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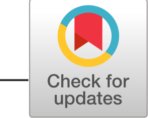

COMMUNICATION TO THE EDITOR

Best practices in plant cytometry

David Galbraith[1,2] | João Loureiro[3] | Ioanna Antoniadi[4] | Jillian Bainard[5] | Petr Bureš[6] | Petr Cápal[7] | Mariana Castro[3] | Sílvia Castro[3] | Martin Čertner[8,9] | Dora Čertnerová[8] | Zuzana Chumová[8,9] | Jaroslav Doležel[7] | Debora Giorgi[10] | Brian C. Husband[11] | Filip Kolář[8,9] | Petr Koutecký[12] | Paul Kron[11] | Ilia J. Leitch[13] | Karin Ljung[4] | Sara Lopes[3] | Magdalena Lučanová[9,12] | Sergio Lucretti[10] | Wen Ma[1,2] | Susanne Melzer[14,15] | István Molnár[7] | Ondřej Novák[4,16] | Nicole Poulton[17] | Vladimír Skalický[16] | Elwira Sliwinska[18] | Petr Šmarda[6] | Tyler W. Smith[19] | Guiling Sun[1,2] | Pedro Talhinhas[20] | Attila Tárnok[15,21,22] | Eva M. Temsch[23] | Pavel Trávníček[9] | Tomáš Urfus[8]

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

[2]State Key Laboratory of Cotton Biology, Key Laboratory of Plant Stress Biology, Henan University, School of Life Sciences, State Key Laboratory of Crop Stress Adaptation and Improvement, Kaifeng, China

[3]Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Coimbra, Portugal

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

[5]Swift Current Research and Development Centre, Agriculture and Agri-Food Canada, Swift Current, Saskatchewan, Canada

[6]Department of Botany and Zoology, Faculty of Science, Masaryk University, Brno, CZ, Czech Republic

[7]Institute of Experimental Botany of the Czech Academy of Sciences, Olomouc, Czech Republic

[8]Department of Botany, Faculty of Science, Charles University, Prague, Czech Republic

[9]Czech Academy of Sciences, Institute of Botany, Průhonice, Czech Republic

[10]Green Biotechnology Laboratory, Biotechnology and Agroindustry Division, Casaccia Research Center, ENEA - Italian National Agency for New Technologies, Energy and Sustainable Economic Development, Rome, Italy

[11]Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada

[12]Department of Botany, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

[13]Department of Comparative Plant and Fungal Biology, Royal Botanic Gardens, Richmond, UK

[14]Clinical Trial Centre Leipzig, University Leipzig, Leipzig, Germany

[15]LIFE-Leipzig Research Center for Civilization Diseases, University of Leipzig, Leipzig, Germany

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

[17]Center for Aquatic Cytometry, Bigelow Laboratory for Ocean Sciences, East Boothbay, Maine, USA

[18]Laboratory of Molecular Biology and Cytometry, Department of Agricultural Biotechnology, UTP University of Science and Technology, Bydgoszcz, Poland

[19]Ottawa Research and Development Centre, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada

[20]LEAF, Linking Landscape, Environment, Agriculture and Food, Instituto Superior de Agronomia, Universidade de Lisboa, Lisbon, Portugal

[21]Department of Precision Instruments, Tsinghua University, Beijing, China

[22]Department for Therapy Validation, Fraunhofer Institute for Cell Therapy and Immunology IZI, Leipzig, Germany

[23]Department of Botany and Biodiversity Research, University of Vienna, Vienna, Austria

David Galbraith and João Loureiro contributed equally to this work.

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Correspondence
David Galbraith, School of Plant Sciences, BIO5 Institute, Arizona Cancer Center, Department of Biomedical Engineering, University of Arizona, Tucson, AZ, 85721.
Email: galbraith@email.arizona.edu

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Flow cytometry (FCM) and flow cytometric sorting (FCS) systems have developed as experimental tools of remarkable power and are enjoying an ever-increasing impact in the general field of biology [1]. Application of these tools to plant biology has developed more slowly given that the natural form of plants infrequently resembles that of the single cell suspension, prototypically the hematopoietic system that drove the original development of FCM/FCS. Nevertheless, these systems have had a profound influence at all levels of plant biology, from the study of single cells and subcellular organelles, to the behavior of populations of plants, and ultimately to the performance of ecosystems. It is safe to say their impact has not plateaued, as further applications of this unique technology are increasingly developed by innovative scientists around the world to address questions both in the basic sciences, and to increasingly confront emerging problems in the applied sector. For example, in addressing the challenges of sustainable production of sufficient food resources based on plant breeding involving ploidy-based approaches (e.g., induction of polyploidy) [2] for the needs of our future global citizens, FCM, and FCS systems will play central roles in this effort.

The degree to which FCM and FCS systems have impacted plant biology and applied agricultural sciences must not be understated. The major applications of DNA FCM are ploidy level and genome size estimations, and cell cycle analysis/endoreplication (with the later included in a lower percentage of studies). Indeed, FCM is currently/extensively and almost exclusively employed as the method of choice for measurement of plant genome sizes [3, 4]. Measurements of this type impact agriculture in terms of ploidy estimation, with applications ranging from plant biotechnology, breeding and seed quality testing to taxonomy and population biology. They also impact the fundamental plant sciences in terms of biosystematics, ecology, evolution, genomics, and conservation, among other applications. One of the most startling observations of the angiosperms is the bandwidth occupied by genome size, which spans almost 2400-fold.

Flow sorting of higher plant chromosomes has provided invaluable information regarding the organization of DNA sequences within plant species. It has also greatly facilitated the process of whole-genome sequencing by permitting subdivision of large genomes into samples comprising entire chromosomes or chromosome arms [5]. FCS methods applied to wall-less cells (protoplasts) expressing fluorescent proteins (FPs) in a cell type-specific manner have allowed elucidation of patterns of co-regulated gene expression and plant hormone gradients identification [6, 7] within organized tissues, such as roots [8, 9].

The trigger to develop this virtual issue came from the publication, in 2017, of an article entitled "Guidelines for the use of flow cytometry and cell sorting in immunological studies" in the European Journal of Immunology [10]. As noted in that article, one of the advantages of FCM/FCS systems is that they are relatively simple to implement, with some qualifications, which coupled with the development of user-friendly devices and software during the last 15 years led to increasing applications in other areas, such as plant sciences. However, it is also simple to implement and operate the instruments inappropriately. This calls for a comprehensive and collective summary of the best practices when applying FCM/FCS to plants, as was done for immunology.

The first consideration addresses the problem that plants, particularly the vascular plants, in their commonly recognized and utilized forms, exist not as single cell suspensions (typical of immunology) but as complex three-dimensional tissues comprising cells of irregular shapes, different types and functions, that collectively cooperate to produce the final plant form. Optimal methods for producing suspensions of cells, subcellular organelles and other components appropriate for FCM/FCS from these plant tissues and organs, are therefore one of the challenges discussed in this virtual issue. We are fully aware of the mantra that "junk in equals junk out" and having samples of the highest quality prior to FCM/FCS is a critical concern we also addressed here.

The second consideration relates to the vast variety of different plant species found globally, and the recognition of the consummate ability of plants to produce secondary metabolites/products, affecting DNA staining and resulting fluorescence. Again, methods for recognizing and handling the different challenges provided to FCM/FCS methods by the biochemistries of the source samples are required.

The third consideration focuses on the problem of addressing the non-critical application of FCM/FCS methods developed for mammalian cell systems (typically hematopoietic) to plants without careful consideration of their appropriateness. As it will be detailed in this virtual issue, application of FCM/FCS methods to mammalian cell systems almost exclusively occurs in the context of analysis of samples that comprise a majority, often close to 100%, of single cells in suspension. For plants, particularly when using these instruments and methods for the analysis of organelles in tissue homogenates, the objects of interest comprise a very minor subpopulation of the total particles passing through the instrument. Concepts such as placing initial gates around populations defined by forward scatter (FS) versus side scatter (SS), as routinely used to define leukocytes or other mammalian cells in culture, are at best meaningless and at worst can seriously hamper proper use of the instruments to provide meaningful results. Again, plants are sources of many forms of autofluorescence; in vascular plants, chloroplasts are intensely fluorescent in the red due

to the presence of chlorophyll. Phycoerythrin, a red protein-pigment complex from the light-harvesting phycobiliprotein family found in red algae and cryptophytes, is commercially employed as a fluorescent label for antibodies in cytometry. The presence of autofluorescence can restrict the wavelength bandwidths for fluorescence excitation and emission, and this can affect how best to set up FCM/FCS instruments.

In order to define and enunciate best practices, we drew together a network of volunteer authors, experienced in the application of FCM/FCS to plants. We have attempted to make this network as comprehensive as possible, to allow recommendations spanning all relevant life-forms, from the simplest photosynthetic microbes, to the more complex lower and vascular plants, and encompassing also the fungi. In this endeavor, we gratefully acknowledge the support of Wiley and Attila Tarnok, EIC of Cytometry.

As indicated for the Guidelines in Immunology article [9], we do wish to keep our recommendations updated. Therefore, please send us your critical comments, new ideas, practical suggestions regarding best practices, and new articles that could be useful for possible future versions of this virtual issue.

To end, we would like to remember that this virtual issue reflects the vision and dream of the late Jan Suda. He has been an inspiration for all of us, and, most certainly, he left us too soon. We are sure that his legacy will persist, not only in his home country, the Czech Republic, but also across the world. We sincerely hope this virtual issue of Cytometry Part A provides an appropriate tribute.

1 | SETTING THE STAGE

Describing how FCCS can be optimally applied to plants requires information in two general areas (a) concerning the samples being prepared and analyzed, in our case focusing on the relevant physical features of plants as organisms, and (b) concerning the instrumentation being used for this analysis, centering on sample requirements imposed by engineering design and implementation.

2 | VASCULAR AND NONVASCULAR PLANTS

Green plants (Viridiplantae) constitute a monophyletic clade within the tree of life and comprise oxygenic photosynthetic eukaryotes [11]. The group encompasses green algae and land plants, and further splits into major clades: the Chlorophyta [12], comprising only algae, and Streptophyta formed by several algal groups (such as Zygnematophyceae and Charophyceae; [13, 14]), and the land plants (Embryophyta). Land plants further split into several groups: the possibly paraphyletic assemblage of three bryophyte lineages (Bryophyta—mosses, Marchantiophyta—liverworts, and Anthocerotophyta—hornworts) and three sequentially-splitting lineages of vascular plants: lycopods (Lycopodiophyta), ferns and horsetails (Monilophyta) and seed plants (Spermatophyta). The latter group further splits into gymnosperms (Gymnospermae; i.e., conifers, cycads, *Ginkgo*, and gnetophytes) and angiosperms (Angiospermae; [15-17]). The current review is primarily but not exclusively focused on flow cytometric applications in flowering plants, as they represent the most diverse and economically important, and therefore best studied, group of green plants. However, we mention the other green plant lineages where necessary and we also include other organisms that are found in various parts of the Tree of Life (algae in the traditional sense, fungi) and that share certain features of body organization and life style with plants (such as complex tissues or photosynthesis), and have for a long time been a subject of Botany in the broadest sense.

The life cycles of algal groups are highly variable and may comprise stages only with haplophasic (*n*) or diplophasic (*2n*) chromosome numbers, although in other species both stages are present but in separate generations [18]. All land plants exhibit a characteristic life cycle which alternates between a haplophasic gametophyte and a diplophasic sporophyte. Still, the relative importance of each stage in the life cycle differs between groups: while the gametophyte stage dominates in bryophytes (and is usually the tissue that is analyzed by FCM), the sporophyte stage dominates in the vascular plant groups and is the main focus of flow cytometric investigations. Despite a significant reduction in the size of the gametophyte (comprising only up to 3–4 cells/nuclei in flowering plants), there are flow cytometric applications focused on either the independent gametophyte or the spores of ferns or on pollen grains of seed plants [19]. Unlike vascular plants, fungal life cycles are mostly haplophasic, with a short (often single-celled) diplophasic stage, although most fungi (the Dikarya, i.e., the Ascomycota and the Basidiomycota) are dikaryotic (n + n) in part of their life cycles.

The evolution of plant genomes is dynamic, particularly in angiosperms, encompassing a range of genomic processes including multiple rounds of whole genome duplication (polyploidization, [20, 21], chromosomal rearrangements [22, 23] and the turnover and evolution of repetitive DNA (including mobile elements and satellite DNA) [24, 25]. This is mirrored in the tremendous variation in nuclear genome sizes across green plants in general (c. 11,850-fold; 2) and flowering plants in particular (2,400-fold variation; 3,4). This has crucial implications for flow cytometric applications both with respect to technical issues (a series of internal standards of different genome size is required) and also as a study topic per se (e.g. what are the mechanisms driving genome size evolution?). Similarly, the relative content of AT versus GC base pairs is highly variable in green plants, although this variation does not strictly correlate with nuclear DNA-content (e.g., 26).

While the algal groups are mostly unicellular, or comprise a rather simple multicellular thallus (e.g., *Ulva*, *Cladophora*, or *Chara*), land plants form complex tissues and organs. The sporophyte of vascular plants typically differentiates into roots, stems and leaves (note that the floral parts of flowering plants are derived from the leaves). Similar (yet haplophasic and thus non-homologous) structures are found in the gametophytes of bryophytes: rhizoids, cauloids, and phylloids. The specific morphology and anatomy of green plants, as distinct from other eukaryotes, naturally has multiple implications/challenges for

flow cytometric analysis. Firstly, we encounter cells having thick cell walls that render flow cytometric analysis of individual cells impossible. Instead, isolated protoplast and, more commonly, nuclear suspensions are used for the analysis of plant tissues [26–28]. Secondly, two types of endosymbiotic organelles, each with their own genomes, are present in most plant cells, mitochondria and plastids, and FCM applications have been designed to analyze those organelles [29–31]. Lastly, plants present a wide array of chemical compounds, so-called secondary metabolites, conferring protection against factors both abiotic (e.g., UV-light, frost) and biotic (e.g., herbivores, parasites). Some of these chemical compounds (for example, tannins) directly co-interact with the DNA-binding stains used in FCM, and significantly affect the quality and reliability of such analyses [32].

3 | OVERVIEW OF INSTRUMENTATION AND PRINCIPLES

Flow cytometry and cytometric sorting systems are assembled from distinct engineering modules which collectively function to determine the optical properties of suspensions of biological particles, and selectively isolate these particles, or subsets thereof, for subsequent analysis and processing. The particles are typically constrained hydrodynamically within an aqueous stream to flow singly through regions of intense light, almost exclusively provided by lasers that are focused on the stream. On illumination, the particles absorb and scatter light and, if associated with fluorochromes, subsequently emit fluorescence. The intensities of the scattered and fluorescent light pulses coming from each particle are then measured. Key elements in these modules are (a) a flow cell, which spatially positions and aligns the flow stream containing the particles with the excitation light and detection axes, (b) light scatter and fluorescence detectors, screened by wavelength-appropriate filters and oriented orthogonally to the direction of the flow stream and the excitation light path, (c) electronic circuitry including analog-to-digital converters (ADCs) which convert the voltage pulses emerging from the detectors into digital values corresponding to the outputs from the individual particles, (d) computational architecture to process and store the information from these pulses for further analysis, or to use them immediately for processing sort-related decisions, and (e) mechanisms to implement individual, high-speed sorting of the particles, based on preselected combinations of optical characteristics.

One of the first implementations of flow sorting, and one of the most influential, was described by Bonner et al. [33] for characterization and isolation of various mammalian cell types including those of the hematopoietic system. To date, immunological applications represent the largest fraction of cytometric activities, worldwide. Most flow sorters employ a version of this original implementation, which involves precise conversion of the flow stream into a series of individual droplets, electromechanically synchronized to appear at a fixed distance below the point of laser interception (Figure 1). Based on the degree of sample dilution, some of these droplets contain the cells of interest, and can be selectively displaced into collection vessels by a process of charging the droplet at the point of its detachment from the flow stream followed by passage through a fixed electrostatic field. The rates of sorting depend on the size of the cells, which determines the size of the flow tip, and the rate of flow of the fluid stream [34].

Advances in the area of instrument development have included multiplexed excitation and detection modalities to comprehensively cover the excitation and fluorescence emission spectra of the available fluorochromes [35]. Recently, spectral analysis has been demonstrated as an alternative to conventional light filters in FCM [36, 37]. Other advances include the use of flow tips that accommodate cells and biological particles that are larger, and sometimes much larger, than mammalian blood cells, drastic reductions in overall instrument sizes, footprints, and purchase costs, full replacement of analog by digital signal processing and the use of miniaturized fluidics systems with corresponding improvements in accuracy and reliability, and accompanied by reductions in costs of maintenance.

4 | RATIONALE AND TARGET

Flow cytometry and flow cytometric sorting are not new methods. However, their use in Plant Biology has grown dramatically in the last decades, and in some cases, such as genome size measurements, these technologies have come to dominate. At the same time, instruments and associated protocols continue to be improved and expanded (e.g., bead beating, the use of tissues other than leaves, dry tissue). The literature now includes many resources outlining methods, theoretical issues, and limitations of methods (e.g., the "Flow Cytometry with Plant Cells" book [38], ESACP guidelines http://www.classimed.de/esacflow.html). However, despite this progress, it is clear from some recent publications that experimental design and manuscript review have not always kept pace with what we know about the application of FCM and cytometric sorting to plants, and this has adversely affected the quality of the results and the conclusions drawn.

Contributing factors include:

1. The practical need of carrying out experiments at centralized flow facilities that are not primarily concerned with, or understand, the characteristics of the input plant materials, or the types of questions (e.g., the large amounts of samples used in population biology) that are being addressed.
2. Effects of "lab culture," in which poor practices that have become established in laboratories are taught to uncritical novices.
3. Recommendations for "best practices" being scattered across the existing scientific literature: thus, a comprehensive article summarizing key rules for the reliable application of FCM and FCS to plants is still lacking, even for widely used applications such as DNA content measurements (but see 28).

Examples of poor practices and erroneous theories developed as a consequence of these practices, identified by Jan Suda in the

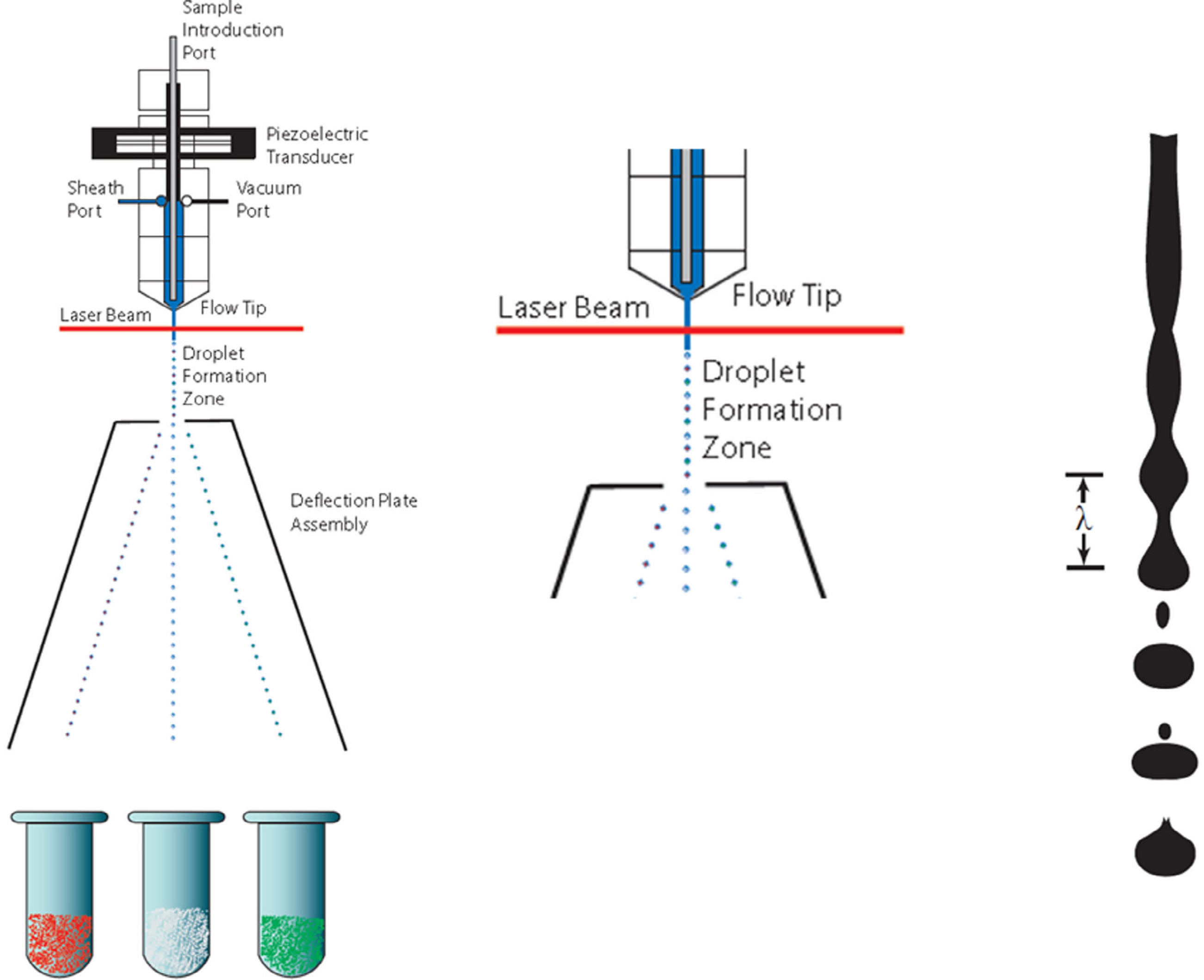

FIGURE 1 Schematic of the process of droplet formation for a typical droplet-in air flow sorter. Droplet formation is synchronized below the point of interception of the flow stream by the laser illumination. The undulation wavelength (λ) is defined by the velocity of the flow stream, and the drive frequency of the piezo-electric oscillator attached to the flow cell. A constant high DC voltage is maintained across the deflection plates. Precise switching of the charge applied to the flow stream at the time of droplet break-off retains that charge on the droplet, which then can be predictably deflected by the electric field

original draft, include flax genotrophs and problems with intraspecific variation reports, as reviewed in Greilhuber [35]. Recent tendencies in manuscripts to justify the use of dry tissue based on existing literature frequently lack acknowledgment of necessary precautions from the prior literature.

The main objective of this virtual issue is to outline key experimental issues and associated guidelines (under the heading of "Best Practices") that researchers are recommended to follow, and to provide the rationale for these recommendations, such that the guidelines may be modified with confidence as new applications emerge. We also identify those areas where the establishment of clear guidelines will require additional empirical data or theoretical work. Such guidelines will benefit researchers, facility managers, journal editors, and reviewers, since they should serve to guarantee high-quality results through elimination (or, at the very least, minimization) of artifactual variation from future research submitted for publication, as well as providing a means to identify artifacts within the published literature.

5 | THE SCOPE OF THIS VIRTUAL ISSUE IS

1. Applications based on the staining of DNA (ploidy, genome size, AT/GC content, cell cycle, including endoreduplication, nuclei and chromosome sorting) which represent a majority of uses.
2. Applications based on sorting single cells (protoplasts) and organelles (nuclei, mitochondria and plastids), based on use of FPs or fluorescent dyes in protoplast/organelle sorting for downstream omics analyses at the cell-type-specific or organelle-specific level.

3. A focus predominantly on plants, but with separate sections devoted to algae, and to fungi. However, in many cases, the general principles should apply across all organisms; wherever possible, we will extrapolate to other organisms.

The emphasis will be on providing guidelines for reviewers and for experimental design. This will NOT be a methods virtual issue in the sense of providing protocols: these are well-covered elsewhere (e.g., [27], "Flow Cytometry with Plant Cells" book [38], the supplemental material of Kron et al. [39], online resources).

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

David Galbraith: Conceptualization; writing-original draft; writing-review and editing. **João Loureiro:** Conceptualization; writing-original draft; writing-review and editing. **Ioanna Antoniadi:** Writing-review and editing. **Jillian Bainard:** Writing-review and editing. **Petr Bureš:** Writing-review and editing. **Petr Cápal:** Writing-review and editing. **Mariana Castro:** Writing-review and editing. **Sílvia Castro:** Writing-review and editing. **Martin Čertner:** Writing-review and editing. **Dora Čertnerová:** Writing-review and editing. **Zuzana Chumová:** Writing-review and editing. **Jaroslav Dolezel:** Writing-review and editing. **Debora Giorgi:** Writing-review and editing. **Brian Husband:** Writing-review and editing. **Filip Kolar:** Writing-review and editing. **Petr Koutecký:** Writing-review and editing. **Paul Kron:** Writing-review and editing. **Ilia Leitch:** Writing-review and editing. **Karin Ljung:** Writing-review and editing. **Sara Lopes:** Writing-review and editing. **Magdalena Lučanová:** Writing-review and editing. **Sergio Lucretti:** Writing-review and editing. **Wen Ma:** Writing-review and editing. **Susanne Melzer:** Writing-review and editing. **István Molnár:** Writing-review and editing. **Ondřej Novák:** Writing-review and editing. **Nicole Poulton:** Writing-review and editing. **Vladimír Skalický:** Writing-review and editing. **Elwira Sliwinska:** Writing-review and editing. **Petr Šmarda:** Writing-review and editing. **Tyler Smith:** Writing-review and editing. **Guiling Sun:** Writing-review and editing. **Pedro Talhinhas:** Writing-review and editing. **Attila Tárnok:** Writing-review and editing. **Eva Temsch:** Writing-review and editing. **Pavel Trávnícek:** Writing-review and editing. **Tomas Urfus:** Writing-review and editing.

ORCID

David Galbraith https://orcid.org/0000-0003-4020-1635
João Loureiro https://orcid.org/0000-0002-9068-3954
Paul Kron https://orcid.org/0000-0002-1734-5019

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