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:

- Optional **tetramer staining**.
- 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.

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.

Tetramer staining is a specific exception (see below).

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.

Approximate timing

- Tetramer staining (optional): 20 min
- Plate setup and Fc block: 10–15 min
- Surface staining: 30 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) — *for tetramer staining only.*
   - **MACS buffer** (PBS + 2% FCS + 2 mM EDTA) — *default buffer for all other steps.*
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 – Tetramer staining (optional, if applicable)

This step applies when staining with MR1 or CD1d tetramers.

1. Prepare a **tetramer staining mix in FACS buffer (no EDTA)**:
   - Fc block: **1:100**
   - Tetramer(s): per manufacturer recommendation
   - Final volume: **50 µL per well**
2. Add **50 µL** tetramer mix to each well.
3. Incubate for **20 min on ice**, protected from light.

> **Note**
>
> Tetramer 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 tested within the lab.

> **Note**
>
> MR1–5-OP-RU tetramer can be added together with surface antibody staining.
> For CD1d tetramers, compatibility with simultaneous surface staining is currently unclear and will be empirically tested.

4. Without washing, proceed directly to Step 3.

Step 3 – Surface staining (with live/dead)

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**.

3.1 If tetramer staining was performed (from Step 2)

1. Prepare a **2× surface staining master mix in MACS buffer** containing:
   - Live/dead dye (2× final concentration)
   - Surface antibodies (2× final concentration)
2. Add **50 µL** of the 2× surface mix to each well already containing **50 µL tetramer mix** → **Final volume: 100 µL per well (1× final concentration)**.
3. Incubate for **30 min at 4 °C**, protected from light.

3.2 If no tetramer staining was performed

1. Prepare a **1× surface staining master mix in MACS buffer** containing:
   - Live/dead dye (1× final concentration)
   - Surface antibodies (1× final concentration)
2. Add **50 µL** of the 1× surface mix directly to each well.
3. Incubate for **30 min at 4 °C**, protected from light.

3.3 Wash steps (both cases)

1. Top up each well to **200 µL** with MACS buffer.
2. Centrifuge at **400 g for 2 min**.
3. Flick off the supernatant.
4. Wash once more with **200 µL MACS buffer**, centrifuge, and flick.

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

3.1 Surface staining only

1. Resuspend pellets in **100–200 µL 4% PFA in PBS**.
2. Store at **4 °C**, protected from light, until acquisition.

Step 4 – Fixation and permeabilization

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

4.1 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 and flick.
5. Resuspend in **200 µL** PermWash.
6. Centrifuge and flick.

4.2 Optional pause point

1. Resuspend pellets in **200 µL MACS buffer**.
2. Store at **4 °C overnight**, protected from light.

> **Note**
>
> This pause point is compatible with most intracellular workflows, but antigen stability should be confirmed for each marker.

Step 5 – 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 and flick off the supernatant.
6. Wash once more with **200 µL PermWash**, centrifuge, and flick.

7. Resuspend each pellet in **100–200 µL 4% PFA in PBS**.
8. 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 (tetramers only)
- **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