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MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

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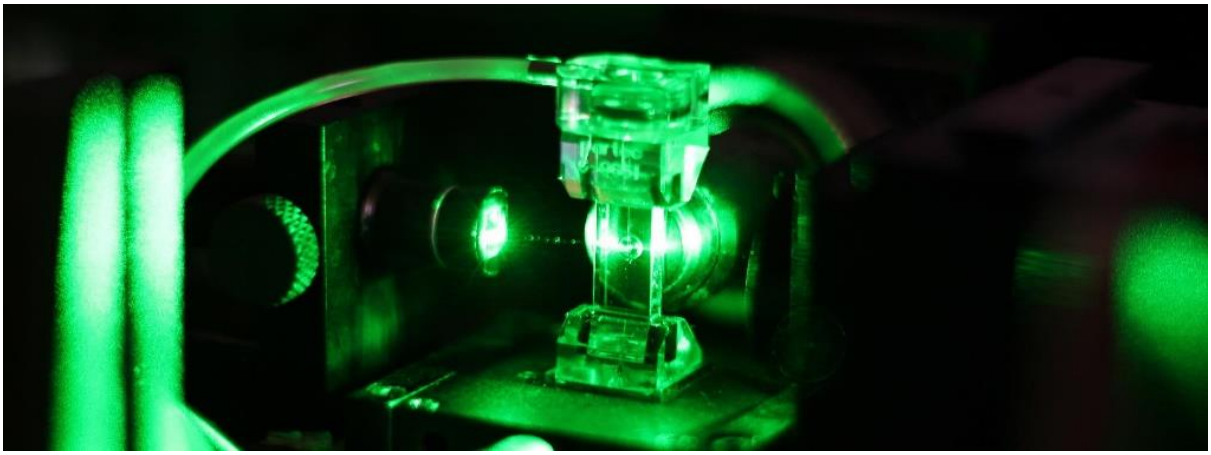
PRACTICAL COURSE OF KARYOLOGICAL AND PALYNOLOGICAL METHODS

Urfus T., Urfusová R. & Suda J. (†)



Flow cytometric part of practical course is held in the Suda's laboratory of flow cytometry (https://www.natur.cuni.cz/biologie/botanika/struktura/cyto) in the Department of Botany, Faculty of Science, Charles University. Our lab is predominantly using Partec (Sysmex) instruments and that is why the manual is specifically focused on their operating.

Flow cytometry in plants is a fast and effective way of analyzing optical properties (fluorescence, light scatter) of single particles in suspension as they move in a narrow liquid stream through a powerful beam of light. The majority of applications in plant sciences focuses on recording fluorescence intensities of nuclei stained with DNA-selective dyes. The typical result is genome size (absolute or relative).

ADVANTAGES AND LIMITATIONS

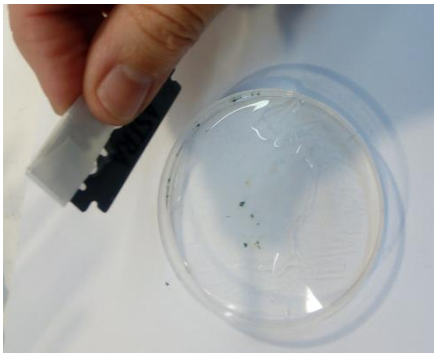
PROS	CONS
high speed	fixation not possible
easy sample preparation	secondary metabolites
non-destructiveness	reference standards
analysis of mitotically-inactive cells	instability
mixed samples detection	fluorochrome interference
low price	no visual control
	histogram interpretation
	karyological calibration

BOTANICAL APPLICATIONS

- determination of closely related species
- determination of hybrid plant (heteroploid and homoploid)
- detection of rare cytotypes
- cytotype distribution pattern at various spatial scales
- sympatric growth of various cytotypes
- detection of aneuploid individuals (incl. B-chromozomes)
- sex in dioecious plants
- endopolyploidy (incl. partial endoreduplication)
- agmatoploidy
- breeding mode detection
- genome composition in allopolyploid taxa
- base composition (AT / GC ratio)
- genome size and C-value determination
- nuclear DNA content in pollen grains

SAMPLE PREPARATION

1. nuclei isolation (razor blade chopping in hypotonic buffer), sample together with internal standard
    - Two step procedure Otto I + II (Otto 1990) – Otto I nontoxic, predominantly used
    - LB01 (Doležel et al. 1989)
    - Tris-$MgCl_2$ (Pfosser et al. 1995)
    - Matzk (seeds) (Matzk et al. 2000)
2. filtering through 42 µm nylon mesh
3. incubation in room temperature (min 5 minutes)
4. staining (DAPI or PI)
5. fluorescence analysis



An essential step is to select suitable internal standard. The most used standard species are listed below: taxon, cultivar, 2C-values, measurement method, the method for calibration (see above), calibration standards, references and some pros and cons are given.

Species	**Cultivar**	**2C-value**	**Measurement method**	**Reference**
Arabidopsis thaliana	ecotype Columbia	0.32 pg	flow cytometry	Bennett et al. 2003
Carex acutiformis	wild clone	0.82 pg	flow cytometry	Lipnerová et al. 2013
	wild clone	0.818 pg	flow cytometry	Šmarda et al. 2014
Oryza sativa	Nipponbare	0.795 pg	flow cytometry	Šmarda et al. 2014
Raphanus sativus	Saxa	1.11 pg	flow cytometry	Doležel et al. 2007
	Saxa	0.997 pg	flow cytometry	Šmarda et al. 2014
Solanum lycopersicum	Stupické polní rané	1.96 pg	flow cytometry	Doležel et al. 2007
	Stupické polní rané	1.735 pg	flow cytometry	Šmarda et al. 2014

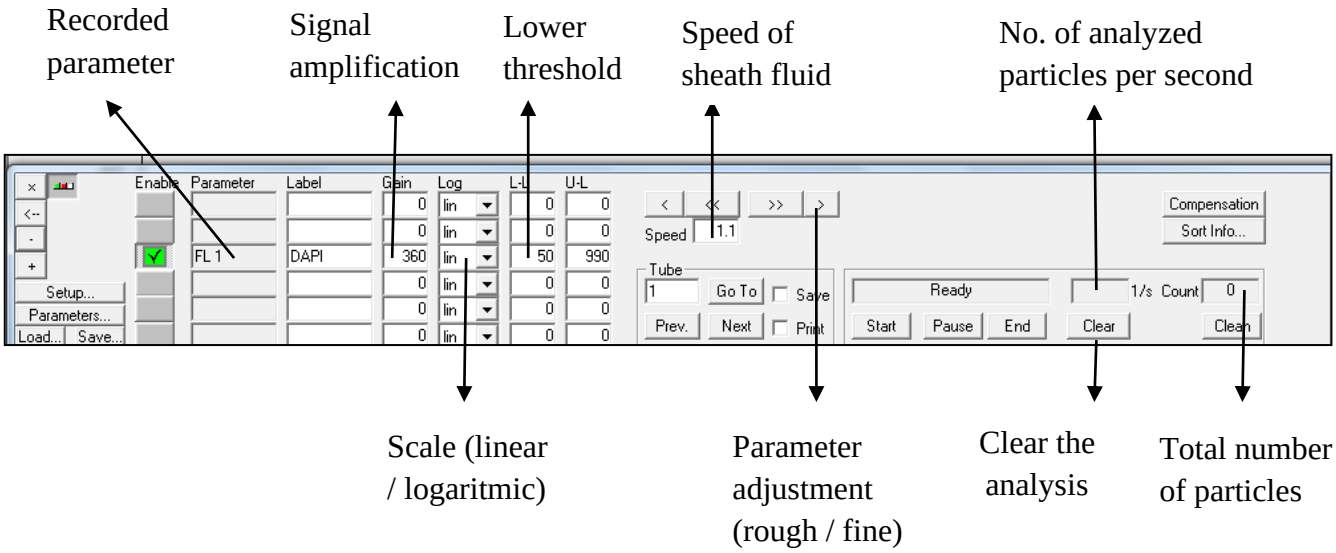
Glycine max	Polanka‘	2.50 pg	flow cytometry	Doležel et al. 2007
Solanum pseudocapsicum	commertial clone	2.59 pg	flow cytometry	Temsch et al. 2010
Bellis perennis	wild clone	3.38 pg	flow cytometry?	Schönswetter et al. 2007
	wild clone	3.159 pg	flow cytometry	Šmarda et al. 2014
Zea mays	CE-777	5.43 pg	flow cytometry	Doležel et al. 2007
Pisum sativum	Ctirad	9.09 pg	flow cytometry	Doležel et al. 2007
	Ctirad	8.018 pg	flow cytometry	Šmarda et al. 2014
	Dwarf Gray Sugar	8.77 pg	flow cytometry	Bai et al. 2012
	Kleine Rheinländerin	8.84 pg	flow cytometry	Greilhuber & Ebert 1994
Hordeum vulgare	Hitchcock	10.68 pg	flow cytometry	Tuna et al. 2001
Secale cereale	Daňkovské	16.19 pg	flow cytometry	Doležel et al. 2007
Vicia faba	Inovec	26.90 pg	flow cytometry	Doležel et al. 2007
		23.796 pg	flow cytometry	Šmarda et al. 2014
Allium cepa	Alice	34.89 pg	flow cytometry	Doležel et al. 2007
		30.745 pg	flow cytometry	Šmarda et al. 2014

FLOMAX SOFTWARE

INSTRUMENT CONTROL

Opens new screen

Opens Instrument setting panel



Gain - position the peak of the standard on the appropriate channel (e.g., 200)

- tune the instrument so that the gain is as low as possible (appropriate range 200-600)

Log - set linear for most measurements, logarithmic for analysis of endopolyploidy

Lower threshold - adjust so that (i) it cuts off most debris, (ii) is not higher than 0.4-times of the position of the first peak

Speed - adjust so that the number of analyzed particle per second is ~20 - 50 (use fine adjustment)

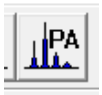
Clear - clear the analysis until the speed is uniform and distinct peaks appear on the screen

HISTOGRAM ANALYSIS

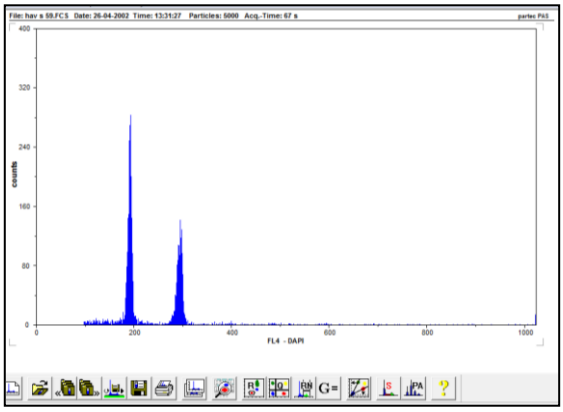
Following manual focuses on operating of the Partec software and subsequent analysis of resulting histograms and other dotplots.

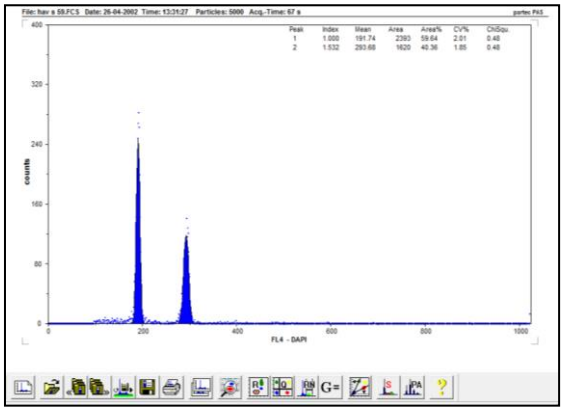
1-parameter measurements

I. DISTINCT PEAKS, LOW BACKGROUND - DIRECT PEAK ANALYSIS

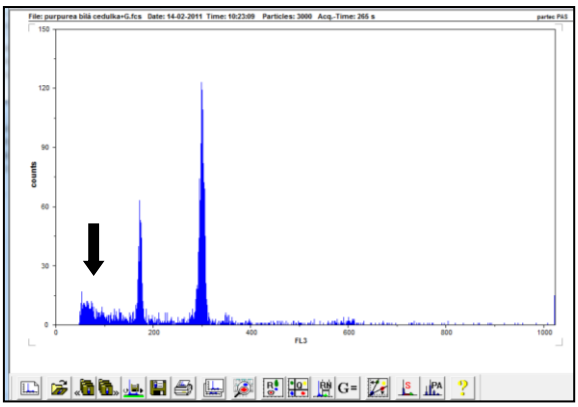
1)  or **Analysis - Peak analysis**...

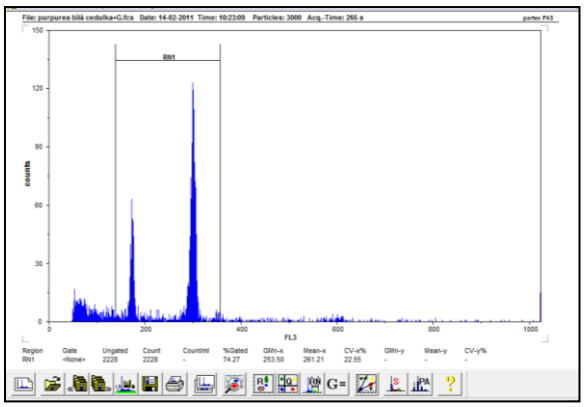
2) Fit Gauss Peaks





II. HIGHER BACKGROUND - SELECTION OF PEAKS, ELIMINATION OF DEBRIS (GATING)

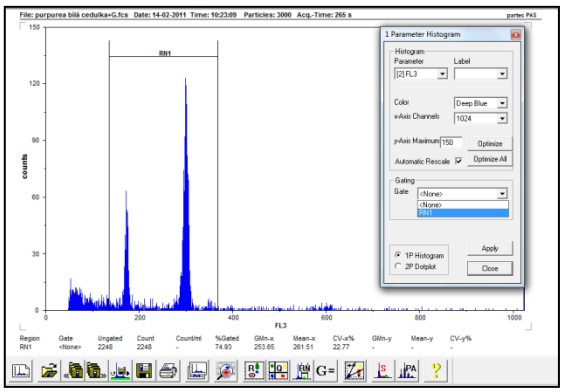


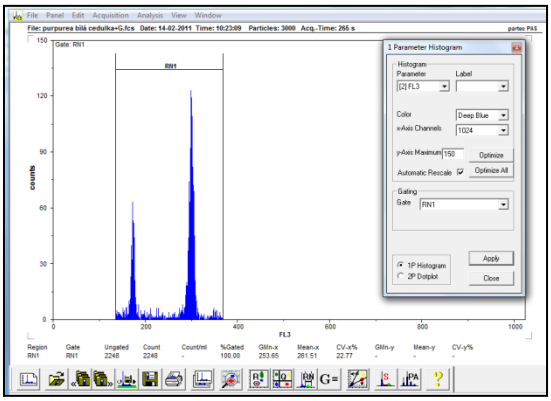


1)  or **Analysis - Gating regions - Region range...**

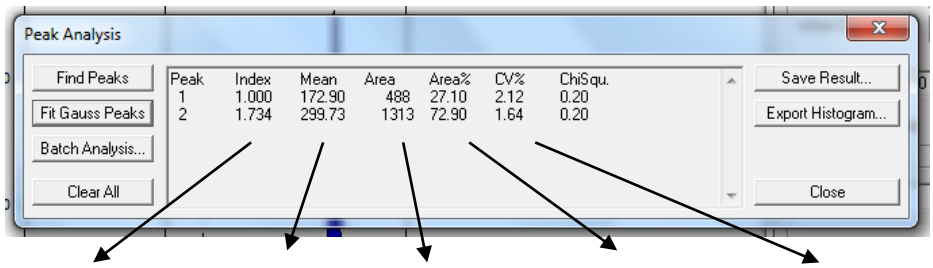
2) Selection of lower (left) gate - selection of upper (right) gate

3) Double click - selection of the gate (RN1) - Apply





4) Peak analysis (as above)



Peak ratio Position on the x-axis Number of particles in the peak Proportion of particles **Coefficient of variation**

III. LESS DISTINCT PEAKS - PEAK SELECTION

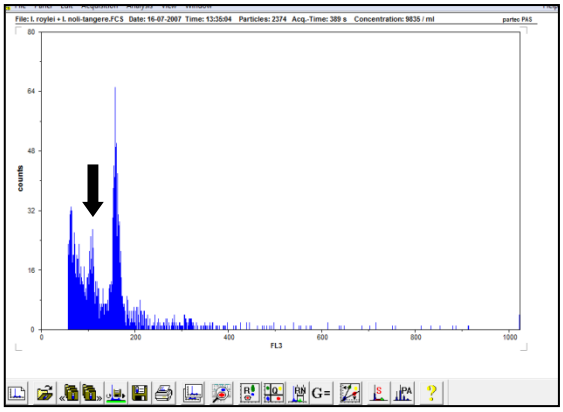
1)  or **Analysis - Peak analysis...**

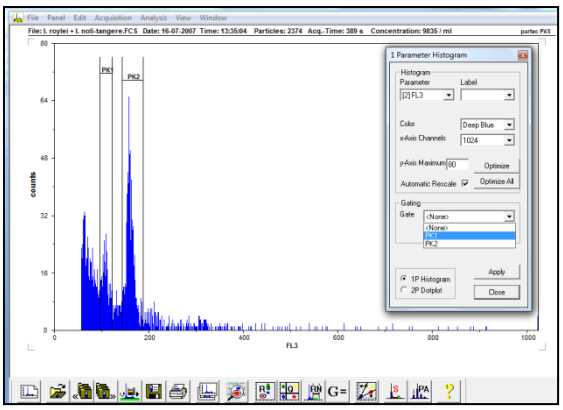
2) Find peaks

3) Selection of the first peak = double click - PK1 gate - Apply - Fit Gauss Peak; the same for the second peak

4) Comparison of peak statistics

Both peaks can also be gated manually





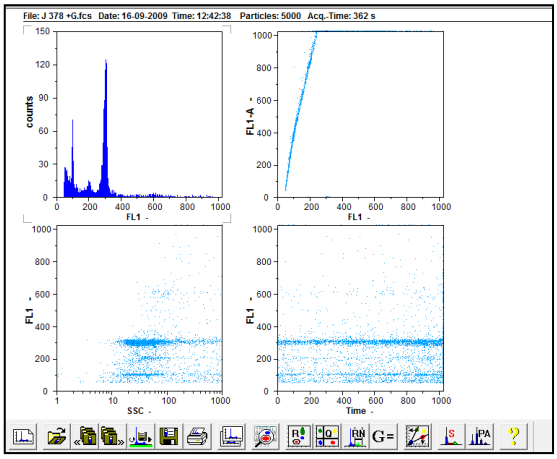
Multi-parameter measurements
(only in laser-based instruments or Beckman Coulter CytoFLEX instrument)

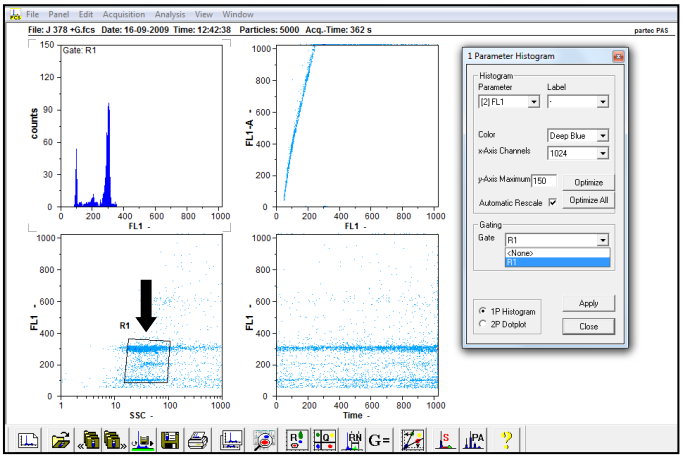
Debris elimination using SSC / FL gating

1)  or **Analysis - Gating regions - Polygon region...**

2) Select intact nuclei of SSC / FL scattegram

3) Go to the FL histogram - double click - R1 gate - Apply - Fit Gauss Peak





Plant samples for the practical session

- "easy" plant species, e.g., internal standards *Pisum sativum, Bellis perennis* or selected model taxa (genus *Pilosella*)
- identification of heteroploid hybridization (genus *Pilosella*)
- species with endopolyploidy, e.g., Brassicaceae (*Arabidopsis*), Cucurbitaceae, succulents - the effect of different tissues (leaves / flowers)
- species with polyphenolics (e.g., Rosaceae, ferns) - the effect of different fluorescent stains
- species with mucilage (e.g., Hyacinthaceae, Violaceae, Lythraceae, Malvaceae) - the effect of different tissues
- reproduction mode in seeds – embryo/endosperm ploidy ratio (sexual vs. apomictic), e.g. selected model taxa (genus *Pilosella, Taraxacum*)
- analysis of silica-dry samples
- progressively partial endoreduplication (Orchidaceae)