Supplement 2 to the article: Skaptsov M.V., Kutsev M.G., Smirnov S.V., Vaganov A.V., Uvarova O.V., Shmakov A.I. "Standards in plant flow cytometry: an overview, polymorphism and linearity issues"

Protocols and reagents

There is no strict protocol in cytometry. In many respects, everything is species specific and often for a new plant group it is necessary to empirically select a buffer, sample amount, components and other conditions. In most of the work, Otto buffers, Tris-$MgCl_2$ and LB01 buffers are used. It is worth making sure that the sample is not insect infested. For example, whitefly eggs give a peak of about 0.5 pg and the insects themselves give a peak of about 1 pg.

Protocol using Tris-$MgCl_2$ buffer (Prosser et al., 1995) or LB01 buffer (Doležel et al., 1992). Simultaneous extraction and staining.

- Prepare a working buffer for nuclear extraction according to the number of samples.
- Add 5 µl PI (final concentration 50 µg/ml) or 4 µl DAPI (final concentration 4 µg/ml), 5 µl RNase (final concentration 50 µg/ml), 10 µl/ml sodium thiosulfate per 1 ml extraction buffer (e. g. for 20 samples take 20 ml buffer, add 100 µl PI, 100 µl RNase and 200 µl sodium thiosulfate). Plus 1 % PVP for woody plants.
- Place a small amount (1–2 cm^2) of the plant material in a petri dish and add 1 ml of buffer. Chop with a razor blade (sandwich-like, with short cutting movements, in a plastic Petri dish). Filter through a 50 (30) micron nylon filter or equivalent. Use a cut-off tip to collect a sample from a Petri dish.
- Incubate for 5–15 minutes at room temperature.
- Examine relative DNA content using a green laser (532 nm) / blue laser (488 nm) for PI or a UV lamp (365 nm) / DAPI as light source.

Notes: - The prepared staining buffer can be used in larger or smaller amounts depending on the amount of sample and the cytometer. For example, on Cytoflex, we chop the sample in 0.7 ml of buffer, on Partec – 1.5 ml.

- Do not take too much material; exogenous nucleases, metabolites and pigments can interfere with the analysis. The amount of sample is species specific, and the amount selected for cytometry depends on the cell density in the same volume of tissue.
- Typically, 1–2 minutes are required to stain the sample. Incubation is required for RNA digestion. An average of 15 minutes at room temperature is usually sufficient. Incubation on ice for 30 minutes is also possible. Incubation for 15 minutes at 37–42 °C may be helpful for difficult samples with metabolites, high RNA content or poor histograms with noise and high CV. Other organs and tissues should be used

instead of leaves (petioles, stems, petals, etc.) and seeds or seedlings if the quality of the histograms does not improve.

- Instead of sodium thiosulphate, 2 µl/ml mercaptoethanol, up to 10 mM DTT or 10 mM sodium metabisulphite can be used. Sodium thiosulphate is convenient because it has a long shelf life, is odorless and is available in vials from any pharmacy. Stock solution of sodium thiosulphate pentahydrate 300 mg/ml (1.2 M). It is useful to add 1 % **polyvinylpyrrolidone** when working with woody plants.

Protocol with Otto buffers (Otto, 1990). Extraction and staining in separate buffers.

Nuclear extraction

- Place a small amount (1–2 cm^2) of the plant material in a petri dish.
- Add 500 µl of cold extraction buffer (Otto I). Chop with a razor blade.
- Filter through a nylon filter with a pore diameter of 50(30) microns or similar. Centrifuge the suspension at 150–200 g (1000–1500 rpm) for 5 minutes.
- Carefully resuspend the pellet in 200 µl Otto I. Leave on ice for 10 minutes and up to 1–2 hours. Or proceed directly to staining.

Nuclear staining

- Prepare the staining buffer by adding 10 µl PI (or 8 µl DAPI), 10 µl RNase, 20 µl sodium thiosulphate to 1 ml Otto II buffer (based on the calculation that Otto I is diluted 2-fold). For example, for 20 samples, take 3 ml Otto II buffer, add 15 µl PI, 15 µl RNase, 30 µl sodium thiosulphate).
- Add 200 µl staining buffer to the isolated nuclear suspension.
- Incubate for 15–30 minutes at room temperature.
- Examine the relative DNA content using a green laser (532 nm) / blue laser (488 nm) in the case of PI with a 610 nm filter (ECD channel). Or use a UV lamp (365 nm) in the case of DAPI as a light source with a 465 nm filter.

Note: 1. This protocol has been optimized by us for cytometers with a peristaltic sampler (Cytoflex and others). If you are using Partec, the volumes should be larger; at the post centrifugation stage, resuspend the nuclear pellet in 750 µL Otto I and add 750 µL Otto II staining buffer.

2. There are many other modifications of the protocol with Otto buffers: without centrifugation or with a different ratio of Otto buffers (one to one, or one volume of Otto I and two volumes of Otto II). If the ratio of one volume of Otto I to two volumes of Otto II is used, the concentrations of dye and RNase must be recalculated. For example, for 1 ml of Otto II buffer, use 7.5 µl PI, 7.5 µl RNase and 15 µl thiosulphate.

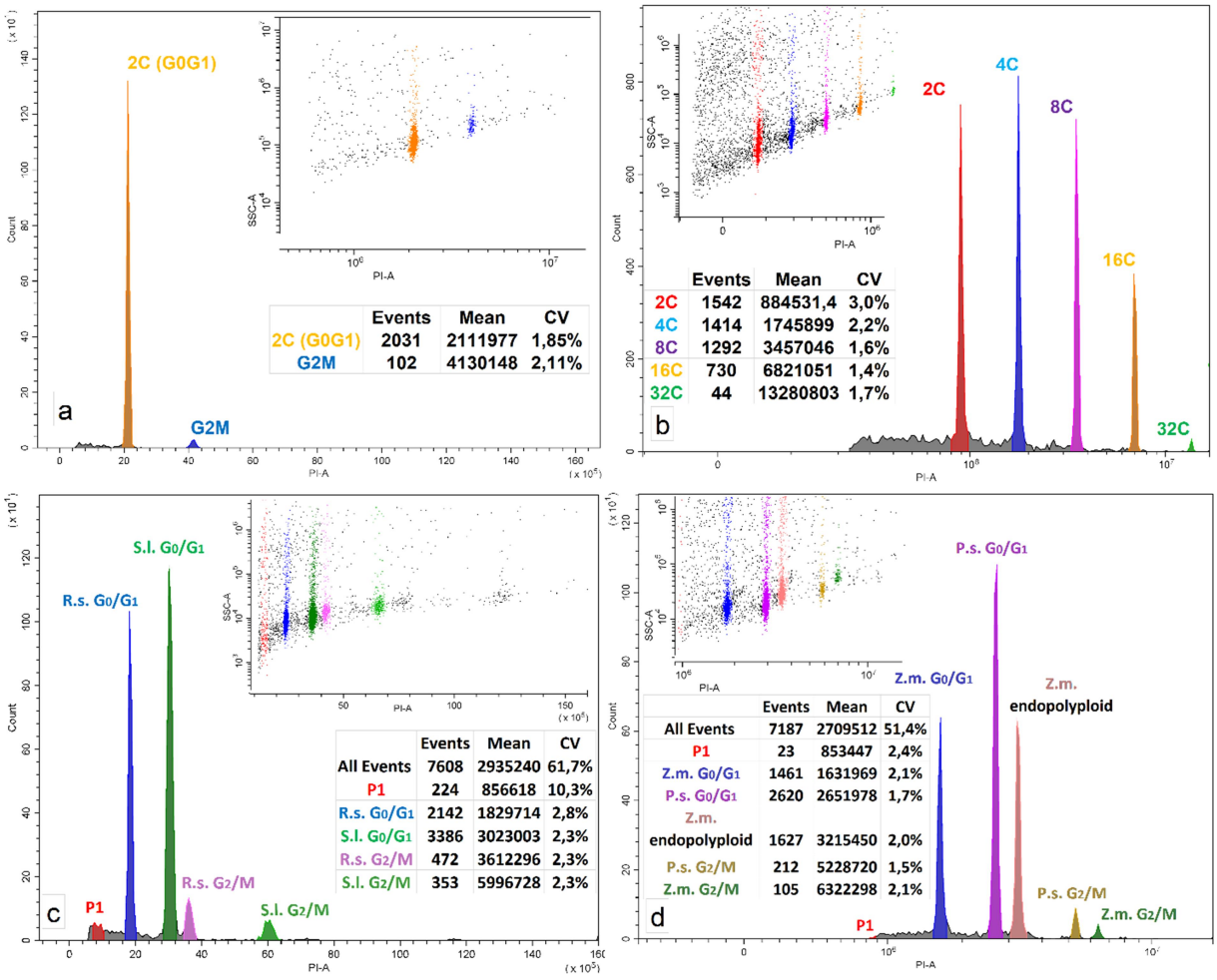

Figure 1. Examples of ungated histograms: a – Typical histogram for a living sample, *V. faba*; b – Typical histogram for an endopolyploid sample, *Phalaenopsis sp.*; c – Histogram on a linear scale, internal standardization of *R. sativus* and *S. lycopersicum*; d – Histogram on a logarithmic scale, internal standardization of *Z. mays* and *P. sativum*. Basic requirements for histograms. From the histograms it is clear that: internal standardization was used; the CV of the G_0/G_1 peaks is less than 3 %; there are more than 1000 nuclei in the main peaks used for DNA content calculations; the main G_0/G_1 peaks do not overlap and the G_2/M peaks and endopolyploid peaks do not overlap with them; the ratio of the number of nuclei of the main peaks (peak heights) is comparable, the differences are less than 2 times; there are no peaks in the P1 region, i.e. there are no nuclei 2 times to the left of the left peak.

Pulse of fluorescence

A pulse of fluorescence has three characteristics labelled -area, -height and -width. These characteristics refer to the pulse of fluorescence and characterize the pulse in terms of width (duration), height (intensity) and area (total fluorescence) (Pulse-W, Pulse-H, Pulse-A). Analyses can be performed in all three

characteristics and the data can be different. In plant cytometry, the pulse-a is the most commonly used. Since in other characteristics linearity is lost. Figure 2 shows an example of loss of linearity, increase in CV and data corruption at PI-Height.

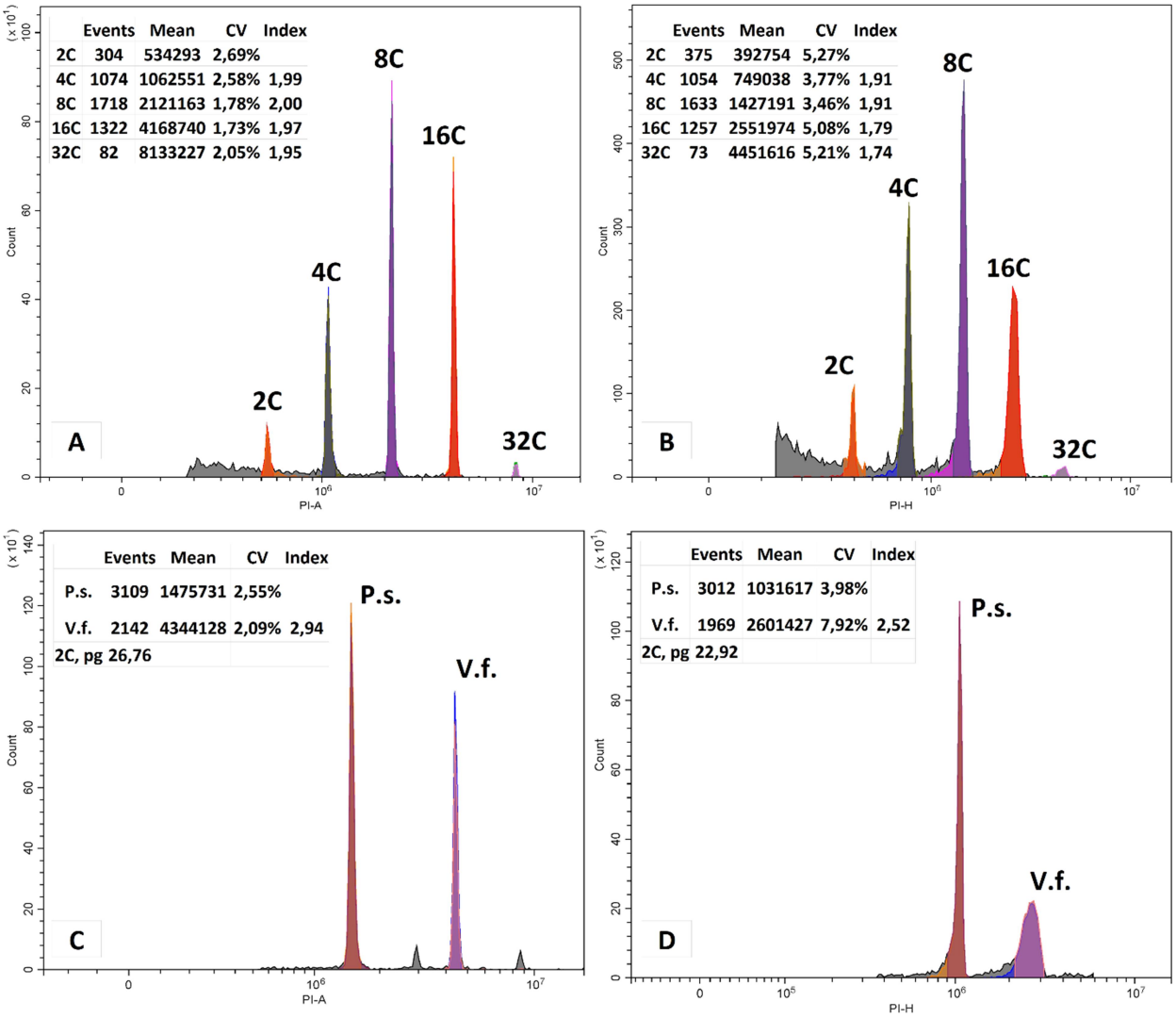

Figure 2. Examples of ungated histograms on PI-Area signal (a, c) and PI-Height signal (b, d). Logarithmic scale: a – endopoliploid nuclei of *Phalenopsis* sp. Peaks ratio (Index) is closer to 2 and CV is less than 3%; b – endopoliploid nuclei of *Phalenopsis* sp. Peaks ratio (Index) is less then to 2 and CV is more than 3%; c – measuring of the DNA content of *V. faba* 'Inovec' (26.9 pg) using *P. sativum* 'Ctirad' (9.09 pg) as an internal standard. CV less than 3 % and 2C-value closer to literature data; d – measuring of the DNA content of *V. faba* 'Inovec' (26.9 pg) using *P. sativum* 'Ctirad' (9.09 pg) as an internal standard. CV more than 3% and 2C-value much lower than literature data.

Principle of peak search

Create a one-parameter histogram on the channel that is optimal for propidium iodide (ECD, PI or others close to 610 nm). Ensure that all channels are open on the scale (e. g. Cytoflex has channels from 100 to 1,000,000 open by default, full scale more 16 million channels (24bit)). On Partec the channels are programmatically divided into bins (256, 1024 or more).

Set Gain and Threshold to minimum value.

Minimum flow rate.

Run the analysis.

Cut off the fast rising signal on the left part of histogram by increasing Threshhold.

Gradually increase the gain until peaks appear and reduce the noise with the Threshold until the peaks are comparable on the linear and logarithmic scalese.

Ensure that there are no peaks with 2 times less fluorescence by increasing the Gain or decreasing the Threshold, which is important if the sample is endopolyploid. Often the 4C peak of an endopolyploid sample can be confused with the 2C peak because the 4C peak may be higher than the 2C peak. Classically, samples have two peaks, the main peak and a peak corresponding to the G_2 stage of mitosis. Plants with endopolyploidy will have 3 or more peaks.

Generate a two-parameter histogram (dot plot) of side scatter/fluorescence on a logarithmic scale when a large number of noise or extraneous populations select positive signals using gating.

Buffers and components

Otto buffers (Otto, 1990)

Per 100 ml Otto I

0.1 M citric acid monohydrate	2.1 g
0.5 % Triton X-100 (originally Tween 20)	0.5 ml

Store at 4 °C.

Per 100 ml Otto II

0,4M $Na_2HPO_4 \times 2H_2O$	7.11 g

Store at room temperature. If $Na_2HPO_4 \times 12H_2O$ is used, crystallization may occur.

LB01 buffer (Doležel et al., 1992) per 100 ml.

15 мM Tris-HCl	236 mg
2 мM Trilon B ($Na_2EDTA \times 2H_2O$)	74 mg
0,5 мM spermine tetrahydrochloride	17.4 mg
80 мM KCl	596 mg
20 мM NaCl	116 mg
0.1 % Triton X-100	100 µl

Adjust the pH to 7.5 with 1M sodium hydroxide. Adjust volume to 100 ml. Store at –20 °C in 10–50 ml aliquots. Spermine can be replaced with spermidine at the same concentration.

Tris-$MgCl_2$ buffer (Pfosser et al., 1995) per 100 ml.

0.2 M Tris-HCl	3.15 g
4 мM $MgCl_2 \times 6H2O$	81 mg
0.5 % Triton X-100.	0.5 ml

Adjust the pH to 7.5 with 1M sodium hydroxide. Adjust volume to 100 ml. Store at 4 °C.

It is convenient to prepare 1M Tris-HCl (pH 7.5) and 1M $MgCl_2$ separately and prepare the required volume of buffer immediately before use.

DAPI stock solution (1 mg/ml, 250x) Preparation: Dissolve 1 mg of DAPI in 1 ml of deionized H_2O. Store at –20 °C in 200 µl aliquots. Add 4 µl per 1 ml of buffer.

Propidium iodide PI stock solution (10 mg/ml, 200x) Preparation: dissolve 100 mg propidium iodide in 10 ml 50 % DMSO. Store at –20 °C in 0.5 ml aliquots. Add 5 µl per 1 ml of buffer.

RNase A stock solution (10 mg/ml)

If the RNase is DNAse free grade: dissolve 100 mg RNase A in 10 ml deionized water. Store at –20 °C in 1 ml aliquots.

If RNase contains DNase impurities: dissolve 100 mg of RNase in 9 ml of sodium acetate buffer (10 mM, pH = 5.2), heat for 15 minutes at 95–100 °C (or in a boiling water bath), cool to room temperature and add 1 ml 1 M Tris-HCl (pH 7.5). Store at –20 °C in 1 ml aliquots. Add 5 µl per 1 ml of buffer.

Acetate buffer for RNAse, 100 ml

10 mM sodium acetate trihydrate	154 mg

Adjust the pH to 5.2 with glacial acetic acid (approx. 5–7 µl).

Sodium thiosulfate 1.2 M, 10 ml

Sodium thiosulfate anhydrous	1,897 g

Adjust volume to 10 ml.

Or use pharmacological sodium thiosulphate 300 mg/ml (1.2 M, pentahydrate) from pharmacy. Add 10 µl per 1 ml of buffer.

Polyvinylpyrrolidone (20 %), 100 ml

Polyvinylpyrrolidone	20 g

Adjust volume to 100 ml. Add 50 µl per 1 ml of buffer.

Sodium metabisulphite 1M, 10 ml

Sodium metabisulphite	1.90 g

Adjust volume to 100 ml. Add 10 µl per 1 ml of buffer.