**Antibody Staining of Intracellular Antigens in Flowcytometry**

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This guideline works in connection with the FACS Core Facility Guidelines

* Panel Design in Flow Cytometry (1)
* Titration in Flow Cytometry (2)
* Controls in Flow Cytometry (3)
* Compensation in Flow Cytometry (4)

**Notes**

This is a general guideline based on intracellular staining of cell lines or other single cell suspensions.

It does not concern specific antibodies; however, it takes into consideration: Incubation, washing and fixation.

You may need to adjust the procedure.

We strongly recommend that you evaluate if blocking is essential for your experiment (6). It depends on the cell type you are working with. If in doubt, make a parallel experiment with and without blocking.

In this protocol the antibody incubation is performed at +4 °C. The incubation time should be 30-60 minutes and the centrifuge should be cold (+4 °C).

There are several detergent possibilities. Triton X100 being the most harsh and Saponin the mildest.

**Materials**

1. Single cell suspension.
2. Tubes 1.5 ml, 2.0 ml Eppendorf tube or 5 ml FACS tubes
3. Viability dye.
4. Fixation: 4% paraformaldehyde or normal buffered formalin (NBF). If nuclear antigens are your target use acetone or ethanol (or methanol) as fixation. You can also use Zn fixation that does not cross bind the proteins (5).
5. Blocking Reagent: Species relevant serum, commercial Fc Block or blocking reagent all with detergent added if needed (6).
6. Stain and wash buffer: PBS pH 7.4 with 0.5% BSA or 2-5% FBS or Non sticky stain buffer. All with detergent added if needed.

Detergent: e.g. 0.3% Triton X100, 0.05% Tween 20, 0.2% Saponin or commercial permeabilization reagent (7, 8).

1. Fluorochrome conjugated antibodies.

**Procedure**

Be cautious at any time not to mix fluid from one tube to another. Even a tiny bit of antibody can stain your cells.

1. Adjust your single cell suspension to 1-10x106/ml and fix your cell pellet by slowly adding fixative while vortex mixing. Fixation time and temperature is dependent on fixative and cells. If you use ethanol or acetone as fixation, bear in mind that the cell pellet will be fluffy.
2. Wash out the fixative with 500 µl wash buffer.
3. Centrifuge the tube at 500-800xg for 3-5 min. cold. Speed (and time) is dependent of cell size and fixative.
4. Pour off or pipette off the supernatant and loosen the cell pellet by knocking the tube gently.
5. Repeat the wash twice.
6. Blocking Reagent can be added to your cell pellet now, mix and incubate at +4 °C for at least 15 minutes.
7. Add antibodies to the tube and mix.
8. Incubate in the dark 30-60 min at +4 °C.
9. Add washing buffer (according to the table below) to each tube.
10. Centrifuge the tubes at 500-800xg for 3-5 min cold.
11. Pour or pipette off the supernatant.
12. Loosen the cell pellet by gentle knocking the tube.
13. Repeat from 9-12 twice.
14. Resuspend the cells in at least 300µl.
15. Keep your samples cold and dark until analysis.
16. We strongly recommend analysing/sorting as quickly as possible.

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| Tube | Number of cells | Final stain volume | Wash volume |
| 1,5 ml eppendorf tube | ≤ 1x10^6 | 50-100 μl | 500 μl1 |
| 2 ml eppendorf tube | ≤ 5x10^6 | 50-100 μl | 1 ml1 |
| 5 ml FACS tube | ≤ 5x10^6 | 50-100 μl | 2 ml2 |
| 15 ml tube | ≤ 50x10^6 | 100-500 μl | 5-10 ml2 |

1 Carefully aspirating closely to pellet.

2 Pour or aspirate carefully.

**References:**

1. [FACS Core Facility Guidelines, Panel Design in Flow Cytometry](https://facs.au.dk/facscorefacilityguidelines/)
2. [FACS Core Facility Guidelines, Titration in Flow Cytometry](https://facs.au.dk/facscorefacilityguidelines/)
3. [FACS Core Facility Guidelines, Controls in Flow Cytometry](https://facs.au.dk/facscorefacilityguidelines/)
4. [FACS Core Facility Guidelines, Compensation in Flow Cytometry](https://facs.au.dk/facscorefacilityguidelines/)
5. Zn fixation: [Zinc fixation for flow cytometry analysis of intracellular and surface epitopes, DNA content, and cell proliferation.](https://pubmed.ncbi.nlm.nih.gov/21732310/)
6. Blocking: Elimination of erroneous results in flow cytometry caused by antibody binding to Fc receptors on human monocytes and macrophages by Andersen et al 2016, [Cytometry A.](https://www.ncbi.nlm.nih.gov/pubmed/27731950) 2016 Nov;89(11):1001-1009 <https://www.ncbi.nlm.nih.gov/pubmed/27731950>
7. <https://www.biolegend.com/en-us/bio-bits/phospho-staining-and-intracellular-flow-cytometry>
8. <https://www.thermofisher.com/dk/en/home/references/protocols/cell-and-tissue-analysis/protocols/staining-intracellular-antigens-flow-cytometry.html>