Staining $CD4^+$ T cells with oligomers of class II MHCs

Prologue:

We have limited experience in this arena, but I think that is true for everyone in the field. Every cell/cell line seems to behave differently. What I detail below is what I generally recommend. On this last page of this document, is a more detailed, specific (and limited) protocol.

In my experience, some cells will stain while on ice, while others require elevated temperatures (for which 37º C is preferred). In my hands, the "4º C stainers" stain more brightly at 37º C, so I always recommend staining at 37º C. You might try both, if you have time, reagent, and cells. The advantage of elevated temperature is that reagent is internalized by the cell and subsequently trapped inside while receptors might recycle and gather more reagent. Thus, I find that longer incubation times yield brighter staining (although > 5 hrs seems to be overkill). Higher concentrations of tetramer also seem to give brighter stainings, but in the interest of saving this valuable reagent I perform most experiments between 10 and 50 micrograms/ml.

Importantly, be careful which oligomerization reagent you buy. I find that per microgram of DR, R-phycoerythrin conjugated streptavidin (SA-PE) from BioSource (lot 1101 or 1301) gives the brightest staining (also note that both the Kappler and Kwok labs use this same reagent). Reagents from other companies are either less bright or almost completely ineffectual. I believe that this is a function of oligomericity. SA-PE conjugates are probably never one-to-one SA to PE. They are made by amine-amine crosslinking which can yield many different products depending on the ratio of reagents and extent of crosslinking. The SA-PE from BioSource appears to have an average MW of 15,000,000 Da (measured by dynamic light scattering). Thus, I believe this reagent is likely to be of higher valency than four. Reagents from other companies have probably been optimized for bright staining of biotinylated antibodies, for which a conjugate of multiple PE with low biotin-binding valency is optimal. For "tetramer" staining, a balance of high oligomericity and high PE content is desirable.

Reagents:

Biotinylated class II MHC with peptide of choice

(in our lab this is usually DR1 folded from inclusion bodies of alpha and beta subunits produced in E. coli and chemically biotinylated using maleimide-PEO-biotin).

Biotinylated class II MHC with control peptide
SA-PE from BioSource, Inc (lot 1101 or 1301)
Cells, rested 7-14 days
RPMI
Extra media
cold FACS buffer (we use PBS, 1% BSA, 0.1% NaAzide, 0.5 mM EDTA).
FACS tubes and a FACS machine.

Oligomerize:

Oligomerized reagent is stable for several weeks. Eventually it will start to precipitate. I usually store at 4º C, although it can be frozen. Assay your MHC for biotinylation efficiency. Consider unbiotinylated MHC useless and use the concentration of biotinylated MHC for all further calculations. I advise adding the SAPE stepwise to maximize the formation of high order oligomers.

Aliquot desired amount of biotinylated MHC to tube. Add 0.25 µl of SA-PE per µg of MHC and mix by pipette. Wait several minutes. Repeat three more times (until you have added 1 µl SAPE per µg of MHC. (I determined this ratio empirically for my tube of SAPE and my cells. You may want to try a titration yourself). Add required volume of RPMI for a final concentration of 60 µg/ml (where half is MHC and we assume SAPE is 1 mg/ml. ie solution is 30 µg/ml MHC and 30 µg/ml SAPE).

Staining:

Prepare cells in a small volume of media. Aliquot 10 µl cells per well of a round bottom 96 well plate, or an eppendorf tube, or a PCR plate. Use any number of cells that you desire. I have used from 500,000 cells to 5,000 cells in 10 µl and seen no difference in the staining. Add 5 µl of MHC oligomer reagent. Seal wells/tubes with tape to minimize evaporation (or use some other trick to minimize evaporation). Place in a 37º C incubator 2-5 hours in the dark.

Chill on ice. Add secondary antibodies (CD3, CD4?) and proceed with staining as normal except with special attention to keeping cells cold continuously.

I recommend washing the cells in a 96 well plate, resuspending in a small final volume (30-50 µl) and transferring to 1.2 ml microdilution tubes (USA Scientific, Cat # 1412-0400). These tubes can be inserted inside FACS tubes for easy analysis.

Count them on the FACS, gate for live CD4+ cells. I typically find staining intensities of ag-specific tetramers to be 50- to 500-fold brighter than non-specific tetramers.

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Specific Protocol for staining CD4+ T cells with DR Tetramers:

-tom cameron, Dec 2000

Reagents:

DR1pep: Biotinylated DR1-peptide of choice in PBS.
SAPE: R-phycoerythrin conjugated Streptavidin from BioSource, Inc
RPMI and T cell Media
T cells and/or PBMCs
CD4-APC, CD14-PerCP (optional, good for PBMCs), CD3-Fitc (optional, but good, esp for clones).

Make the Oligomer Reagent:

For 50 ul:

Aliquot 1.5 ug DR1pep into a epp. tube
Dilute 2 ul SAPE with 4 ul PBS
Add 2 ul of diluted SAPE to DR1
Wait 2 min
Add 2 ul of diluted SAPE to DR1
Wait 2 min
Add remainder of diluted SAPE to DR1.
Wait 2 min.
Dilute DR1-SAPE oligomer with RPMI or T cell media to a final volume of 50 ul.

Notes: I would call this a 60 ug/ml reagent, half of it is (by weight) DR, and approximately half is SAPE. Since SAPE is a poorly characterized heterogeneous reagent, that isn't very precise. I have determined experimentally that a final ratio of 1.3 ul of SAPE per 1 ug of DR1-pep gives optimal staining when using SAPE Lot 1401 from BioSource. Different lots of SAPE should be titrated with DR1pep to determine optimal ratio (or contact me and ask if I've done that experiment!) although approx 1 ul SAPE per 1 ug DR generally seems pretty good.

Stain the Cells:

Collect cells in a 15 ml conical tube and spin down 5 min 1500 rpm
Aspirate as much of the media as you can.
Resuspend the cells in a very small volume of media (10 ul for every stain that you want to do. So if you're doing only one specific tetramer, and one control tetramer, then resuspend in 20 ul).
Aliquot 10 ul into wells of a rnd bottom 96 well plate (use only inner wells).
Add 5 ul of DR1pep-SAPE oligomer reagent (tetramer) to appropriate wells.
Add 200 ul RPMI or PBS to the outer wells of the plate.
Place plate in CO2 incubator for 3 hours.
Chill plate on ice 5 min.
Prepare cocktail of desired secondary antibodies (I typically use 1 or 2 ul of each 2ndary per well) and dilute with media, RPMI or PBS so that you have 5 ul per sample.
Add 5 ul of 2ndary Ab cocktail to each sample. Pipet mix.
Let sit on ice > 20 min
Add 200 ul cold FACS buffer (PBS, 1% BSA or FBS, 0.02% NaAzide).
Spin plate in cold centrifuge 5 min, 1500 rpm
Aspirate with pipet tip or flick plate into sink.
Add 200 ul cold FACS buffer
Spin
Aspirate
Resuspend in either FACS buffer or PBS/1% paraformaldehyde.
Take to the FACS and count!

Notes: Remember to include plenty of controls including single-stained cells for compensation adjustment, control tetramers, control cells. Try to keep samples cold after the tet-stain incubation (3 hr) is over. Anti-CD3 antibody can stimulate the cells at RT or 37C and do weird things (downregulate TCR, maybe CD4, etc.)