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 **tetramer staining performed together with surface staining**
- 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.

Buffer nomenclature (important)

- **FACS buffer** = PBS + FCS
- **MACS buffer** = PBS + FCS + EDTA

Unless otherwise specified, **MACS buffer is used throughout this protocol** to minimise cell clumping during staining and washing.

When tetramers are included, all surface staining (including tetramers and live/dead dye) is performed in FACS buffer (no EDTA).

Critical notes (read before starting)

- Keep cells and antibody mixes cold (on ice or at 4 °C) and protected from light whenever possible.
- Default staining range: $\mathbf{2 \times 10 - 1 \times 10}$ **cells per well** (96-well format).
- Always include appropriate controls (unstained, live/dead only, FMO, single-colour controls).
- Fixation and permeabilization conditions must be compatible with antibodies and fluorochromes used.
- Commercial fixation/permeabilization kits should be used according to manufacturer instructions.

> ⚠ Warning
>
> Improper fixation/permeabilization or incompatible buffers can destroy epitopes and fluorochromes, leading to misleading intracellular or intranuclear staining.

> ⚠ Warning
>
> Antibodies and tetramers are **protein reagents and mechanically sensitive**.
> Do **not vortex**, as this can denature proteins and promote aggregation.
> Instead, mix gently by pipetting or inversion. If needed, briefly spin reagents in a **mini "pulse" centrifuge (quick spin)** to collect liquid and pellet aggregates before use.

> 💡 Tip
>
> Surface markers (including live/dead dyes and tetramers) should generally be stained **before fixation/permeabilization** for best performance.

> 💡 Tip
>
> Surface staining master mixes can be prepared in advance (e.g. the day before) to save time; however, it is recommended to add the live/dead dye immediately before use.

Approximate timing

- Surface ± tetramer staining: 30 min
- Plate setup and Fc block: 10–15 min
- Fixation only (no intracellular): 15–20 min
- Fix + permeabilization + intracellular/intranuclear staining: 1.5–2 h
- Optional overnight intranuclear stain: +1 day

Total (surface only): ~1–1.5 h
Total (full workflow): ~3–4 h (or overnight)

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Procedure

Step 1 – Plate setup and buffers

1. Prepare buffers:
    - **FACS buffer** (PBS + 2% FCS) — *use only if tetramers are included*
    - **MACS buffer** (PBS + 2% FCS + 2 mM EDTA) — *default buffer*
2. Prepare single-cell suspensions.
3. Count cells and adjust concentration to plate **2 × 10 − 1 × 10 cells per well**.
4. Aliquot cells into a 96-well U- or V-bottom plate.
5. Keep plates on ice or at 4 °C.

Step 2 – Surface staining (with live/dead ± tetramers)

Surface staining is performed **in a single master mix**.

- **If tetramers are included** → use **FACS buffer (no EDTA)**
- **If no tetramers are included** → use **MACS buffer**

Antibody titration guidance (applies to all surface staining): - Surface antibodies: default **1:200** if titration data are unavailable. - Transcription factors or weakly expressed markers: default **1:50**.

2.1 Prepare surface staining master mix

Prepare a **1× surface staining master mix** in the appropriate buffer containing:

- Fc block: **1:100**
- Live/dead dye (1× final concentration)
- Surface antibodies (1× final concentration)
- **Optional:** Tetramer(s), per manufacturer recommendation

Final volume: **50 µL per well**

> **Note**
>
> When tetramers are included, staining is performed in **FACS buffer without EDTA** to avoid potential interference with multimer–TCR interactions.
> Whether EDTA exclusion is strictly necessary remains under investigation and will be empirically evaluated within the lab.

> **Tip**
>
> After flicking off the supernatant, residual liquid in the dead volume can be used to resuspend cell pellets by briefly vortexing the plate at low speed to agitate the wells. This helps achieve uniform resuspension before adding the next reagent.

2.2 Staining

1. Resuspend each cell pellet in **50 µL** surface staining master mix.
2. Incubate for **30 min on ice**, protected from light.

2.3 Wash steps

1. Top up each well to **200 µL** with **MACS buffer**.
2. Centrifuge at **1000 g for 2 min at 4 °C**.
3. Flick off the supernatant.
4. Wash once more with **200 µL MACS buffer**, centrifuge at **1000 g for 2 min at 4 °C**, and flick.

> **Note**
>
> All antibodies and tetramers should be titrated for optimal performance. Default dilutions are provided as a starting point only.

2.4 Surface staining only

1. Resuspend pellets in **100–200 µL 4% PFA in PBS**.
2. Wrap the plate in **aluminium foil** to protect from light and **clearly label with name and date**.
3. Store at **4 °C**, protected from light, until acquisition.

Step 3 – Fixation and permeabilization

This step assumes use of: - **BD Cytofix/Cytoperm** (cytoplasmic targets), or
- **eBioscience FOXP3 fix/perm** (intranuclear targets).

3.1 Initial fixation/permeabilization

1. Add **100 µL** fix/perm solution per well.
2. Incubate **20 min on ice**, protected from light.
3. Top up to **200 µL** with 1× PermWash.
4. Centrifuge at **1000 g for 2 min at 4 °C** and flick.
5. Resuspend in **200 µL** PermWash.
6. Centrifuge at **1000 g for 2 min at 4 °C** and flick.

3.2 Optional pause point

1. Resuspend pellets in **200 µL MACS buffer**.
2. Wrap the plate in **aluminium foil** to protect from light and **clearly label with name and date**.
3. Store at **4 °C overnight**, protected from light.

> **Note**
>
> If the protocol is paused at this step, the recovery steps below must be performed before proceeding to intracellular or intranuclear staining.

3.3 Restarting the protocol after pause point

1. Centrifuge the plate at **1000 g for 2 min at 4 °C**.
2. Flick off the supernatant.
3. Resuspend pellets in **200 µL PermWash**.
4. Incubate for **30 min on ice**, protected from light.
5. Centrifuge at **1000 g for 2 min at 4 °C** and flick.
6. Proceed directly to **Step 4 – Intracellular or intranuclear staining**.

Step 4 – Intracellular or intranuclear staining

1. Prepare an **intracellular or intranuclear antibody master mix** in the appropriate **PermWash** buffer (kit-specific):
   - Final volume: **50 µL per well**
2. Resuspend each cell pellet in **50 µL** of the antibody master mix.
3. Incubate **30 min on ice**, protected from light, **or** incubate **overnight at 4 °C**.

> **Tip**
>
> For **intranuclear targets** (e.g. **FOXP3, Helios, T-bet, Eomes**), overnight antibody incubation at **4 °C** can improve signal intensity, particularly for low-abundance or epitope-masked transcription factors.
> Ensure plates are **well sealed, clearly labelled, and protected from light** during overnight incubation.

> **Warning**
>
> Overnight antibody incubation is **not recommended for cytoplasmic intracellular targets** (e.g. cytokines, granzymes, perforin). Prolonged incubation can increase background staining and does not typically improve signal for these antigens. Use **30–60 min** incubations for intracellular (non-nuclear) targets.

4. Top up each well to **200 µL** with PermWash.
5. Centrifuge at **1000 g for 2 min at 4 °C** and flick off the supernatant.
6. Wash once more with **200 µL PermWash**, centrifuge at **1000 g for 2 min at 4 °C**, and flick.
7. Resuspend each pellet in **100–200 µL 4% PFA in PBS**.
8. Wrap the plate in **aluminium foil** to protect from light and **clearly label with name and date**.
9. Store at **4 °C**, protected from light, until acquisition.

Materials

Reagents

Reagent	Notes
PBS 1×	Ca^2 /Mg^2 -free
FCS (heat-inactivated)	2% v/v
EDTA	2 mM (MACS buffer only)
Fc block	Mouse: anti-CD16/32
Live/dead dye	As per manufacturer
Tetramers (e.g. MR1–5-OP-RU, CD1d)	Optional
Surface antibodies	Titrate individually
Intracellular/intranuclear antibodies	Kit-compatible
Fix/perm kits	BD or eBioscience
4% PFA	Fixation

Buffers used

- **FACS buffer** – PBS + 2% FCS (used when tetramers are included)
- **MACS buffer** – PBS + 2% FCS + 2 mM EDTA (default)

Version history

Version	Date	Author	Change summary
v1.0	2025-11-21	Dillon Corvino	Initial version
v1.1	2025-12-29	Dillon Corvino	Added tetramer workflow, clarified buffers, titration defaults, overnight intranuclear staining
v1.2	2025-12-30	Dillon Corvino	Unified tetramer and surface staining into single master mix; buffer choice conditional on tetramer inclusion
v1.3	2025-12-30	Dillon Corvino	Added conditional restart steps after fix/perm pause point and explicit plate centrifugation conditions