PREPARATION FOR TISSUE HARVEST

At least 1 to 2 days prior, identify all samples to be collected. You will need a lot of plastics and tubes. It is good practice to label tube sides and caps in case of mix-ups. Assume all plasticware (tubes, pestles, syringes, etc.) to be sterile for handling and processing cells. Example catalog numbers provided from 2022 purchases. Some available at the tissue culture facility (TCF) like pen-strep, FBS, ACK or HEPES buffer – but not all plastics or exact media. i.e., RPMI with HEPES must be ordered at this time whereas the TCF sells RPMI and HEPES separately.

1. Complete Media Recipe: Complete cell media is RPMI1640 with 25 mM HEPES (Fisher, 10-041-CV), 2 mM L-glutamine or stable L-alanyl-L-glutamine alternative (Gibco, 35050061), 100 U/mL or 1% v/v pen-strep (Gibco, 15140122), and 10% v/v heat-inactivated fetal bovine serum FBS (VWR, 97068-085).
    - HEPES buffer helps maintain neutral pH during processing steps. Supplement with cell-culture grade HEPES if RPMI is unavailable with it preformulated.
    - Reminder that L-glutamine degrades quickly even at 4°C over a month or so. Media should be replenished with either L-glutamine or shelf-stable version per preparation.[1]
    - Suggest media filtration with a 500 mL filter system and 0.2 µm PES membrane (Fisher, FB12566504). Add supplements first then top with media before vacuum. If you need cold media fast, filter all supplements first then fill with ~450 mL cold media before finishing filtration.

2. Supplies for General Mouse Harvest
    - 70% ethanol in spray bottles
    - ~20G needles and Styrofoam lids for pinning and dissecting mice
    - Autoclaved tweezers and surgical scissors
    - Sharpies for label corrections
    - Teamwork!

3. Supplies for Spleen and Lymph Node (LN) Harvest
    - A 15 mL centrifuge tube (VWR, 21008-216) with 2 mL cold complete media for each organ or LN set to be collected
    - Ice buckets full of crushed ice for holding your samples. Refill as needed.
        - Note that samples on ice in the cold room may freeze or get close to doing so. If long-term storage needed, store samples off ice in the cold room or a refrigerator.

4. Supplies for Spleen Processing
    - A 6-well well plate (TC-treated acceptable; Fisher, 08-772-1B) for every 6 samples
    - A 40 and 70 µm filter for each sample (VWR, 75799-940 and 21008-952)
        - Note that these filters are made for 50 mL centrifuge tubes. Small diameters can be purchased for 15 mL size. Some have side-wall mesh, some clip on to the top of a tube. The 50 mL (normal) size fits in a 6-well plate nicely.
    - A 3 mL syringe to use as pestle (Fisher, 14-823-435) or purpose-bought pestle (maybe more expensive).
    - A 15 or 50 mL centrifuge tube (VWR, 21008-242) for final filtered cell slurries (post-40 µm filter). Label ahead
    - Swinging bucket centrifuge with 15 mL tube rotor buckets. Prechill at 4°C.
    - An additional 15 to 20 mL complete media per sample
    - ACK lysing buffer for red blood cell removal (VWR, 10128-808)
    - Plenty of serological pipettes (5, 10, 25, and 50 mL; plugged ends)
    - Either 2 mL microfuge tubes (pre-labelled) or a 2+ mL deep well plate(s) for final cell dilutions.

- Cell counter (Moxi Orflo Z) with enough cassettes for all samples

5. Supplies for Lymph Node Processing
    - Same as spleen processing except without the 70 µm filter, second 15/50 mL tube, nor ACK buffer.

6. Supplies for Bronchoalveolar Lavage (BALs) and/or Nasal Washes
    - 2 mL tubes, labelled per mouse
    - Either fresh PE20 catheters or Disposable Safelet I.V. Catheters (Fisher, 14-841-20)
    - Wash solution (2+ mL per mouse for both BAL and nasal wash)
        - Per 50 mL tube preparation, 1 tablet of protease inhibitor cocktail (Sigma, 11697498001)
        - 0.01% v/v Triton X-100 (10% in water stock; Sigma, 93443-100ML)
        - 1× DPBS to dilute

7. Supplies for ELISA and ELISPOT Experiments
    - Reminder that the day before harvest, ELISPOT plates (Fisher, S2EM004M99) must be coated with capture antibody after wetting. Keep bottom film/lid on all ELISPOT plates until end of assay (drying).
    - ELISAs use Greiner 96-well plates, non-TC, U-bottom, clear, sterile ((VWR, 82050-634).
    - ELISPOTs use Millipore MultiScreen-IP Filter Plate, 0.45 µm Hydrophobic PVDF membrane, white, sterile, (Fisher, S2EM004M99)
    - We typically run IL-2 and IFN-γ ELISPOTs (Fisher, BDB551876 and BDB551881).
        - These BD kits come with unlabeled capture antibody and biotinylated detection antibody.
        - Enzyme conjugate and AEC are purchased separately (Fisher, BDB557630 and BDB551951, respectively)
    - Use reagents/antibodies from matched lots and follow certificates of analysis (CoA).
    - Diluents typically DPBS for coating and 10% heat-inactivated FBS in DPBS for detection
        - 50 mL reagent reservoir (VWR, 89094-686)

HARVEST DAY

1. Notes and Reminders
    - All steps need to be performed with relative aseptic technique, including the harvest. Make sure cells are kept on ice throughout the entire procedure. Performing dissection in a ducted biosafety cabinet is optional but will help with the smell.
    - Bring a Bluetooth speaker to help pass the time.
    - For multiple harvest steps, assign people based on specialty or experience for different harvests.
        - E.g., one person on cervical lymph nodes and spleens, one or two people on BALs and nasal wash (since *much* slower)
        - Spleens alone are quick to harvest, as short as 30s per mouse.
        - BALs are one of the most difficult techniques for mouse harvesting in the group and take a lot of time per mouse.
        - Whoever does BALs should tag team if more than 10 mice. Same person runs BALs and nasal wash on that mouse. Can hand off for spleens and LNs to another person.
    - Tissues should be processed immediately. Cell slurries can be kept on ice throughout the day without much issue.
        - Do not store on ice in the cold room for long, since it will go below freezing in your cell tubes.
        - It's fine to store off ice in the cold room or a fridge though.

- References
  - Nasal wash[2]
  - BALs[3]
  - Wash solution (need ref for surfactant+protease inhibitor in wash solution)

2. Initial Harvest

- Euthanize the mice using CO_2 asphyxiation following posted guidelines.
  - Spray mouse and tools heavily with 70% ethanol before any dissection step. Wipe tools dry before proceeding with dissection steps.
  - For secondary, if you **do not need** access to the trachea (i.e., for BALs, nasal wash) then quickly perform cervical dislocation on the mice.
  - For secondary, if you **do need** access to an intact trachea or surrounding tissue then carefully open mouse chest cavity.
    - Follow by exsanguination via caudal vena cava or cardiac puncture (careful not to puncture lungs). You can access the caudal vena by pushing GI organs aside and finding it near the base of the spinal cord.
    - *Technical Note*, cardiac puncture can be acceptable for final blood collection but is not as robust as submandibular bleeds (done day prior) by inexperienced researchers. It should be done with a beating heart – quickly after asphyxiation.
- Harvest spleens, being careful not to puncture with your tweezers or scissors. Remove excess fat and connective tissue. Place in 2 mL cold complete RPMI 1640 media (1.1 Complete Media Recipe) and keep on ice.
  - For lymph nodes, store in separate tube with 2 mL cold complete media for each set and mouse.

3. Tissue Processing

- After harvest and return to lab, take a short break to set up biosafety cabinet space and ensure all plastics are labelled and ready to go. Drink some water, have a snack. Keep it going!
  - **Do you have enough media?** Estimate 1 L for 60 samples (40 spleens and 20 lymph nodes), including processing and standard ELISA+ELISPOT plating (2 mL per sample at final dilution).
- **Before processing**, block your ELISPOT plates with complete media at room temperature for 2+ h.
- Isolate cells into a 6-well plate using a 70 µm cell strainer with the flat, back side of a syringe plunger from 3 mL syringes. Do not touch the plunger with your gloved hands.
  - Open the syringe packaging from the syringe tip side. Remove syringe body while holding plunger within the packaging. Handle the plunger from just below the rubber seal (has slip agents on the seal) while keeping the flat end sterile for use as a pestle in the filter.
- *Optional for low yielding tissues*
  - Rinse the strainer with 3 mL complete media.
- Pipet up and strain 3–5 times. Transfer the solution into a clean 15 or 50 mL tube by pipetting through the same 70 µm strainer used above.
  - Our normal filter purchases are for 50 mL tube diameter but can be held at angle for 15 mL.
  - For transfer at end, you can minimize transfer loss by hold the pipette tip end flush with the filter then slowly move across as you dispense. This forces the slurry through the mesh.
- Centrifuge the cells at 350–500 ×g for 10 min at 4°C (swinging bucket only).
- Decant the tubes into a common waste container. Resuspend the pellet by dragging capped tubes along the grate back and forth for turbulent mixing. Make sure the entire pellet is resuspended.

    - Take care not to be too aggressive since the plastic wears and can break.
- For spleens only
    - Add 1 mL ACK buffer to lyse red blood cells for 2 minutes (perform in sets of 5 tubes).
        - Cap and mix by inversion. Remove or loosen caps when done.
    - Add 10 mL complete media and mix well (to quench the ACK buffer action).
- For lymph nodes or other, dilute to 2 mL as a starting point. Dilution strongly depends on size and number of lymph nodes. No ACK treatment is required for lymph nodes.
    - Lymph nodes are now ready for counting!
        - You do not need to dilute before counting on the Moxi Z system for lymph nodes… usually.
        - Start at 1:10 dilution if needed.
        - If count is too low, spin down and dilute as needed.
        - It helps to have someone else processing lymph nodes and count separately
- Continuing with spleens… centrifuge at 350–500 ×g for 10 min at 4 C.
    - Decant the tubes into a common waste container. Resuspend the pellet by dragging capped tube along the grate. Make sure the entire pellet is resuspended.
        - Some particulates may remain. This is mostly fat aggregate.
    - Add 2 mL complete media and mix well.
- Put the spleen cell suspension (~2 mL) through a 40 µm filter to get rid of extracellular matrix/fat aggregates.
    - Again, place the tip flush to mesh to fully push your suspension through without it pooling.
    - *Optional* wash with 3 mL fresh RPMI media through the strainer.

4. Assay Preparations

- You may now count the cells using the cell counter (see above note for LNs df).
- For count preparation, dilute cells at 1:40 dilution in non-TC 96-well plate (i.e., 195 µL media + 5 µL slurry).
    - *Note*, wipe pipettors and use filter tips to minimize cross-contamination.
    - You should expect anywhere from 50–150 million splenocytes per mouse.
    - ELISA and ELISPOT plating will use 100 µL/well of cell seeding.
        - Keep suspensions on ice.
        - Flu crew commonly prepares 2 mL tube preparations at 10×10^6 cell/mL (**1×10^6 cell/well**)
            - For small sample number, the electronic repeater is useful with a slower dispense flow rate
            - The semi-electronic (‘manual’) repeater is too fast and will easily splash over
        - A 2+ mL 96-deep well plate or two can be very useful as a template for ELISA and ELISPOT seeding/transfer. Use this approach for larger sample numbers.
            - Use a lid and keep on ice until ready to seed plates.
            - Dilute suspensions to desired density in well then briefly mix and dispense.
- Dispense cells to ELISA (n=2+ technical replicates) and ELISPOT plates (n=1+ technical replicates).
    - Reminder, each mouse is a biological replicate for any given treatment. Anything else is a technical replicate in the strictest interpretation.
    - ELISPOT plates are seeded at two densities:
        - 2×10^6 cell/mL and 10×10^6 cell/mL or alternatively,
        - 2×10^5 cell/well and 1×10^6 cell/well (200k or 1 million cells per well!)
    - ELISA plates are only seeded at the higher density.

        - All plates should have media only controls and ConA (positive) controls. Pool vehicle or PBS groups for positive controls before seeding.
        - Lymph nodes are not always very high yielding so, you can usually plan to only include them in plates designated for ELISAs only. Pool lymph node vehicle or PBS groups for positive controls separately.
        - Once seeded, mix gently (tilting/rocking North-South-East-West) and thoroughly for incubation.
            - Mix after finished seeding in biosafety cabinet
            - Mix again after walking plates over to the incubator (e.g., open the door, place well plates inside, then rock your plates in sets of 3 to 5 while keeping the door mostly closed or lightly latched).
- Prepare treatments for antigen recall experiment (100 µL/well).
    - i.e., OVA vaccination can include:
        - cell-free, media only
        - unstimulated cells: media-only
        - stimulated (10 µg/mL): full OVA, MHC-I (OVA 257–264) SIINFEKL, and MHC-II (OVA 323–339)
        - positive control, ConA at 5 µg/mL
    - Most non-OVA antigen recall experiments will probably only have one antigen (stimulated) and media (unstimulated) – two treatment groups.
    - *Note1* for COBRA HA, we typically use 10 µg/mL final well concentration for stimulation, which can be resource intensive.
    - *Note2* account for df=2, for stimulating at 10 µg/mL, you will need working stocks at 20 µg/mL.
    - *Note3,* quoting previous protocol here regarding full OVA treatments:
        - Cells can be activated and produce cytokines even without antigens present. (media reference)
        - Also, use endotoxin-free (tested) materials for stimulation.
        - OVA (257–264) SIINFEKL will be the CD8 epitope
        - Full OVA can be used for “CD4” but in my opinion is actually both CD4 & CD8, and
        - The immunodominant MHC-II peptide for CD4 which is OVA (323–339) ISQAVHAAHAEINEAGR.
- Dispense your materials for antigen recall at 100 µL/well (final 200 µL/well):
    - Suggestions:
        - Reagent reservoirs are particularly useful for this step. Use with multichannels.
        - Avoid bubbles
        - Use filter tips to prevent cross-contamination. This is a valuable experiment, use filter tips to guarantee no foreign stimulant contaminates your samples (keep it endo-free).
    - Antigen recall treatment group reminder:
        - Unstimulated, media only (all mouse samples)
        - Stimulated with antigen (all mouse samples)
        - Positive control with ConA (pooled vehicle treated mice)
        - Negative control, media only (200 µL/well media final)
- Mix gently and place well plates inside a reserved or low traffic incubator (≥95% RH, 5% CO_2, 37°C).
    - Stack only one or two plates high. Minimize vibrations or moving during stimulation period (typically 36 h). Any extra movement will blur your ELISPOT spots and worsen the imaging data quality.
    - The 36 h stimulation period is *slightly* flexible but do not go outside of an hour or so. Time your stimulation start and ask yourself, could I reasonably arrive here 12 h from now… 36 h from now?

    - If unreasonable (too early, too late, parking situation, etc.), adjust so you can shoot for between 7 to 9 AM (stimulating at 7 to 9 PM beforehand). This is reasonable if you like to work 9–5 shifts.
- Refer to the BD manufacturer's ELISPOT protocol for additional information.
    - JR has included some technical notes or lab-specific suggestions in the Teams folder for antigen-recall.