

Estimation of nuclear DNA content in plants

Attune® Acoustic Focusing Cytometer

Introduction

Flow cytometry has been widely utilized as an expeditious and accurate method for the estimation of plant nuclear genome sizes (C-values). The flow cytometric approach releases nuclei through the homogenization of the plant materials using a simple chopping procedure. The homogenate is then filtered to remove large debris, stained with a DNA-specific fluorochrome, and subjected to flow cytometric analysis. The amount of fluorescence emitted by the individual nuclei provides an estimate of the DNA content, and this can be converted into absolute amounts by comparison to standards.

This application note will describe the preparation and staining of nuclei, setting up the Attune® Acoustic Focusing Cytometer for acquisition, and finally, acquiring and analyzing the data.

Materials

Plant material: fresh, intact leaves of *Arabidopsis thaliana* Columbia-1 (Col-1)

Galbraith's buffer (45 mM MgCl₂, 30 mM sodium citrate, 20 mM MOPS, 0.1% (v/v) Triton® X-100, pH 7.0)

1 mg/mL RNase A (Cat. No. AM2270)

1 mg/mL propidium iodide (PI, Cat. No. P21493)

60 x 15 mm plastic petri dishes

30 µm nylon mesh filters

Ceramic tile

Surgical carbon steel, single-edged razor blades

Attune® Acoustic Focusing Cytometer in the blue/violet configuration (50 mW laser at 405 nm and a 20 mW laser at 488 nm, Cat. No. 4445315)

Attune® Performance Tracking Beads (Cat. No. 4449754)

Preparation and staining of nuclei

Important: all manipulations and incubations must be performed on ice.

1. Place 50–100 mg of excised plant tissue into a 60 x 15 mm plastic petri dish sitting on a pre-chilled ceramic tile that is sitting on ice.
2. Add 750 µL of ice-cold Galbraith's buffer to the plant tissue (1.5 mL per 100 mg of tissue).
3. Chop the tissue with a fresh razor blade for 2–3 min (discard the razor blades after a single use).
4. Filter the homogenate to remove large debris with a 30 µm nylon filter, add 750 µL of ice-cold Galbraith's buffer to the dish, rinse the chopped tissue, and filter the suspension as before.
5. Treat the filtered homogenate with 10 µL/mL RNase A (1 mg/mL stock) for 10 min prior to staining.
6. Nuclear DNA is stained by adding 50 µL/mL of PI (1 mg/mL stock) for a final concentration of 50 µg/mL and then placing the suspension in the dark for 30 min before analysis.

Instrument setup for stained nuclei suspensions

Replace the standard 574/26 BP filter in blue laser channel 2 with a 603/48 BP filter.

Set collection rate to Standard 25 µL/min and acquisition volume to 50 µL. The acquisition volume can be changed before recording data to adjust for the number of events, time, or volume desired as the stop criteria for the analysis.

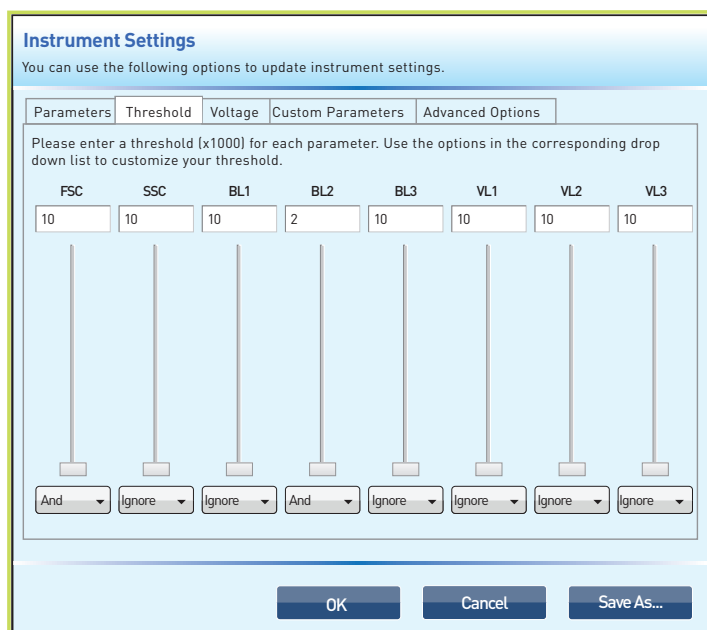


Figure 1. Threshold settings for FSC and BL2 for the experiment presented here. Note: adjustment of thresholds may be necessary.

Select the View tab and then select Instrument Settings. Select the Threshold tab and change the Boolean value under FSC to AND and the value to 10. Change the Boolean value on BL2 to AND and set the value to 2. Figure 1 shows thresholds set as described; note that adjustment of thresholds may be necessary.

With Instrument Settings still open, select the Voltages tab and use the following voltages as a starting point for your experiment (further adjustment will be necessary):

FSC: 2500
 SSC: 3000
 BL2: 1400
 BL3: 1200

Select the Insert tab to view the available plots. Create the following density plots and histograms for your data, making sure that SSC-A is set to logarithmic scaling.

Density plots:
 BL2-A vs. SSC-A
 BL3-A vs. BL2-A
 Histogram:
 BL2-A vs. Count

Begin collecting data, adjusting the SSC, BL2, and BL3 voltages and thresholds to place the nuclei on scale while eliminating noise and debris from the data. This is easiest

to gauge on BL2-A (PI-DNA fluorescence (603/48)) vs. SSC-A (side scatter) and BL3-A (PI-DNA fluorescence ≥ 640) vs. BL2-A (PI-DNA fluorescence (603/48)) density plots (Figure 2). Voltage and threshold settings are sample dependent and will vary. These settings should be optimized for each experiment. Note: Plots in Area will not show the sharp demarcation of the threshold cutoffs. Inserting a set of plots into the workspace using the Height parameter (e.g., SSC-H) will aid in setting voltages and thresholds.

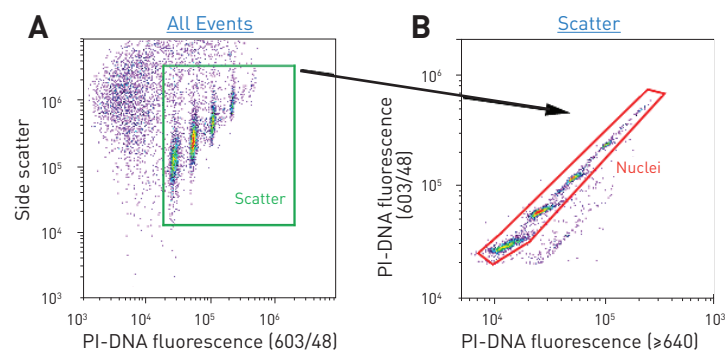


Figure 2. Acoustic focusing cytometric analysis of homogenates prepared from *Arabidopsis thaliana* Col-1 leaf tissue. (A) Biparametric density plot of side scatter vs. PI-DNA fluorescence with scatter gate surrounding the fluorescent nuclei. (B) Biparametric density plot of PI fluorescence (603/48 vs. ≥ 640 nm) of scatter gate population from panel A.

Acquisition and analysis

With the collection rate set to Standard 25 $\mu\text{L}/\text{min}$, change the acquisition volume to 200 μL . Acquisition volumes will depend on the stop criteria used for the analysis. For the data presented here, the stop criterion was set to collect between 5,000–10,000 events (gating for the data is explained below). To set the stop criteria, uncheck the boxes next to the volume and time stop criteria in the collection panel and check the event stop criteria; enter 5,000 as the number of events to be acquired, and select from the drop-down menu the desired gate the 5,000 events represent. Press the Run button to begin the acquisition and the Record button as events begin to show in the displayed plots to record data. The acquisition will stop when 5,000 events have been acquired (according to the set stop criteria). Select the Insert tab to view the Gating Tools. Gates are applied by selecting a plot on the workspace then selecting one of the gates from the Gating Tools ribbon bar and placing it around the population of interest (Figure 2). After gating the populations of interest, insert a Statistics box by clicking the Statistics box icon in

the Insert tab ribbon bar. This creates a global Statistics box. Selecting a plot and clicking the Statistics box icon in the Insert tab ribbon bar will create a Statistics box specific to that plot.

Results

Flow cytometric analysis of RNase-treated, propidium iodide-stained homogenates from *Arabidopsis thaliana* Columbia-1 using the Attune® Acoustic Focusing Cytometer is illustrated in Figure 2. A discrete series of five clusters was found, equally spaced along the abscissa corresponding to 2C, 4C, 8C, 16C, and 32C nuclei. The different clusters of nuclei exhibit broadly similar side-

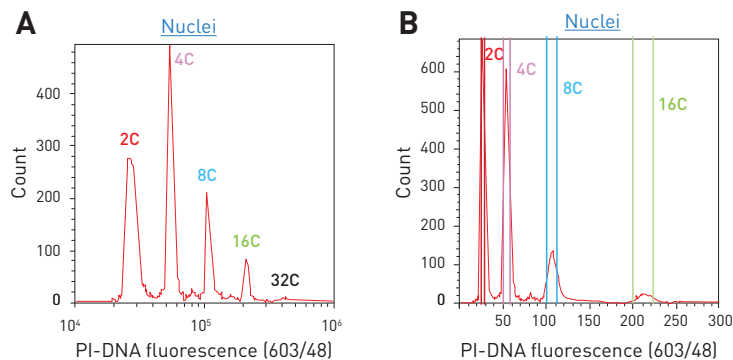


Figure 3. Acoustic focusing cytometric analysis of homogenates prepared from *Arabidopsis thaliana* Col-1 leaf tissue. (A) Logarithmic histogram of PI-DNA fluorescence (603/48 nm) of nuclei gate population from Figure 2B; (B) Linear histogram of PI fluorescence (603/48 nm) of nuclei gate population from Figure 2B. 2C, 4C, 8C, 16C denote the C-values for the respective peaks.

Name	Event Count	Mean X	% C.V.X
All Events	8,516	0	—
Scatter	5,255	66,856	85.84%
Nuclei	5,000	32,899	85.18%
2C	1,230	26,661	4.64%
4C	1,574	54,357	3.61%
8C	569	107,072	2.77%
16C	228	211,920	2.76%

Figure 4. Statistics of acoustic focusing cytometric analysis of homogenates prepared from *Arabidopsis thaliana* Col-1 leaf tissue. Note the incremental doubling of the mean fluorescence intensities of the 2C, 4C, 8C, and 16C peaks and the low associated %CVs.

scatter distributions, with side scatter scaling linearly with respect to DNA content ($n = 4$; r^2 of the means = 0.999996). It was therefore easy to distinguish the nuclei from general cellular debris and to gate only the nuclei to further analyze their fluorescence properties (Figure 2A).

Figure 2B shows the addition of a further gate to define the position of the nuclei, relying on division of the fluorescence emission of PI-DNA into two correlated portions corresponding to fluorescence centered around 603 nm and that produced at wavelengths longer than 640 nm. The resultant uniparametric DNA content distributions (Figures 3A and 3B) had mean fluorescence values of 2C = 25,770 ± 68, 4C = 51,493 ± 190, 8C = 102,803 ± 357, and 16C = 204,726 ± 1,199 ($n = 4$; mean ± SD; $r^2 = 0.999996$).

Conclusions

The *Arabidopsis* 2C nuclear DNA content (0.32 pg) falls toward the lower end of the range of 2C values reported across the angiosperms. *Arabidopsis* displays somatic endoreduplication, and flow cytometric analyses of the nuclear DNA contents of chopped somatic tissues display distinct peaks of fluorescence corresponding to 2C, 4C, 8C, and 16C nuclei. Since the DNA contents of these nuclei are correlated with the mean fluorescence intensity values of these peaks (Figure 4), *Arabidopsis* provides a convenient linear scale for measuring nuclear DNA contents in the range of 0.32–5.12 pg and a convenient model for demonstrating the dynamic range of the Attune® Acoustic Focusing Cytometer. The 32C nuclei (visible in Figure 3A) are measured to contain 5.12 pg of DNA, which is almost exactly the 50th percentile of the 2C values for angiosperms contained in the Royal Botanical Gardens Kew Plant DNA C-value database. Based on the instrument setup for this particular experiment, the highest bin value (8,388,608) would give a 2C value of 105 pg of DNA. Given the adjustable voltages for the PMTs on the Attune® Acoustic Focusing Cytometer, DNA estimates for plants much smaller than *Arabidopsis* 2C nuclei and much larger than 32C nuclei are possible.

Tips and tricks

Use a new razor blade for each plant sample to be chopped and perform all chopping and staining on ice.

Set SSC for logarithmic scaling. This aids in the identification of distinct populations for gating and data analysis.

Setting proper voltages and thresholds will maintain an event rate ≤100 events/second, minimizing the inclusion of coincidence in the data set and maximizing population resolution.

Prior to data acquisition by flow cytometry, checking samples by fluorescence microscopy will confirm that the nuclei are present and stained.

References

1. Galbraith DW, Harkins KR, Maddox JR et al. (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220:1049–1051.
2. Galbraith DW, Harkins KR, Knapp S (1991) Systemic endopolyploidy in *Arabidopsis thaliana*. *Plant Physiol* 96:985–989.
3. Galbraith DW (2009) Simultaneous flow cytometric quantification of plant nuclear DNA contents over the full range of described angiosperm 2C values. *Cytometry* 75A:692–698.
4. Dolezel J, Greilhuber J, Suda J (2007) Estimation of nuclear DNA content in plants using flow cytometry. *Nat Protoc* 2:2233–2244.