Preparing for a metabolomics experiment using tissues

General considerations for stable isotope (e.g. C-13, N-15, H-2 (D)) labeling:
- the minimum labeling duration depends on the metabolic pathway of interest and the tissues of interest.
- you can label animals through i.p. or i.v. injection (bolus) or over time through a catheter into the tail vein, for instance
- optional: use a positive/input control, i.e. blood plasma, to verify that the tracer has gone into the blood stream

Polar metabolites extraction from tissues

Decide the best way to homogenize your tissues:
(I) pulverize the tissue in liquid nitrogen (LN2) using a mortar and pestle or a hammer (see below)
(II) use a bead beating method.
Practice this beforehand so that you know it will work for your tissue types and what challenges there might be!

1. If desired rinse tissue in ice-cold 150 mM NH4OAc pH 7.4\(^{[1]}\) to remove any blood. Use about 20 mg freshly obtained tissue (when using pestle & mortar with LN2; you may be able to use less with other devices).

   Aim for a very similar input amount for all samples! When weighing the tissue pieces, cool the Eppendorf tube on dry ice, place it on the mini scale, zero the scale, add the tissue and record the weight to one decimal (i.e., 17.2 mg).

   Flash-freeze the tissues in LN2. Frozen tissue can be stored at -80 °C for extraction at a later time.

2. Homogenization

   This can be done various ways, depending on what instrumentation you have:
   I. Homogenization with a suitable homogenizer probe like the Kinematica Polytron TP1200E (Fisher 05-400-261 & 05-400-264) in cold 80% MeOH (-80°C) on dry ice
   II. Bead beating with cold 80% MeOH (-80°C)
   III. Pulverization of tissues in liquid nitrogen (WEAR PROPER PPE!)

   There are several ways:
   i. Using mortar and pestle (use about 20 mg)
      • Precold a mortar, pestle, and spatula in a container with LN2 in it.
      • Remove the mortar and pestle from the container and place on the bench. Pour a small volume of LN2 from a clean dewar into the mortar. Place the frozen tissue piece in the LN2 in the mortar, and let cool for 1 min to get brittle. Then carefully pound and grind the tissue into a fine powder. With a precooled spatula, scrape the powder into a heap and transfer to an Eppendorf tube sitting on dry ice. Work quickly so that the mortar, pestle, and spatula do not get the chance to warm up.
• Clean the mortar, pestle, and spatula in between samples by rinsing with 100% methanol and wiping them clean with large Kimwipes.
• Precool the mortar, pestle, and spatula again before proceeding with the next sample.

ii. **Using aluminum foil, a cold hard surface and a hammer** (can potentially use less than 20 mg)
• Precool a thick piece of metal and the end of the hammer on dry ice.
• Place the frozen tissue piece on a small piece of heavy duty aluminum foil (pre-cooled) and wrap it closed.
• Place this in a dewar with LN2 for a minute or two (longer if the tissue was not frozen).
• Remove from LN2 and place on the cold metal surface on dry ice. Open the wrap (or keep closed?) and use the hammer to pulverize.
• Close the wrap and put it back in LN2 for 1 minute, then transfer the ground tissue into a new microcentrifuge tube sitting on dry ice.
• Clean the hammer with 100% MeOH between samples.

iii. **Using a device like the Cellcrusher**: [https://cellcrusher.com/](https://cellcrusher.com/)

3. To the with LN2 pulverized tissues, add 1 ml **80% MeOH (at -80°C/dry ice temperature)** and vortex vigorously for 20 sec.

4. Keep the samples for a minimum of 20 min on dry ice or at -80°C to aid proper quenching and protein precipitation.

5. Optional: Extraction may benefit from one or more freeze-thaw cycles if the homogenization was not 100% efficient. If using LN2, then you will need to use cryovials!

6. Vortex the samples again for 20 s.

7. Spin the samples at top speed (16,000 g) for 15 min @ 4°C.

8. Transfer the entire supernatant to a new Eppendorf tube (or glass vial) and place on ice. Keep the tubes with the remaining pellets on ice as well.

   Optional: Re-extract the pellet with another 0.2 ml cold 80% methanol (-80 °C); spin 15 min at 4°C, and add the supernatant to the first extraction volume.

9. Resuspend the sample pellets in 10 pellet volumes 0.1-0.2 M NaOH (with optional heating at 95°C for 10 min) and determine the protein concentration (by BCA or Bradford methods). Then calculate the total protein content for each sample.

   *Note: Resolubilization can be done with protein lysis buffers, but 0.1-0.2 M NaOH resolubilizes the pellet much better. An overnight incubation at 4 °C may be required to aid complete solubilization when using standard protein lysis buffers! If choosing the latter, store the extracts at -80 °C in the meantime.*
10. Transfer equivalent amounts of the extracts – i.e. 50-100 µg protein equivalent – into either flat bottom glass vials (if using a Genevac) or new Eppendorf tubes (if using speed vac):
   - I.e., the total protein amount of sample #1 = 400 ug.
   - For 100 ug protein equivalent, use 100/400 x 1.0 ml (the total volume of the extract #1) = 250 ul of extract #1.

11. Make all the sample volumes the same by adding fresh 80% methanol to those samples with a smaller sample volume.

12. Dry the samples using the Genevac EZ-2 Elite evaporator at 30 °C using program 3 (aqueous) or use a speed vac.

13. Store the dried samples at -80 °C until ready for LC-MS analysis.

(1) **150 mM ammonium acetate solution, pH 7.4:**
Dissolve 1.1562 g ammonium acetate (Molecular Biology grade) per 100 ml of Millipore Milli-Q water (18 MΩ) to make a 150 mM solution.
Adjust the pH to 7.4 using a few drops of 1 M NH4OH (i.e., a 1:11 dilution of a 21% NH4OH stock solution).
If stored at room temperature, sterilize the solution using a Stericup Vacuum Filter Cup (i.e. Millipore Corp).

Useful reference:
Lu et al, 2018: Metabolite Measurement: Pitfalls to Avoid and Practices to Follow
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5734093/
Equipment, materials and reagents needed for this protocol

- round bottom cryovials, 2 ml:
  - 03-374-22 FisherScientific Corning Internally Threaded Cryogenic Vials
- Eppendorf centrifuge tubes
- dry ice and ice
- MeOH: A456-1 FisherScientific Fisher Methanol (Optima* LC/MS)
- H2O: W5-1 FisherScientific Water, Glass Bottle; 1L
- Norvaline: N7502-25G Sigma DL-Norvaline
  Used as an internal standard: prepare a 100 mM solution in H2O. Make a 10 mM working stock in MeOH. Store both at -20C.
- glass vials: 13-622-351 FisherScientific 1.8 mL Volume; Clear Glass, 12x32 mm, 9 mm thread
- caps: 03-379-123 FisherScientific Rubber/Silicone Septa
  Alternatively: American Chromatography Supplies
  - glass vials: VT009M-1232 ACS 1.8 mL Volume; Clear Glass, 12x32 mm, 9 mm thread
  - caps: C395E-09SB ACS Bonded PTFE/Silicone Septa
  - caps: C394-09SB ACS Bonded PTFE/Rubber Septa

C-13- and N-15-labeled metabolites (from Cambridge Isotope Laboratories if not otherwise stated)
- U13C Glucose: CLM-1396-1 1 g