ml of PBS and analysed to cytofluorimeter
6. Add the conjugated antibody (10 µl/10^6 cells, or a previously titrated amount) and vortex. Incubate the cells for 45 minutes on ice in the dark.
7. Wash the cells twice with Permeabilization / Wash Buffer.

Indirect and subsequent direct staining protocol.

1. Centrifuge cells at 500 xg for 5 minutes and wash three times in PBS with 0.5% BSA to remove any residual of growth factors that may be present in the culture medium. Adherent cell lines may require pre-treatment with 0.5 mM EDTA or trypsin to facilitate removal from their substrates.

2. Add the unconjugated antibody and vortex. Incubate the cells for 30-45 minutes in ice in the dark.

3. Wash cells three times in PBS with 0.5% BSA

4. Add the appropriate secondary antibody (1: 200) 20-30 min on ice

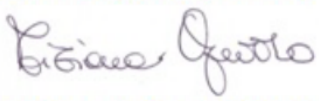
5. Add 2µg/ml of mouse IgG for saturation of nonspecific sites, incubate 20 min ice. Do not wash cells after IgG incubation.

6. Add the conjugated antibody (10 µl per 10^6 cells, or a previously titrated amount) and vortex. Incubate the cells for 30 minutes on ice in the dark.

7. Wash cells three times in PBS with 0.5% BSA

8. Resuspend the cells in 200-500 µl of PBS ready for the analysis

Autor: T. Apuzzo **1/02/2015** **Approved: Giorgio Stassi**



Note: For a negative control, a separate group of cells should be stained with an antibody as isotype control using the procedure described above

Cell cycle with PI solution

1. Centrifuge cells at 500 xg for 5 minutes and wash three times in PBS with 0.5% BSA to remove any residual of growth factors that may be present in the culture medium. Adherent cell lines may require pre-treatment with 0.5 mM EDTA or trypsin to facilitate removal from their substrates.

Note: The staining of surface antigens with antibodies can be done at this point. PI not it can be used for the simultaneous analysis of intracellular markers.

2. Resuspend the cells in 500 ml of PI Buffer for each sample just before of the analysis (from 30 min up to some hours also O/N .)

3. Determine PI fluorescence (using the FL-2 or FL-3 channel) with FACS.

Note: Use the FL-2 channel if staining is performed only with PI. Collect the fluorescence of PI in the FL-3 channel if the cells were stained with FITC or a PE-conjugated antibody.

Note: Do not wash cells after adding the PI staining solution. Cells can be fixed before or after Pi staining with 70% cold ETOH. The fixed cells can be stored in the long term at 4°C.