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FLOW CYTOMETRY PROTOCOL

This method is based on Roberts, Gladis, and Brumme. 2009. Plant Cell Rep. 28: 61-71

For 500 ml lysis buffer (this should be stored at 4C):
50 ml [1M] citric acid
2.5 ml Triton X-100
442.5 ml D.I. water
5 g P.V.P.- listed in original protocol as optional, but has now become standard

For 10 ml P.I. staining solution (for ~26 samples- prepare the same day):
5 ml [0.8M] Na_2PO_4
1 ml [100mM] sodium citrate
1 ml [250mM] sodium sulphate
2922 ul D.I. water
78 ul [1.3 ug/ul] propidium iodide (P.I.)
wrap in foil after preparation

Other supplies:
RNase A
BD Falcon tubes with 5 ul mesh cell strainer cap, 5ml polystyrene round-bottom (ref 352235, 25/pack)
Standard
Petri dishes, single edged razor blades, cold bricks (if hand-chopping)

Bead Mill version:

1) In advance, book the flow cytometry machine and prepare the lysis buffer and staining solution.

2) Place ~4mg of silica-dried leaf material and 2-3 zirconia beads in a 1.5 Eppendorf tube. Material must be fully dried, otherwise it will not fragment. A similar amount of standard can be included and co-chopped, e.g. 3 mg pea.

3) Grind samples for 3 seconds in bead mill (or see below for hand chopping notes). Material must be finely chopped, rather than powdery. Do not over-grind. If material is not sufficiently fragmented, grind for another 3 seconds and check.

4) Place each tube on ice and add 500 ul (up to 1000) cold lysis buffer.

5) Filter suspension through cell culture tube and keep on ice.

6) Transfer 140 ul of filtrate to a new 1.5 ml Eppendorf and add 1 ul of RNaseA (1mg/ml to 10mg/ml). Optional step: incubate for 30 min.

7) Add 350 ul P.I. staining solution and cover tubes with foil to protect from light. Incubate for a least 1 hr at room temperature.

8) Run on flow cytometer. Soltis lab at UF uses the Accuri C6* flow cytometer (see Galbraith Cytometry Part A. 2009. 75A: 692-698).

Hand Chopping version:
Hand chopping results in fewer fragmented cells and less noise. While this method takes longer, it is preferable for older and/or degraded tissue.

1) In advance, book the flow cytometry machine and prepare the lysis buffer and staining solution.

2) Place silica-dried leaf material in half of a petri dish.
The amount of tissue is group specific and rather subjective. Estimate by eyeballing "the size of a pinky nail." 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. For example, try using ¼ the amount of pea, compared to the amount of silica-dried sample.

3) Place the petri dish with the samples on a cold brick, which will serve as a cold chopping surface.
Add 500 ul (up to 1000) cold lysis buffer to the petri dish and begin chopping with a single-edged razor blade. Retain the pipette tip from this step to mix the sample later.
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.

4) Swirl the chopped material in the lysis buffer for ~20-30 seconds until a green tint appears to 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 suspension through cell culture tube and keep on ice.

6) Transfer 140 ul of filtrate to a new 1.5 ml Eppendorf and add 1 ul of RNaseA (1mg/ml to 10mg/ml). Optional step: incubate for 30 min.

7) Add 350 ul P.I. staining solution and cover tubes with foil to protect from light. Incubate for a least 1 hr at room temperature.

8) Run on flow cytometer. Soltis lab at UF uses the Accuri C6* flow cytometer (see Galbraith Cytometry Part A. 2009. 75A: 692-698).

*The University of Idaho uses a BD FACSAria sorting cytometer. UI has nozzle sizes at both 70 and 100 microns and has red and blue lasers. Contact Ann Norton in the Optics Core for an introduction to the machine. Book machine through ilabs account.

Running the flow cytometer
(some notes from April 2014 visit to UF)

The BD Accuri C6 cytometer routinely uses a nozzle of 150-200 microns
Speed: start slow. If it looks good (i.e. low noise relative to peak), can increase to med or fast. Count at least 10,000 events.