

Multimerization of MHC Class I Molecules and Multimer Staining SOP

A. Materials

- Streptavidin-conjugated fluorochromes

It is strongly recommended to use only PE and APC conjugates in the beginning to test your peptide(s) of interest. If screening using a higher number of fluorochromes is necessary (i.e. low cell numbers or large peptide screens), please discuss the specifics with the IML staff and we can provide you with the necessary information for combinatorial coding.

 - ❖ APC-SAV (cat.#: SA1005, Molecular Probes)
 - ❖ PE-SAV (cat.#: 405204, BioLegend)
- Fc Block (cat.#:553142, BD)
- D-biotin (cat.#: 47868, Sigma Aldrich)
- 96 well polypropylene plates
- FACS Buffer
 - PBS, 4% FBS, 0.1% sodium azide
- Antibodies for characterization (we recommend the following):
 - ❖ Zombie NIR live/dead stain (cat.#423105, BioLegend)
 - ❖ anti-CD8 α FITC (cat.#100706, BioLegend)
 - ❖ anti-Thy1.2 PE/Cy7 (cat.#140309, BioLegend)

B. Production of Fluorochrome-conjugated Tetramers

- Spin the vial of exchanged peptide-MHC monomer at 14,000 rpm for 5 minutes to pellet any aggregate
- Transfer 10 μ L of exchanged peptide-MHC monomer to a well of a 96-well V-bottom polypropylene plate on ice being careful not to transfer any of the pellet
- Multimerize the peptide-MHC complexes by the addition of streptavidin-fluorochrome conjugates per the amounts titrated below (*one fluorochrome per one well; if using double color coding or combinatorial coding, you will have 2 separate wells with 10 μ L of monomer, each with an individual fluorochrome at this step*):

APC (Molecular Probes)	0.6 μ L
PE (BioLegend)	1.1 μ L
- Mix well and leave to conjugate protected from light at 4C for 30 minutes
- Add D-biotin (without azide) to a final concentration of 25 μ M to each well and incubate 20 minutes on ice (i.e. add 2.5 μ L of a 100 mM stock to 1 mL PBS then add 1 μ L of diluted D-biotin to each well)
- If using combinatorial coding, mix the complexes as per the plate layout prior to staining

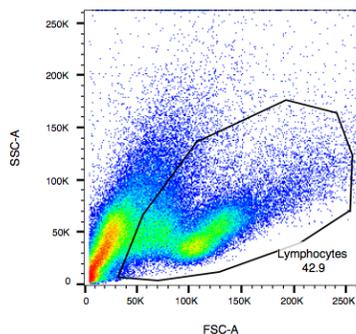
C. Multimer Staining

- Spin down the cells at 2,000 rpm for 2 minutes, tip out buffer and resuspend the pellet in 50 μ L of FACS buffer
- If staining **mouse** cells: Add 1 μ L of Fc block to each well and incubate 5 minutes on ice
- Add the multimer complexes directly to the well per the amounts titrated below (if double color staining with PE and APC, add 3.0 μ L of Tetramer-APC and 2.0 μ L of Tetramer-PE to the each well):

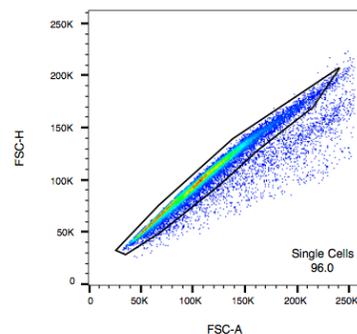
<u>Fluorochrome</u>	<u>TIL</u>	<u>Spleen/LN/Blood</u>
APC	3.0 μ L	2.0 μ L
PE	2.0 μ L	2.0 μ L

- Incubate at 37°C for 15 minutes in the dark
- Add antibodies for cellular characterization and incubate on ice for 20 minutes (*i.e. 0.3 μ L of Thy1.2, 0.5 μ L of CD8, 0.3 μ L of NIR*)
- Wash wells twice with FACS buffer
- Resuspend cells and run on cytometer:
(*a representative gating strategy is outlined below*)

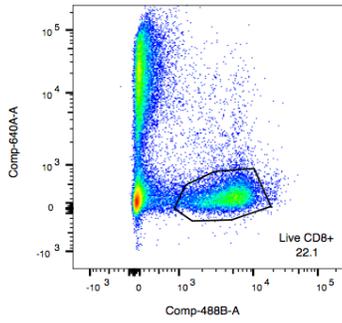
a. Gate on Lymphocyte population:



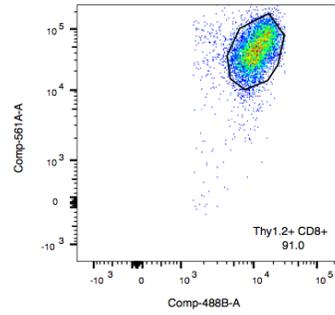
b. Gate out doublets:



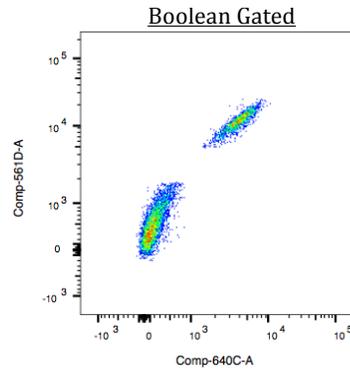
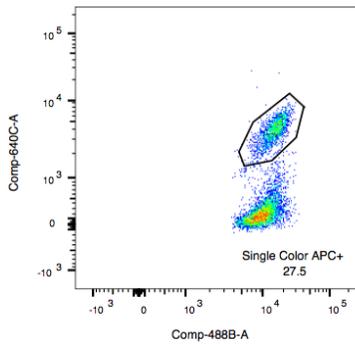
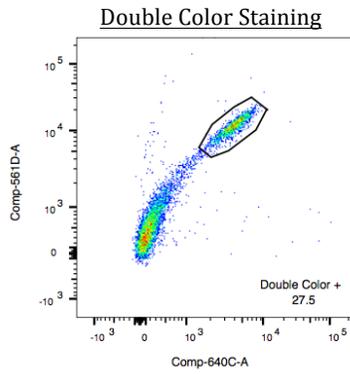
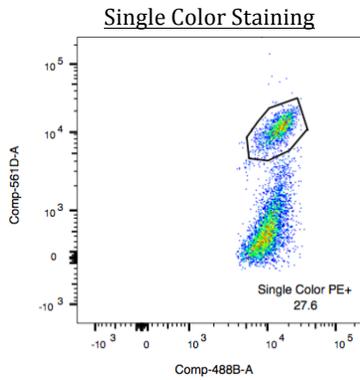
c. Gate on NIR- (640A), CD8+ (488B) cells:



d. From live CD8+ population, gate on Thy1.2+ (561A) cells:



e. Tetramer specific cells should be PE (561D) and/ or APC (640C) positive:



** For assistance with Boolean Gating strategies, please see IML Tetramer Staff*