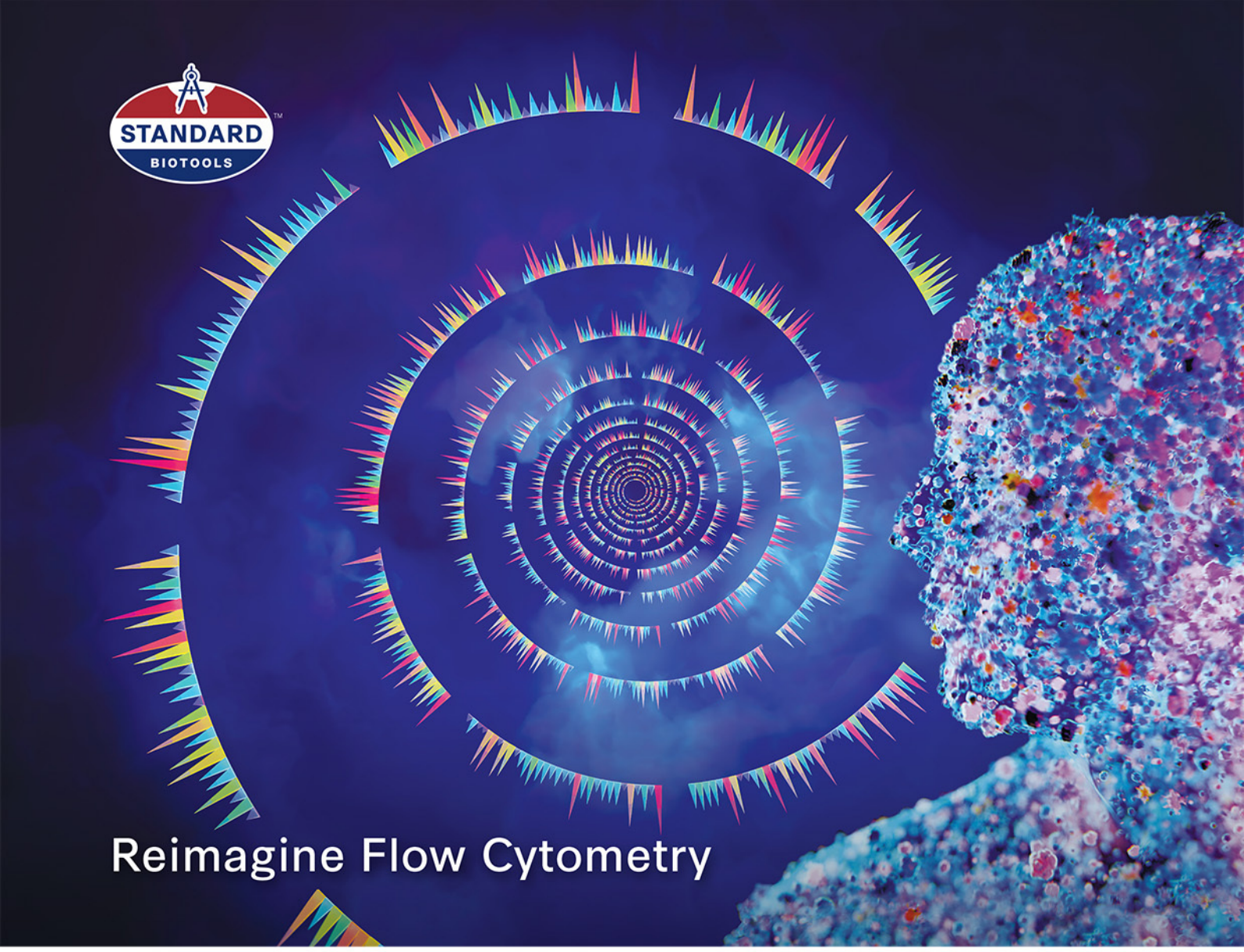

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REVIEW ARTICLE

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Fluorescence activated cell sorting—A selective tool for plant cell isolation and analysis

Ioanna Antoniadi[1] | Vladimír Skalický[2] | Guiling Sun[4] | Wen Ma[4] | David W. Galbraith[3,4] | Ondřej Novák[1,2] | Karin Ljung[1]

[1]Umeå Plant Science Center, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, Umeå, Sweden

[2]Laboratory of Growth Regulators, Institute of Experimental Botany, The Czech Academy of Sciences and Faculty of Science, Palacký University, Olomouc, Czech Republic

[3]Department of Biomedical Engineering, University of Arizona, School of Plant Sciences, BIO5 Institute, Arizona Cancer Center, Tucson, Arizona, USA

[4]School of Life Sciences, Henan University, Institute of Plant Stress Biology, Kaifeng, China

Correspondence
Karin Ljung, Umeå Plant Science Center, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden.
Email: karin.ljung@slu.se

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Abstract

Instrumentation for flow cytometry and sorting is designed around the assumption that samples are single-cell suspensions. However, with few exceptions, higher plants comprise complex multicellular tissues and organs, in which the individual cells are held together by shared cell walls. Single-cell suspensions can be obtained through digestion of the cells walls and release of the so-called protoplasts (plants without their cell wall). Here we describe best practices for protoplast preparation, and for analysis through flow cytometry and cell sorting. Finally, the numerous downstream applications involving sorted protoplasts are discussed.

KEYWORDS
autofluorescence, best practices, plant flow cytometry and sorting, protoplasts, viability and integrity

1 | PROTOPLAST PREPARATION

Flow cytometric analysis and flow sorting in higher plants requires production of single cell suspensions from three-dimensional tissues and organs comprising multiple interconnected cell types of different function. This is achieved by the digestion of the plant cell walls using microbial enzymes that degrade cellulose, hemicelluloses, and pectin, in the presence of a slightly hypertonic osmoticum such as mannitol. Incubation of the plant tissue in the presence of the cell wall degrading enzymes releases the protoplasts, living plant cells without cell wall, and form a single cell suspension. The released protoplasts can then be pelleted using low centrifugal forces, and re-suspended in the osmoticum (Figure 1).

The preparation of protoplasts, first described in 1960 [1], has since that time been tailored to the plant species, the tissue under study, and the developmental stage of the plant. As a consequence, a large number of protocols for protoplast isolation have been developed [2–8]. In protoplast preparation, variable factors between protocols concern mainly the components of the digestion mixture (composition and concentrations) and the incubation conditions [9–12]. Obtaining healthy and unstressed protoplasts derived from all the layers of the tissue under study is a key consideration in terms of the efficiency, accuracy, and relevance of flow cytometry and sorting. Protoplast production issues associated with organ type and structure include the obvious, for example, that some organ elements such as mature xylem elements and phloem sieve tubes lack cellular structures that can emerge as protoplasts. Other issues include that the protoplast preparation solutions may not access equally all internal cell types within organized tissues, will penetrate epidermal surfaces covered with waxy cuticles less easily as compared, for example, to the root, and will differ in effectiveness depending on cell wall composition. These issues further relate to the means whereby the starting tissues are prepared, for example whether as sterile plantlets in culture, as plants in growth chambers, as greenhouse-grown materials, or as field materials. Sterile plantlets in culture containers experience very high levels of humidity, which reduces the accumulation of waxes on aerial surfaces, thereby improving access of the protoplasting solutions. Tissue digestibility of organs can also be a function of developmental age and prior manipulation; for example, an initial sampling of leaves can induce systemic wound responses in samples subsequently taken, with notable changes in protoplast release and overall production. Best-practice recommendations include precisely defining the

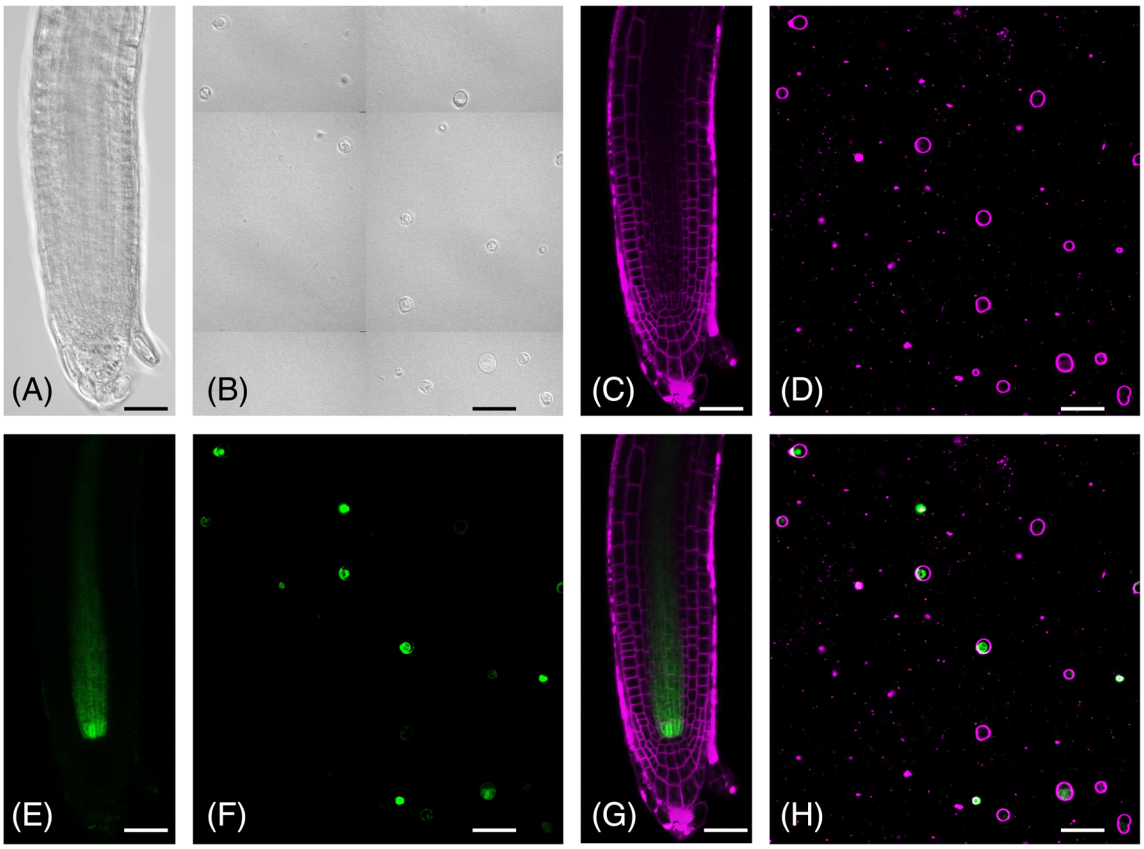

FIGURE 1 Protoplast preparation from the pWOL:GFP transgenic arabidopsis line. Six-day-old pWOL:GFP seedlings were transferred to microscope slides (A, C, E, and G), while the remaining seedling roots were enzymatically digested to release their corresponding protoplasts (B, D, F, and H). The pWOL:GFP-derived protoplasts are shown in bright field (A and B), according to their fluorescence after staining with the FM4–64 plasma membrane dye (C and D), and their endogenous GFP fluorescence (E and F). G and H provide the merged fluorescent images. Scale bar is 50 μm [Color figure can be viewed at wileyonlinelibrary.com]

growth conditions of the starting plant materials used for protoplast preparation and employing consistent methods for sampling, manipulating, and incubating these materials in the enzyme solutions.

Following digestion of the cell walls, the emerging protoplasts are osmosensitive, fragile structures (Figure 1). They are also generally larger in diameter than the mammalian cells of the hematopoietic system, around which flow cytometry instruments were originally designed. They therefore should be manipulated with extreme care in order to maintain their integrity. The first step is passage through a nylon filter (these are commercially available with defined mesh sizes from 20 μm upwards, and should evidently be selected dependent on average protoplast diameter) to remove undigested materials. This is followed by centrifugation and washing steps to eliminate residual enzymes, organelles, and other undesired contaminants. Crucial to maintaining protoplast integrity is centrifugation at very low speed in round-bottomed tubes, using a swing out centrifuge rotor, followed by gentle resuspension using wide bore pipette tips. This is one of the most common features found in all protoplast isolation protocols. Less common, but equally important, is purification of the viable protoplasts from the general tissue digest. Sucrose step-gradient flotation via low-speed centrifugation is a convenient way to selectively concentrate viable protoplasts [13, 14]· This step is significantly improving the efficiency, accuracy, and relevance of downstream applications.

After resuspension in the selected resuspension medium, the isolated protoplasts should be finally filtered through a nylon mesh filter of appropriate size to accommodate the average diameters of the protoplasts, prior to sample injection into the cytometer [10, 15]. Protoplasts can be analyzed directly after isolation, but it may be necessary to culture them for longer periods of time, for example to allow gene expression after transfection. Over prolonged culture periods, under optimal conditions, cell wall regeneration will occur, followed by cell expansion and cell division. Since this is not conducive to flow cytometric analyses of single cells, addition of 2,4-dichlorobenzoic acid can be used to specifically inhibit cell wall regeneration without deleterious effects on cellular metabolism [16]. At all stages, protoplast viability should be determined using fluorescent staining protocols [17–19]. Viability can be measured according to accumulation of a positive signal by viable protoplasts, for example by staining with fluorescein diacetate (FDA, Figure 2, [17–19]). In this case, FDA, which is non-fluorescent and readily permeable to the plasma membrane, is hydrolysed by cytoplasmic esterases to produce fluorescein, which is highly fluorescent, and significantly less permeable to the plasma membrane than FDA. Thus, viable protoplasts accumulate fluorescence, albeit transiently. According to the dye's emission signal and its permeability properties through the plasma membrane, the selection of intact, viable protoplasts for analysis or sorting can also be achieved based on the ability of the intact plasma membrane of viable protoplasts to exclude nuclear staining by 4′,6-diamidino-2-phenylindole (DAPI), Hoechst, and/or propidium iodide (PI). Positive and negative viability staining can be combined [20, 21], as illustrated in Figure 2 using FDA and PI. Several additional viability dyes are now commercially available.

In all of these cases, a best practice recommendation is to always include photographic documentation of the protoplast suspension immediately prior to flow analysis.

2 | ENDOGENOUS AND ACQUIRED PROTOPLASTS' FLUORESCENCE

For the desired cell populations to be analyzed using flow cytometry and isolated through sorting, optical signals are required. These are of two types, light that is scattered and detected either parallel to or orthogonally to the excitation beam path, and light emitted as

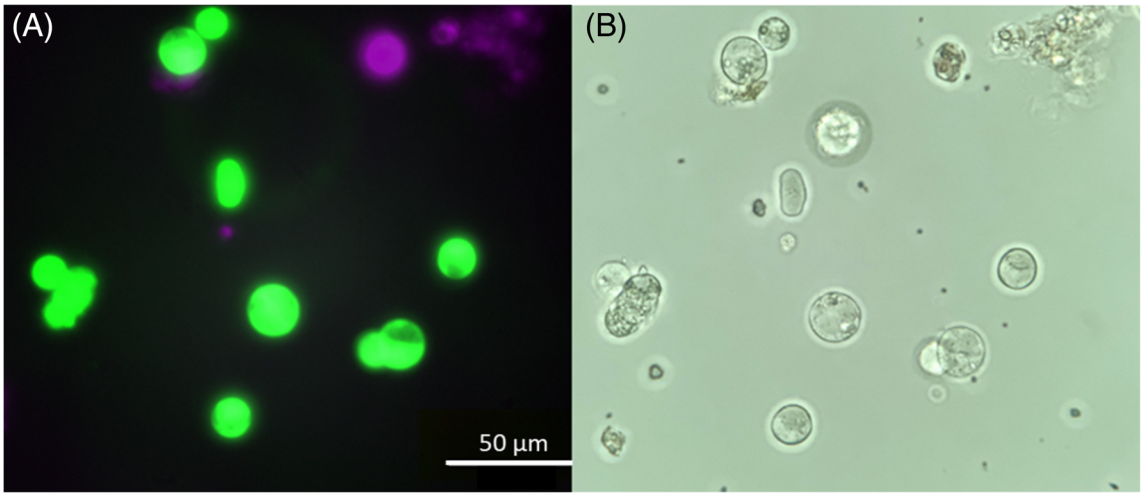

FIGURE 2 Maize epidermal protoplast viability determination via counterstaining with fluorescein diacetate (FDA) and propidium iodide (PI). (A) Protoplasts were stained with 0.20 μM FDA and 74.8 μM PI. Viable protoplasts transiently retain fluorescein, staining green. The plasma membrane of viable protoplasts excludes PI from accessing the nucleus, non-viable protoplasts therefore staining red. Fluorescence microscopy, bar = 50 μm. (B) The same sample under bright-field illumination [Color figure can be viewed at wileyonlinelibrary.com]

fluorescence. Plant protoplasts, as for all cells, contain endogenous compounds that emit autofluorescence when excited, excitation being particularly notable at shorter laser wavelengths [20, 21] (Table 1). Protoplasts derived from green tissues (aerial organs) display high levels of autofluorescence from chlorophyll due to excitation of photopigments within the chloroplasts (Table 1). Taking the above into consideration, fluorophores selection during experimental design is a crucial process that should aim to the reduction of spectra overlap.

Further fluorescent tags can be incorporated into the experimental design by transgenic expression of fluorescent macromolecules (cf. the Fluorescent Proteins; see below) following protoplast transfection or generation of stable transformed lines. As compared to the production of stable transgenic lines, transfection is more fast, efficient, direct, and well-established for some specific cell types, for example mesophyll protoplasts [4] However, the process of transfection is stressful, and additional centrifugation steps are generally required prior to flow sorting. Another limitation of transfection is that efficiencies could vary depending on the tissue or on the plant species.

An alternative method for fluorescent tagging involves staining with exogenous fluorochromes, either in the form of directly reactive species [22, 23], or fluorescent compounds that can be taken up by the plant as fluorescent hormone analogues [24] or attached to antibody ligands, as widely used in flow cytometric analyses of mammalian cells [25]. Although the latter approach, in principle, is rapid and efficient, and can be extensively multiplexed [26], its application is not at the moment possible using plant protoplasts, since a wide variety of antibodies recognizing cell-surface epitopes, equivalent to the CD-markers on mammalian cells [27], have not been identified and made commercially available. As a consequence, the overwhelming majority of reports of fluorescence labeling of plant cells and protoplasts has focused on the expression of the Fluorescent Proteins, starting with the prototypical Green Fluorescent Protein (GFP), described first for transfected maize leaf protoplasts in 1995 [28, 29]. Methods for transgenic and transfected expression of Fluorescent Proteins in several plant species are now in widespread use [4, 30, 31]. The most reliable and reproducible method, and thus the most commonly used in plant cytometric analysis and sorting, involves isolation of protoplasts from genetically modified plants [10–12, 32]. An example of combined fluorescent techniques in roots and root-derived protoplasts is shown in Figure 1. In this case, the endogenous GFP fluorescence of the *pWOL*:*GFP* line (*pWOODEN LEG*:*GFP*; 9) is combined with staining with the fluorescent dye FM4-64.

3 | PROTOPLASTS ANALYSIS THROUGH FLOW CYTOMETRY

Analysis of protoplast populations via flow cytometry was previously highly challenging, since cytometers and sorters were designed for smaller diameter (10–20 μm) mammalian hematopoietic cells, and the diameters of the original flow tips (50–70 μm) were too small to satisfactorily accommodate plant protoplasts. The first reports of successful sorting and recovery of viable tobacco leaf protoplasts demonstrated the use of larger (200 μm) flow tips, which required modification of the instrument configuration to achieve stable droplet break-off and to accurately determine the point of droplet break-off [33]. Most commercial instruments are now configured with a standard 100 μm flow tip, and several can accommodate flow tips up to 130 μm in diameter, which should be sufficient for many protoplast types. The use of even flow tips larger in diameter (up to 400 μm) is also possible. However, it requires significant alterations to the instrument configuration and their handling to achieve stream stability is exceptionally demanding (discussed in detail below).

Prior to cytometric analysis and sorting, the protoplasts should be gently resuspended in an osmoticum that is compatible with the cytometer sheath fluid. Conventionally, cytometers and sorters

TABLE 1 Autofluorescent compounds naturally occurred in plant cells and most commonly used fluorescent proteins

(Auto)fluorescence compounds	Excitation (nm)	Emission (nm)	Excitation laser/lamp
Flavins	380–490	520–560	Violet and Blue
NADH, NADPH	360–390	440–470	Violet
Lignin	488	530	Blue
Chlorophyll	488	685–740	Blue
Tryptophan	280	300–350	UV
Tyrosine	270	305	UV
Advanced glycation end-products (AGEs)	320–370	385–450	Violet
CFP	380–470	450–570	Violet
GFP	430–510	490–560	Blue
YFP	470–530	510–590	Blue
tdTomato	470–580	550–670	Yellow–green
RFP	500–580	550–680	Yellow–green
mCherry	510–610	580–690	Yellow–green

are operated using various formulations of phosphate-buffered saline (typically 0.9% [154 mM] NaCl, [mostly sodium] phosphate, KCl, and a pH between 5.7 and 7.4). Commercial PBS formulations often contain low levels of detergents as well as antibacterial agents, so caution is needed depending on the downstream application following sorting. An important warning is to be aware that many media used for protoplast preparation and resuspension contain significant levels (≥5 mM) of Ca^{2+}. This will readily precipitate inside the cytometer in the presence of PBS, and therefore non-PBS-based sheath fluids are recommended [11, 12, 34, 35]. A best practice recommendation is to

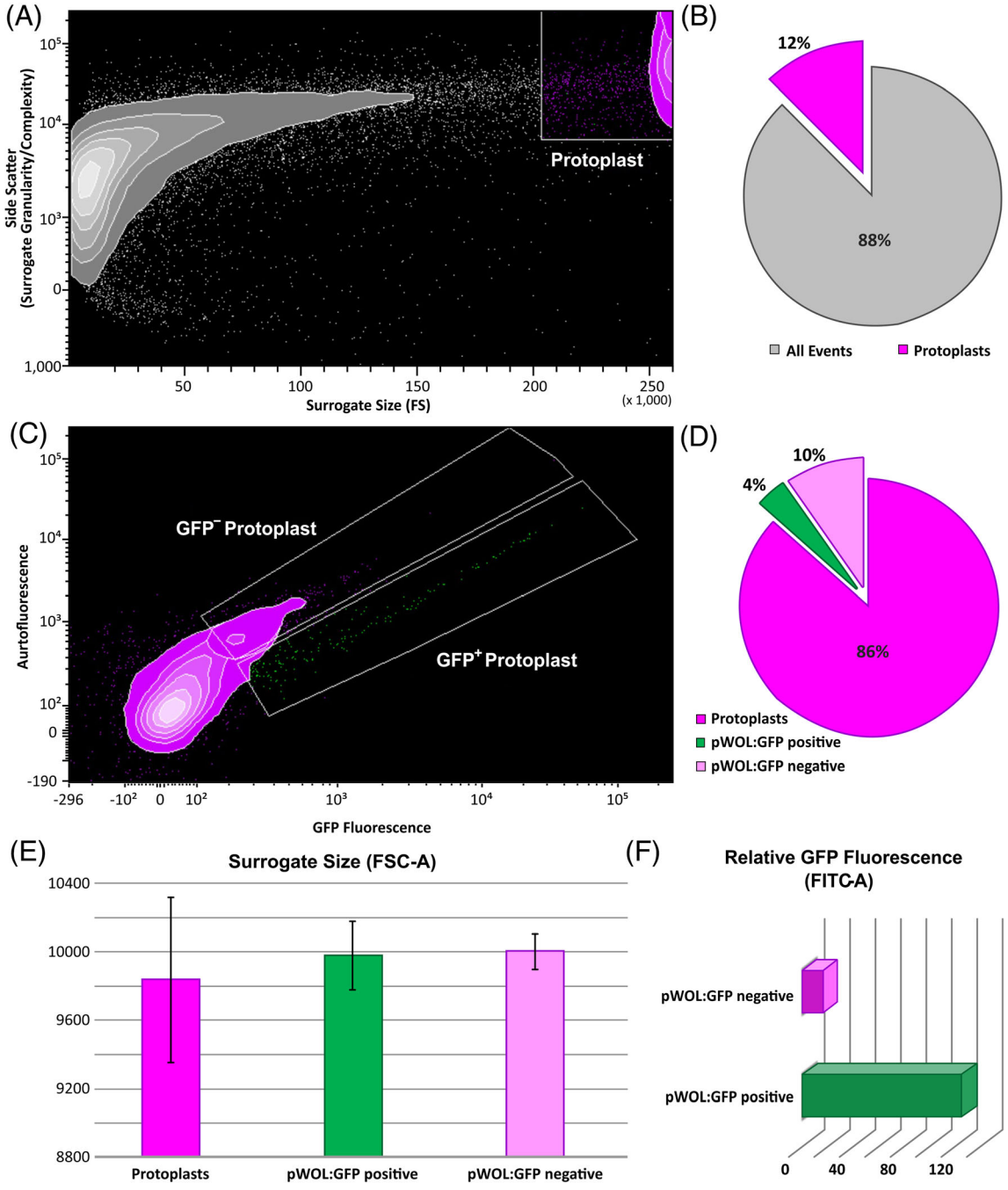

FIGURE 3 Flow cytometric analysis of pWOL:GFP root protoplasts: (A) Biparametric plot of forward and side scatter (surrogate size and granularity/complexity parameters respectively). The protoplasts being the biggest structures in the sample are at the upper-right edge of the chart. (B) The respective abundance percentages of the protoplast population in the sample is shown. (C) The population selected as protoplasts in a (magenta) is then plotted according to its autofluorescence and GFP fluorescence. Two populations are distinguished as GFP-positive (green) and GFP-negative (pink) and are gated respectively. (D) Their respective abundance percentages are shown. (E) The mean of the forward scatters of the three gated populations is plotted, error bars representing standard deviations. (F) The means of the GFP fluorescence of pWOL:GFP positive and negative cells are plotted [Color figure can be viewed at wileyonlinelibrary.com]

empirically explore the effects on protoplast integrity, viability and subsequent growth in culture, when using different sheath fluids and protoplast resuspension media. Osmotica and sheath fluids based on KCl or even NaCl, including 5 mM $CaCl_2$ but excluding phosphate, and instead using one of the "Good" buffers (MES, MOPS, etc.) that maintain a slightly acidic pH, are excellent places to start. Choice of a lower pH reflects our understanding of the microenvironment of the plant wall/plasma membrane interface, which is dominated by the presence of acidic pectins.

When protoplasts are analyzed through flow cytometry, both background noise and clusters of aggregated protoplasts are to be expected, although purification should greatly reduce their contributions. Exclusion of debris, aggregates and dead protoplasts from the data enables cleaner separation and identification of protoplast populations. Background cytometric signals produced by debris (particles approximately up to 2–5 μm in maximum diameter), mainly derives from ruptured protoplasts, organelles (intact or damaged) liberated from these protoplasts, and general fragments of undigested tissue (Figure 3(A)). Debris can be readily distinguished from protoplasts based on the fact that protoplasts are 10–40 times larger in size. Debris is typically excluded from analysis by use of specific thresholds applied to either the forward light scatter (FS) or the orthogonal side scatter (SS) signals typically used to trigger event detection by the cytometer. This renders the instrument "blind" to the events below this threshold. Application of thresholds can also be done using two-dimensional FS-SS scatter plots, since this can improve discrimination of populations of protoplasts from debris. However, the application of all thresholds should be approached cautiously in flow cytometry and sorting since it can lead to inclusion of undesired but non-detected events along with the objects that are detected and desired for sorting. It should be also noted that the forward and side scatter signals are empirically derived and should rather be considered as surrogates (FS) of size and (SS) of granularity/complexity, since the signals are not monotonically increasing, but rather are complex functions of refractive index, absolute size, pigmentation and so-on. As a reasonable approximation, objects of similar functionality and sizes can be compared, such as protoplasts. If a more precise absolute estimation of protoplast size is required, this can be readily determined via analysis of the features of the pulse-width time-of-flight signal [36].

Although the distinction between debris and protoplasts is in most cases possible in the bi-parametric dot plots of forward and side scatter (Figure 3(A)), it is more difficult to discriminate between healthy and severely stressed or even ruptured protoplasts, since these may not show differences in FS and SS signals. In that situation, viability dyes (FDA, PI, DAPI) can be employed, as previously described, for this discrimination, assuming there are no conflicts with other fluorescent tags involved in the experimental designs (Fluorescent Proteins, antibody labels, etc.). A best practice recommendation is always to purify the protoplasts by gradient flotation [13, 14, 19], and if possible, use dyes singly or in combination to identify viable protoplasts during cytometric analysis and sorting (Figure 2).

After the (healthy) protoplasts have been selected, the population can then be projected in biplot according to their autofluorescence (Table 1) and targeted fluorescence properties. Autofluorescence can be projected in several parameters and can be further distinguished from the targeted fluorescence (labeled protoplasts) by application of compensation and proper band pass filters. Therefore, we select the parameter that facilitates the best population separation between the targeted fluorescence and autofluorescence (Figure 3(C)). The daughter gates are then designed within the gated protoplasts, to define the well-separated negatively and positively fluorescent populations (for targeted fluorescence). (Figure 3(C),(D)). Since in the example provided by Figure 2, a viability stain was not employed, identification and selection of the *pWOL:GFP*-negative cells is driven by the autofluorescence properties of the *pWOL:GFP* positive cells. Since the negative population is much more abundant compared to the positive (Figures 1(C),(E),(G) and 3(C),(D)), it becomes possible to be highly selective. A feature of modern FACS instruments is specialized software that, in parallel to analyzing the events, generates a statistical overview of average signal intensities and association deviation statistics. These data can provide additional information about the sample under analysis or the sorted population and can also be used to confirm the stability and reproducibility of the flow sorting method used over different days of sorting. An example is shown in Figure 3(E),(F). Additional data about the *pWOL:GFP* positive and negative cells gated in Figure 3(C) can be plotted to show that, although these two populations differ substantially in GFP fluorescence (Figure 3(F)), they are very similar in terms of their FS size surrogate (Figure 3(E)) as both populations contain protoplasts.

4 | SORTING OF PROTOPLASTS

The protoplast populations that have been selected by gates for sorting can be readily collected in a purified state by flow sorting into different types of collection tubes, well plates and microscope slides using flow sorters. Most sorters employ the jet-in-air sorting principle. When protoplasts are sorted, preservation during the sort process of their health and integrity, or of their contents of experimental interest, is crucial for meaningful results from downstream applications. Best practice recommendations to achieve this are as follows: firstly, and as already discussed in the flow cytometry section, accommodation of the large plant protoplasts demands flow tips larger in diameter. This requires a reduced system/sheath fluid pressure and a lower maximal drive frequency for droplet formation. On the one hand, lower sheath fluid pressures are beneficial for maintenance of protoplast health and integrity during analysis and sorting, which should improve the quality of biologically-relevant downstream applications as the protoplasts experience less physical stress. On the other hand, the physical constraints on droplet formation, apart from demanding more acute technical FACS expertise, inevitably reduce the upper limit of the sort rate, and the lower sorting yield imposes an extension of sorting hours and costs as compared to handling smaller (i.e., mammalian) cells. Prolonged sorting hours should be carefully planned, and should include

control experiments relevant to the downstream application. This follows largely as a consequence of an enlargement of the time that the sample is loaded into the cytometer and required for collection, the conditions for which may well not be optimal for the protoplasts. One option is to maintain sorting at 4°C (using a chilled sample introduction chamber as well as chilled sorting tubes), to globally reduce the activities of cellular enzymatic reactions and physiological processes [10–12, 15]. Sorting into tubes containing appropriate extraction agents compatible with the downstream applications is also highly appropriate. For example, samples that will be used for transcriptomics can be sorted into tubes preloaded with RNA extraction buffer to optimize the recoveries and integrity of transcripts. An additional suggestion is to load fresh sample in the loading port during long sorting processes as low-quality input will greatly reduce sorting efficiency. The gate design takes place on the beginning of the sorting and thus concerns a freshly loaded protoplasts sample. Therefore, the populations selected for sorting (Figure 3(A),(C)) refer to live protoplasts. If the protoplasts in the loaded sample start to be stressed and rupture, then they will have altered forward and side scatters and will not be selected for sorting as they will not be inside the designed gates anymore. This will have negative effects on the efficiency of the sorting but not on the purity of the sorted populations. Finally, caution is needed in establishing optimal droplet breakoff points and droplet delay settings [33], in order to maintain sorting purity and reproducibility. In this respect, the best practice is use of commercially available fluorescent microspheres in larger diameter sizes (−25 to 90 μm)or naturally autofluorescent particles (pollen, fungal spores) that are both indestructible and similar in size to the fragile protoplasts that are being sorted, rather than use of standard (2 and 10 μm) fluorospheres, for optimizing instrument calibration and sort settings [14].

5 | APPLICATIONS OF FLOW CYTOMETRIC PROTOPLAST ANALYSIS IN PLANT RESEARCH

Flow cytometry and sorting of viable protoplasts followed by regeneration into plants was first reported in 1984 [14], and subsequently for isolation of somatic hybrid plants recovered by fluorescence-activated sorting of heterokaryons formed by protoplast fusion [19]. Flow analysis and sorting of protoplasts is now routinely employed in basic and applied plant research (Figure 4).

Over the intervening decades, there has been a tremendous increase in FACS-derived data in plant research. Analysis of sorted protoplasts has significantly deepened our understanding in

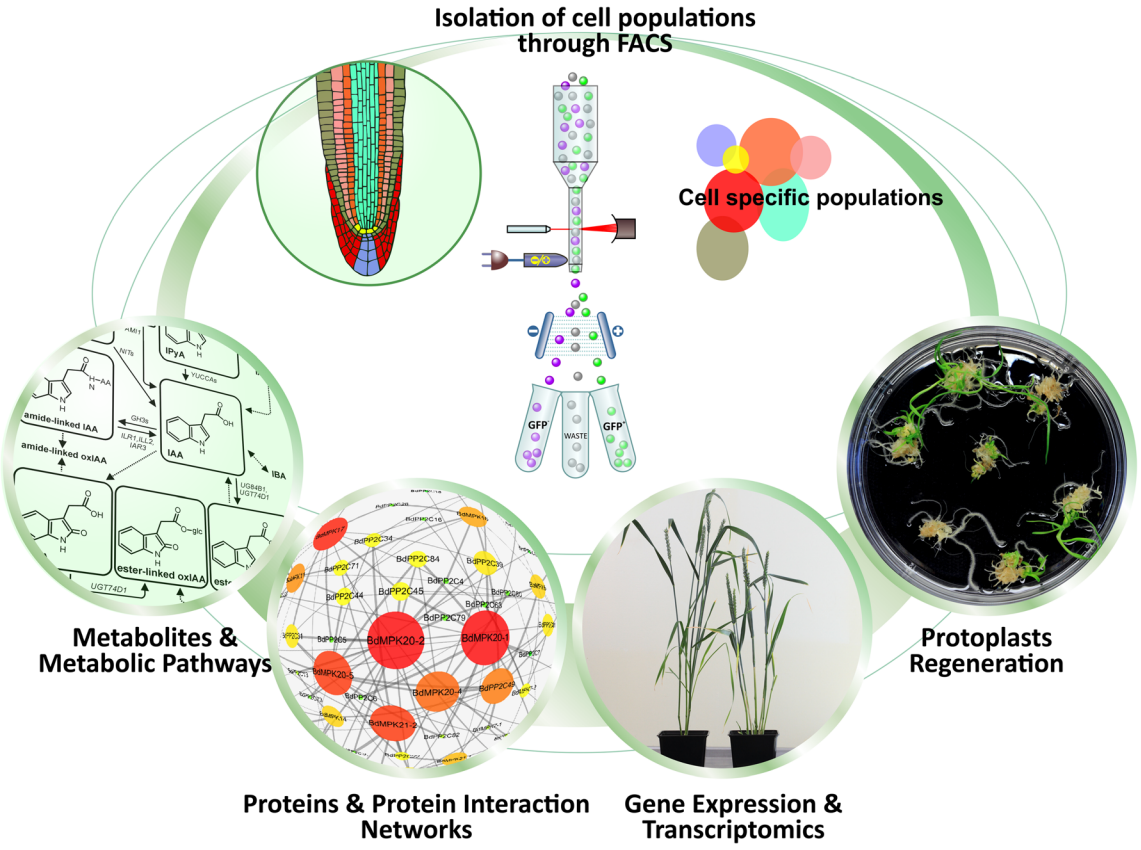

FIGURE 4 Schematics of downstream applications following protoplast isolation and sorting . Isolation of root cell-specific populations by FACS can be followed by applications in protoplast regeneration and different omics technologies, such as transcriptomics, proteomics, and metabolomics [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Selected examples of conditions for preparation and flow sorting of protoplasts

Plant species	Tissue, conditions	Flow cytometry tip	Sorting tip	Downstream application	Reference
At	Root Apex	Sheath fluid: 0.7% NaCl, 20 psi, 100 μm nozzle	Flow rate 2500–5000 events/s. Sort precision "Purity" mode.	Hormone analysis (auxin and cytokinin metabolites)	[11, 12, 47, 48]
At	Root	30 psi, 100 μm nozzle	Flow rate 2000–5000 events/s. Protoplasts sorted into Qiagen RLT lysis buffer.	Gene expression profiling (microarrays)	[9, 10, 32]
Nt	Leaf	Machine sterilization, 76 μm nozzle - 24.5 kHz and 100 μm nozzle - 14.5 kHz	Flow rate 700 events/s. Sorting in conjunction with the operation of the Autoclone. Protoplasts sorted into KOM medium.	Protoplast growth and plant regeneration	[14]
Nicotiana spp.	Leaf	All settings as Reference [14]. Laser power output 200 mW	All settings as Reference [14]. Flow sorter at 457 nm.	Isolation of somatic hybrids	[22]
Zm	Leaf	Sheath fluid: 0.47 M mannitol, 50 mM KCl, 10mM$CaCl_2$, 4 mM MES, pH 5.7. Sheath and flow pressures: 8.0 and 7.3 psi. Sort-sense flow with 100 μm orifice −15 kHz. Laser power output 15 mW	Flow rate 50–200 events/s. Sort rate 25–40 cells/s	GFP labeling	[28, 29]
Nt	Leaf	Sheath fluid ×3: DI water, KOM medium and PBS buffer. Fluorescein diacetate stained events selection (viability stain)	Sterile sorting. Laser output 200 mW. Sorting based on time of-flight analysis of chlorophyl autofluorescence.	Gene expression	[8]
Os and *At*	Root /Salt stress	Sheath fluid: PBS buffer, 70 μm (or 100 μm) nozzle	Sort precision "Purity" mode. Protoplasts sorted into RNA extraction buffer (RLT)	Gene expression profiling (microarrays)	[51]
At	Root apex and emerging lateral root/ Response to Nitrogen	N.S.	Protoplasts sorted into lysis buffer	Gene expression profiling (microarrays)	[52]
At	Leaf	Sheath fluid: TEX buffer, 9 psi, 200 μm nozzle	Flow rate 6000–15,000 events/s	Gene expression profiling (microarrays)	[54]
At	Roots/Salt and Iron Stress	N.S.	N.S.	Gene expression profiling (microarrays)	[53]
At	Infected and non-infected cells/downy mildew	All settings as Reference [42]	All settings as Reference [42]. Protoplasts sorted into Qiagen RLT lysis buffer with 1% β-mercaptoethanol.	Gene expression profiling (microarrays)	[49]
At	Aerial tissue and seedlings (stomata)	All settings as Reference [51]. 100 μm nozzle. Forward scatter cutoff: 5000.	Flow rate 2500–3500 events/s, Sort precision "Purity" mode. Protoplasts sorted into RNA extraction buffer (RNeasy™ Micro Kit, QIAGEN).	Gene expression profiling (RNA sequencing and microarrays)	[46]
At and Os	Root tips (root hair)	All settings as Reference [43]	All settings as Reference [43].	Gene expression profiling (RNA sequencing)	[45]

(Continues)

TABLE 2 (Continued)

Plant species	Tissue, conditions	Flow cytometry tip	Sorting tip	Downstream application	Reference
At	Root tip, Root epidermis	N.S.	N.S.	Gene expression profiling (microarrays)	[43]
At	Embryo. Nuclei sorting	Sheath fluid: 1× PBS pH 7.0, ~60.5/~60.0 psi, 70 μM nozzle, ~95 kHz,	1–2 single drop envelope.	Gene expression profiling (microarrays)	[44]
At	Leaf	Sheath fluid: 20 psi (sheath) and 21–21.5 psi (sample), 100 μm nozzle, 39.2 kHz	Flow rate < 4000 events/s. Protoplasts sorted into a lysis buffer containing a reducing agent. Sort time of a single sample limited to 20–30 min.	Gene expression analysis (qRT-PCR) and visual analysis (confocal microscopy)	[42]
At	Roots	Settings as Reference [32]. 20 psi, 70-μm nozzle	Settings as Reference [32]. Flow rate 5000 events/s.		[40]
At	Inflorescence	Settings as Reference [56]. Sheath fluid: 25 psi, 100-μm nozzle. Doublets removal- single cells selection, PI stained events exclusion (viability stain)	Settings as Reference [56]. Flow rate 10,000 events/s. Protoplasts sorted into Trizol (Invitrogen/Life Technologies). Occasionally agitated during ~40 min of sorting.	Gene expression profiling (mRNA sequencing)	[41]
At	Root apex	Sheath fluid: 0.7% NaCl, 20 psi, 100 μm nozzle.	Flow rate 2000–3000 events/s	Metabolomics analysis	[55]
At	Root/biotic stress (flg22, Pep1)	Sheath fluid: BD FACSFlow, 20 psi and 21–21.5 psi, 39.2 kHz, 100 μm nozzle	Flow rate < 4000 events/s. Protoplasts sorted into Qiagen RLT lysis buffer containing 1% (v:v) β-mercaptoethanol.	Gene expression profiling (RNA sequencing)	[50]
At	Root apex	All settings as References [10, 56]	All settings as References [10, 56].	Proteomics analysis (GeLC-MS/MS)	[38]
At	Root Hair	Sheath fluid: 25 psi, 100 μm nozzle	Flow rate 7000 to 8000 events/s.	Gene expression profiling (RNA sequencing)	[37]
Nt	Leaf	Sheath fluid: 6 psi, 204 μm nozzle, Flow velocity 7.9 m/s	Flow rate 50 events/s.	Chlorophyll content and protoplasts size determination	[36]

Abbreviations: *At*, *Arabidopsis thaliana*; *Os*, *Oryza sativa*; *Nt*, *Nicotiana tabacum*; *Zm*, *Zea mays*; N.S., for conditions not specified.

developmental aspects of highly specific plant tissues and organs [8, 9, 11, 12, 32, 37–50] as well as their interaction with biotic [49, 50] and abiotic [51–55] factors.

Most of these detailed studies on sorted protoplasts have investigated the transcriptome, developmentally, in root cell types [32, 37, 43, 45], shoot apical meristem [9], carpel margin meristem [41], embryo [44] and stomatal lineage cells [48]. Transcriptomic analysis has been also performed in sorted protoplasts subjected to environmentally stressful conditions [53] such as responses to salt [51], nitrogen [52], light [54] and pathogen infections [50, 51]. These data are not only shedding light into plant functions and tissues at uniquely high resolution, but they also certify/confirm that RNA, a molecule that is highly sensitive to degradation, can be reproducibly recovered from sorted protoplasts. This provides additional evidence that FACS, despite its invasive nature as a technique, is a credible method for obtaining results that can represent endogenous signals in planta discussed also in References [10, 39].

Research does not stop at the transcriptional level though, various groups reporting analysis of proteomes and metabolomes of root protoplast populations isolated via flow sorting. This has included proteome analysis within protoplasts from the different cell types of the root body and in root hairs [38, 45], as well as metabolomics studies [40, 56]. Finally, the distributions of a subgroup of small but highly active molecules, the plant hormones auxin and cytokinin, has been quantified in protoplasts from different cell types of the root apical region [11, 12] revealing high resolution hormonal gradients that are an essential feature of plant growth and development.

The above-mentioned publications, that have equipped FACS technology to achieve high-resolution analyses, have some common settings (Table 2). These being the best practices for protoplasts

sorting include low flow rates, low sheath fluid pressure and sorting of protoplast directly into the buffer that corresponds to the downstream application.

Future challenges will be to combine different 'omics techniques in specific organs and under different treatments or stress conditions in plant lines of specific genotypes, in order to understand their regulation and, for example, identify molecular switches during plant development.

6 | SUMMARY OF BEST PRACTICE RECOMMENDATIONS

- Growing healthy plants is a prerequisite for production of healthy protoplasts. Precise definition of growth conditions is essential for providing sources of protoplasts that respond reproducibly in downstream assays.
- Predefined optimized conditions during protoplast extraction (solutions, incubation times and handling) are essential for success and reproducibility. Verifying in a documented form is essential of the viability and purity of the protoplast populations that will be analyzed via flow cytometry and sorting. The simplest approach involves light and fluorescence microscopy.
- Round-bottom containers and swing-out centrifuges contribute to optimum gentle isolation of protoplasts.
- Choosing the appropriate fluorescent tagging method at the beginning of the experimental setup is a critical factor in experimental design.
- Protoplasts are highly autofluorescent, so selection of fluorochromes with limited spectra overlap is critical.
- Gating strategies should be carefully considered. This includes application of threshold(s) to limit analysis and sorting to the protoplast populations of interest, the use of viability stains for selection of healthy protoplasts, and appropriate positioning of daughter gates to accurately define desired sub-populations.
- Sorting protoplasts directly into an appropriate buffer for the downstream analysis increases the integrity of the investigated molecules and thus the quality of the results.

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AUTHOR CONTRIBUTIONS

Ioanna Antoniadi: Conceptualization; visualization; writing-original draft; writing-review & editing. **Vladimir Skalický:** Conceptualization; visualization; writing-original draft; writing-review & editing. **Guiling Sun:** Visualization; writing-original draft; writing-review & editing. **Wen Ma:** Visualization; writing-original draft; writing-review & editing. **David Galbraith:** Conceptualization; visualization; writing-original draft; writing-review & editing. **Ondřej Novák:** Conceptualization; visualization; writing-original draft; writing-review & editing. **Karin Ljung:** Conceptualization; funding acquisition; visualization; writing-original draft; writing-review & editing.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ORCID

Ioanna Antoniadi https://orcid.org/0000-0001-9053-2788
David W. Galbraith https://orcid.org/0000-0003-4020-1635
Ondřej Novák https://orcid.org/0000-0003-3452-0154
Karin Ljung https://orcid.org/0000-0003-2901-189X

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