Flow cytometry

basic principles
protocol

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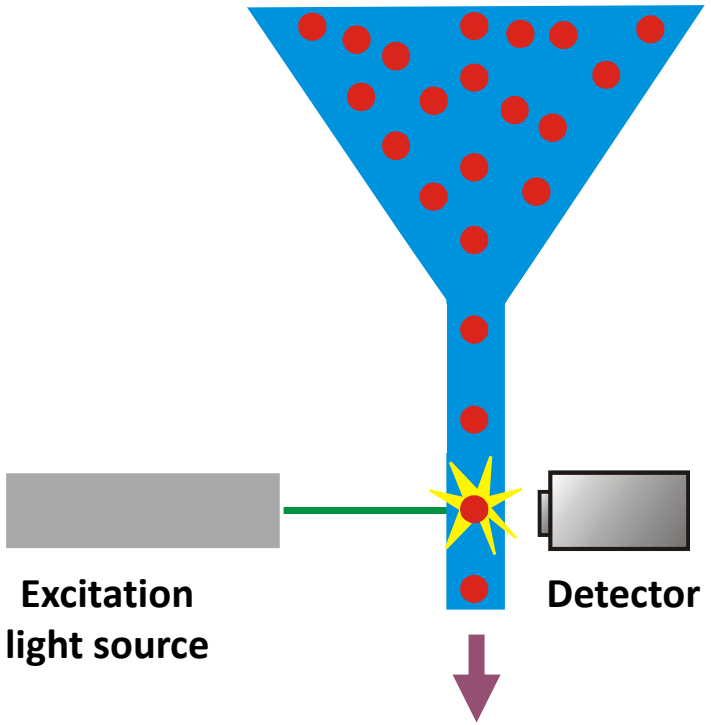
DNA flow cytometry

DNA flow cytometry involves the analysis of fluorescence intensity of nuclei/chromosomes (particles) in flow, after staining with a DNA-specific fluorescent dye

The sample for flow cytometry should be a suspension of single nuclei (no clumps allowed)



DNA flow cytometry

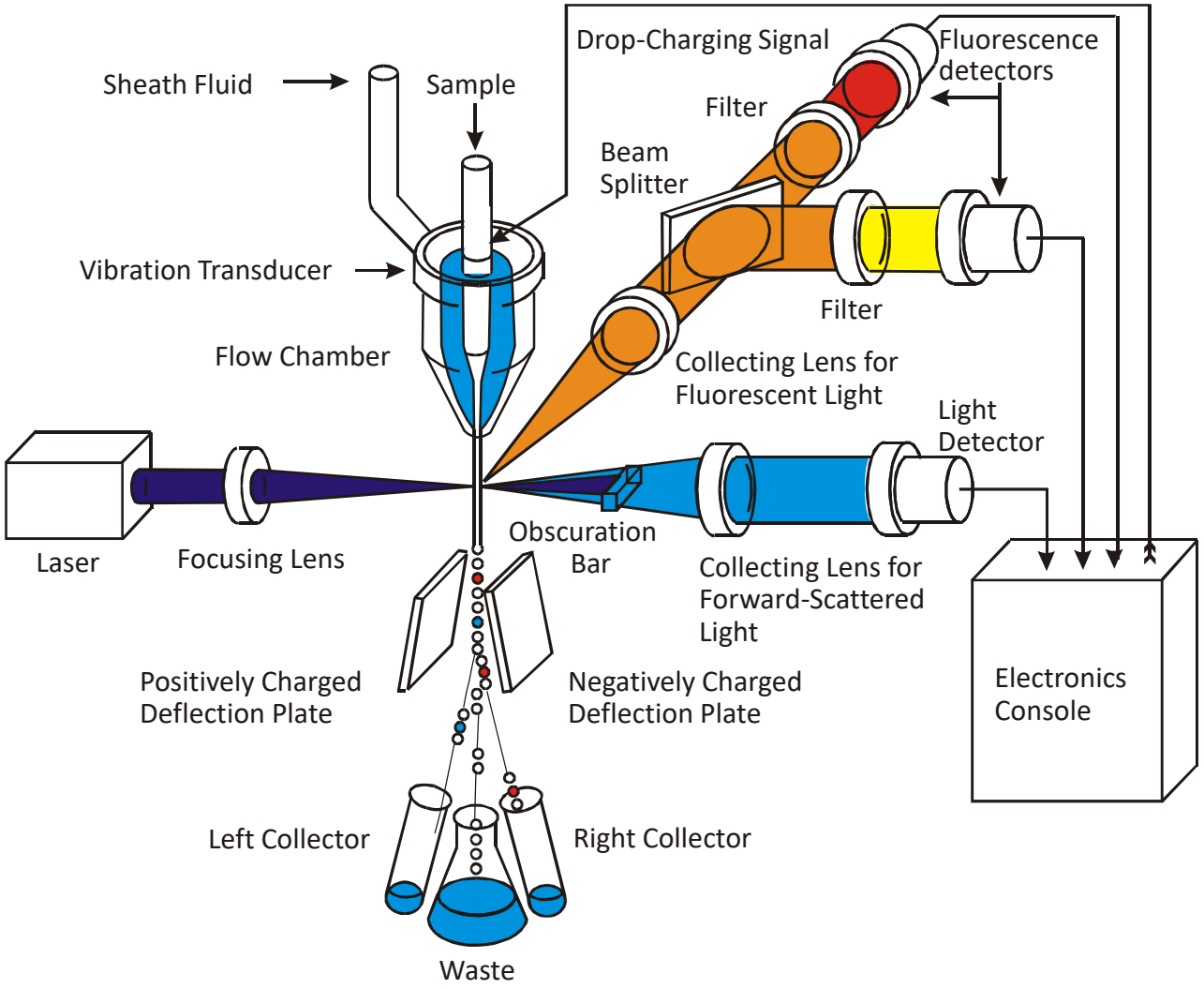
- unique feature - ability to analyse multiple optical properties of single particles at the rate of several hundreds or thousands per seconds

- flow cytometers - sophisticated instruments utilizing fluidics, lasers, optics and electronics

Basic principles:

- population of single particles, suspended in a medium are injected into a stable stream that forces particles to travel one by one to be interrogated by the flow cytometer
- particles pass individually through a beam of light (laser)
- after interaction between the particles and light (light scatter and fluorescence) the optical signal is drived/directed to the detector (photomultiplier) where optical pulses are transformed into electrical pulses and then processed by electronic processing
- results are displayed as monoparametric frequency distribution histogram, biparametric (2D) dot plots or multiparametric dot plots

DNA flow cytometry



DNA flow cytometry

Basic principles:

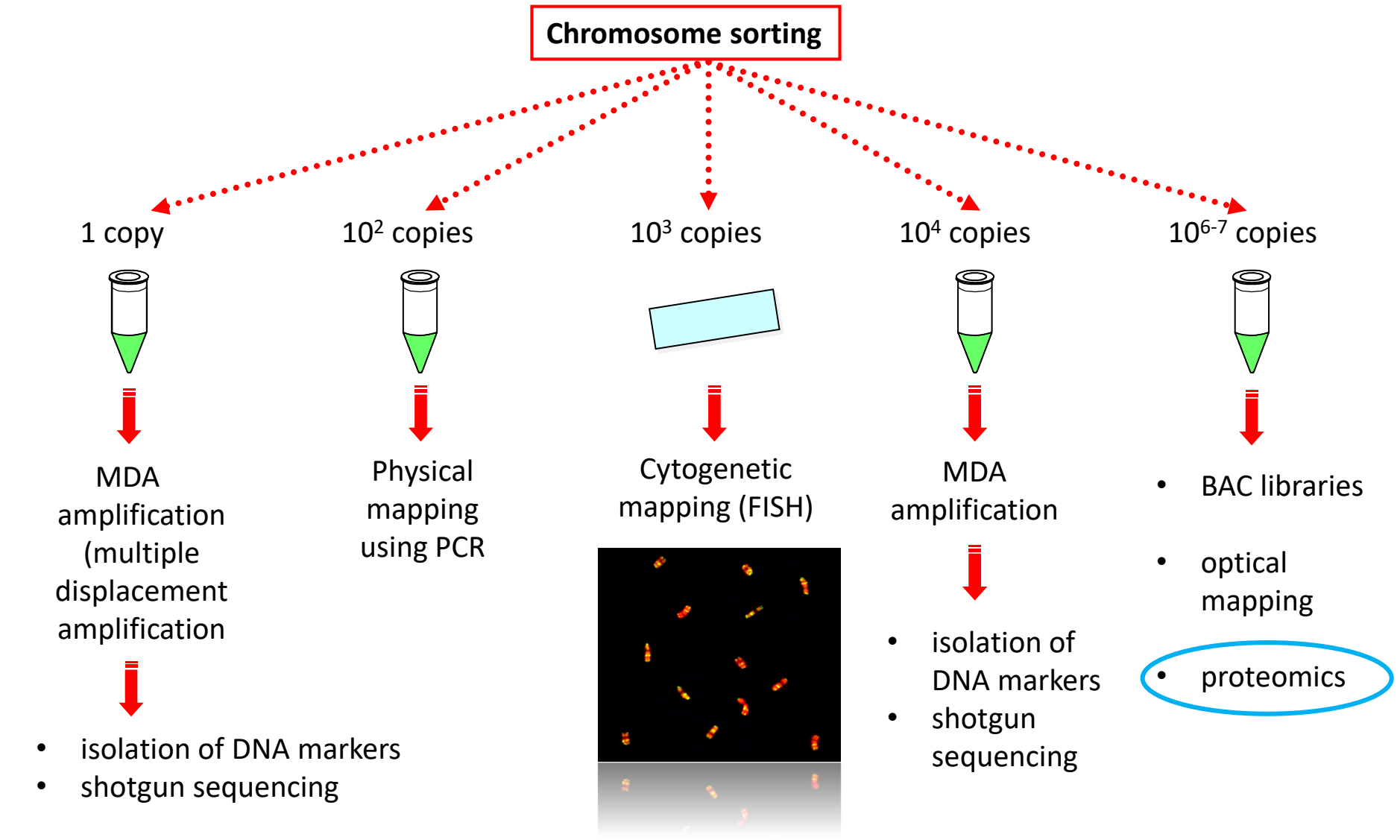
- sorters can physically isolate particles of interest for further analyses
- they can do it simultaneosly with the analysis and at high speed

- when particle of interest (in droplet) is detected, instrument electrically charges the droplet containing particle
- further downstream, the charged droplet is electrostatically deflected and collected in a tube (or on microscopical slide).

Flow cytometry has found numerous applications in biomedical research, but its extenstion to plant science has been hindered by problems surrounding sample preparation (Doležel et al., 2007).

The requirement to generate a suspension of single particles is difficult to manage in most **plant materials**, because the cells are typically joined to one another by a robust extracellular matrix, and their rigid cell walls hamper the release of the cellular content.

A portfolio of applications of sorted chromosomes



Protocol

Plant material:

dried mature seeds of barley (Hordeum vulgare L., cv. Morex)

Reagents and solutions:

1. Tris buffer: 10 mM Tris/HCl, pH 7.5, 10 mM Na2EDTA, 100 mM NaCl

2. Formaldehyde fixative: 2% (v/v) formaldehyde in the Tris buffer. Prepare just before use!

3. 10x LB01-P stock solution: 15 mM Tris/HCl, pH 9.0, 2 mM Na2EDTA, 0.5 mM EGTA, 80 mM KCl, 20 mM NaCl, 0.2 mM spermine·4HCl, 0.5 mM spermidine·3HCl, 0.1% (v/v) Triton X-100

4. LB01-P buffer: Mix 1 mL of the 10x LB01-P stock solution with 9 mL deionized water. Add 10 µL of 2-mercaptoethanol and mix well. Prepare just before use!

Protocol

Reagents and solutions:

5. DAPI stock solution: 0.1 mg/mL DAPI
6. Phenylmethanesulfonylfluoride (PMSF) stock solution: 100 mM PMSF dissolved in isopropanol
7. Sheath fluid: 10 mM NaCl
8. P5 buffer: 10 mM Tris/HCl, pH 8.0, 50 mM KCl, 2 mM MgCl2.6H2O, 5% (w/v) sucrose

Note:
The highest available purity of all chemicals is a prerequisite for achieving best results.

Protocol

Consumables, tools and instruments:

1. Non-powdered protective gloves.
2. Pipettes and appropriate tips.
3. Plastic tubes (0.5, 1.5, and 2 mL) with appropriate racks.
4. Biological incubator with a temperature control.
5. Glass Petri dishes (18 cm diameter) with filter paper cut to fit the diameter for seed germination.
6. Mechanical tissue homogenizer.
7. Nylon mesh filters (50 and 20 μm pore size), cut to 4 x 4 cm squares.
8. Sample tubes for flow cytometer.
9. Sample tube holder.
10. Ice container.
11. pH meter.
12. Flow cytometer and sorter equipped with blue (488 nm, 100 mW) and UV (355 nm, 100 mW) lasers.
13. Microscopic slides with coverslips.
14. Fluorescence microscope with optical filter sets for DAPI fluorescence (for checking of nuclei integrity after flow sorting).

Protocol

Seed germination:

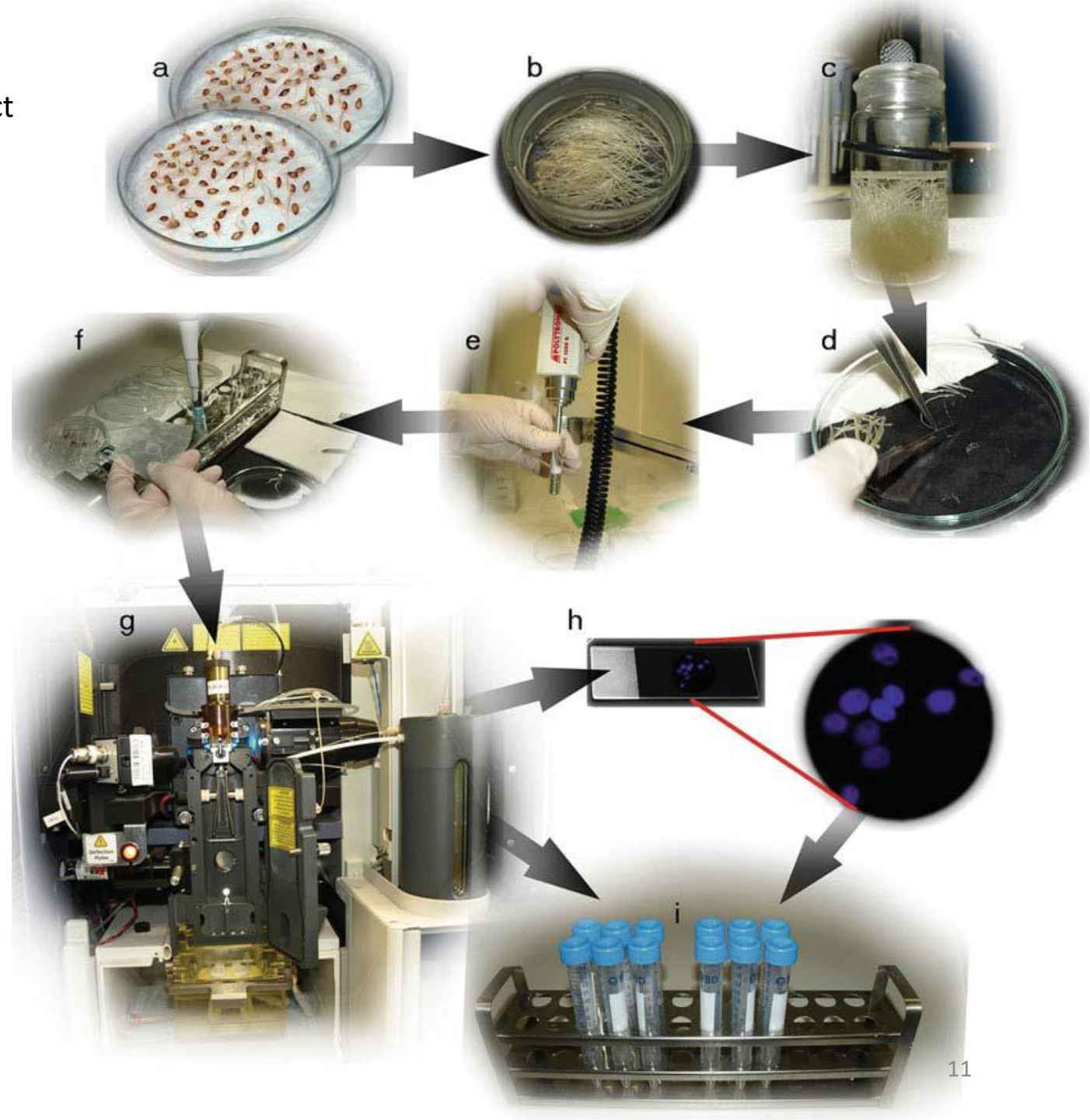
1. Leave seeds in a beaker filled with deionized H_2O and let them to soak at laboratory temperature for 15 min. Keep the seeds aerated in the dark at 25 C (in a biological incubator) overnight.

2. Germinate the seeds in a glass Petri dish on a layer of wet paper towels sandwiched by two layers of filter paper in the dark at 25 ^{o}C (in a biological incubator), until the root length reaches 2–3 cm (Fig. 1a).

Notes:
The use of viable and healthy seeds is necessary !!!
The number of seeds, which are needed for the preparation of a 1 mL sample, depends on the number of roots per seedling and size of root tips.

Fig. 1: Preparation of intact barley nuclei suspension

(a) Seed germination in a Petri dish.
(b) Cutting of roots and their transfer into a beaker with deionized water.
(c) Fixation of roots in formaldehyde solution.
(d) Cutting of meristem root tips prior to their mechanical homogenization.
(e) Release of nuclei into LB01-P lysis buffer by mechanical homogenization.
(f) Filtration of the suspension of nuclei.
(g) Nuclei sorting and collection into tubes
(h) or onto microscopic slides for integrity verification **(i)**



Protocol

Preparation of suspension of intact nuclei:

1. Cut out the roots and transfer them into a beaker with deionized H2O (Fig. 1b). The number of barley roots needed for the preparation of a 1 mL nuclei sample (see further) is estimated to a value of 60.

2. Place the roots into a beaker with the formaldehyde fixative solution and keep them immersed at 5 ^{o}C for 10 min (Fig. 1c).

3. Rinse the roots three times in the Tris buffer with EDTA at 5 ^{o}C for 5 min. After the last rinse, keep the roots in the Tris buffer on ice.

4. Cut root tips (in a length of 1–2 mm) and transfer them into a 5 mL polystyrene tube containing 1 mL of LB01-P buffer (Fig. 1d).

5. Grind the root tips using a mechanical homogenizer at 15,000 rpm for 13 s (Fig. 1e).

Protocol

Preparation of suspension of intact nuclei:

6. Filter the crude suspension of nuclei through a 50 µm nylon mesh and collect the filtrate into another 5 mL polystyrene tube (Fig. 1f).

7. Keep the suspension on ice until the flow sorting.

Notes:
During homogenization of root tips and the subsequent flow sorting, nuclei are exposed to shearing forces. The preceding mild fixation of roots with formaldehyde thus makes the nuclei more resilient.
Formaldehyde-fixed roots should be stored on ice and processed within a few hours.

Always wear non-powdered protective gloves and work in a bio-safety hood when using formaldehyde.

Protocol

Nuclei sorting using flow cytometry:

1. Set up the flow sorter according to the manufacturer's instructions.

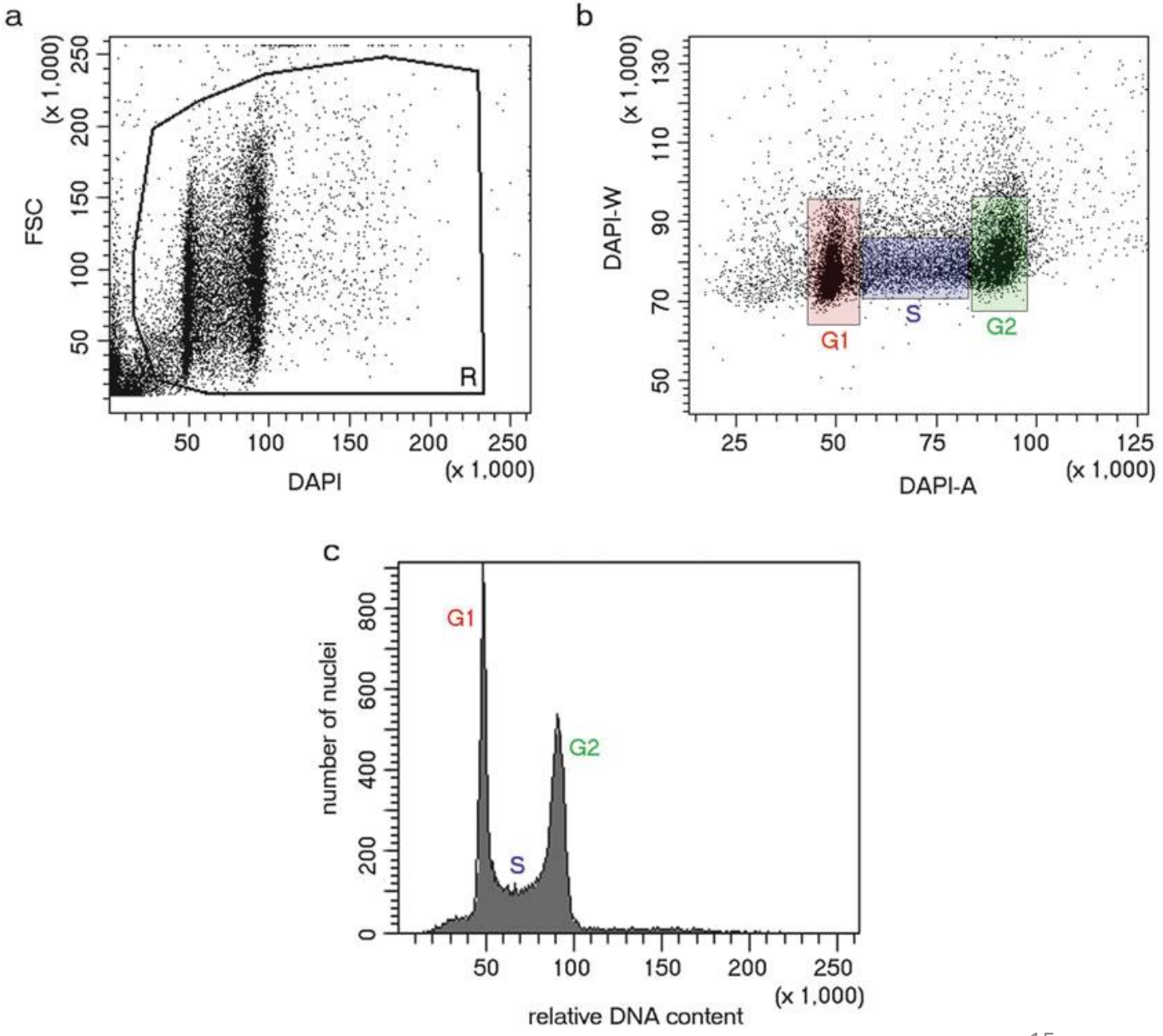
2. Filter the suspension of nuclei through a 20 µm nylon mesh.

3. Add DAPI to the filtered suspension of nuclei to achieve a final concentration of 2 µg/mL (e.g., 20 µL of the DAPI stock solution is used for 1 mL nuclei sample).

4. Launch the control software of the flow sorter to create all appropriate histograms and dot plots. First, use a dot plot of forward-scattered light (FSC) vs. DAPI to visualize populations representing nuclei (Fig. 2a). Mark a region surrounding the population of nuclei (R) and use this gating on the remaining dot plots and histograms. For sorting, build dot plots DAPI-W vs. DAPI-A (Fig. 2b). Make a histogram for DAPI staining showing the distribution of relative DNA content among nuclei (Fig. 2c).

Fig. 2: Flow cytometric analysis of barley nuclei

(a) A dot plot FSC vs. DAPI, which is used for separating populations of nuclei (region R).
(b) A dot plot DAPI-W vs. DAPI-A created for analysis and sorting nuclei stained with DAPI. DAPI-A and DAPI-W represent the total fluorescence and size of the particle, respectively. There are three regions G1, S, and G2 chosen for sorting nuclei of interest.
(c) A histogram of relative fluorescence intensity of DAPI-stained nuclei. It consists of the three groups of nuclei (according to the G1, S, and G2 phase of the cell cycle)



Protocol

Nuclei sorting using flow cytometry:

5. Run the sample. Analyze at least 20,000 nuclei and save the data.

6. Create sorting regions (G1, S, and G2, according to the respective cell cycle phase) surrounding the population of nuclei of interest (Fig. 2b).

7. Sort the nuclei in a required number (Figs. 1g and 2c) and collect them in appropriate collection tubes containing LB01-P supplemented with 10 µM PMSF (Fig. 1h), and onto microscopic slides into P5 buffer for a verification of nuclei integrity (Fig. 1i).

8. Analyze the prepared slide using a fluorescence microscope

Notes:
Sorting of approx. five million nuclei is usually performed into 1 mL of LB01-P with 100 µM PMSF in 15 mL collection tubes.