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 Cytofix/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 ~5 × 10 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.

> **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	eBioscience/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 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. Cytek 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.