Flow Cytometry for Ploidy and Genome Size analysis

1. Functional principles

The technique of plant ploidy and genome size analysis in a flow cytometer is based on the fluorescent labelling of the nuclear DNA of individual nuclei and the individual fluorescence analysis in a flow stream. For the detection of such kind of DNA fluorescence a small, easy-to-use one-parameter flow cytometer can be used. In a flow cytometer fluorescent particles are transported in single file through an excitation light beam. Here the fluorescent dye in the individual nucleus is excited and emits fluorescence light.

This fluorescence light is collected by an optical system, detected by a photodetector, quantified and subsequently displayed and stored by the Flow Cytometer (FC, see figure 1).

Figure 1: Principle of a flow cytometer
A typical example of a flow cytometer tailored to the requirements of plant ploidy analysis and genome size determination is the **Partec CyFlow® Ploidy Analyser**. The heart of the Ploidy Analyser is its Flow Cell. It is a quartz glass capillary with a rectangular flow channel of only 320 x 200 µm side length. During operation a liquid for sample transportation (hereafter called “sheath fluid”) is flowing through the glass capillary called the flow channel. The suspended particles (hereafter called “sample”) are injected into the flow of sheath fluid through an even smaller metal capillary. The sheath fluid moving with high speed (1m/s) is stabilizing the sample flow which becomes hydrodynamically focussed in the centre of the flow cell. Two distinct non-mixing liquid flows (sheath and sample) are passing the flow channel.

The resulting sample flow dimension covered by the sheath fluid flow can be as small as a few microns. By this effect, also known as hydrodynamic focussing, the nuclei in the sample flow are individualised and made to pass **in single file** the excitation light beam (see figure 2).

The fluorescent dye on the individual nucleus is exposed to excitation light and the emitted fluorescent light is collected by an optical lens system, and subsequently filtered through a specific wavelength band to be detected by a photo sensor (photomultiplier tube, PMT) and transferred into an electronic signal.

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**Figure 2: Flow Cell and hydrodynamic focussing**
In order to detect fluorescent signals three system components have to be in-line:

- **Sample flow** (= position of the analysed particle)
- **Focus of the excitation light beam**
- **Focus of the light collection optics**

In order to supply a perfect steady supply of sheath fluid and sample the liquids are indirectly driven by an air pressure applied to their containers (sample port and sheath/waste fluid container, figure 3). After particle analysis sample and sheath fluid are mixed and jointly collected in the waste container. A pressure sensor controlled precision pump is generating the pressure applied to the sheath or waste container (negative pressure in the waste container), a computer-controlled syringe pump is pressing air into the sample port and thus is indirectly pushing the sample suspension.

Valves for sheath and waste line as well as for air pressure are regulating the liquid flow during different phases of operation.

![Figure 3: Typical fluid system of a Ploidy Analyser](image-url)

1. sample port  
2. flow Cell  
3. syringe pump  
4. pressure gauge and regulator  
5. membrane pump  
6. sheath fluid valve  
7. waste fluid valve  
8. air valve  
9. waste container  
10. sheath fluid container
2. Instrument start-up procedure

To start your Partec flow cytometer for Ploidy or genome size analysis please refer to the User Manual. For daily quality control (before analysing samples) it is recommended to check the instrument’s performance with Partec alignment products (Partec DNA Control UV or DNA Control PI or Partec Calibration Beads, figure 4). For instructions of use check the product information leaflets.

Figure 4: Partec UV Calibration Beads (left) and DNA Control UV (right)

3. Sample Preparation

The preparation of plant specimen for nuclear DNA analysis starts usually with the extraction of intact nuclei from plant tissue material. Subsequently the extracted nuclei are stained with DNA specific fluorochromes (see the table 1) and the intensity of fluorescence of the individual nuclei is analyzed in the flow cytometer. For the flow cytometric analysis basically all vital plant tissue may be used. Tissue structure and content of secondary metabolites or storage substances may influence the quality of the fluorescence staining procedure.
a) Preparation of a nuclei suspension

Alternative methods:

- production of plant protoplasts with subsequent lysis in a hypotonic buffer
- manual destruction of plant tissue (chopping) with a razor blade in a lysis buffer (nuclei extraction buffer)
- destruction of the plant tissue by bead mills (type TissueLyser)
- enzymatic digestion of cell walls after ethanol/acetic acid fixation
- squeezing or homogenisation of formalin fixed material in a lysis buffer

For almost all applications the chopping procedure (figure 5) is representing the best and easiest extraction method. A small, defined amount of plant material is added to a small amount (few drops) of lysis buffer in a plastic petri dish. Using a sharp(!) razor blade the material is chopped. Cells are destroyed and nuclei are released into the lysis buffer.

The nuclei are separated by filtration (30µm-50µm, Partec CellTrics® disposable filter units) from bigger fragments of tissue. Subsequently the staining solution is added. Time of incubation and further treatment is depending on the used DNA fluorochrome.

![Figure 5: Lysis and staining procedure by chopping](image-url)
b) Lysis buffer

The essential components of the lysis buffer are determined by the necessity of reducing nuclease activity, by maintaining the nuclei integrity and by establishing the optimal chemical conditions for the used fluorochromes (pH, salt concentration, metal ions). Depending on the used plant material more specific additives may be necessary (e.g. reducing or anti-oxidation agents, table 1).

c) DNA-specific fluorochromes

The selection of the used fluorochrome is depending on the experimental aim and of course is influenced by the instrumental configuration with respect to light sources and detection wavelength.

For the determination of absolute DNA amount per nuclei intercalating dyes like ethidium bromide and propidium iodide are suited. These fluorochromes are characterised by base-unspecific intercalating binding to the DNA. Such base-pair ration independent binding allows the use of references with different base composition concerning A-T and G-C basepairs.

For analysis of relative differences in DNA (e.g. ploidy level or an-euploidy determination) fluorochromes like DAPI are recommended. Due to the superior data resolution and ease-of-use these UV excited dyes are the better choice for this type of analysis.

Our first recommendation for plant nuclear DNA analysis is the DAPI fluorochrome. DAPI is excited by UV light from a UV-LED and emits blue fluorescent light. The optical configuration of the CyFlow® Ploidy Analyser filer set up is depicted in the appendix of the user manual.

As alternative to DAPI the FC may run also with the intercalating dye propidium iodide (PI) for direct genome size determination. In this case a different optical configuration with green excitation light of a 532 nm solid state laser and red fluorescence emission has to be used. The CyFlow® Ploidy Analyser and all other Partec FC for Plant DNA Analysis (CyFlow Space, CyFlow Cube8) are capable to be configured to run DAPI as well as propidium iodide stained samples.

d) Remarks

During tissue disruption and nuclei extraction secondary metabolites located in the cytoplasm and the cell vacuole (i.e. polyphenolic substances, organic acids, mucilage) might be released and getting into direct contact with the nuclei. Such substances disturb the binding of fluorochromes to the DNA or aggressively destroy nuclei integrity. In general such effects may be inhibited or reduced by adding chelating and reducing agents.

Oxidation leads to the degradation of DNA and therefore creates broad DNA histograms and
instable (shifting) peak positions. Oxidation can be reduced by mercaptoethanol (0.1 %) or PVP.

Table 1: DNA binding fluorescent dyes

<table>
<thead>
<tr>
<th>Fluorescent Dye</th>
<th>Binding Mode</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium bromide</td>
<td>Intercalation</td>
<td>530 605</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>Intercalation</td>
<td>540 615</td>
</tr>
<tr>
<td>Hoechst 33258</td>
<td>AT-selective</td>
<td>365 465</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>AT-selective</td>
<td>360 460</td>
</tr>
<tr>
<td>DAPI</td>
<td>AT-selective</td>
<td>365 450</td>
</tr>
<tr>
<td>DIPI</td>
<td>AT-selective</td>
<td>365 450</td>
</tr>
<tr>
<td>Chromomycin A3***</td>
<td>GC-selective</td>
<td>445 570</td>
</tr>
<tr>
<td>Mithramycin***</td>
<td>GC-selective</td>
<td>445 575</td>
</tr>
<tr>
<td>Acridine orange</td>
<td>Intercalation</td>
<td>502 525</td>
</tr>
</tbody>
</table>

Table 2: Additives to be added to lysis and staining buffer.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Function</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>Chelating agent</td>
<td>2 mM</td>
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<td>EGTA</td>
<td>Chelating agent</td>
<td>2 mM</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>Reducing agent</td>
<td>15 mM / 0.1 %</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>Reducing agent</td>
<td>15 mM / 0.1 %</td>
</tr>
<tr>
<td>Polyvenylpyrrolidone (PVP-10, PVP-20)</td>
<td>Complexing agent</td>
<td>0.5 – 2 %</td>
</tr>
<tr>
<td>Spermine</td>
<td>Chromatine stabilizer</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Mg 2+, Ca2+</td>
<td>Chromatine stabilizer</td>
<td></td>
</tr>
</tbody>
</table>

The incubation time of the sample in the lysis buffer may have also strong influence on the staining result. For most plant species short incubation periods of 30 to 120 seconds (Partec CyStain UV precise P kit) or up to 5 minutes (Partec CyStain UV precise T kit) are well suited whereby some species may require incubation of up to 2 hours. In literature different opinions regarding the temperature at preparation can be found. The preparation at 4°C leads to a reduction of
DNAse activity and oxidation effects but also to a slower speed of fluorochrome diffusion and binding to the DNA. Often preparation with buffers at room temperature is more advantageous than on ice.

4. Analytical strategies and data analysis for plant DNA in flow cytometers

a) Regarding the handling of the instrument please refer to the user manual.

b) System calibration – selection of reference method

As the flow cytometer delivers relative DNA values a calibration of the individual measurement has to be done. Basically we do distinguish an internal and an external reference method. Both are used for different analytical targets.

In the internal reference methodology the individual sample is mixed with a piece of material of a reference plant and both pieces of tissue are co-chopped together.

In the external reference method the reference plant material is chopped separately from the samples and is used to calibrate the instrument. Table 3 summarizes the use of reference methodology according to the intended application. The result of analysis is finally calculated by comparing the DNA peak position of the reference with the DNA peak position of the sample (figure 6).

\[
\text{Sample DNA content} = \frac{\text{Peak Position Sample}}{\text{Peak Position Reference}} \times \text{DNA content reference}
\]

\[
\text{Sample Ploidy level} = \frac{\text{Peak Position Sample}}{\text{Peak Position Reference}} \times \text{Ploidy level reference}
\]

DNA content can be expressed in genome size as picogramm DNA, base pairs or as ploidy level.
Table 3: DNA determination and reference method

<table>
<thead>
<tr>
<th>Application</th>
<th>Reference method</th>
<th>Nature of reference</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ploidy Analysis</td>
<td>External</td>
<td>Same species as sample with known ploidy level</td>
<td>In case peak position is stable and allows safe determination of ploidy level</td>
</tr>
<tr>
<td>Genomes Size Analysis</td>
<td>Internal</td>
<td>Plant sample with 1.2 – 4 fold genome size, not overlapping with sample</td>
<td>Co-chopping, avoid reference containing secondary metabolites</td>
</tr>
<tr>
<td>Small genome Size Variation, genome size aberrations</td>
<td>Internal</td>
<td>Plant with regular genome size</td>
<td>Mix the “normal” plant with the sample, co-chopping</td>
</tr>
<tr>
<td>Small genome Size Variation, genome size aberrations</td>
<td>Internal</td>
<td>Any plant with 1.2 – 4 fold genome size</td>
<td>Mix reference with normal plant and with test plant, establish DNA ratio and compare</td>
</tr>
</tbody>
</table>

An external reference method should only be applied for ploidy analysis or when bigger variations in DNA content are determined and no special problems during analysis or between different sample preparations do occur (like shifting peak positions).

For plant DNA analysis it is good laboratory practice to use reference material originated from plants. Other available DNA reference material like fish, chicken or human blood cells must be avoided. Ploidy reference material usually is sourced from own plant material, genome size reference material must be cultivated from approved seed lots. Literature cites suited DNA standards for flow cytometry for different genome size ranges. Raphanus sativus, Lycopersicon esculentum, Glycine max, Zea mays, Pisum sativum, Secale cereal,
c) General considerations for the set-up of the flow cytometer

1. In genome size analysis the data usually are displayed on a linear scale. Only in the presence nuclei with several different C-levels (from endopolyploidyidization or several endo-reduplication steps) the display on a logarithmic axis is recommended.

2. Reference peak position should be maintained equal within one single experiment, even when samples are analysed on different days. Instrument GAIN values can be adapted in order to keep reference positions.

3. For calculation of results peaks outside a range of < 10% and > 80% of the total DNA scale should be avoided (e.g < 100 and >800 on a scale of 1024 channels).

4. Amplification (GAIN) < 200 Volts and > 700 Volts should be avoided.

5. For high-resolution analysis sample speed should be lower than 1 µl/s and sample rate should be < 100 nuclei/s

6. Background peak height should not exceed the height of sample/reference peak. Use threshold (lower level LL) to cut off high background noise. Background usually is caused by defective, fragmented nuclei and can only be avoided by improving the sample preparation procedure and reagents.

7. Genome size determination requires repetitions of the analysis in order to establish statistically valid data.

Literature recommendation:
