Using the Attune NxT Flow Cytometer for rapid and accurate analysis of nuclear DNA content in plants

Introduction

Nuclear DNA content and genome size values are important in studying characteristics of biodiversity and are useful in a variety of applications in basic and applied plant biology [1,2]. Fundamental to these studies is the C-value, defined as the amount of DNA contained in a haploid nucleus of a eukaryotic organism [3,4]. In eukaryotes, genome size is largely independent of organism complexity, and plant genomes are characterized by large variations in genome size and ploidy level [5,6]. Despite their importance, C-values are known for only a fraction of all plant species [4,7,8]. Providing a simple, fast, accurate, and reliable methodology, flow cytometry has become the method of choice to determine C-values in plant homogenates, and the use of flow cytometry in plant biology has increased rapidly [5-12]. *Arabidopsis thaliana* is a small, fast-growing plant that exhibits extensive endoreduplication, which consists of genome amplification in the absence of mitosis. This species contains the smallest genome of flowering plants at ~157 Mb with a known C-value of 0.321 pg [5,13-15]. These characteristics make *A. thaliana* particularly useful as an internal standard for genome size calibrations and may provide a means for quality control of instrument linearity.

Plant samples for flow cytometry are typically prepared from a small amount of tissue (Figure 1). The plant tissue is macerated in buffer using a standard razor blade. The homogenate containing plant nuclei is filtered to remove large particles, labeled with a DNA-specific fluorophore, and then acquired on the flow cytometer. The intensity of the fluorescence emitted by each of the individual nuclei provides an estimate of the DNA content. This intensity can be converted to absolute amounts by comparison to a known standard. *A. thaliana* is often used as an internal standard, where the nuclei of the standard and the unknown sample are isolated, stained, and analyzed simultaneously [4].

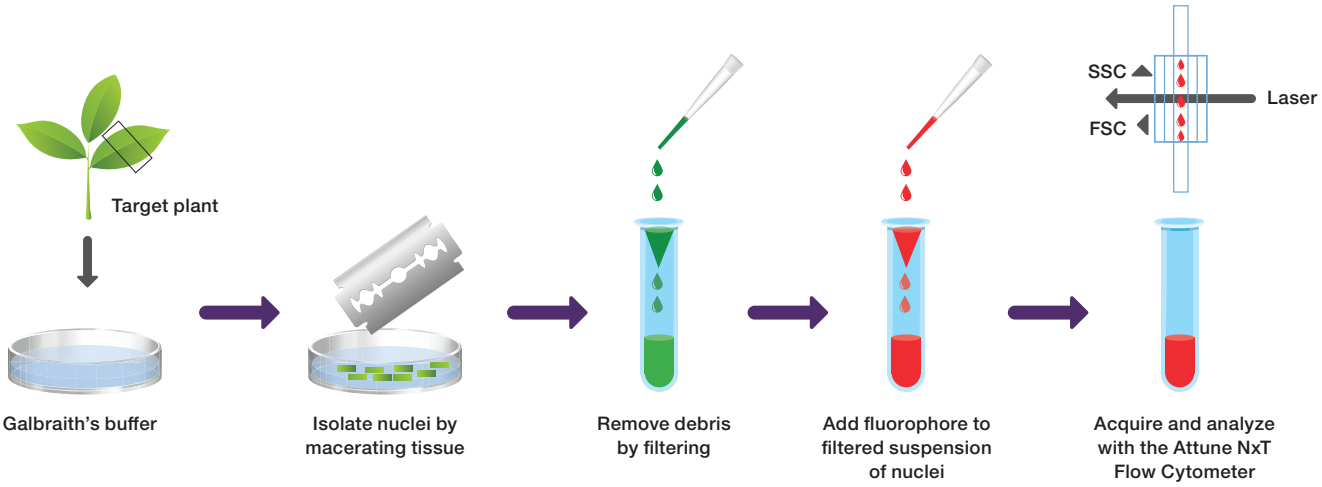


Figure 1. Overview of sample preparation.

The Invitrogen™ Attune™ NxT Flow Cytometer is ideally suited for DNA content evaluation. Any of the standard configurations may be used, including the most affordable single-laser system. This application note describes the preparation and labeling of plant nuclei using Invitrogen™ FxCycle™ PI/RNase Staining Solution with analysis using the 488 nm, 532 nm, or 561 nm excitation laser, or FxCycle Violet Stain with analysis using the 405 nm excitation laser (Table 1).

Table 1. Filter configurations compatible with the indicated DNA-binding dyes.

Product	Laser (nm)	Attune NxT channel	Attune NxT bandpass filter (nm, configuration dependent)
FxCycle PI/RNase Staining Solution (propidium iodide)	488	BL2	574/26, 590/40, or 620/15*
	532	GL2	620/15*
	561	YL2	620/15*
FxCycle Violet Stain (DAPI)	405	VL1	440/50 or 450/50*

* Data shown in this application note use these bandpass filters.

Materials

- Plant material: fresh, intact leaves of *A. thaliana* Columbia-1 (Col-1), obtained from Oregon State University Department of Horticulture
- Galbraith's buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% (v/v) Triton™ X-100, pH 7.0)
- FxCycle PI/RNase Staining Solution (Thermo Fisher Scientific Cat. No. F10797)
- FxCycle Violet Stain (Thermo Fisher Scientific Cat. No. F10347)
- Fisherbrand™ Sterile Polystyrene Petri Dishes, 60 x 15 mm (Fisher Scientific Cat. No. AS4052)
- Falcon™ Cell Strainers, 40 µm nylon mesh (Fisher Scientific Cat. No. 08-771-1)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with 0.2 µm PES Membrane (Thermo Fisher Scientific Cat. No. 565-0020)
- Fisherbrand™ Razor Blades, single-edged, surgical carbon steel (Fisher Scientific Cat. No. 12-640)
- Attune NxT Flow Cytometer (Thermo Fisher Scientific)—all standard configurations are compatible with FxCycle PI/RNase Staining Solution; a configuration with the 405 nm laser is required for using FxCycle Violet Stain
- Invitrogen™ Attune™ Performance Tracking Beads (Thermo Fisher Scientific Cat. No. 4449754)
- Corning™ Falcon™ Round-Bottom Polystyrene Tubes, 12 x 75 mm (Fisher Scientific Cat. No. 352025)

Methods: reagent preparation

1. Prepare Galbraith's buffer. You will need approximately 1.5 mL for each 1 cm^2 piece of tissue. Filter the buffer using a 0.2 µm sterile filter and place on ice.
2. If using the violet 405 nm laser, make a 1 mg/mL stock solution of FxCycle Violet Stain by adding 100 µL deionized water to one vial of the stain. Mix well.
3. If using the 488 nm, 532 nm, or 561 nm excitation laser, the FxCycle PI/RNase Staining Solution is ready to use and formulated with DNase-free RNase A and a permeabilization reagent in DPBS.

Methods: sample preparation

Important: All procedures and incubations should be performed on ice for optimal results.

1. Prechill the petri dish by placing on ice.
2. Place 50 mg of freshly excised plant tissue into a prechilled petri dish. A tissue fragment of approximately 1 cm^2 is generally sufficient.
3. Add 750 µL ice-cold Galbraith's buffer to the plant tissue (1.5 mL per 100 mg of tissue).
4. Macerate the tissue with a fresh razor blade for 2–3 minutes. (Discard the razor blade after a single use.)
5. Filter the homogenate through a 40 µm nylon filter to remove large debris. Add 750 µL of ice-cold Galbraith's buffer to the petri dish and rinse the chopped tissue. Filter the suspension into the same tube that contains the first filtrate, for a total volume of ~1.5 mL.
6. Treat the filtered homogenate with either FxCycle PI/RNase Staining Solution or FxCycle Violet Stain in the following manner:
   - For FxCycle PI/RNase Staining Solution, add 1.5 mL of staining solution to 1.5 mL of filtered homogenate. Mix well. Place the suspension on ice, protected from light, for 30 minutes.
   - For FxCycle Violet Stain, add 3 µL of stain to 1.5 mL of filtered homogenate. Mix well. Place the suspension on ice, protected from light, for 30 minutes.

Methods: sample acquisition and analysis

The following protocol was used for sample acquisition and analysis on the Attune NxT Flow Cytometer. Please see our user guides and application notes for detailed instructions on setting up an experiment and analyzing samples [16-19]. Refer to Table 1 for instrument configurations and recommended emission filters.

1. Create a new experiment and set up the workspace and gating hierarchy as described below and indicated in Figure 2.
   A. Insert one plot of log side scatter area (SSC-A) vs. log fluorescence, and a gate to include the labeled nuclei (labeled as "Scatter" in this example). Events outside the scatter gate are subcellular debris and compromise most of the objects in the analysis.
   B. Insert one logarithmic dual-parameter plot for the two fluorescence detectors used, gated on the events in the scatter gate. This gate will be used to identify labeled nuclei based on the expected 1:1 correlation of intensity of the DNA-binding dye's emission in the two channels; populations lacking the 1:1 correlation will not represent nuclei. Objects inside this gate can be used for further DNA content analysis.
   C. Insert one logarithmic histogram fluorescence plot that is a daughter plot of the nuclei events. This plot will show distinct peaks that are typical of the nuclear DNA content found with endoreduplicated plant tissues. Markers can be drawn that include events within each peak for further statistical analysis.

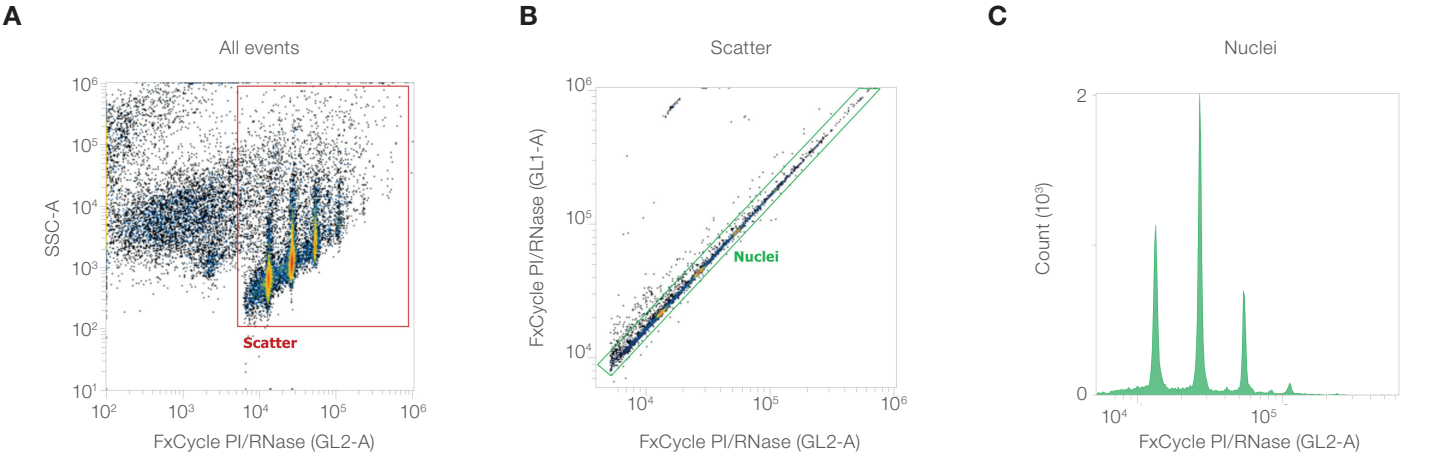


Figure 2. Gating and flow cytometric analysis of plant nuclei prepared from *A. thaliana* Col-1 leaf tissue and labeled with FxCycle PI/RNase Staining Solution. Data were collected with the 532 nm laser. **(A)** Biparametric density plot of side scatter vs. FxCycle PI/RNase fluorescence, with a scatter gate surrounding the fluorescent nuclei. **(B)** Biparametric density plot of FxCycle PI/RNase fluorescence to gate on singlet nuclei. **(C)** Logarithmic histogram of FxCycle PI/RNase fluorescence of nuclei-gated population, showing multiple peaks.

2. Acquire the sample on the Attune NxT Flow Cytometer, and adjust voltages to ensure that scatter and nuclei can be seen using the appropriate channels.

3. Adjust the fluorescence threshold to 10 (x1,000 in the Attune NxT Software) to eliminate noise and subcellular debris from the analysis. Adjust the fluorescence threshold setting empirically while viewing the data collected in the fluorescence detector to ensure no events in the nuclei gate are excluded from analysis. A dual threshold using forward scatter (FSC) and the fluorescence detector may be used by setting the Boolean gating to "AND" for both parameters.

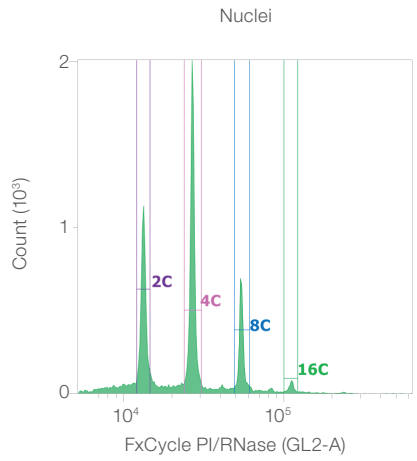
4. Adjust the stop criterion based on the appropriate number of events or volume required for the analysis. Collect 20,000 events in the scatter gate.

5. Acquire the data using a preferred flow rate. In this example, we show data collected with a 25 µL/min flow rate (Figures 2–5) and comparing 12.5, 25, and 100 µL/min flow rates (Figure 6 on page 6).

6. Place markers around each distinct peak in the histogram, and insert statistics for the experiment workspace that include median and %rCV (Figures 3–5).

7. Acquire and record data. Maintain an event rate of ≤100 events/sec to minimize the inclusion of coincidence in the data set.

A



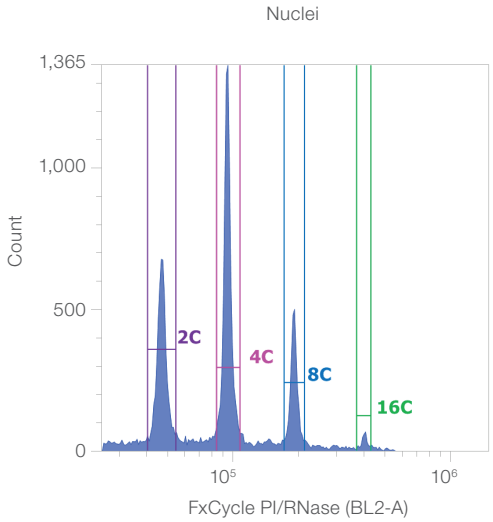
B

Experiment: **Arabidopsis PI-RNase 532 nm laser**

Name	Count	%Gated	X Median	X %rCV
All Events	29,228	100.000	N/A	N/A
Scatter	20,577	70.402	26,541	70.72
Nuclei	20,319	98.746	26,562	70.69
2C	5,047	24.839	13,370	3.30
4C	7,673	37.763	26,997	2.83
8C	2,669	13.135	54,313	2.72
16C	370	1.821	111,380	3.69

Figure 3. Flow cytometric analysis of plant nuclei prepared from *A. thaliana* Col-1 leaf tissue and labeled with FxCycle PI/RNase Staining Solution. Data were collected with the 532 nm laser. **(A)** Logarithmic histogram of FxCycle PI/RNase fluorescence of the nuclei-gated population, with markers placed around the 2C, 4C, 8C, and 16C peaks. **(B)** Gating hierarchy with statistics showing count, percent gated, median, and percent robust CV (%rCV) for each gated peak. Note the incremental doubling of the median fluorescence intensities of the 2C, 4C, 8C, and 16C peaks, and the low associated %rCV.

A

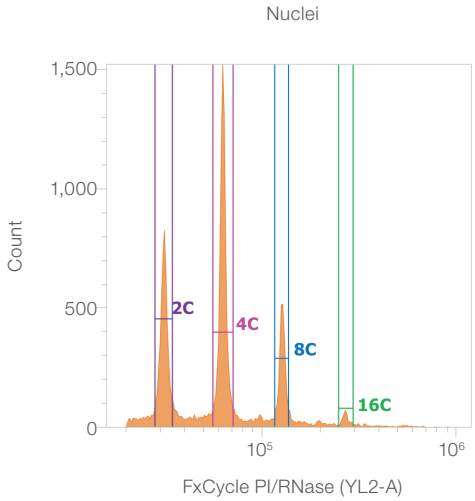


B

Experiment: **Arabidopsis PI-RNase 488 nm laser**

Name	Count	%Gated	X Mean	X %rCV
All Events	23,515	100.000	N/A	N/A
Scatter	20,224	86.005	109,437	68.94
Nuclei	19,215	95.011	106,022	67.52
2C	5,028	26.167	47,220	4.75
4C	7,519	39.131	94,671	3.48
8C	2,660	13.843	190,872	3.50
16C	314	1.634	401,616	2.99

C



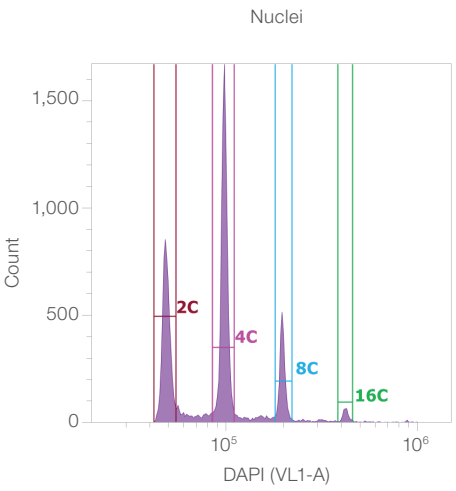
D

Experiment: **Arabidopsis PI-RNase 561 nm laser**

Name	Count	%Gated	X Mean	X %rCV
All Events	23,515	100.000	N/A	N/A
Scatter	19,464	82.773	78,253	68.37
Nuclei	19,282	99.065	75,696	67.99
2C	4,703	24.391	31,449	3.92
4C	7,543	39.119	63,304	3.42
8C	2,565	13.303	127,568	3.15
16C	345	1.789	270,866	3.48

Figure 4. Flow cytometric analysis of plant nuclei prepared from *A. thaliana* Col-1 leaf tissue and labeled with FxCycle PI/RNase Staining Solution. Data in A and B were collected with the 488 nm laser; data in C and D were collected with the 561 nm laser. **(A, C)** Logarithmic histogram of FxCycle PI/RNase fluorescence of the nuclei-gated population, with markers placed around the 2C, 4C, 8C, and 16C peaks. **(B, D)** Gating hierarchy with statistics showing count, percent gated, mean, and %rCV for each gated peak. Note the incremental doubling of the mean fluorescence intensities of the 2C, 4C, 8C, and 16C peaks, and the low associated %rCV.

A



B

Experiment: **Arabidopsis DAPI 405 nm laser**

Name	Count	%Gated	X Mean	X %CV
All Events	22,618	100.000	N/A	N/A
Scatter	20,000	88.425	103,142	85.59
Nuclei	18,143	90.715	107,541	74.40
2C	5,316	29.301	48,972	4.38
4C	8,328	45.902	98,260	3.91
8C	2,201	12.131	197,432	3.37
16C	326	1.797	422,183	3.39

Figure 5. Flow cytometric analysis of plant nuclei prepared from *A. thaliana* Col-1 leaf tissue and labeled with FxCycle Violet Stain (DAPI). Data were collected with the 405 nm laser. **(A)** Logarithmic histogram of FxCycle Violet Stain fluorescence of the nuclei-gated population, with markers placed around the 2C, 4C, 8C, and 16C peaks. **(B)** Gating hierarchy with statistics showing count, percent gated, mean, and %rCV for each gated peak. Note the incremental doubling of the mean fluorescence intensities of the 2C, 4C, 8C, and 16C peaks, and the low associated %rCV.

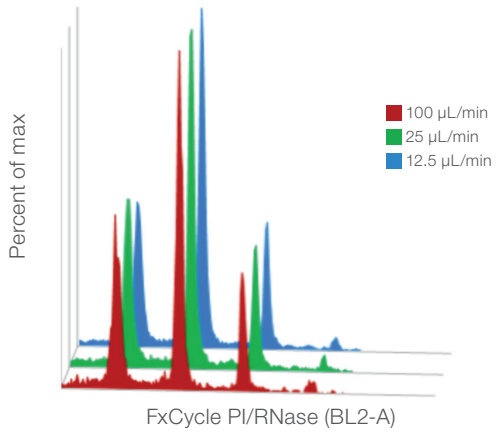


Figure 6. Overlay of 12.5, 25, and 100 µL/min flow rates. Plant nuclei prepared from *A. thaliana* Col-1 leaf tissue were labeled with FxCycle PI/RNase Staining Solution and analyzed by flow cytometry. Data were collected with the 488 nm laser. Data are consistent across all three flow rates, so no trade-off between speed and data quality is observed as the flow rate increases.

Results

The Attune NxT Flow Cytometer demonstrates distinct peaks of fluorescence corresponding to 2C, 4C, 8C, and 16C nuclei of *A. thaliana* Col-1 leaf tissue labeled with the FxCycle PI/RNase Staining Solution or FxCycle Violet Stain, as indicated in Figures 3–5. Data were collected with the 488 nm laser, 532 nm laser, and 561 nm laser for nuclei labeled with FxCycle PI/RNase Staining Solution, and with the 405 nm laser for nuclei labeled with FxCycle Violet Stain, demonstrating utility across all instrument configurations. The Attune NxT Flow Cytometer provides data consistency across all laser options. Instrument optimization for small particles such as nuclei is made easier using an instrument that permits user adjustment of threshold settings and windows extension.

The *A. thaliana* 2C nuclear DNA content (0.32 pg) falls toward the lower end of the range of 2C values reported across angiosperms. *Arabidopsis* displays somatic endoreduplication, and flow cytometric analyses of the nuclear DNA content of macerated somatic tissues display distinct peaks of fluorescence corresponding to 2C, 4C, 8C, and 16C nuclei. As the DNA content of these nuclei correlates with the mean fluorescence intensity, *A. thaliana* provides a convenient scale for measuring nuclear DNA content in the range of 0.32–5.12 pg, and a convenient model for demonstrating the dynamic range of the Attune NxT Flow Cytometer. 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 photomultipliers (PMTs) on the Attune NxT Flow Cytometer, DNA estimates that are smaller than *A. thaliana* 2C nuclei and larger than 16C nuclei are possible.

Since flow cytometry analyzes relative fluorescence intensity, genome size may be determined only after comparison with nuclei of a reference standard whose genome size is known. The use of *A. thaliana* as an internal standard can be done by macerating, filtering, and staining plant material, and acquiring data for the test species together with *A. thaliana* and then comparing the results.

Tips on technique

- Use a new razor blade for each plant sample to be macerated, and perform all maceration and staining on ice.
- Use log scaling for scatter settings to aid identification of distinct populations for gating and data analysis.
- Set proper voltages and thresholds, and maintain an event rate of ≤100 events/sec to help minimize the inclusion of coincidence in the data set and maximize population resolution.
- Prior to data acquisition by flow cytometry, check samples by fluorescence microscopy to confirm that the nuclei are present and stained.
- To better resolve populations, it may be necessary to adjust the window extension (WE) setting under the advanced settings section in the instrument settings menu.
- Labeling with a DNA-binding dye that is not base-specific (such as a dye that intercalates into DNA, such as DAPI) is best to quantify the size of the genome as accurately as possible.
- Poor CVs can result from excessive tissue maceration, or using dull razor blades. Blades that are best suited for this application have an edge thickness of 0.2 mm or less. Use a fresh blade for each preparation.
- Drifting peak positions may be caused by insufficient staining time; 15–30 minutes are sufficient. To evaluate staining efficiency, analyze successive samples over time from a single labeled homogenate and compare results.
- This technique can be performed with any plant material: leaves, seeds, roots, seedlings, flowers, or fruit peels.

Conclusions

The Attune NxT Flow Cytometer is ideal for performing nuclear DNA content analysis on plant material. The syringe-driven volumetric sample delivery system and larger flow cells help prevent instrument clogging and other common fluidic problems often seen with flow cytometers that require sheath pressure. There is no need to concentrate samples; the acoustic focusing technology easily allows the use of dilute samples. Low %rCVs are demonstrated, even at increasing flow rates, enabling consistent and reliable results. The Attune NxT Flow Cytometer is ideal for plant nuclear DNA content analysis, ploidy analysis, or other investigations requiring C-value determinations. This application note demonstrated efficient detection with multiple laser options, including a blue (488 nm), green (532 nm), yellow (561 nm), and violet (405 nm) laser. The Attune NxT Flow Cytometer is efficient and flexible, and configurable as a 1-, 2-, 3-, or 4-laser system to meet simple to diverse application needs.

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