

Standard Flow Cytometry Staining (Surface, Intracellular, Intranuclear)

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Purpose

This protocol describes a standard flow cytometry staining workflow for suspension cells, including:

- Surface staining with Fc block and live/dead dye.
- Optional intracellular staining (e.g. BD Cytfix/Cytoperm-based).
- Optional intranuclear staining (e.g. eBioscience FOXP3 fix/perm-based).

It is intended as a general framework for murine or human samples and can be adapted to specific panels and antibody combinations.

Critical notes (read before starting)

- Keep cells and antibody mixes cold (on ice or at 4 °C) and protected from light whenever possible.
- Use FACS buffer containing EDTA to reduce cell aggregation during staining and washing.
- Do not exceed the recommended maximum of $\sim 5 \times 10^5$ cells per well in 96-well plates to ensure effective staining and washing.
- Always include appropriate controls (unstained, live/dead only, FMO, single-colour controls, etc.) for panel design and compensation.
- Fixation and permeabilization conditions must be compatible with the antibodies and fluorochromes used (consult datasheets).
- If using commercial fixation/permeabilization kits, follow the manufacturer's instructions for dilution and incubation times.

Warning

Improper fixation/permeabilization or using incompatible buffers can destroy epitopes and fluorochromes, leading to poor or misleading intracellular/intranuclear staining. Verify kit compatibility with your antibodies and fluorochromes before use.

Tip

Where possible, stain surface markers (including live/dead) first, then fix and permeabilize for intracellular/intranuclear staining. This often yields more robust surface staining and simplifies panel design.

Approximate timing

- Plate setup and Fc block + live/dead: 20–30 min
- Surface staining: 30–45 min
- Fixation only (no intracellular): 15–20 min
- Fix + permeabilization + intracellular/intranuclear staining (same day): 1.5–2 hours
- Optional overnight pause (after initial fix/perm): adds 1 day

Total approximate time (surface only): ~1 hour.

Total approximate time (full intracellular/intranuclear workflow): ~3–4 hours over 1–2 days.

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Procedure

Step 1 – Plate setup and FACS buffer

1. Prepare FACS buffer (BUF-GEN-FACS-BUF-001):
 - PBS, 1×
 - 2 % FCS (heat-inactivated)
 - 2 mM EDTA
2. Prepare single-cell suspensions of the desired samples.
3. Count cells and adjust concentration so that you can plate up to **5 × 10 cells per well** of a 96-well U-bottom or V-bottom plate.
4. Aliquot cells into the plate (typically 5 × 10 cells per well or less).
5. Keep plates on ice (or at 4 °C) until staining begins.

Step 2 – Fc block and live/dead staining

1. Prepare a staining mix in FACS buffer containing Fc block and live/dead dye:
 - Fc block: **1:200** dilution.
 - Live/dead dye: **1:100** dilution.
 - Final volume: **50 µL per well**.
2. Add **50 µL** of this Fc block + live/dead mix to each well containing cells.
3. Gently tap or briefly pulse-centrifuge the plate if needed to ensure mixing.
4. Incubate on ice (or at 4 °C) for the time recommended by the live/dead reagent manufacturer (typically 15–20 min), protected from light.
5. Centrifuge the plate at **1000 g for 2 min**.
6. Quickly flick off the supernatant into a suitable waste container.

Warning

Live/dead dyes are light-sensitive and can be toxic at high concentrations or with prolonged incubation. Avoid direct light and adhere to the manufacturer's recommended dilution and incubation times.

Step 3 – Surface staining

1. Prepare a surface staining master mix in FACS buffer with all desired surface antibodies:
 - Total volume: **50 µL per well**.
 - Antibody dilutions according to titration data (commonly 1:50–1:200).
2. Resuspend cell pellets in **50 µL** surface stain master mix per well.
3. Incubate the plate on ice for **30 min**, protected from light.
4. After incubation, top up each well to **200 µL** with FACS buffer.
5. Centrifuge the plate at **400 g for 2 min**.
6. Flick off the supernatant.
7. Wash the cells by adding **200 µL** FACS buffer to each well, gently resuspending the pellet, and centrifuging again at **400 g for 2 min**.
8. Flick off the supernatant.

3.1 If only surface staining is required

1. Resuspend each pellet in **100–200 µL** of 4 % PFA solution (or appropriate fixative) in PBS.
2. Store plates at 4 °C, protected from light, until acquisition.

Step 4 – Fixation and permeabilization for intracellular or intranuclear staining

This step assumes the use of:

- **BD** intracellular staining kit (e.g. Cytofix/Cytoperm) for cytoplasmic targets, or
- **eBioscience FOXP3** or equivalent intranuclear staining kit for transcription factors.

Always consult the kit manuals; the steps below mirror the original SOP structure.

4.1 Initial fix/perm

1. Add **100 µL** of BD fix/perm solution (e.g. Cytofix/Cytoperm) **or** eBioscience FOXP3 fix/perm solution (typically 1:4 concentrate with 3:4 diluent) to each well.
2. Incubate for **20 min on ice**, protected from light.
3. Top up each well to **200 µL** with 1× PermWash (diluted in water, according to the kit instructions).
4. Centrifuge the plate and flick off the supernatant.
5. Resuspend each pellet in **200 µL** 1× PermWash.
6. Centrifuge the plate and flick off the supernatant.

4.2 Pause point (optional, overnight)

1. Resuspend each pellet in **200 µL** FACS buffer.
2. Seal and label the plate clearly.
3. Store at **4 °C** overnight, protected from light.

i Note

An overnight pause at this stage can be convenient for scheduling, but confirm that your antigen of interest and fluorochromes are stable under these conditions.

4.3 Re-permeabilization before intranuclear staining (if using FOXP3 kit)

1. The following day, centrifuge the plate at **1000 g for 2 min** and flick off the supernatant.
2. Resuspend pellets in **200 µL** 1× PermWash (FOXP3 kit).
3. Incubate for **30 min on ice**, protected from light.
4. Centrifuge and flick off the supernatant.

Step 5 – Intracellular or intranuclear staining

1. Prepare an intracellular or intranuclear staining master mix in the appropriate PermWash (kit-specific):
 - Total volume: **50 µL per well**.
 - Antibody dilutions according to titration data (typically 1:50–1:200).
2. Resuspend each pellet in **50 µL** of the master mix.
3. Incubate on ice for **30 min**, protected from light.
4. Top up each well to **200 µL** with PermWash.
5. Centrifuge and flick off the supernatant.
6. Wash once more with **200 µL** PermWash and centrifuge.
7. Flick off the supernatant.
8. Resuspend each pellet in **100–200 µL** of 4 % PFA solution (or other appropriate fixative) in PBS.
9. Store plates at 4 °C, protected from light, until acquisition.

Materials

Reagents

| Reagent | Supplier | Cat. # | Notes |
|---|------------------------------|-----------|--------------------------------------|
| PBS, sterile, 1× | Various | – | Base for FACS buffer |
| Fetal calf serum (FCS), heat-inactivated | Various | – | 2 % v/v in BUF-GEN-FACS-BUF-001 |
| EDTA, 0.5 M stock | Various | – | 2 mM final in BUF-GEN-FACS-BUF-001 |
| Fc block (e.g. anti-CD16/CD32 for mouse) | Various (e.g. BD) | – | Used at 1:200 in FACS buffer |
| Live/dead viability dye | Various (e.g. Invitrogen) | – | Used at 1:100 in FACS buffer |
| Surface antibodies (various fluorochromes) | Various | – | Titration required for each antibody |
| Intracellular antibodies | Various | – | Compatible with BD fix/perm |

| Reagent | Supplier | Cat. # | Notes |
|--|-------------------------|-----------|------------------------------------|
| Intranuclear antibodies (e.g. FOXP3) | Various | – | Compatible with FOXP3 fix/perm kit |
| BD fix/perm kit (e.g. Cytofix/Cytoperm) | BD Biosciences | – | For intracellular staining |
| eBioscience FOXP3 fix/perm kit | eBio- science/Thermo | – | For intranuclear staining |
| 1× PermWash (BD, FOXP3 kit) | From kits | – | Used according to manufacturer |
| 4 % PFA in PBS | Various / in-house | – | For fixation prior to acquisition |

Disposables

| Item | Specification |
|---------------------------------------|---|
| 96-well U-bottom or V-bottom plates | Sterile, for staining |
| Pipette tips (10 µL, 200 µL, 1000 µL) | Sterile |
| Serological pipettes | For buffer and media handling |
| Microcentrifuge tubes | For master mixes and controls |
| Tube strips or FACS tubes | For compensation/single-colour controls |
| Waste containers | For liquid and solid biohazard waste |

Equipment

| Equipment | Specification / Notes |
|---------------------------|---|
| Benchtop plate centrifuge | Able to reach 1000 g for 96-well plates |
| CO ₂ incubator | If cells require incubation before/after staining |
| Biological safety cabinet | For safe handling of biological samples |
| Vortex mixer | For mixing master mixes (low speed for cells) |
| Flow cytometer | e.g. Cytex Aurora, BD FACSCanto, etc. |

Buffers used

- **BUF-GEN-FACS-BUF-001** – FACS buffer (PBS + 2 % FCS + 2 mM EDTA).
- Commercial kit buffers:
 - BD fix/perm and 1× PermWash (per manufacturer).
 - eBioscience FOXP3 fix/perm and 1× PermWash (per manufacturer).

Troubleshooting

| Problem | Possible cause | Suggested solution |
|--|--|---|
| High background staining | Antibody over-titration; inadequate washing; Fc block insufficient | Titrate antibodies; ensure thorough washes; increase Fc block if appropriate. |
| Poor separation of positive/negative populations | Suboptimal antibody concentration; insufficient incubation time | Re-titrate antibodies; extend incubation (e.g. up to 40–45 min). |
| Extensive cell clumping | No EDTA; high cell density; dead cells present | Use FACS buffer with EDTA; reduce cells per well; filter cells if necessary. |
| Poor intracellular/intranuclear staining | Inadequate fixation/permeabilization; incompatible buffers | Confirm kit protocol; ensure correct fix/perm times; use recommended buffers. |
| Loss of surface staining after fixation | Antibodies or epitopes not fixation-stable | Move sensitive markers to “post-fixation” staining if compatible, or use fix-stable clones/fluorochromes. |
| Low viability | Over-handling; extended time at room temperature | Minimize processing time; keep cells on ice when possible; avoid harsh pipetting. |

Safety (brief)

- Handle all biological samples under appropriate biosafety conditions (e.g. BSL2) and follow institutional guidelines.
- Wear standard PPE (lab coat, gloves, eye protection) when handling cells, antibodies, and fixatives.
- 4 % PFA and commercial fixatives should be handled in a fume hood or well-ventilated area; avoid skin and eye contact.
- Dispose of plates, tubes, tips, and liquids contaminated with biological material or fixatives as chemical/biological hazardous waste according to local regulations.

Version history

| Version | Date | Author | Changes |
|---------|------------|----------------|---|
| v1.0 | 2025-11-21 | Dillon Corvino | First Quarto protocol version for standard flow cytometry staining. |