A tutorial to plant flow cytometry using BD Accuri C6

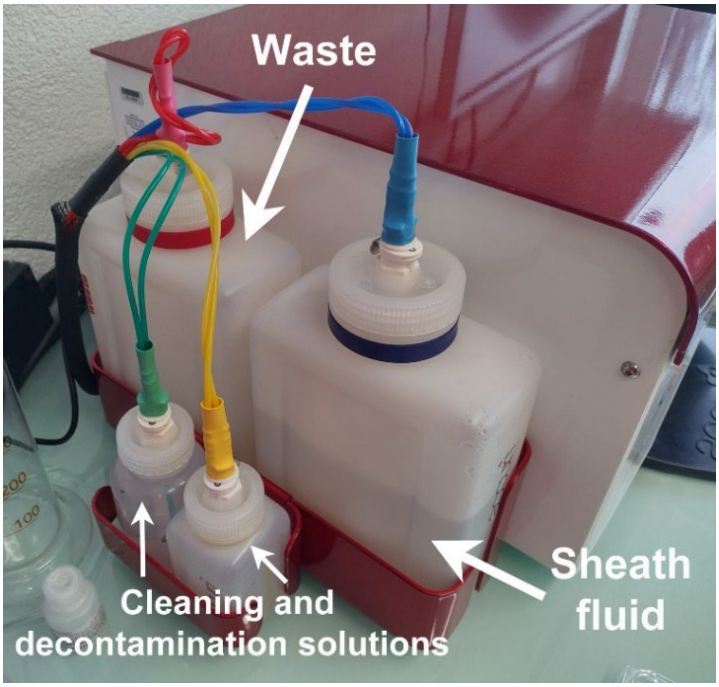
Martin Čertner, January 2023

Before starting:

- **Check that you have** all of the following: sufficient volumes of the Otto I and Otto II buffers, propidium iodide stock solution (and optionally also RNase stock solution), β-mercaptoethanol, small plastic petri dishes (may be re-used many times), a well plate compatible with the automated sampler (e.g. Costar 48-well plate, product no. 3548; may be washed and re-used), double-edged razor blades (each can be used twice – once from each side of the blade), Partec CellTrics filters (30 µm, green; may be washed and re-used many times), 1000 µL pipette + blue tips, sharp laboratory scissors.

- **Prepare the staining solution** in a falcon tube: add 20 mL of the Otto II buffer, 1 mL of the propidium iodide stock solution, 50 µL of β-mercaptoethanol, optionally also 1 mL of the RNase stock solution, and mix well before use.

- **Check** that **the flow cytometer** has enough sheath fluid (carries the stained nuclei throughout fluidic system of the machine), remove any remaining waste from previous measurements. The sheath fluid is distilled water mixed with additives from the manufacturer (Sarah will provide more details on that). Once in a while, the cleaning and decontamination solutions will need a refill (these are prepared from commercial concentrates provided by the manufacturer).



Plant sample preparation:

1. To a petri dish, add a small amount of selected plant tissue (the first-choice material are fresh young leaves; for an extensive account on alternative tissues and material preservation strategies see Čertner et al. 2021); approximately 0.5 – 1 cm^2 is usually enough to obtain 5,000 – 10,000 nuclei. If applicable, add the appropriate amount of reference standard tissue (the amount should be determined empirically, as the aim is to get two clearly separated peaks of similar size). See Temsch et al. 2021 for an overview of widely applied plant genome size standards.

2. Add 0.5 mL of ice-cold Otto I buffer.

3. Chop tissues of both the sample and the standard with a sharp razor blade until the material is finely chopped into equal pieces.

4. Place a green CellTrics filter on top of a well in the Costar plate and pass the homogenate through using a pipette. I recommend cutting off the tips to prevent their clogging with plant material. If convenient, note the position (identity) of your samples within the well plate on a piece of paper / on the plastic lid.

CellTrics filter

5. Incubate the nuclei suspension in a well for 5 – 30 min at room temperature. Prepare more samples in the meantime.

6. Add 1 mL of the staining solution (Otto II buffer + propidium iodide + mercaptoethanol) and incubate the sample for 5 – 20 minutes at room temperature before the flow cytometric analysis. With the Costar 48-well plate, the volume of each well is 1.6 mL and it is crucial they don´t spill over when the plate is handled (across sample contamination!) – it may be convenient to add just 0.8 – 0.9 mL of the staining solution.

Some useful notes:

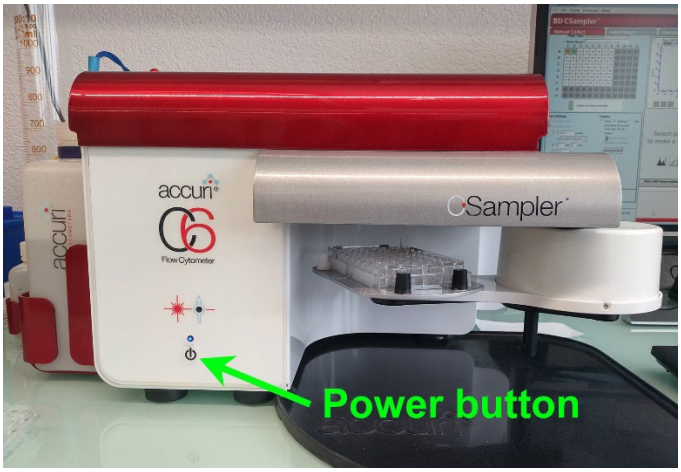
- These plant standards I brought from Prague and left in Fribourg. You can use *Solanum* and *Glycine* for ploidy analysis in *Biscutella laevigata* (2C = ca 1.8 Gbp for 2x *B. l. kerneri*); their peaks will likely overlap with 3x *Biscutella* (does not hinder the detection of triploids) but they are optimal for telling apart 2x and 4x *Biscutella*.

Species	Genome (2C)	Reference	Availability
Solanum pseudocapsicum	2.533 Gbp	Temsch et al. 2010 Preslia 82:63-80	plantlet
Pisum sativum cv. 'Ctirad'	8.89 Gbp	Doležel et al. 1998 Ann Bot 82:17-26	seeds
Glycine max cv. 'Polanka'	2.45 Gbp	Doležel et al. 1994 Biol Plant 36:351-7	seeds

- I also brought some LB01 buffer which is an alternative to Otto I and II buffers. The LB01 is a universal buffer that allows both nuclei isolation and staining. You can use it as it is for nuclei isolation (instead of Otto I) and then take an aliquot and mix it with propidium iodide and mercaptoethanol (the same concentrations as with Otto II) to produce a staining solution. In our experience the Otto buffers usually provide higher precision measurements but may not work with every plant species, then you can try the LB01 instead (its chemical composition is somewhat different and has a higher pH).

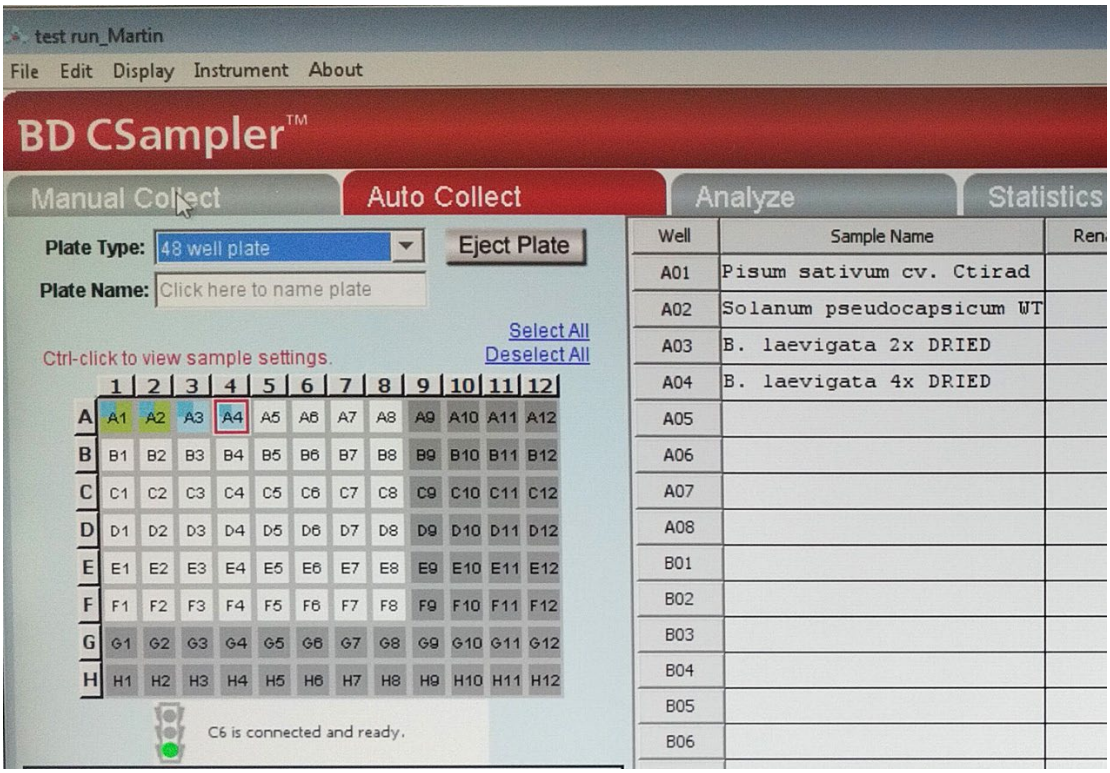
Flow cytometric analysis of samples:

1. Turn on the computer, then run VirtualBox to start an older version of Windows which is compatible with the flow cytometer operating software. Start the program BD CSampler (the operating software must be running before the machine!). Turn on the flow cytometer using the power button in the front.

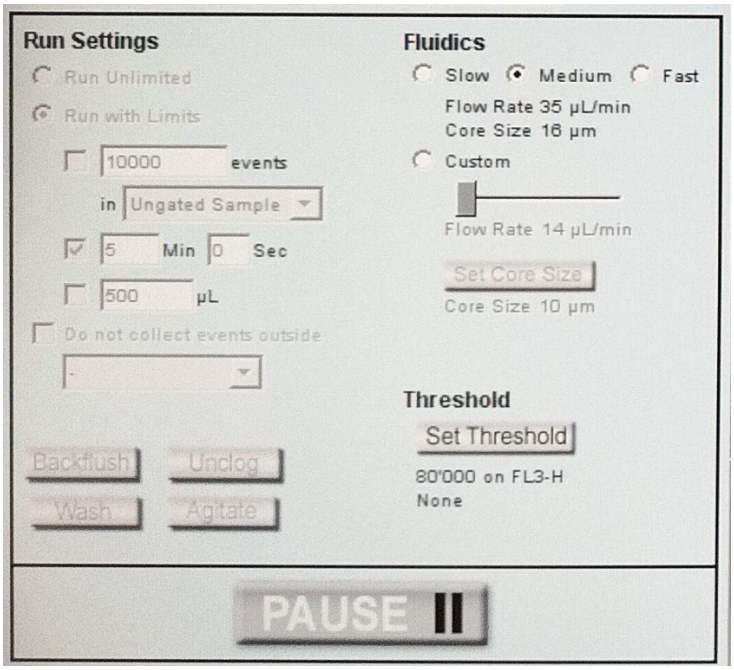


2. Lower the arm of an automated sampler via the main software menu (Instrument / Align CSampler). Place a well plate with the stained samples on the automated sampler as shown in the picture below.

3. Select either “Manual Collect” to run a particular sample or “Auto Collect” to let the flow cytometer analyse all the samples (one by one). Check that you have correctly specified Plate Type = 48 well plate. On the Auto Collect tab you can specify the names of your samples in particular wells (A1 – F8) after clicking in a table on the left (representing your plate with samples).



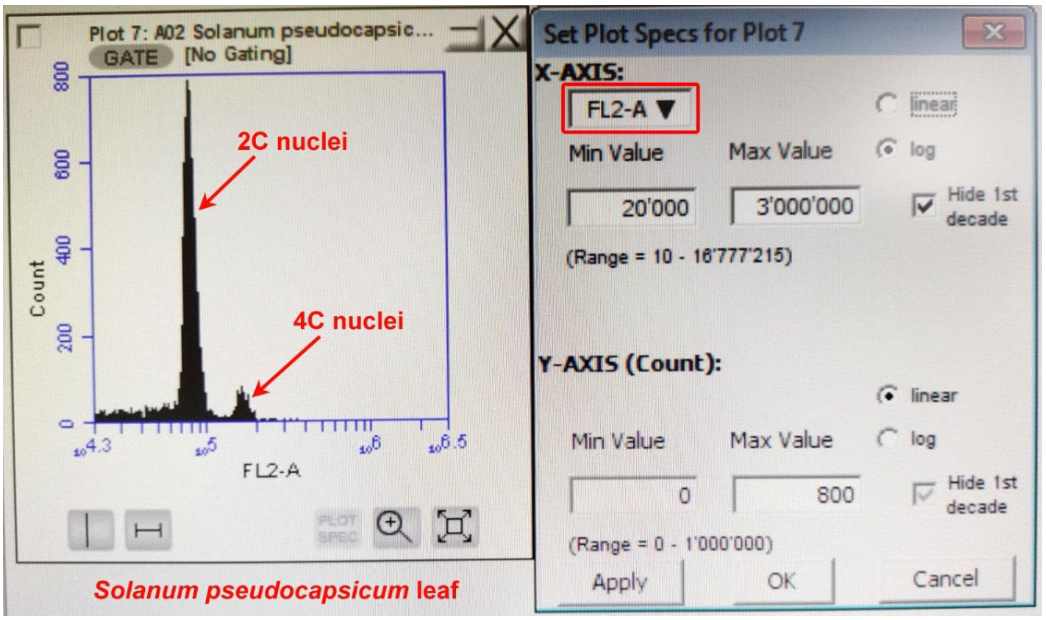
4. There are a few things you can set before running your samples. In the "Run Settings" menu, I recommend putting some thresholds on when the analysis will be terminated ("Run with limits"; e.g. 10,000 events or 5 – 10 minutes). In "Fluidics" you can alter the flow rate, last time I preferred medium over slow (which took way too long though in theory should result in more precise measurements).

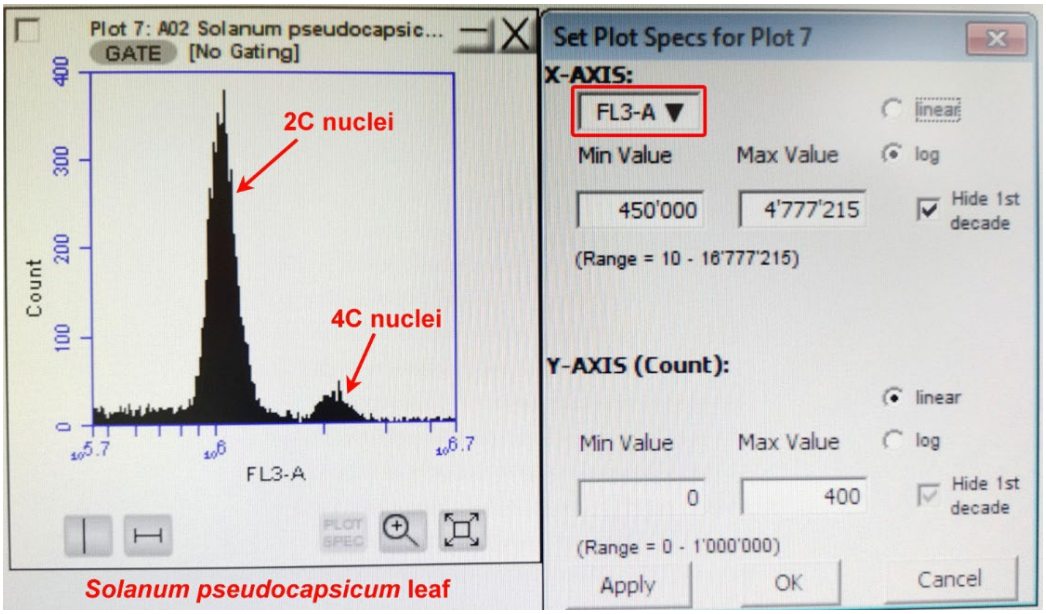


5. At this point, you can initiate Run / Autorun and analyse your samples. Plots will appear, where you will see the events being recorded (and you can change their number and type). Even if you don´t see anything in the plots (nuclei are outside the bounds delimited by the axes), all information will be recorded, and you can recover it later. (In your first runs you can load the workspace "test

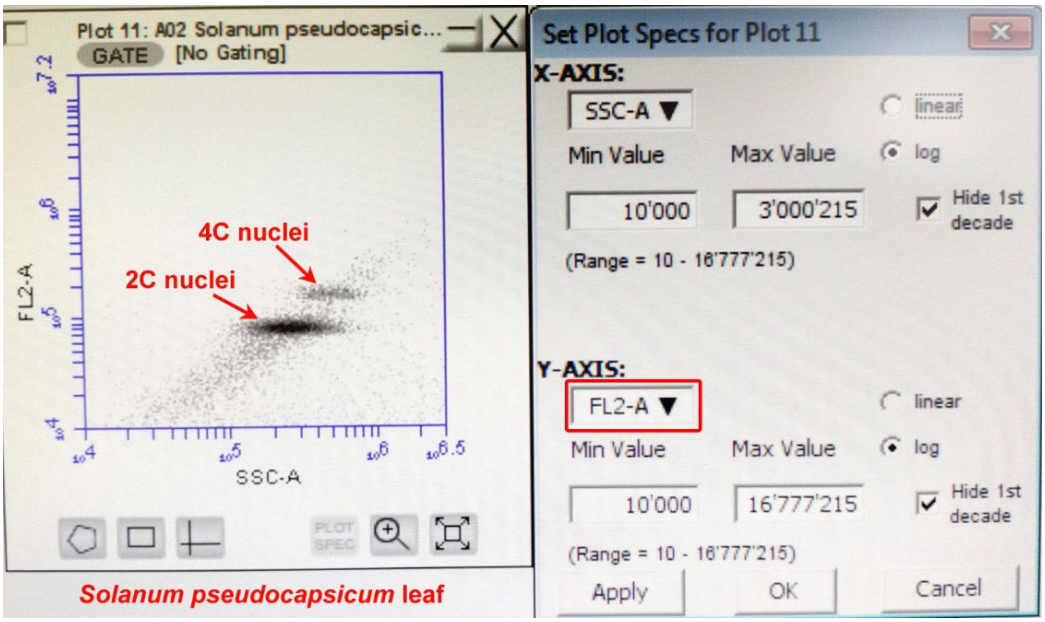
run_Martin.c6" that I created, and which contains a reasonable set of parameters for ploidy analysis in *Biscutella laevigata*.) Be careful with setting any thresholds as you will not be recording data within the specified intervals (loss of information), it is safe to set them at least an order of magnitude lower than where you expect your peaks (e.g. to remove bacterial / organellar genomes).

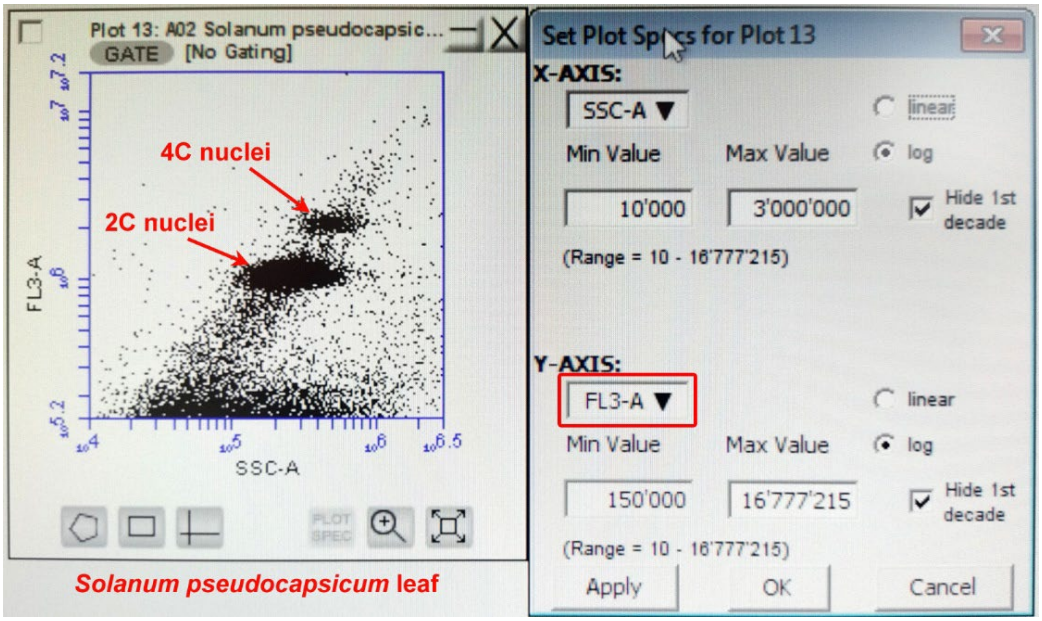
6. On the "Analyze" tab, you can visualize and explore your results. Click on "Make a new plot" to add a fluorescence histogram, dot plot or a density plot. The propidium iodide fluorescence can be detected on two channels, FL2-A and FL3-A, but I would recommend using FL2-A which seemed to provide a better resolution in my pilot analyses. Using the "PLOT SPEC" button you will have to adjust the channels being plotted and limits of the X and Y axes, for *Biscutella laevigata* you can start with the same setting as in the pictures below.



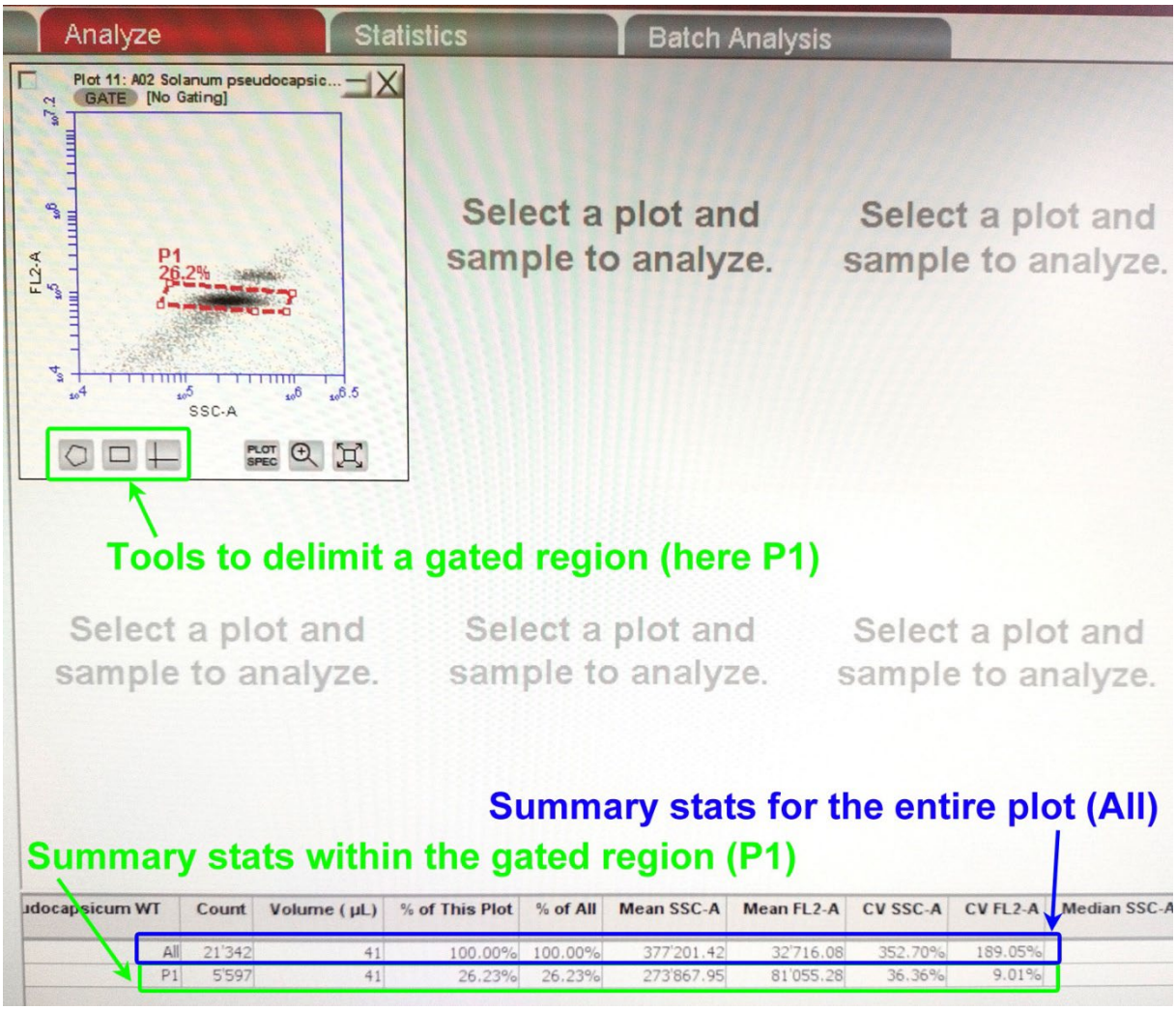


You can also plot this channel against the side scatter of light (SSC-A), which is more appropriate for precise gating and data analysis (though rather useful for genome size estimation than ploidy level analysis).





7. To extract information from the plots, you can use a procedure called “gating”. Usually, you are interested in the mean fluorescence of both your sample and standard nuclei (to calculate a sample / standard ratio; = relative genome size) and in relative precision of your analysis (coefficients of variation (CVs) for your sample and standard nuclei). In the lower left corner of each fluorescence diagram, there are designated tools to delimit the nuclei of interest using intervals (in a histogram) or polygons (in a dot plot / density plot). Each gate will add a new line in the summary table at the bottom of the software window (from which you can easily copy paste the data). You can remove an existing gate by pressing the Del key.



8. You can save your document (incl. all the measurements, analysis results and instrument setting) as a workspace (File / Save workspace as…) and gradually collect your data on multiple occasions. An autosave function is turned on by default.

9. Diagrams may be exported as a FCS file (universal format to store flow cytometric data) and opened in a different software, probably there is also a possibility to export them as raster images. On the "Statistics" tab, there are some options for extracting fields from analysis tables across samples and combining that in a single master table, which might be useful.

10. The software is very simple and user friendly, feel free to explore it on your own. There is also a thorough software documentation, available for example at:

https://www.bdbiosciences.com/content/dam/bdb/products/instruments/flow-cytometers/research-cell-analyzers/accuri-c6/BD_Accuri_QuickStartGuide_CSamplerSoftware.pdf

https://www.univ-reims.fr/media-files/22088/bd_accuri_csampler_software_user_guide.pdf

https://rai.unam.mx/manuales/ucf_BD_Accuri_C6_Software_User_Guide.pdf

When the analysis is finished:

1. Lower the arm of the automated sampler (Instrument / Align CSampler) and take out the well plate with your analysed samples. Pour off the remains of your samples (in a special container or down the drain) and wash the well plate with distilled water for re-use (and do the same with the used CellTrics filters and petri dishes).

2. When you are finished with your measurements for the day, it is important to clean the fluidic system of the flow cytometer via Instrument / Run cleaning fluid cycle (for at least 10 min.). From time to time, you may also use other cleaning options (particularly the decontamination fluid cycle, extended clean of flow cell). Ask Sarah for more details on the cleaning routines she is applying.

3. After the cleaning fluid cycle is finished, turn off the machine using the Power button in the front. The machine will be working for a while, making odd sounds. Only then you can exit the BD CSampler software, VirtualBox, Windows and turn off the PC.

References and further reading:

Čertner M, Lučanová M, Sliwinska E, et al. (2021) Plant material selection, collection, preservation, and storage for nuclear DNA content estimation. Cytometry Part A, 101: 737-748. https://doi.org/10.1002/cyto.a.24482 **[a review of how to choose and sample optimal tissues for flow cytometry, incl. long-term material preservation strategies]**

Doležel J, Binarová P, Lucretti S (1989) Analysis of nuclear DNA content in plant cells by flow cytometry. Biol Plant 31:113–120. https://doi.org/10.1007/BF02907241 **[LB01 buffer citation]**

Otto FJ (1992) Preparation and staining of cells for high-resolution DNA analysis. In: Radbruch A (ed) Flow Cytometry and Cell Sorting. Springer Lab Manual. Springer, Berlin, Heidelberg 65–68. https://doi.org/10.1007/978-3-662-02785-1_8 **[Otto buffers citation]**

Sliwinska E, Loureiro J, Leitch IJ, et al (2021) Application-based guidelines for best practices in plant flow cytometry. Cytom Part A. https://doi.org/10.1002/cyto.a.24499 **[a review of best practices in ploidy level assessment and genome size estimation using flow cytometry]**

Temsch EM, Koutecký P, Urfus T, et al. (2021) Reference standards for flow cytometric estimation of absolute nuclear DNA content in plants. Cytometry Part A, 101: 710-724. https://doi.org/10.1002/cyto.a.24495 **[a review of how standardisation works in flow cytometry, list of widely used plant standards]**