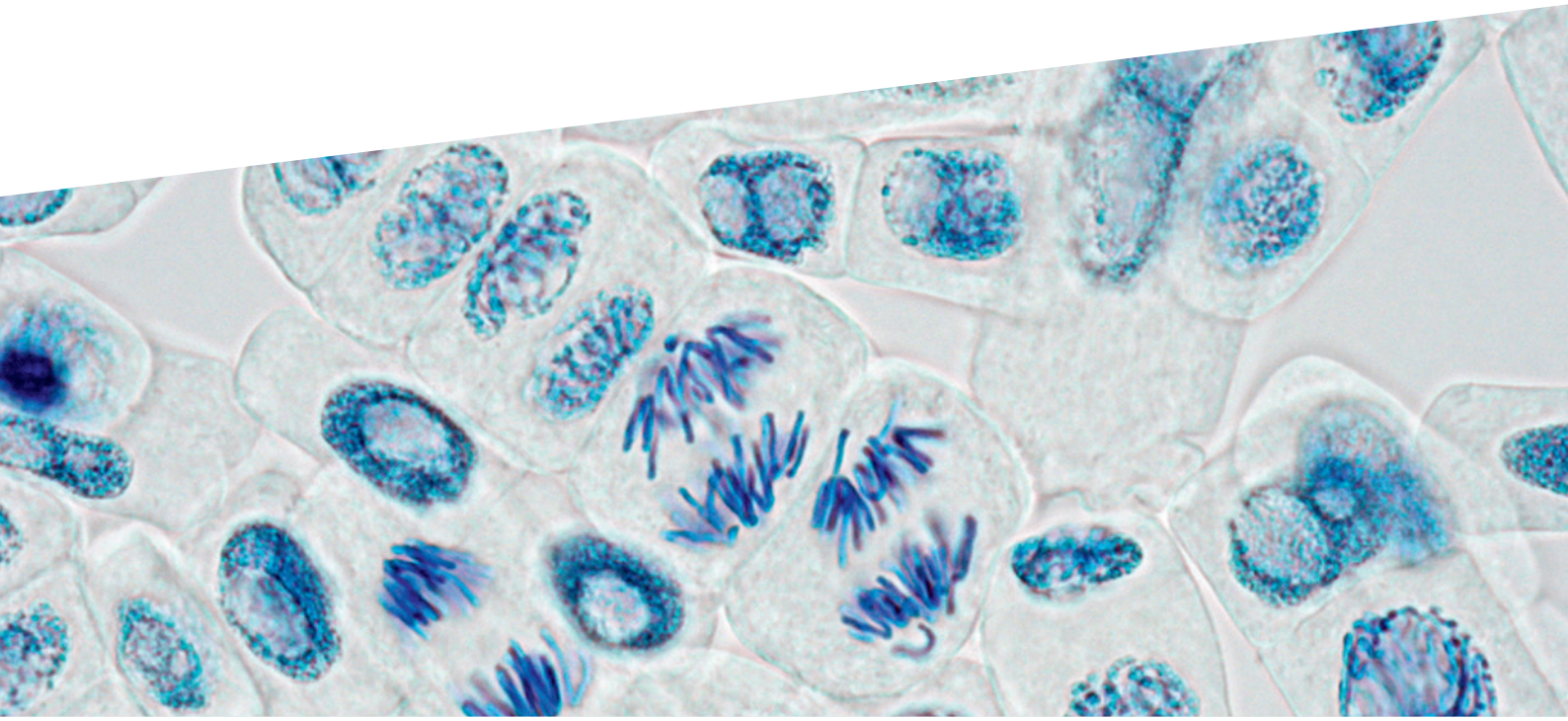
WHITE PAPER CYTOMETRY



Flow cytometry for analysis of the ploidy and the size of the genome in plants and other organisms

Introduction:

Right from the very beginning, flow cytometry was developed for measuring variations in the quantity of genetic material in dysplastic cells. This technology remains the reference method in plant biology for assessing the size of a genome and the level of ploidy.

Principle of the method:

The cell nuclei are mechanically removed from a tissue fragment measuring approximately 1 cm^2 (or 20 mg) in the presence of an extraction buffer. Following filtration, the cell nuclei are stained with CyStain® solutions from Sysmex Partec, containing either propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI) (Figure 1). This preparation is then analysed on a CyFlow® flow cytometer, comprising one or more sources of green (PI), blue (PI), ultraviolet (DAPI) excitation ...

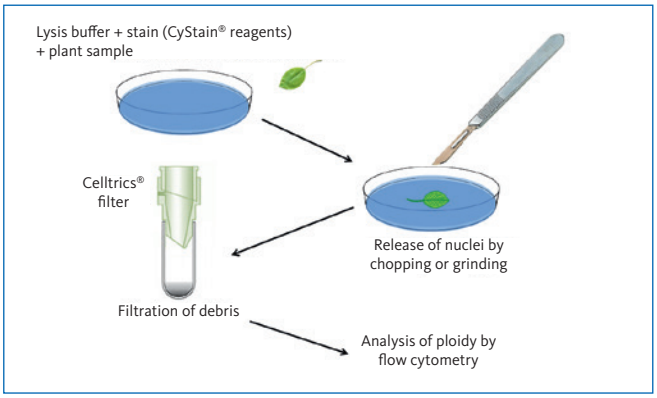


Figure 1: *Preparation stage of plant sample for analysis of ploidy.*

The cell cycle:

Under physiological conditions, the cells naturally present a DNA profile corresponding to a cell cycle with different phases (Figures 2 & 3):

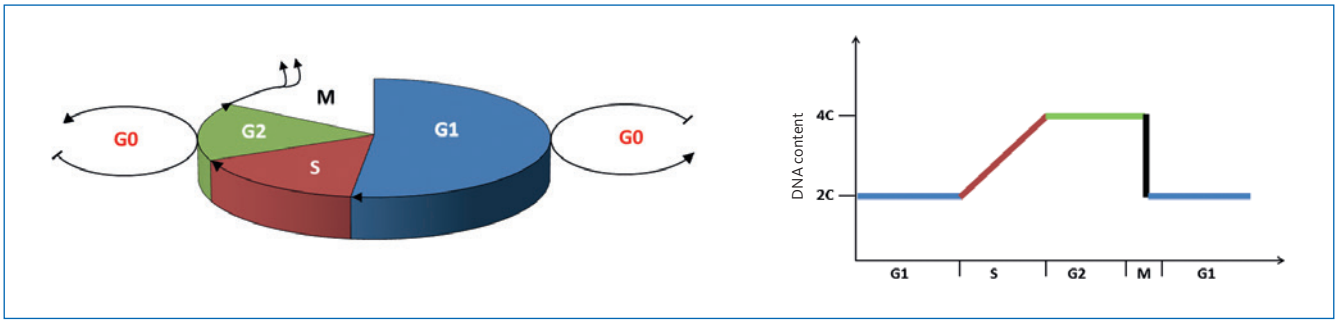


Figure 2: *Cell cycle. Key: Go phase = resting, G1 phase = first gap, S phase = synthesis, G2 phase = second gap and M phase = mitosis.*

Suspension of the nuclei:

The filtration stage removes the tissue debris during release of the nuclei. Nevertheless, small fragments not eliminated during the filtration stage, such as chloroplasts, mitochondria and soluble phenolic compounds as well as DNases and RNases ..., can all impair the quality of the analysis.

The addition of antioxidants (bisulfite, 2-Mercaptoethanol), tannin-binding agents (PVP), chloroplast lysis (Triton), RNA lysis (RNases) as well as preparation in an ice bath can correct the interactions due to undesirable substances.

Use of an in-house reference:

It is sometimes recommended to use a reference composed of nuclei from a species whose genome is of a similar size to that of the sample being analysed. This reference is prepared at the same time as the sample so that the factors have the same effects on the two species. The relative position of the peaks between the reference and the sample makes it possible to measure the sample accurately, under certain conditions, knowing the characteristics of the DNA of the in-house reference in picograms (pg) or base pairs (bp), knowing that:

DNA content (base pairs)
= 0.978 10^9 x DNA content (pg)

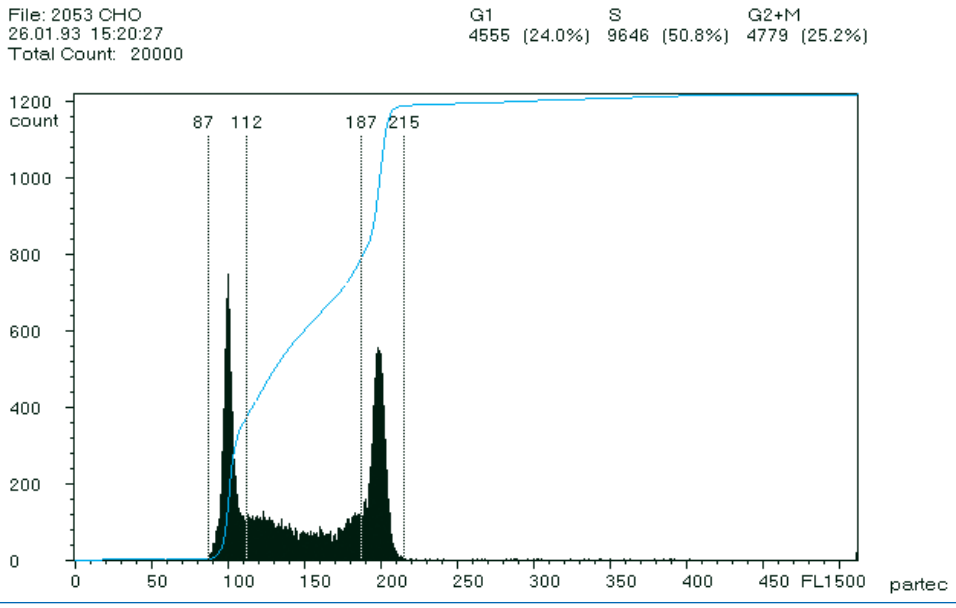


Figure 3: *Cytometric analysis of cell cycle.*

Analysis by flow cytometry:

In order to eliminate the debris which passed through the filtration, cytometry makes it possible to initiate an analysis of a fluorescence parameter and to use this fluorescence analysis to determine a gate created on the basis of a cytogram of granularity 'SSC' versus fluorescence 'DAPI or PI' (Figures 4A & 4B).

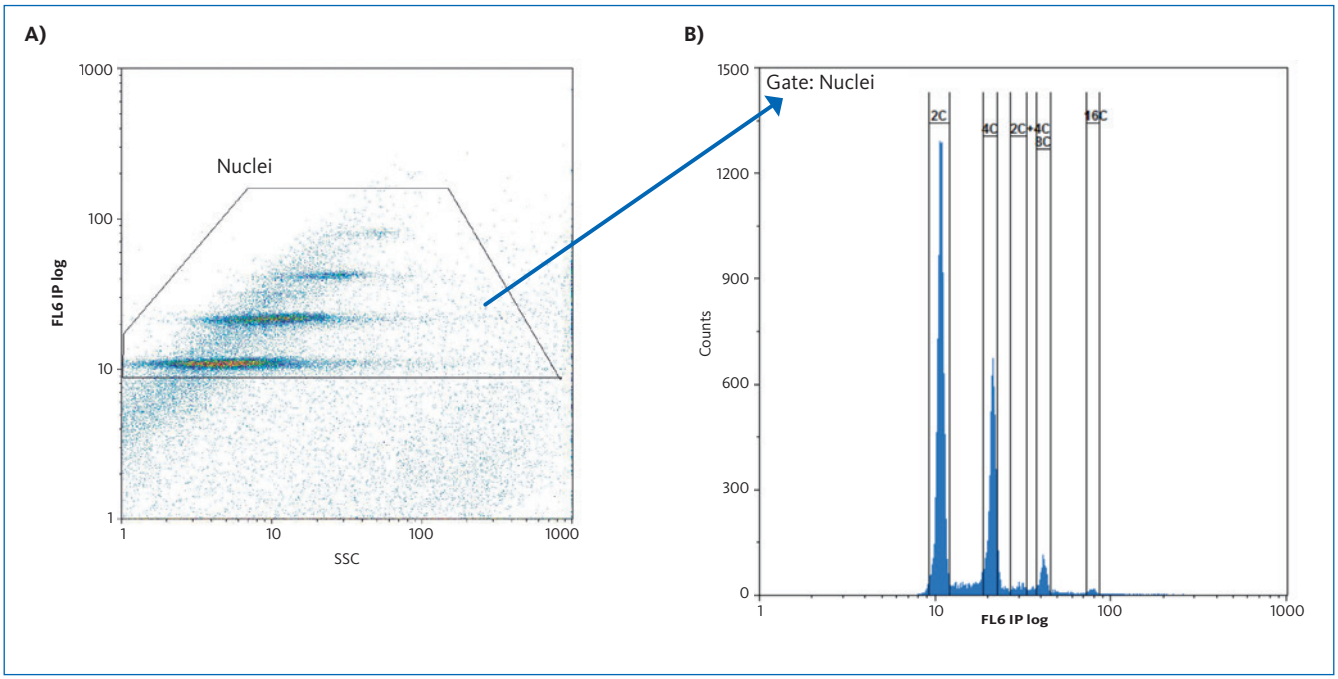


Figure 4: *Analysis of the ploidy of a tomato sample (Lycopersicum esculentum); 4A cytogram showing granularity (SSC) vs FL6 fluorescence emitted by the PI, the 'Nuclei' gate is applied to Figure 4B; 4B histogram of FL6 fluorescence emitted by PI and conditioned on the 'Nuclei' gate showing the ploidy peaks.*

Assessment of the degree of endoreduplication:

The example above (Figures 4) using a tomato *(Lycopersicum esculentum)* demonstrates the phenomenon of endoreduplication corresponding to a DNA replication, such as mitosis, but without division of either the nucleus or the cell itself. The endoreduplication peaks are superimposed over the peaks of the classic cell cycle:

$$2C \rightarrow 4C \rightarrow 8C \rightarrow 16C$$

It is also possible to view the most abundant nuclei analysed simultaneously; their coincidences create a peak artefact '2C + 4C' (Figures 5 & 6).

Region	Gate	% Gated	Count	Mean-x	CV-x %
2C	Nuclei	54.49	18367	10.80	4.17
4C	Nuclei	26.64	9648	21.23	3.76
8C	Nuclei	4.32	1460	41.58	3.72
16C	Nuclei	0.74	260	78.68	3.87
2C + 4C	Nuclei	1.95	649	30.40	5.55

Figure 6: *Statistics of regions corresponding to different endoreduplication or coincidence peaks.*

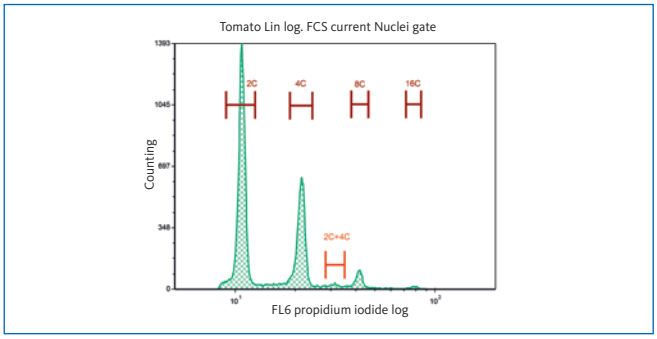
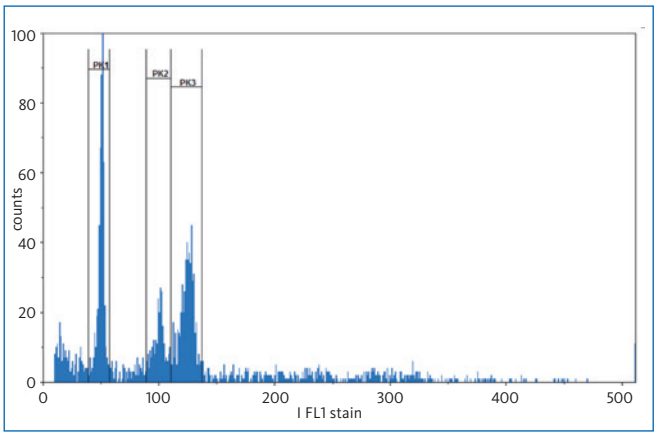


Figure 5: *Ploidy analysis of tomato sample (Lycopersicum esculentum) showing the endoreduplication peaks.*

Measurement of size of genome:

The quantification of DNA by cytometry using an in-house reference requires the use of a staining which is not base-specific, a true DNA intercalator, to quantify the sizes of the genome as accurately as possible. These intercalating fluorochromes are generally propidium iodide or ethidium bromide (in the example below: RNase and propidium iodide, Figure 7).



Region	Count	% Gated	GMn-x	Mean-x	CV-x %
PK1	500	25.00	49.50	49.59	6.03
PK2	251	12.55	99.60	99.73	5.11
PK3	534	26.70	123.97	124.11	4.74

Figure 7: *Analysis of the DNA content of a sample of horsetail (Equisetum sp.) using fragments of garlic (Allium sativum) with a determined DNA content as a reference.*

The PK1 and PK2 regions correspond respectively to peaks 2C and 4C of garlic (*Allium sativum*) used as a reference, based on the knowledge that 2C = 34.80 pg. Region PK3 of horsetail (*Equisetum sp.*) makes it possible to calculate the DNA index compared to PK1 = 123.97/49.50 = 2.50, and thus value 2C of the Equisetum sp. is 2.50 x 34.80 = 87.15 pg.

Measurement of AT/GC ratio:

The principle is based on the use of a DNA intercalator versus a fluorochrome specific to base repetitions, e.g., 5 x AT for Hoechst 33342 and 3 x GC for mithramycine.

Measurement of AT/GC ratio

R_{IP} = Intensity $_{of\ test}$ / Intensity $_{of\ reference}$ Measured with propidium iodide

or R_{HO} = Intensity $_{of\ test}$ / Intensity $_{of\ reference}$ Measured with Hoechst 33342

or R_{CA} = Intensity $_{of\ test}$ / Intensity $_{of\ reference}$ Measured with chromomycin A3

$\%AT_{test} = \%AT_{reference} \times (RHO/RIP)^{1/5}$ and/or $\%GC_{test} = \%GC_{reference} \times (RCA/RIP)^{1/3}$

When $\%AT_{test} + \%GC_{test}$ is checked, the result should be 100 %

Apomixis and aneuploidy:

Apomixis is the substitution of normal sexual reproduction by asexual reproduction without fertilisation but producing seeds. Apomictic plants are generally identical from one generation to the next.

The peaks generated by flow cytometry thanks to the embryo and endosperm cells of the seeds specify apomixis or not, as well as the type of apomixis (Figure 8).

Sexual profiles **Apomitic profiles**

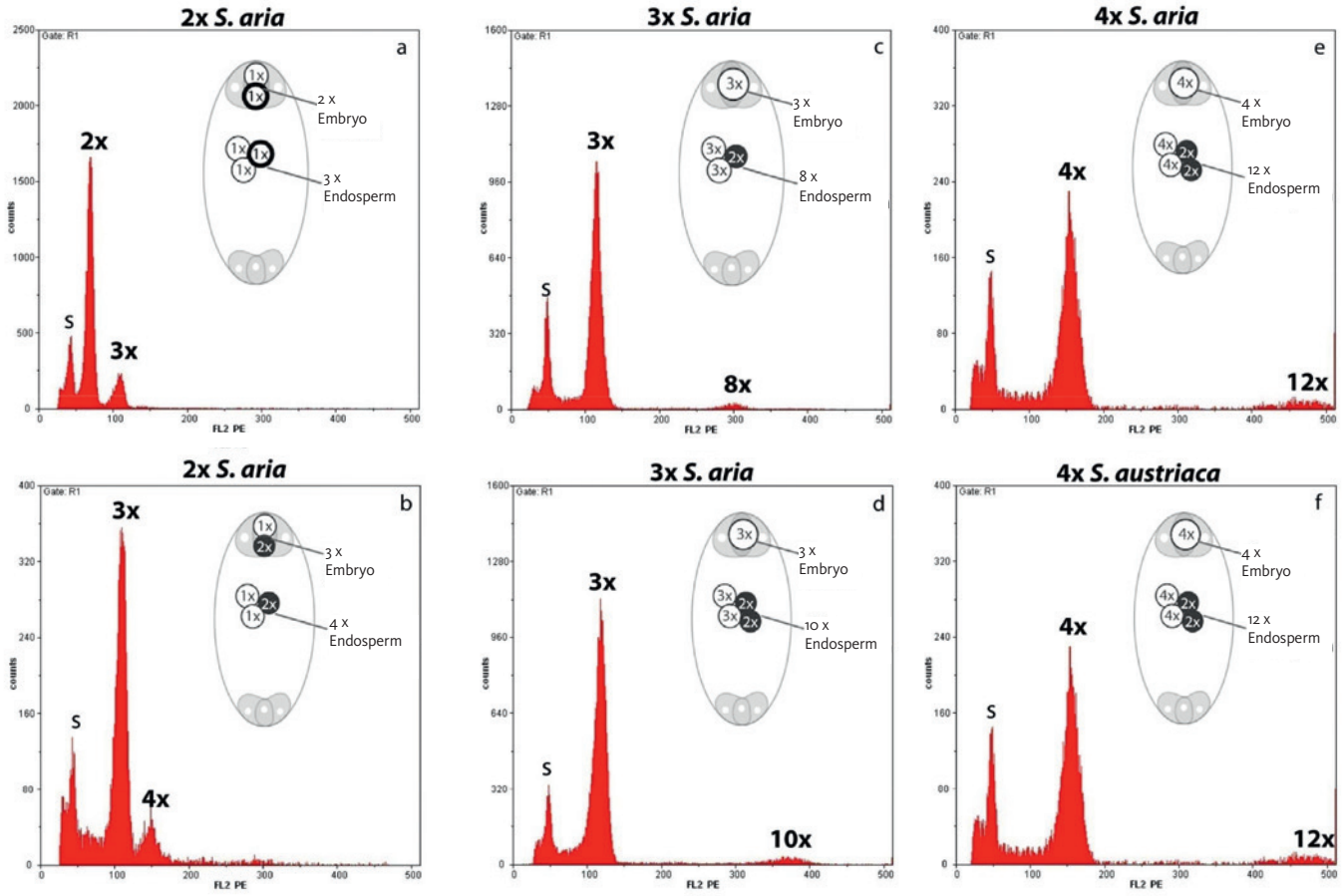


Figure 8: *Study of the methods of reproduction according to the position of the peaks on the fluorescence histograms by flow cytometry (Matzk & al. 2000).*

In case of aneuploidy, the position of the peaks will be affected and will appear as in the following example: rapeseed seedlings (*Brassica napus*) with a number of chromosomes n = 16 and n = 19 creating double peaks.

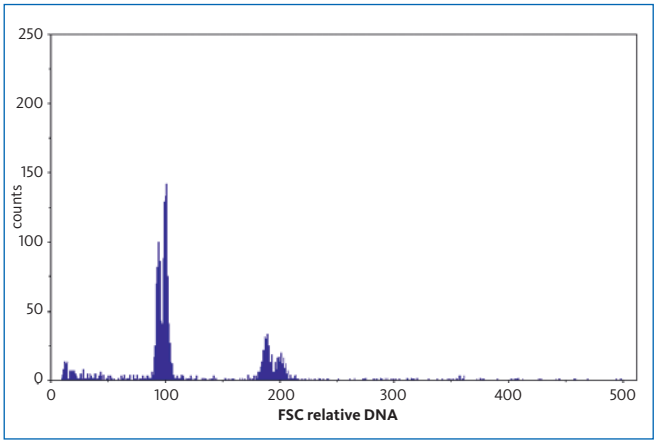


Figure 9: *Analysis of ploidy of a sample of rapeseed (Brassica napus) and presenting an aneuploidy profile with splitting into two of the 2C and 4C peaks.*

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Sysmex Partec GmbH
Am Flugplatz 13, 02828 Görlitz, Germany · Phone +49 3581 8746-0 · Fax +49 3581 8746-70 · info@sysmex-partec.com · **www.sysmex-partec.com**