Extraction of metabolites from adherent tissue culture cells

Preparing cells for a metabolomics experiment
- Plate 2e5-5e5 cells per well in 6-well plates, or up to 1e6 cells in a 60 mm dish. Target confluency at the time of extraction is typically 70-80%. Aim for a similar cell numbers (or similar confluency when cell types are different in size) per well/dish at the time of extraction!
- Prepare at a minimum 3 wells/plates per condition for LC-MS analysis.
- Prepare additional wells/plates per condition for cell counts at the time of harvest; AND/OR determine the protein/DNA content of the sample pellets after extraction. The cell count or protein/DNA content can be used for slight normalization of the input amounts.
- Incubate o/n.

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. Metabolites in the glycolysis pathway take roughly 30 minutes to reach isotopic steady-state for many cell types, while other metabolic pathways (e.g. nucleotides) might take 24-48 hours.
- plate the cells in regular medium containing dialyzed FBS (check beforehand if this affects your phenotype!).
- 3-24 h before extraction (depending on your labeling time), switch to medium containing dialyzed FBS and the isotope tracer that is replacing the normal nutrient in the medium (i.e., 13C6 glucose instead of normal glucose).
A volume of 1.5-2.0 ml is sufficient to cover the cells in a 6-well plate for 24h, but keep in mind that the cells might deplete the labeled nutrient when long labeling times are used! Replacing part (i.e. 1/3th) of the medium with fresh medium 3-6 hours prior to extraction may be required.

Polar metabolites extraction with 80% MeOH
You can also use MeOH, ACN, and H2O in a 40:40:20 ratio. This might be better for extraction of some less polar metabolites like fatty acid precursors. If using this, work at -20 °C rather than -80 °C.

Keep plates in incubator until ready for extraction. Work in small batches as needed and is manageable, i.e. one 6-well plate at the time!

1. Place the plate(s) on ice. Aspirate the medium.
2. Rinse the cells quickly with ice-cold 150 mM NH4AcO, pH 7.3 and aspirate.
3. Place the plate on dry ice (or on a pre-cooled metal pan turned upside down over dry ice).
4. Add 1 ml [80% MeOH/20% water] precooled in -80 °C freezer – keep on dry ice while processing the plates.
5. Optional: add 1 nmol norvaline to each sample as an internal standard
   - prepare a fresh 1:50 dilution in H2O from a 10 mM stock solution (see below)
   - add 5 ul of the freshly prepared 0.2 mM solution to each well/plate.
6. Incubate the plate(s) in the -80 C freezer for 30-60 min to aid proper quenching and protein precipitation.
7. On ice (or dry ice), scrape the cells from the plate.
8. Transfer the entire content of the well into a prepared Eppendorf tube placed on ice.
10. Centrifuge at top speed (16,000 g) for 15 min at 4 °C.
11. Transfer supernatant into a glass vial if using a Genevac EZ-2 Elite evaporator (or high quality Eppendorf brand centrifuge tubes if using a speed vac).

12. Usually not required and thus optional: do a 2nd extraction of the pellet
- resuspend the pellet in i.e. 200 µl 80% MeOH and vortex to break up the pellet
- centrifuge at top speed for 10 min at 4 °C; add the supernatant to the same glass vial as the 1st extraction volume.

13. Keep the centrifuge tubes with the pellets if normalization by protein content is desired. Briefly air dry the tubes to remove any remaining 80% MeOH. Resuspend the pellets in relatively large volumes of 0.2 M NaOH, i.e. 100 - 200 ul, of and heat for 20 min at 95 °C. Flick tubes to ensure complete resuspension of the pellets. Cool to RT. Spin the tubes for 5 min at top speed. Determine the protein content of the supernatant using the BCA method, using an i.e. 1:10 dilution of the samples. If desired, instead of protein content, the DNA content can be determined.

14. Dry the metabolite extracts down without heat in an evaporator – i.e., the Genevac EZ-2 Elite using program 3 (aqueous) or a speed vac. Choose an appropriate drying time for your sample volume and remove the samples promptly when the program is finished.

15. Store the dried extracts at -80 °C.

(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 uΩ) 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/

Supplies needed:
- Ammonium acetate A1542-500G FisherScientific molecular biology grade, ≥98%
- MeOH: A456-1 FisherScientific 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 Fisher Scientific Thermo Scientific™ Chromacol™ GOLD-Grade Inert Glass Vials; Thermo 2SVWGK
- caps: 03-379-123 Fisher Scientific 9 mm autosampler vial screw thread caps (PTFE,Silicone) / Thermo Scientific C500054A

C-13- and N-15-labeled metabolites, 99% pure (from Cambridge Isotope Laboratories or Sigma)
- U13C Glucose: CLM-1396-1 1 g