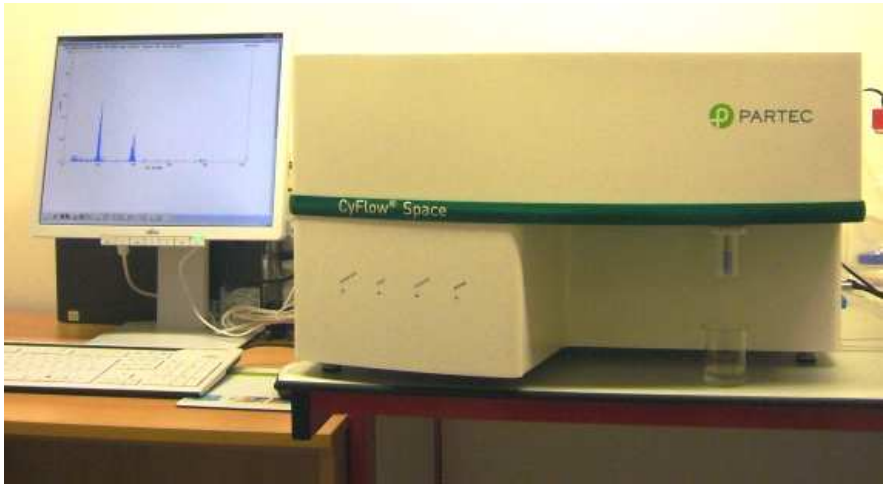
CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

FACULTY OF TROPICAL AGRISCIENCES

Department of Crop Sciences and Agroforestry

Laboratory of Plant Tissue Cultures

Book of practicals: Flow cytometry (Partec CyFlow Space)



Prague 2013

Acknowledgments

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CONTENT

1. Safety rules and regulations in Laboratory of plant tissue cultures

- At the beginning of the work in the laboratory, students have to know laboratory safety rules and regulations in Laboratory of plant tissue cultures.
- It is forbidden to eat, drink and smoke in the laboratory.
- Students have to wear laboratory coats, slippers and rubber gloves.
- Unauthorized experiments are strictly forbidden.
- The laboratory must be kept clean and organized.
- Check the proper installation of the equipment. If there is any problem with equipment, do not use it and inform the supervisor.
- If a piece of equipment fails while being used, report it immediately to your laboratory assistant or tutor. Never try to fix the problem yourself because you could harm yourself or the others.
- Clean up your work area before leaving the laboratory.
- Turn off all electric devices before leaving the laboratory.
- Before leaving the laboratory, wash your hands.
- Ask the supervisor if you are in doubt.
- Read labels carefully.
- Never “smell” a solvent directly! Read label on the solvent bottle to identify its contents. Chemicals must never be tasted!
- Check where the laboratory fire extinguisher and wash station are located and how to use them.
- Always give the chemicals and reagents you used back to the place where you had taken them from.
- Special care should be taken while working with open fire, combustibles, corrosives and toxic substances.
- Do not work in the laminar box with UV light on.
- Hazardous chemicals and their containers have to be dispose by proper authorized procedures.
- Always inform the teacher about any accident or injury and provide the first aid if necessary.
- Manipulation with irritating, smelling and toxic substances is allowed only in well aired and functional hood.
- Throw toxic and nontoxic waste into the appropriate containers.
- Everybody who work in the laboratory, have to respect all the rules mentioned above.

2. Sample preparatin using Partec CyStain UV Precise P Kit

Check if the CyStain UV Precise P Kit contents following reagents

- **Extraction Buffer, 125 ml**
- **Staining Buffer, 500 ml**

Storage conditions

- Store Extraction Buffer and Staining Buffer at 4 °C in the dark.

Additional material required

- Pipettes (1000 µl and 5000 µl) with compatible tips
- 55 mm plastic Petri dishes
- Razor blades
- 50 µm mesh filter
- Sample tubes

Sample preparation

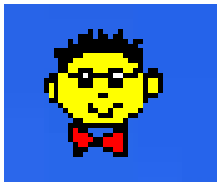
- Put approximately 0.5 cm^2 leaf tissue in a 55 mm plastic Petri dish.
- Add 400 µl Extraction Buffer.
- Chop the plant material using a sharp razor blade for 30-60 seconds (razor blades have to be exchanged after 5 to 10 samples).
- Incubate sample for 30 seconds up to 5 minutes (test the best incubation time for each individual species).
- After incubation in the Extraction Buffer filter the sample through a 50 µm filter.
- Add 1.6 ml Staining Buffer to the sample tube.
- Incubate for 30 to 60 seconds.
- Analyse the sample in the flow cytometer.

3. Starting a measurement in the flow cytometer

- Check SHEATH and WASTE bottles
    - Make sure SHEATH bottle is filled with no more than 1600 ml of Sheath Fluid. Make sure the WASTE bottle is empty.

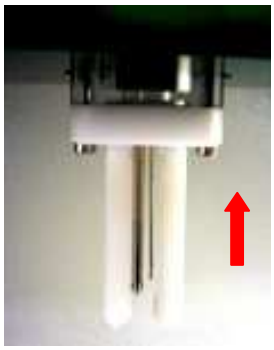
- Switch on the instrument
    - Switch on main power at the left side of the instrument. The laser has its own power button closeby, please switch it on, too.

- Switch on computer.
- Start instrument operating software
    - Doubleclick on the icon with left mouse button, the display shows the FloMax Welcome window. By confirming with “OK” the CyFlow space is initialized and the last used instrument settings are loaded.



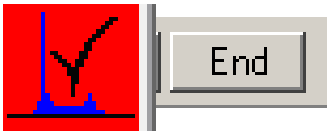
- Make sure the flow cytometer is ready for analysis and the operating software is prepared for the measurement.
- Fill up sample tube with 1-2 ml of the ready prepared sample suspension. The tube should not be filled up more than 2/3 of its volume.

- Insert sample tube onto the sample port until you recognize a “click”. The sample should be fully mounted within a second.

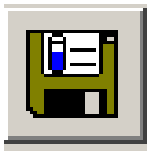


- The measurement starts automatically – FlowMax software indicates the **Prerun**, **Stabilize**, **Run** and **Counts**.
    - In the **Run** phase, cells are analyzed and classified into histograms on the display in realtime*.

- To finish the measurement, click to the icon or “End” button, or simply remove the sample tube from the sample port.



- To save results, click on the disk icon or select “File-Save As..”.

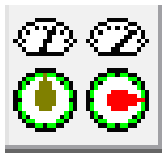


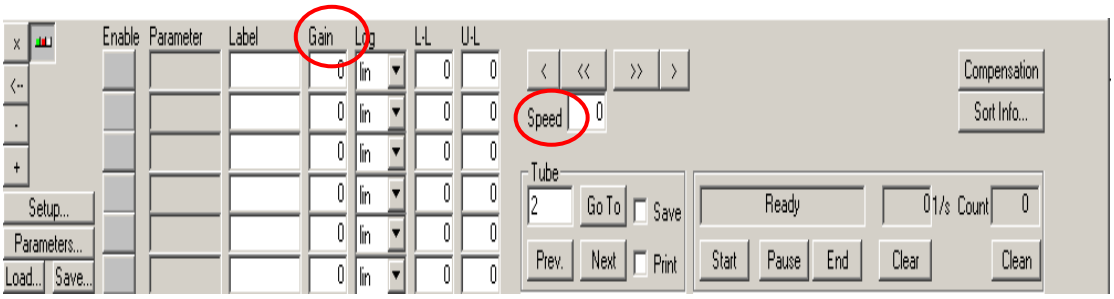
- To analyze the next sample tube repeat previous steps.
- Clean the flow cuvette and connecting tubes.
    - Use speed 15.
    - **Cleaning procedure:** Run 1.6 ml of Partec Cleaning Solution until sample tube is empty. Run 1.6 ml of Partec Decontamination Solution for one minute. Press “Clean” button once to start manually a cleaning cycle. Run 1.6 ml Partec Sheath Fluid for two minutes. Press “Stop”.
- Protect the sample port. Leave the final sample tube connected to the sample port- this avoids drying and crystallizing of any remaining material.

- Quit the operating software.
- Shut down the computer.
- Switch off instruments. Switch off main power and laser at the left side of the instrument.

NOTE: * In order to optimize the acquisition for the particles of interest, the instrument settings can be used in this phase. Instrument settings will be explained in Chaper 4.

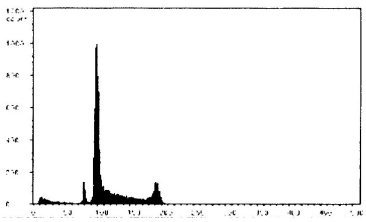
4. Operating basics – Instrument settings

- Open the Instrument settings box using the icon 
or select “Acquisition–Instruments settings” in the menu. The Instruments settings box appears.

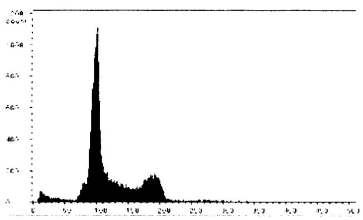


- **Gain**
  - By means of the gain, the signal amplification can be adjusted in a wide range for each parameter separately.
  - The gain value can be increased (decreased) by clicking into the corresponding gain value field and using the Up (Down) buttons. In case of linear amplification is used, peaks are moved to the right (compressed to the left) when increasing (decreasing) the gain. Use gain to move the peaks of interest to a suited position in the histogram.
- **Sample speed**
  - By means of the speed value, the sample speed (in term of µl/s) is set.
  - The speed value of a parameter can be increased (decreased) by clicking into the corresponding field and using the Up (Down) buttons.
  - The histogram is built up faster (slower). If the speed is increased too much, peaks in the histogram may become wider as a result of decreasing accuracy.

Typical effect of speed change



Speed = 3µl/s

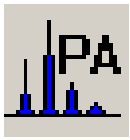


Speed = 20µl/s

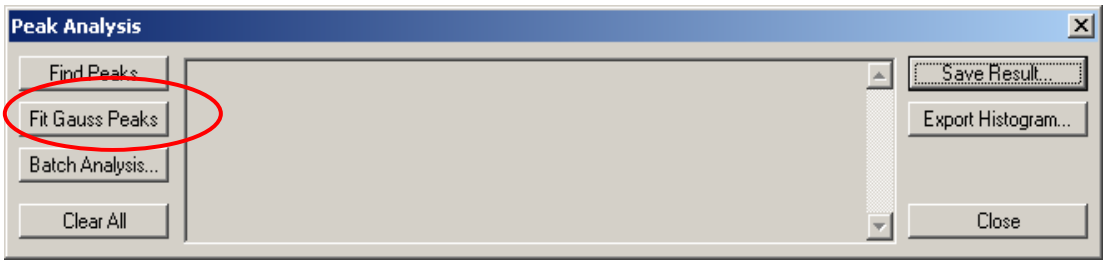
- NOTE: For more information about instrument settings see Operating Manual-Instrument Control and Acquisition in the Laboratory of plant tissue cultures.

5. Peak analysis

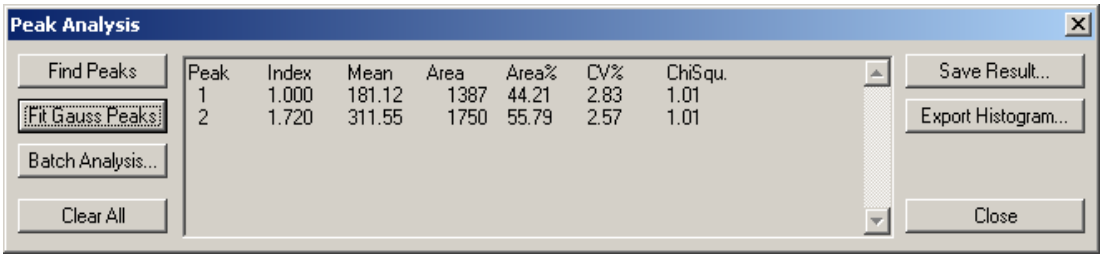
By the numerical fit method a mathematical model is fitted to the histogram data. In the model, each peak is represented by a Gaussian distribution with given position, width, and height.



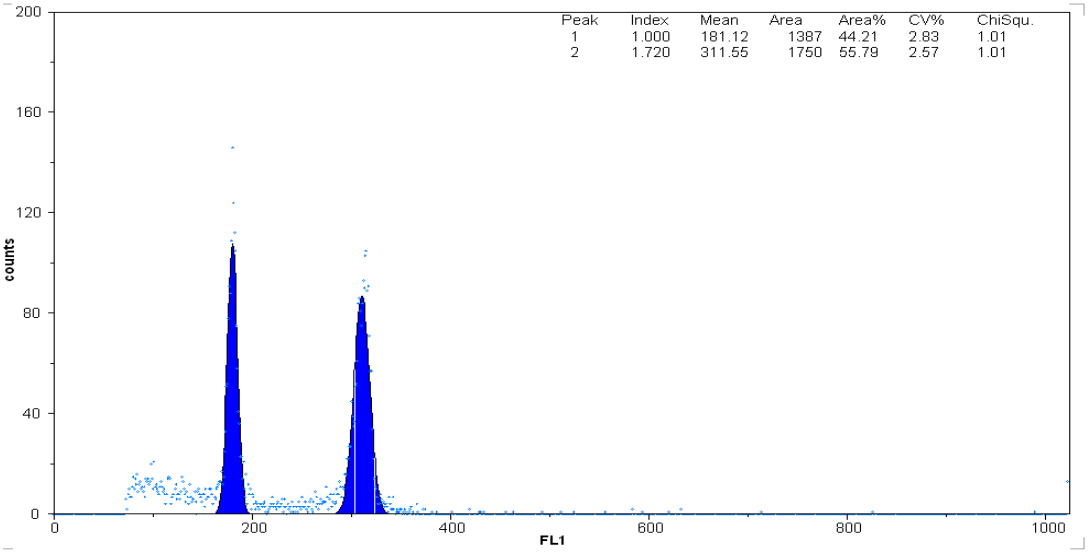
- Click the “Peak Analysis” button in the toolbar or select “Analysis–Peak Analysis”.
- The Peak analysis box is opened.
- Click “Fit Gauss Peaks”.



- The numerical result is shown in the Peak analysis box.



- Press “Close” to close the Peak analysis box. Results stay displayed in the histogram.



- **Quantitative Peak Results:**
  - **Peak:** peak number (from left to right)
  - **Index:** relative peak position related to the first peak (or a reference channel)
  - **Mean:** mean channel position of the peak
  - **Area:** peak area (of Gaussian peak). Corresponds to the number of particles belonging to that peak.
  - **Area (%):** percentage of peak area related to all sum of all peak areas.
  - **ChiSqu.:** measure of the variation between experimental data and the fitted mathematical model.

- NOTE: For more information about data processing see Operating Manual-Data Analysis in the Laboratory of plant tissue cultures.

6. General recommendation for ploidy level analysis

- Internal standardization should always be used.
- Nuclear DNA contents of the internal standard and the sample should be reasonably closed (but not overlapping or extremely close).
- The peaks of both the internal standard and the sample should be symmetrical (non-skewed) and of approximately the same height. The fluorescence of 5000 - 7000 particles should be recorded.
- The coefficient of variation of G0/G1 peaks should be reasonably low (generally below 3 %, although higher values can be regarded as acceptable e.g. in plants possessing very small nuclear DNA content).

Plant DNA standards

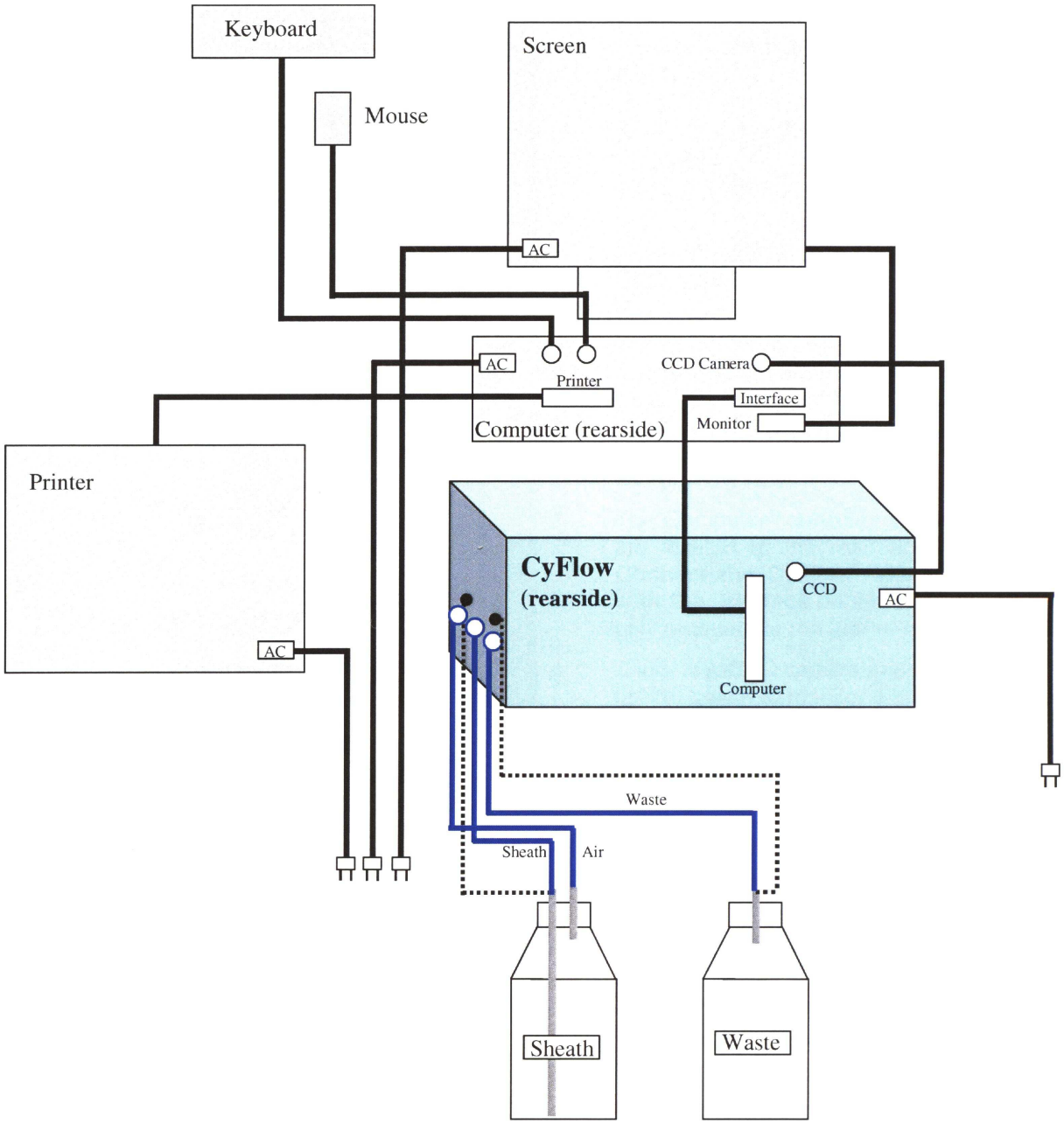
Species	**2C DNA content (pg)**
Raphanus sativus cv. Saxa	1.11 pg
Lycopersicon esculentum cv. Stupické polní tyčkové rané	1.96 pg
Glycine max cv. Polanka	2.50 pg
Zea mays cv. CE-777	5.43 pg
Pisum sativum cv. Ctirad	9.09 pg
Secale cereale cv. Dankovské	16.19 pg
Vicia faba cv. Inovec	26.90 pg
Allium cepa cv. Alice	34.89 pg

(source: Institute of Botany AS CR, Prague – Průhonice, Olomouc)

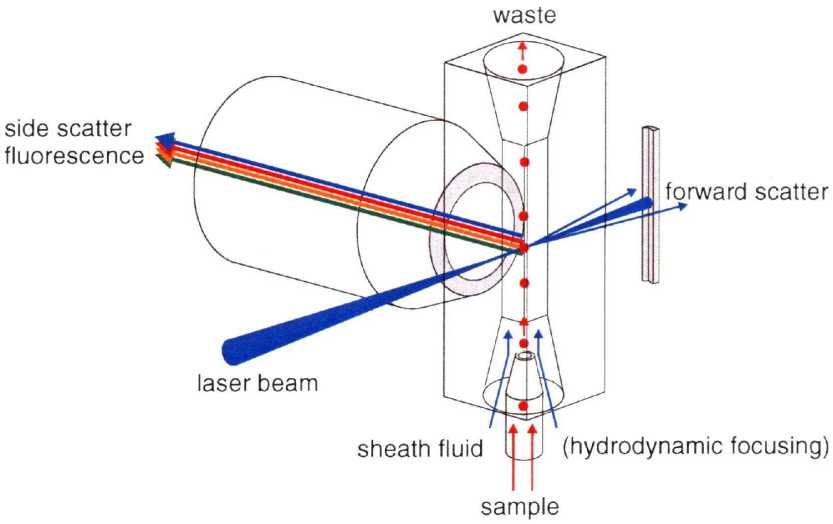
7. Appendix: Instrument setup - overview

Typical flow cytometer consists of five basic operational units:

1) light source (laser or arc lamp)
2) flow chamber* and fluidic system
3) optical assembly (lenses, filters, mirrors)
4) signal processing part (photodetectors, converters)
5) computer part



***Flow chamber:**



Flow chamber represents central part of the instrument. Its mission is to adjust the measured particles in a narrow central stream and to deliver them one after another into a focal point of the light source. This is achieved by so-called hydrodynamic focusing: the sample is injected into a stream of sheath fluid (mostly water or saline solution) moving with a greater velocity and thus confining the sample within a central core. Induced acceleration at the narrowing flow chamber orifice forces the particles to move singly, and they are delivered to the point of excitation. (source: Institute of Botany AS CR, Prague – Průhonice)