**Flow Cytometry Protocol**

Flow cytometry protocol with modifications based on Roberts, Gladis, & Brumme (2009) and Loureiro et al. (2007); from Pelosi et al., (in review, Appendix 1). Developed and modified by Bethany A. Zumwalde.

1. Place leaf material in petri dish. The amount of tissue is group specific and rather subjective. Estimate by eyeballing “the size of a pinky nail” – aim for 0.25g fresh weight tissue. ***Note:*** This may be increased to improve reads, if necessary. If desired, include standard tissue to be co-chopped. A smaller amount of standard (relative to sample) may be used to prevent the standard signal from swamping out the sample.
2. Place the petri dish with the samples on a cold brick, which will serve as a cold chopping surface, or in a dish of ice.
3. Add 1000µl cold lysis woody plant buffer (Loureiro et al., 2007) to the petri dish and chop tissue with a single-edged razor blade for 60 seconds. Retain the pipette tip from this step to mix the sample later. ***Note:*** Again, the amount of chopping (fine vs. course) may be adjusted depending on the tissue. Finer chopping may result in more noise. Use a new razor blade for each sample. Do not over-chop!
4. Swirl the chopped material in the lysis buffer for approximately 20-30 seconds until a green tint appears on the liquid.
5. Remove the end of the retained pipette tip with the razor blade and use the larger opening to mix the material by pipetting up and down a couple of times.
6. Filter 800µl of suspension through Falcon® 5 mL Round Bottom Polystyrene Test Tube with 35µm nylon mesh strainer cap and keep on ice.
7. Transfer 500µl of filtrate to a new 1.5mL Eppendorf and add 2.5µl of RNase A (1-10 mg/mL). Incubate for 10 minutes.
8. Add 6µl PI stock solution for 500µl of filtrate (for an estimated final concentration of 50µg/mL) and cover tubes with foil to protect from light. Incubate on ice and analyze within 10 minutes for fresh tissue or keep on ice and protect from light; dry tissue may require incubation for 20-30 min.
9. Run on Accuri C6 flow cytometer (see Galbraith Cytometry Part A. 2009. 75A: 692-698).