

Cytometry and Plant Sciences: a Personal Retrospective

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The plant kingdom is divided into the lower plants (blue-green algae, green algae, the bryophytes consisting of mosses and liverworts, and the euglenophytes) and the higher plants (which mainly comprise the vascular plants). Higher plants are grouped into the pteridophytes (ferns) and their relatives, and the two classifications of seed plants, the angiosperms and the gymnosperms. The former comprises the monocotyledons and the dicotyledons, and the latter comprises the conifers and cycads (1). In this retrospective, I have restricted my coverage to the seed plants.

Plants differ fundamentally from animals in a few general ways, one of which is the almost universal presence of a cellulosic cell wall. This is coupled to a method of cell division that involves partitioning of the daughter nuclei after mitosis by a phragmoplast, which expands and elaborates to join the parental cell walls, thereby effecting cytokinesis. Consequently, the cell walls of daughter cells remain topologically continuous with one another. Given that the entire mature form of a plant is achieved through regulated cell division and cell expansion, all cell walls can be viewed as a single continuum, termed the *apoplast*. The concept of flow cytometry and cell sorting arose in the late 1960s and 1970s from the study of natural single-cell suspensions, particularly those of the hematopoietic system. At first glance, these technologies did not seem applicable to higher plants, comprising complex three-dimensional tissue architectures of interlinked cells. In fact, only in the last few weeks did I become aware of what appears to be the first report, in German, of the use of flow cytometry for analysis of fluorescence signals from higher plant nuclei prepared from fixed tissues (1a), which involved use of ISAC member Wolfgang Gohde's flow cytometer (2).

My interest in flow cytometry and cell sorting started in 1976, when I took up a NATO postdoctoral fellowship at Stanford University. I was working in the laboratory of Dr. Peter Ray in the Department of Biological Sciences, about 300 yards away from the medical school. One of the topical interests of plant biologists at that time was the production from plant tissues of single-cell suspensions (termed *protoplasts*, prepared by enzymatic hydrolysis and solubilization of the cell wall) and their use for somatic hybridization. Kao and coworkers at Saskatoon had described the use of polyethylene glycol for the induction of high-frequency fusion of protoplasts (3), an observation that translated to animal cells and became a major technical basis for the emerging hybridoma technology. One persistent problem for plant biologists interested in so-

matic cell fusion was how to recognize the two parental sets of protoplasts that would be employed as fusion partners. Genetic mutants and transgenic lines resistant to various chemicals were not at that time available. In part of my graduate work at Cambridge, I had explored the use of generating antibodies directed against plant protoplasts with the hope of being able to find antibodies directed against plasma membrane proteins. At Stanford I became aware that Len Herzenberg had developed an interesting machine that was capable of recognizing and sorting cells based on surface fluorescence. I remember walking over to his laboratory to see a version of the fluorescence-activated cell sorter (I think a FACS IV) installed there. I recognized the possibilities offered by the instrument, but also realized that it would be necessary to devise means to label specific plant cells by using fluorescent tags before the fluorescence-activated cell sorter could be used for sorting hybrid protoplasts. There also was a variety of technical questions that would require solving, including how to accommodate protoplasts having diameters close to, or in some cases greater than, that of the flow tips. To label protoplasts, it would be necessary to find two pairs of fluorochromes that had distinct absorption and emission spectra. Keller et al. (4) in 1977 had described the synthesis of lipid-linked derivatives of fluorescein and rhodamine. I and my colleagues synthesized these molecules and found that they could be used to prelabel cell cultures from which fluorescent protoplasts could subsequently be produced (5). Alternatively, we found it was possible to label protoplasts after preparation by using fluorescein isothiocyanate and rhodamine isothiocyanate (6), which are required for protoplasts prepared from tissues other than suspension cultures. It therefore seemed feasible that flow cytometry and cell sorting might be used for heterokaryon identification and cell sorting for their purification (7,8).

For the work to be successfully accomplished, of course, we would need a flow sorter. In 1979, I put together a multi-user equipment proposal to the National Science Foundation requesting funds to purchase a cell

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sorter, with the major purpose being for the sorting heterokaryons for production of somatic hybrid plants. The grant was approved later that year, and I started the necessary homework to find out what instruments were available for the \$126,000 amount of the award. Three manufacturers were producing suitable instruments: Coulter, Becton-Dickinson, and Ortho. Ortho took themselves out of the running early on, based on cost, and we chose Coulter based on the availability of a their new model, the EPICS V. It turned out we were to get the seventh instrument off the production line.

The EPICS V was delivered around September 1980. Gary Durack was the Coulter field service representative at the time, and remembers calling Coulter to find out how to program the MDADS. Unfortunately, the software had not been completed at that time, so we were loaned a one-parameter pulse height analyzer, which at least enabled us to learn the basics of flow cytometry. The fact that I was forced into single-parameter analyses over this initial period had the unforeseen and, in retrospect, highly productive effect of forcing our attention onto one-dimensional analysis of the plant cell cycle.

FLOW ANALYSIS OF THE PLANT CELL CYCLE

I had already been interested in the idea of analyzing the plant cell cycle, based on work that we had started to investigate the behavior of tobacco leaf protoplasts placed in culture. Over a period of 2 days or so, the protoplasts initiated cell wall formation and entered into the cell division cycle, producing clusters of undifferentiated cells. By using Hoechst 33258 staining of fixed protoplasts and quantitative measurement of nuclear fluorescence with a jury-rigged photomultiplier attached to a fluorescence microscope, I was able to follow the onset of DNA synthesis and found that leaf protoplasts initiated the cell cycle within about 30 h and, after resynthesizing a cell wall, entered into cell division (9). Measurements made in this way were inaccurate and time consuming, so, with the availability of the flow cytometer, we started to examine fluorescence emission from fixed protoplasts. The EPICS was equipped with a 5-W multiline argon laser and separate optics for ultraviolet light, which were inconvenient to switch out. Because mithramycin could be excited at 457 nm, mithramycin had been successfully employed in combination with other dyes (ethidium bromide or propidium iodide) for animal cell cycle analyses, and plant cells generally lacked pigments absorbing and emitting in this part of the spectrum, we chose this DNA-specific fluorochrome for our work. We were able to establish rather quickly that fixed tobacco leaf protoplasts produce a readily distinguishable signal representing the nuclear DNA fluorescence, with a reasonable coefficient of variation of approximately 7%.

Immediately before our work, ISAC member Awtar Krishan (10) had described the use of hypotonic citrate for mammalian cell lysis before flow cytometric cell cycle analysis, and Christensen et al. (11) had introduced the use of detergents, so we also examined the suitability of similar approaches using plant protoplasts. The eventual

composition of the eventual lysis buffer included 45 mM $MgCl_2$ (required for mithramycin binding), 30 mM sodium citrate, 0.1% Triton X-100, and 20 mM MOPS for buffering at pH 7. Freshly prepared protoplasts treated with this lysis buffer and stained with mithramycin produced DNA histograms of very high quality, with coefficients of variation for the G_1 peak at approximately 2–3%. Using this methodology, we then went back to analysis of tobacco protoplasts during the 2-day period of culture, with the additional use of 2,6-dichlorobenzonitrile as an inhibitor of cell wall formation. 2,6-Dichlorobenzonitrile acts at the level of cellulose synthase by preventing formation of a coherent cellulose cell wall. It has little or no effect on re-initiation of the cell cycle by leaf protoplasts in culture (12). To release free nuclei from the cultured protoplasts, it was important to prevent cell wall formation around the protoplasts. Having charted this process to our satisfaction, we sent a manuscript to *Plant Physiology* describing the method of plant cell cycle analysis using flow cytometry, a first of its type, and the use of this method for the analysis of the initial stages of leaf protoplast development in culture. The paper came back with the major criticism that our method was not general to plant tissues, i.e. we could not ensure that protoplasts would be released from all cells that were present in the tissue of interest. To address this criticism, I realized that it was not necessary to make protoplasts at all—nuclei could be released from plant tissues simply by homogenization, assuming the process of homogenization was sufficiently gentle. We devised such a method by using single-edged razor blades and manual chopping. Each slice of the razor blade has the effect of cutting open the cells, thereby releasing the nuclei into suspension, along with the remaining cellular organelles and a variety of other forms of debris. Large material is then removed with nylon filters, and the clarified homogenate is then stained with mithramycin and run through the flow cytometer. Armed with a couple of razor blades, over a couple of hours my technician at the time (Kristi Harkins) and I reduced a large number of plant species within the teaching greenhouse to homogenates, and we were able to measure genome sizes for most of them by flow cytometry, including chicken red blood cells as an internal standard. The resultant publication describing the method, which appeared in *Science* in 1983 (13), has since been cited more than 415 times. We did not at all appreciate the impact of this method would have on basic and applied plant biology and agriculture. Before this time, analyses of ploidy, genome size, and the cell cycle involved some kind of light microscopy requiring counting of chromosomes or was based on quantitative microspectrophotometry using Feulgen staining. These techniques were very time consuming and were comparatively inaccurate and less sensitive. Now, flow cytometric methods are used routinely for all of these measurements and forms the major methodologic underpinning of the searchable database of plant nuclear DNA contents established at the Royal Botanical Gardens at Kew (<http://www.rbgekew.org.uk/cval/homepage.html>). The ease of sampling means that large populations can be routinely

analyzed, and we extended the method to the analysis of haploids in tissue culture (14), of natural variation in cytotype distributions (15), and for addressing issues in angiosperm evolution (16). Flow cytometry also can be used for quality control monitoring of the ploidy of commercial seeds and of different germ plasm accessions, for analysis of novel crosses and identification of wide-hybrids, and for monitoring euploidy in plants emerging from protoplast fusion, tissue culture, and genetic engineering procedures; for a complete discussion, see Galbraith et al. (17).

Our laboratory and other investigators since 1983 have extended the scope of ploidy-based measurements, based on their high accuracy, to include the use of different fluorochromes (18,19) and analysis of systemic and tissue-specific endoreduplication (20–25). The contributions of ISAC members Dick Kowles and Friedrich Srienic in the identification of endoreduplication in maize endosperm are particularly noteworthy, as are those of Spencer Brown and Kathiravetpillai Arumuganathan in applying the flow methods to large numbers of plant species (26–29). ISAC member Mike Fox was involved in early flow analyses of developing pollen (30). Flow cytometry also has been used for the identification of aneuploidy, mixoploidy, and layer chimeras; for a review, see Galbraith et al. (17). Under optimal conditions of alignment, it can be used for classification of sex in dioecious plants (31).

Accurate determination of plant genome sizes using flow cytometry is technically more demanding than that of ploidy, because it requires use of internal standards having precisely defined DNA contents. A thorough analysis of this approach was developed by ISAC member Spencer Johnston using various plant standards (32). Accurate analysis of plant genome sizes also requires that the method of fluorochromatic staining be insensitive to base-pair composition and/or the state of chromatin compaction. ISAC member Spencer Brown at Gif-sur-Yvette did pioneering work in this area (33,34).

In terms of analysis of the cell cycle, the DNA histograms produced by flow cytometry can be used for extraction of cell cycle parameters, and early work from ISAC members Spencer Brown and Catherine Bergounioux, and their collaborators led to further development of cytometric methods in this area (18,35,36), including simultaneous characterization of nuclear RNA and DNA contents (35) with acridine orange, the particularly interesting examination of transcriptional activity of sorted nuclei as a function of cell cycle stage (20,37), and analysis of the regulation of the cell cycle (37–39).

In terms of instrumentation, the contributions of Partec GmbH and ISAC member Wolfgang Gohde have been particularly important, because their development of low-cost flow cytometers has facilitated the movement of methods of plant nuclear DNA analysis into the laboratory and industrial setting. It should be emphasized that the plant-chopping method does produce homogenates within which the objects of interest, the nuclei, comprise a minor population. This is rather different than the situation encountered in flow analysis of animal cell suspen-

sions, within which the objects of interest (the cells) comprise the vast majority of the total population of particles detected by the flow cytometer. For flow operators entering this field for the first time, defining instrument settings for detection of the plant nuclei within homogenates can be tricky. It should parenthetically be noted that the chopping method (and the original plant chopping buffer) also produce excellent DNA histograms from insects and from mammalian tissues (Galbraith and coworkers, unpublished observations).

The DNA within nuclei, of course, is packaged into chromosomes. In comparison with human chromosomes, plant chromosomes in general do not exhibit differential staining when using fluorochromes with different base-pair specificities. For many species, the chromosomes are also numerous and of similar sizes. Nevertheless, a good deal of progress has been made in the development of techniques for flow analysis and sorting of plant chromosomes. Early workers selected plants with uniquely small chromosome numbers such as *Happlopappus gracilis* ($2n = 4$) (40), that had chromosomes of unique sizes such as the sex chromosomes in *Melandrium album* (synonym *Silene latifolia*) (41), or that were easy to manipulate in cell culture (42). ISAC member Spencer Brown was involved in early attempts to sort petunia chromosomes (42) and in the development of spreadsheet programs for the prediction in silico of the flow karyograms for different species (43). ISAC member Kathiravetpillai Arumuganathan was one of the first to attempt chromosome sorting from the crops tomato and maize (44,45), and ISAC member Nigel Miller provided flow expertise in similar work by using wheat chromosomes (46).

ISAC members Sergio Lucretti and Jaroslav Dolezel and their coworkers have made some of the greatest progress in this area, through devising methods for isolation of large numbers of intact chromosomes from seedlings of crop species, including wheat, pea, maize, barley, chickpea, and broad bean, and for flow sorting of many, and in some cases all, pairs of chromosomes (47–55). For some species, this requires the use of specific genotypes carrying multiple defined chromosomal translocations that have the effect of producing karyotypes within which all chromosome pairs are of different sizes and therefore can be sorted (47). Alternatively, in situ hybridization with repetitive sequences can be used to selectively differentiate chromosomes (56). Ultimately, chromosome-specific libraries can be prepared from the sorted chromosomes (53). Dolezel and coworkers have been developing methods for physical mapping, for localizing specific sequences to the individual sorted chromosomes (54,55,57), and for preparation of large insert libraries (58).

FLOW SORTING OF LIVING PLANT CELLS

Our original grant proposal for purchase of the EPICS V in 1980 had as its primary purpose the sorting of fused protoplasts (heterokaryons) for isolation of somatic hybrid plants. For this to be possible, evidently, it was necessary that protoplasts should survive passage through the flow cytometer. Standard flow tips at that time were 50–70 μm

in diameter, clearly a problem for protoplasts that, for the *Nicotiana* species (tobacco and relatives) we were using in our work, approximated 30–50 μm in diameter. Obtaining specially ordered 100- μm flow tips from Coulter, and then 150- and 200- μm tips, led to our studying the process of droplet formation by the piezoelectric bimorph and ultimately devising a set of SOPs for sorting large particles (59–61). These include reductions in the system pressure and in the bimorph drive frequency, somewhat jury-rigged alterations to the sort deflection area to allow visualization of the point of droplet break-off, inclusion of larger-sheath tanks to accommodate the large volumes of sheath fluid consumed by the large flow tips, devising indestructible size markers (pollen) that could be used for sort setup, and exploring appropriate (non-saline) osmotica as sheath fluids. My group found, to my pleasant surprise, that protoplasts could survive passage through the flow cytometer, could be sorted under sterile conditions, and could be regenerated in culture back into normal plants (62,63). ISAC member Jim Jett and coworkers described using viscous solutions as a means to impede sample sedimentation (64), a particular problem with large particles such as maize pollen or mammalian cell clusters.

SORTING OF HETEROKARYONS AND REGENERATION OF SOMATIC HYBRID PLANTS

The idea underlying this project was the fluorescent labeling of the two parental protoplast populations with distinguishable fluorescent dyes. These protoplasts would then be mixed, fused, analyzed by flow cytometry, and the heterokaryons sorted according to the presence of both dyes. The F18/R18 labeling method was not compatible with methods of leaf protoplast production, but we found that simply incubating the protoplasts during protoplast preparation with low levels of fluorescein isothiocyanate and rhodamine isothiocyanate led to the production of protoplasts that could be readily distinguished under the fluorescence microscope, and that these differences could be detected by the flow cytometer. It was then a matter of inducing protoplast fusion and employing the cell sorter to identify and sort the desired heterokaryons containing both sets of fluorochromes. These were subsequently regenerated into plants and were characterized as somatic hybrids at the molecular level (65). The methods described above were general in applicability, because all that is required are pairs of source protoplasts that can be regenerated into plants. ISAC member Jim Jett and coworkers concurrently reported flow analysis and sorting of heterokaryons (66). Later work from Nottingham, including contributions from ISAC members Andrew Lister and Nigel Blackhall, illustrated the general applicability of flow sorting for isolation of heterokaryons (67). Many reports of the practical application of flow sorting for somatic hybridization in the agricultural sector have emerged; for review, see Waara and Glimelius (68).

In terms of instrumentation, the commercial development of a highly effective means for droplet formation by using large flow tips has followed the recognition that

there exist increasing applications, beyond the plant kingdom, for sorting large cells and cell clusters. The MacroSort, produced by Becton-Dickinson, particularly exemplifies this.

DEVISING NOVEL MARKERS FOR PLANT FLOW CYTOMETRY

The types of markers that I have discussed for heterokaryons identification and sorting are relatively crude molecular paints, reacting covalently or non-covalently with the plant cells to provide fluorescent tags for protoplasts. We found that endogenous fluorescent markers could also be used for protoplast analysis and sorting; thus, chloroplasts contained within the cells of photosynthetic tissues themselves contain chlorophyll, which is highly fluorescent in the red area and can be conveniently excited with a variety of laser wavelengths. We found that flow analysis of chlorophyll autofluorescence could be used to directly measure the amounts of chlorophyll within protoplasts and to determine protoplast diameters based on time of flight (69). We then used these properties to flow sort protoplasts from different cell types (epidermis and mesophyll) and to demonstrate cell type-specific patterns of gene expression (70).

ISAC member Iona (O'Brien) Weir has pioneered the development of flow cytometric methods for analysis of apoptosis and programmed cell death in plants. This work primarily uses protoplasts for analysis of the presence of subgenomic levels of nuclear DNA measured through propidium iodide staining, of phosphatidylserine exposure to the external face of the plasma membrane measured through Annexin V binding, and the occurrence of nicks within genomic DNA measured through terminal dUTP nick end labeling analysis (71). More recently, she has teamed up with ISAC member David Hedley for flow cytometric analyses of reactive oxygen species and of membrane potential to implicate the involvement of the mitochondrial respiratory chain in protoplast apoptosis induced by camptothecin (72). Spencer Brown and Catherine Bergounioux were the first to use flow cytometry for analysis of the endogenous fluorescence of protoplasts of cell cultures of *Vinca rosea* (*Catharanthus roseus*), with the idea of employing successive rounds of protoplast sorting for the enrichment of cultures producing high levels of these important alkaloids (20,73,74). Brown and collaborators also devised flow cytometric methods to examine the fluidity of protoplast plasma membranes (75).

From 1985 to the present, my group has put considerable effort into the development of molecular markers that could be manipulated with molecular techniques to identify any plant cell type of interest. These included the production of monoclonal antibodies directed against plasma membrane components (76), analysis of the targeting behavior of mammalian plasma membrane markers (such as the vesicular stomatitis virus G protein) within plants (77), and, the expression and subcellular targeting of the green fluorescent protein (GFP) of *Aequorea victoria* (78,79). Of these, GFP (and the related fluorescent

proteins) appear particularly suited for flow cytometric analysis sorting. They provide a means for highlighting individual cells and/or organelles that can be cell or tissue type-specific based on expression that is regulated by appropriate promoter and enhancer sequences. This means that one can use flow sorting to purify protoplasts in a cell type-specific manner, which can then be used for further analyses, such as global gene expression, using microarrays (Birnbaum et al., unpublished observations) and global protein or metabolite contents.

An evident concern of this type of approach is that the process of protoplast production should not affect the measurements that are subsequently to be used. In part, this appears to be the case (70). However, the hypertonic conditions used for protoplast production and the interruption of signaling between cells and between the plasma membrane and cell wall of individual cells are factors that inevitably must perturb cellular processes (80). As previously noted, it is not always possible to prepare protoplasts from all cell types. For this reason, we have also pursued the cell type-specific labeling of nuclei by using GFP, with the idea of using flow analysis and sorting of fluorescent nuclei within homogenates for characterization of gene expression. This approach, which is covered in the next section, builds on the flow methods for nuclear genome size analysis described previously (13) and avoids the potential for perturbation because the method of homogenization and nuclear sorting is rapid and can be done on ice. Moreover, flow analysis and sorting of nuclei are much simpler than those of protoplasts due to the smaller size of nuclei.

FLOW ANALYSIS AND SORTING OF ORGANELLES

Of the variety of organelles found in higher plant cells, only the chloroplasts are naturally autofluorescent due to the presence of chlorophyll. ISAC member Bob Ashcroft did some of the first work (81) in the flow analysis of chloroplasts and was able to characterize intact chloroplasts and thylakoids prepared from spinach and maize by using forward and 90° light scatter and fluorescence emission signals. ISAC member Patrice Petit took this work forward by using forward angle and 90° light scatter and chlorophyll autofluorescence measurements to characterize the integrity of isolated spinach chloroplasts and, in combination with labeling with fluorescein isothiocyanate-linked lectins, to distinguish intact chloroplasts from thylakoid membranes and from various chloroplast membrane subfractions (82). ISAC member Julie Auger was involved in the further characterization of the fluorescence signals produced by chloroplasts (83). Flow analysis and sorting methods have been developed since for purification of thylakoids isolated from mesophyll and bundle sheath cells of maize (84) based on differences in chlorophyll fluorescence spectra. Amyloplasts (starch grains), which are generally grouped with chloroplasts because both represent subsets of the classification of plastids, can be rapidly analyzed using flow cytometry, as described by ISAC member Friedrich Srenc and his coworkers (85).

Flow analysis of mitochondria, which are non-fluorescent in their natural state, requires use of added fluorochromes or through *in vivo* targeting of GFP (86). Patrice Petit was the first to describe the use of lectins for the detection of glycosyl residues at the mitochondrial surface (87) and rhodamine 123 to monitor mitochondrial membrane potential and changes induced by addition of succinate and adenosine triphosphate and in response to treatments with metabolic inhibitors (88). Recent work by ISAC member Iona Weir on the role of mitochondria in apoptosis was mentioned earlier (72).

The nucleus similarly lacks endogenous fluorescence in its natural state. We have found that GFP can be effectively targeted to nuclei of various higher and lower plant species by using a nuclear localization signal from an orphan tobacco transcription factor (89-91). This requires inclusion of β -glucuronidase as a passive stuffer to increase the size of the chimeric GFP containing the nuclear localization signal beyond that of the passive exclusion limit of nuclear pores. The fluorescent nuclei can be readily detected and sorted in homogenates of transgenic tobacco plants (91). Further improvements in nuclear retention of the transgenic product are achieved by fusing GFP to chromatin-associated proteins (Zhang and Galbraith, unpublished observations). We have recently developed AFLP methods for analysis of transcription within isolated nuclei (92). Alternatively, nuclear transcripts can be used for production of fluorescent targets that can be hybridized to microarrays (80,93). Given the ability to produce transgenic plants expressing nuclear-targeted GFP within specific cell types, such as phloem companion cells and guard cells (94), it soon should be possible to categorize the gene expression profiles of any and all plant cell types. We are confident that these methods are applicable to animal cells.

CONTRIBUTIONS OF ISAC MEMBERS OVER 25 YEARS TO PLANT CYTOMETRY

Given the explosion in the number of journals that has occurred over the past 25 years, it is difficult to ensure that one is comprehensive in identifying all contributions of ISAC members to the published plant flow literature over that period (Table 1). Phil Dean has been most helpful in establishing the identities of past ISAC members. I have arbitrarily restricted myself to publications in journals indexed by the ISI Web of Science (<http://isiknowledge.com>) and apologize for any inadvertent omissions. For more details and for further references in the field of plant cytometry, the reader is referred to the following reviews (1,93,95,96). For specific methods, the following references may prove useful (97-103).

THE ROLE OF ISAC IN PLANT FLOW CYTOMETRY

The plant kingdom in general has interested a minority of ISAC members. However, since 1986, most ISAC congresses have had active platform and poster sessions focusing more or less narrowly on plants, albeit occasionally including "other life forms." Plants have provided the

Table 1
Contributions of Present and Past Members of the International Society of Analytic Cytometry to the Development of Plant Flow Cytometry

Name	City, country	E-mail	Topic
Kathiravetpillai Arumuganathan	Seattle, U.S.A.	karu@vmresearch.org	DNA content measurements; chromosome sorting
Robert Ashcroft	Black Rock, Australia	bashcrof@bigpond.net.au	Chloroplast physiology
Julie Auger	Chicago, U.S.A.	jauger@flowcity.bsd.uchicago.edu	Chloroplast physiology
Catherine Bergounioux	Paris, France	catherine.bergounioux@ibp.u-psud.fr	Cell cycle analysis; chromosome sorting
Nigel Blackhall	Nottingham U.K.	nigel.blackhall@nottingham.ac.uk	Heterokaryon sorting
Spencer Brown	Paris, France	spencer@isv.cnrs-gif.fr	Protoplast physiology; chromosome sorting; cell cycle analysis; DNA content measurement; flow karyotyping
Jaroslav Dolezel	Olomouc, Czech Republic	dolezel@ueb.cas.cz	DNA content/cell cycle; chromosome sorting
Michael Fox	Fort Collins, U.S.A.	mfox@colostate.edu	Pollen nuclear DNA analysis
David Galbraith	Tucson, U.S.A.	galbraith@arizona.edu	Protoplast and heterokaryon sorting; large-particle sorting; plant cell cycle and ploidy measurements; analyses of transgenic gene expression and targeting
Wolfgang Gohde	Munster, Germany	gohde@uni-muenster.de	DNA content measurement; cell cycle analysis
Kristi Harkins	Ames, U.S.A.	Kharkins@AATI-US.com	DNA content measurement; protoplast and large-particle sorting; transcriptional regulation
Jim Jett	Los Alamos, U.S.A.	jett@lanl.gov	Heterokaryon and large-particle sorting
Spencer Johnston	College Station, U.S.A.	spencerj@tamu.edu	DNA content standardization
Dick Kowles	Winona, U.S.A.	dkowles@smumn.edu	Maize endosperm endoreduplication
Andrew Lister	England	andrew_lister@europe.bd.com	Heterokaryon sorting
Sergio Lucretti	Rome, Italy	lucretti@casaccia.enea.it	Protoplast and large-particle sorting; chromosome sorting
Nigel Miller	Cambridge U.K.	ngam2@mole.bio.cam.ac.uk	Wheat chromosome sorting
Patrice Petit	Paris, France	pxpetit@cochin.inserm.fr	Organelle physiology
Sergio Sgorbati	Milan, Italy	Not known	Nuclear DNA contents; cell cycle
Friedrich Srienc	Minneapolis, U.S.A.	fried@cbs.umn.edu	Maize endoreduplication
Iona Weir (O'Brien)	Auckland, New Zealand	iweir@biodiscovery.co.nz	Apoptosis/PCD

impetus to the development of aspects of flow cytometry and cell sorting other than those that I have indicated. For reasons of space, I have not attempted to include lower plants in this article, but it is worth noting that the development of a fully automated flow cytometric monitoring station (the Cytobuoy) by ISAC member George Dubelaar (104) allows remote monitoring of the myriad different organisms (phyto- and zooplankton) found in the world's oceans.

In terms of future developments, within the plant sciences the role of flow cytometry and cell sorting in particular and of cytometry in general will only continue to increase. These techniques provide unique means to analyze and purify populations of cells and of subcellular organelles. As our attention increasingly focuses on the different component cells of plants and of the subcellular components within these different cells, the importance of these techniques to advance

development of the field will no doubt continue to expand.

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LITERATURE CITED

- Galbraith DW. Flow cytometry and cell sorting: applications to higher plant systems. *Int Rev Cytol* 1989;116:165-227.
- Heller FO. DNS-bestimmung an keimwurzeln von *Vicia faba* L. mit hilfe der impulszytometrie. *Ber Deutsch Bot ges Bd* 1973;86: S437-S441.
- Dittrich W, Gohde W. Impulse fluorometry in single cells in suspensions. *Z Naturforsch C* 1969;B24:360-361.

3. Kao KN, Michayluk MR. Method for high-frequency intergeneric fusion of plant protoplasts. *Planta* 1974;115:355–367.
4. Keller PM, Person S, Snipes W. A fluorescence enhancement assay of cell fusion. *J Cell Sci* 1977;28:167–177.
5. Galbraith DW, Galbraith JEC. A method for the identification of fusion of plant protoplasts derived from tissue cultures. *Z Pflanzen* 1979;93:149–158.
6. Galbraith DW, Mauch TJ. Identification of fusion of plant protoplasts II. *Z Pflanzen* 1980;98:129–140.
7. Galbraith DW. Identification and sorting of plant heterokaryons. Patent 4,300,310. Washington, DC: US Department of Commerce; 1981.
8. Galbraith DW, Harkins KR. Cell sorting as a means for isolating somatic hybrids. In: Fujiwara A, editor. *Plant tissue culture 1982*. Tokyo: Maruzen Press; 1982. p 617–618.
9. Galbraith DW, Mauch TJ, Shields BA. Analysis of the initial stages of plant protoplast development using 33258 Hoechst: reactivation of the cell cycle. *Physiol Plant* 1981;51:380–386.
10. Krishan A. Rapid flow cytofluorimetric analysis of mammalian-cell cycle by propidium iodide staining. *J Cell Biol* 1975;66:188–193.
11. Christensen IJ, Hartmann NR, Keiding N, Larsen JK, Noer H, Vindelov LL. In: Lutz D, editor. *Pulse cytophotometry, part III*. Ghent: European Press Medicion; 1978. p 71–78.
12. Galbraith DW, Shields BA. The effects of inhibitors of cell wall synthesis on tobacco protoplast development. *Physiol Plant* 1982;55:25–30.
13. Galbraith DW, Harkins KR, Maddox JR, Ayres NM, Sharma DP, Firoozabady E. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 1983;220:1049–1051.
14. Sharma DP, Firoozabady E, Ayres NM, Galbraith DW. Improvement of anther culture in *Nicotiana glauca*: media, culture conditions and flow cytometric determination of ploidy levels. *Z Pflanzen* 1983;111:441–450.
15. Keeler KH, Kwankin B, Barnes PW, Galbraith DW. Polyploid polymorphism in big bluestem (*Andropogon gerardii* Vitman). *Genome* 1987;29:374–379.
16. Bharathan G, Lambert GM, Galbraith DW. Nuclear DNA contents of monocotyledons and related taxa. *Am J Botany* 1994;81:381–386.
17. Galbraith DW, Bartos J, Dolezel J. Flow cytometry and cell sorting in plant biotechnology. In: Sklar LA, editor. *Flow cytometry in biotechnology*. Oxford: Oxford University Press; 2004. Forthcoming.
18. Bergounioux C, Perennes C, Miegue C, Gadal P. The effect of male sterility on protoplast division in *Petunia hybrida*—cell-cycle comparison by flow cytometry. *Protoplasma* 1986;130:138–144.
19. Sgorbati S, Levi M, Sparvole E, Trezzi F, Lucchini G. Cytometry and flow cytometry of 4',6-diamidino-2-phenylindole (DAPI)-stained suspensions of nuclei released from fresh and fixed tissues of plants. *Physiol Plant* 1986;68:471–476.
20. Bergounioux C, Brown SC, Petit PX. Flow cytometry and plant protoplast cell biology. *Physiol Plant* 1992;85:374–386.
21. DeRocher EJ, Harkins KR, Galbraith DW, Bohnert HJ. Developmentally-regulated systemic endopolyploidy in succulents with small genomes. *Science* 1990;250:99–101.
22. Galbraith DW, Harkins KR, Knapp S. Systemic endopolyploidy in *Arabidopsis thaliana*. *Plant Physiol* 1991;96:985–989.
23. Kowles RV, Srien F, Phillips RL. Endoreduplication of nuclear DNA in the developing maize endosperm. *Dev Genet* 1990;11:125–132.
24. Kowles RV, McMullein MD, Yerck G, Phillips RL, Kraemer S, Srien F. Endosperm mitotic activity and endoreduplication in maize affected by defective kernel mutations. *Genome* 1992;35:68–77.
25. Kowles RV, Yerck G, Haas KM, Phillips RL. Maternal effects influencing DNA endoreduplication in developing endosperm in developing endosperm of *Zea mays*. *Genome* 1997;40:798–805.
26. Arumuganathan K, Earle ED. Nuclear DNA content of some important plant species. *Plant Mol Biol Rep* 1991;9:208–218.
27. Blondon F, Marie D, Brown S, Kondorosi A. Genome size and base composition in *Medicago sativa* and *Medicago truncatula* species. *Genome* 1994;37:264–270.
28. Cerbah M, Mortreau E, Brown S, Siljak-Yakovlev S, Bertrand H, Lambert C. Genome size variation and species relationships in the genus *Hydrangea*. *Theor Appl Genet* 2001;103:45–51.
29. Martel E, DeNay D, Siljak-Yakovlev S, Brown S, Sarr A. Genome size variation and basic chromosome number in pearl millet and fourteen related *Pennisetum* species. *J Hered* 1997;88:139–143.
30. Anderson LK, Stack SM, Fox MH, Zhang CS. The relationship between genome size and synaptonemal complex length in higher plants. *Exp Cell Res* 1985;156:367–378.
31. Dolezel J, Gohde W. Sex determination in dioecious plants *Melandrium album* and *M. rubrum* using high-resolution flow cytometry. *Cytometry* 1995;19:103–106.
32. Johnston JS, Bennett MD, Rayburn AL, Galbraith DW, Price HJ. Reference standards for determination of DNA content of plant nuclei. *Am J Botany* 1999;86:609–613.
33. Godelle B, Cartier D, Marie D, Brown SC, Siljak-Yakovlev S. Heterochromatin study demonstrating the nonlinearity of fluorometry useful for calculating genomic base composition. *Cytometry* 1993;14:618–626.
34. Marie D, Brown SC. A cytometric exercise in plant DNA histograms, with 2C values for 70 species. *Biol Cell* 1993;78:41–51.
35. Bergounioux C, Perennes C, Brown S, Gadal P. Nuclear RNA quantification in protoplast cell-cycle phases. *Cytometry* 1988;9:84–87.
36. Bergounioux C, Perennes C, Brown SC, Sarda C, and Gadal P. Relation between protoplast division, cell-cycle stage and nuclear chromatin structure. *Protoplasma* 1988;142:127–136.
37. Segers G, Gadisseur I, Bergounioux C, Engler JD, Jacqmar A, Van-Montagu M, Inze D. The *Arabidopsis* cyclin-dependent kinase gene *cdc2bAt* is preferentially expressed during S and G(2) phases of the cell cycle. *Plant J* 1996;10:601–612.
38. Perennes C, Qin LX, Glab N, Bergounioux C. *Petunia* P34 (CDC2) protein-kinase activity in G(2)M cells obtained with a reversible cell-cycle inhibitor, mimosine. *FEBS Lett* 1993;333:141–145.
39. Trehin C, Planchais S, Glab N, Perennes C, Tregear J, Bergounioux C. Cell cycle regulation by plant growth regulators: involvement of auxin and cytokinin in the re-entry of *Petunia* protoplasts into the cell cycle. *Planta* 1998;206:215–224.
40. De Laat AMM, Blaas J. Flow-cytometric characterization and sorting of plant chromosomes. *Theor Appl Genet* 1984;67:463–467.
41. Veuskens J, Marie D, Brown SC, Jacobs M, Negrutiu I. Flow sorting of the Y sex-chromosome in the dioecious plant *Melandrium album*. *Cytometry* 1995;21:363–373.
42. Conia J, Bergounioux C, Perennes C, Muller P, Brown S, Gadal P. Flow cytometric analysis and sorting of plant chromosomes from *Petunia hybrida* protoplasts. *Cytometry* 1987;8:500–508.
43. Conia J, Muller P, Brown S, Bergounioux C, Gadal P. Monoparametric models of flow cytometric karyotypes with spreadsheet software. *Theor Appl Genet* 1989;77:295–303.
44. Arumuganathan K, Slattery JP, Tanksley SD, Earle ED. Preparation and flow cytometric analysis of metaphase chromosomes of tomato. *Theor Appl Genet* 1991;82:101–111.
45. Lee JH, Arumuganathan K, Kaepler SM, Kaepler HF, Papa CM. Cell synchronization and isolation of metaphase chromosomes from maize (*Zea mays* L) root tips for flow cytometric analysis and sorting. *Genome* 1996;39:697–703.
46. Schwarzach T, Wang ML, Leitch AR, Miller N, Moore G, Heslop-Harrison JS. Flow cytometric analysis of the chromosomes and stability of a wheat cell-culture line. *Theor Appl Genet* 1997;94:91–97.
47. Dolezel J, Lucretti S. High-resolution flow karyotyping and chromosome sorting in *Vicia faba* lines with standard and reconstructed karyotypes. *Theor Appl Genet* 1995;90:797–802.
48. Dolezel J, Cihalikova J, Lucretti S. A high-yield procedure for isolation of metaphase chromosomes from root-tips of *Vicia faba* L. *Planta* 1992;188:93–98.
49. Gualberti G, Dolezel J, Macas J, Lucretti S. Preparation of pea (*Pisum sativum* L) chromosome and nucleus suspensions from single root tips. *Theor Appl Genet* 1996;92:744–751.
50. Kubalakov M, Vrana J, Cihalikova J, Simkova H, Dolezel J. Flow karyotyping and chromosome sorting in bread wheat (*Triticum aestivum* L.). *Theor Appl Genet* 2002;104:1362–1372.
51. Lucretti S, Dolezel J, Schubert I, Fuchs J. Flow karyotyping and sorting of *Vicia faba* chromosomes. *Theor Appl Genet* 1993;85:665–672.
52. Lysak MA, Cihalikova J, Kubalakov M, Simkova H, Kunzel G, Dolezel J. Flow karyotyping and sorting of mitotic chromosomes of barley (*Hordeum vulgare* L.). *Chromosome Res* 1999;7:431–444.
53. Macas J, Gualberti G, Nouzova M, Samec P, Lucretti S, Dolezel J. Construction of chromosome-specific DNA libraries covering the whole genome of field bean (*Vicia faba* L.). *Chromosome Res* 1996;4:531–539.
54. Neumann P, Pozarkova D, Vrana J, Dolezel J, Macas J. Chromosome sorting and PCR-based physical mapping in pea (*Pisum sativum* L.). *Chromosome Res* 2002;10:63–71.
55. Vlaciлова K, Ohri D, Vrana J, Cihalikova J, Kubalakov M, Kahl G, Dolezel J. Development of flow cytogenetics and physical genome mapping in chickpea (*Cicer arietinum* L.). *Chromosome Res* 2002;10:695–706.
56. Pich U, Meister A, Macas J, Dolezel J, Lucretti S, Schubert I. Primed in situ labeling facilitates flow sorting of similar sized chromosomes. *Plant J* 1995;7:1039–1044.
57. Kejnovsky E, Vrana J, Matsunaga S, Soucek P, Siroky J, Dolezel J,

- Vyskot B. Localization of male-specifically expressed MROS genes of *Silene latifolia* by PCR on flow-sorted sex chromosomes and autosomes. *Genetics* 2001;158:1269-1277.
58. Simkova H, Cihalikova J, Vrana J, Lysak MA, Dolezel J. Preparation of HMW DNA from plant nuclei and chromosomes isolated from root tips. *Biol Plant* 2003;46:369-373.
 59. Galbraith DW. Large particle sorting. In: Radbruch A, editor. *Flow cytometry and cell sorting*. Berlin: Springer-Verlag; 1992. p 189-204.
 60. Galbraith DW, Lucretti S. Large particle sorting. In: Radbruch A, editor. *Flow cytometry and cell sorting*. 2nd ed. Berlin: Springer-Verlag; 2000. p 293-317.
 61. Harkins KR, Galbraith DW. Factors governing the flow cytometric analysis and sorting of large biological particles. *Cytometry* 1987;8:60-71.
 62. Galbraith DW, Afonso CL, Harkins KR. Flow sorting and culture of protoplasts: Conditions for high-frequency recovery and growth of sorted protoplasts of suspension cultures of *Nicotiana*. *Plant Cell Rep* 1984;3:151-155.
 63. Harkins KR, Galbraith DW. Flow sorting and culture of plant protoplasts. *Physiol Plant* 1984;60:43-52.
 64. Freyer JP, Fillak D, Jett JH. Use of xanthan gum to suspend large particles during flow cytometric analysis and sorting. *Cytometry* 1989;10:803-806.
 65. Afonso CL, Harkins KR, Thomas-Compton M, Krejci A, Galbraith DW. Production of somatic hybrid plants through fluorescence-activated sorting of protoplasts. *Biotechnology* 1985;3:811-816.
 66. Alexander RG, Cocking EC, Jackson PJ, Jett JH. The characterization and isolation of plant heterokaryons by flow cytometry. *Protoplasma* 1985;128:52-58.
 67. Hammatt N, Lister A, Blackhall NW, Gartland J, Ghose TK, Gilmour DM, Power JB, Davey MR, Cocking EC. Selection of plant heterokaryons from diverse origins by flow cytometry. *Protoplasma* 1990;154:34-44.
 68. Waara S, Glimelius K. The potential of somatic hybridization in crop breeding. *Euphytica* 1995;85:217-233.
 69. Galbraith DW, Harkins KR, Jefferson RA. Flow cytometric characterization of the chlorophyll contents and size distributions of plant protoplasts. *Cytometry* 1988;9:75-83.
 70. Harkins KR, Jefferson RA, Kavanagh TA, Bevan MW, Galbraith DW. Expression of photosynthesis-related gene fusions is restricted by cell-type in transgenic plants and in transfected protoplasts. *Proc Natl Acad Sci USA* 1990;87:816-820.
 71. O'Brien IEW, Baguley BC, Murray BG, Morris BAM, Ferguson IB. Early stages of the apoptotic pathway in plant cells are reversible. *Plant J* 1998;13:803-814.
 72. Weir IE, Pham NA, Hedley DW. Oxidative stress is generated via the mitochondrial respiratory chain during plant cell apoptosis. *Cytometry* 2003;54A:109-117.
 73. Brown S, Renaudin JP, Prevot C, Guern J. Flow cytometry and sorting of plant protoplasts - technical problems and physiological results from a study of pH and alkaloids in *Catharanthus roseus*. *Physiol Veg* 1984;22:541-554.
 74. Renaudin JP, Brown SS, Barbier-Brygoo H, Guern J. Quantitative characterization of protoplasts and vacuoles from suspension-cultured cells of *Catharanthus roseus*. *Physiol Plant* 1986;68:695-703.
 75. Gantet P, Hubac C, Brown SC. Flow cytometric fluorescence anisotropy of lipophilic probes in epidermal and mesophyll protoplasts from water-stressed *Lupinus albus* L. *Plant Physiol* 1990;94:729-737.
 76. Meyer DJ, Afonso CL, Galbraith DW. Isolation and characterization of monoclonal antibodies directed against plant plasma membrane and cell wall epitopes: Identification of a monoclonal antibody that recognizes extensin and analysis of the process of epitope biosynthesis in plant tissues and cell cultures. *J Cell Biol* 1988;107:163-175.
 77. Galbraith DW, Zeiher CA, Harkins KR, Afonso CL. Biosynthesis, processing and targeting of the G-protein of vesicular stomatitis virus in tobacco protoplasts. *Planta* 1992;186:324-336.
 78. Galbraith DW, Grebenok RJ, Lambert GM, Sheen J. Flow cytometric analysis of transgene expression in higher plants: green fluorescent protein. *Methods Cell Biol* 1995;50:3-12.
 79. Sheen J, Hwang S, Niwa Y, Kobayashi H, Galbraith DW. Green fluorescent protein as a new vital marker in plant cells. *Plant J* 1995;8:777-784.
 - 79a. Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN. A gene expression map of the Arabidopsis root. *Science* 2003;302:1956-1960.
 80. Galbraith DW. Global analysis of cell type-specific gene expression. *Comp Funct Genomics* 2003;4:208-215.
 81. Ashcroft RG, Preston C, Cleland RE, Critchley C. Flow cytometry of isolated chloroplasts and thylakoids. *Photobiochem Photobiophys* 1986;13:1-14.
 82. Schroeder WP, Petit PX. Flow cytometry of spinach chloroplasts: determination of intactness and lectin-binding properties of the envelope and the thylakoid membranes. *Plant Physiol* 1992;100:1092-1102.
 83. Xu C, Auger J, Govindjee. Chlorophyll *a* fluorescence measurements of isolated spinach thylakoids using single-laser-based flow cytometry. *Cytometry* 1990;11:349-358.
 84. Pfundel E, Meister A. Flow cytometry of mesophyll and bundle sheath chloroplast thylakoids of maize (*Zea mays* L.). *Cytometry* 1996;23:97-105.
 85. Jones RJ, Srienec F, Roessler J. Flow cytometric determination of light-scattering and absorbency properties of starch granules. *Starch* 1992;44:243-247.
 86. Kohler RH, Zipfel WR, Webb WW, Hanson MR. The Green Fluorescent Protein as a marker to visualize plant mitochondria in vivo. *Plant J* 1997;11:613-621.
 87. Petit P, Diolez P, Muller P, Brown SC. Binding of concanavalin A to the outer membrane of potato tuber mitochondria detected by flow cytometry. *FEBS Lett* 1986;196:65-70.
 88. Petit PX. Flow cytometric analysis of Rhodamine 123 fluorescence during modulation of the membrane potential in plant mitochondria. *Plant Physiol* 1992;98:279-286.
 89. Afonso CL, Galbraith DW. The callus associated protein (CAP) gene of *Nicotiana tabacum*: isolation, characterization, and evidence for possible function as a transcriptional factor. *In Vitro Cell Dev Biol Plants* 1994;30:44-54.
 90. Grebenok RJ, Lambert GM, Galbraith DW. Characterization of the targeted nuclear accumulation of GFP within the cells of transgenic plants. *Plant J* 1997;12:685-696.
 91. Grebenok RJ, Pierson EA, Lambert GM, Gong F-C, Afonso CL, Halde-man-Cahill R, Carrington JC, Galbraith DW. Green-fluorescent protein fusions for efficient characterization of nuclear localization signals. *Plant J* 1997;11:573-586.
 92. Macas J, Lambert GM, Dolezel D, Galbraith DW. NEST (nuclear expressed sequence tag) analysis: a novel means to study transcription through amplification of nuclear RNA. *Cytometry* 1998;33:460-468.
 93. Galbraith DW, Elumalai R, Gong F-C. Integrative flow cytometric and microarray approaches for use in transcriptional profiling. *Methods Mol Biol* 2003. Forthcoming.
 94. Chytilova E, Macas J, Galbraith DW. Green fluorescent protein targeted to the nucleus, a transgenic phenotype useful for studies in plant biology. *Ann Botany* 1999;83:645-654.
 95. Dolezel J, Lucretti S, Schubert I. Plant chromosome analysis and sorting by flow cytometry. *Crit Rev Plant Sci* 1994;13:275-309.
 96. Fox MH, Galbraith DW. The application of flow cytometry and sorting to higher plant systems. In: Melamed MR, Lindmo T, Mendelsohn ML, editors. *Flow cytometry and cell sorting*. New York: John Wiley & Sons; 1990. p 633-650.
 97. Dolezel J, Lysak MA, Kubalaková M, Simkova H, Macas J, Lucretti S. Sorting of plant chromosomes. *Methods Cell Biol* 2001;64:3-31.
 98. Galbraith DW. Flow cytometric analysis of plant genomes. *Methods Cell Biol* 1990;33:549-562.
 99. Galbraith DW. Isolation and flow cytometric characterization of plant protoplasts. *Methods Cell Biol* 1990;33:527-547.
 100. Galbraith DW. Flow cytometry and sorting of plant protoplasts and cells. *Methods Cell Biol* 1994;42:539-561.
 101. Galbraith DW, Dolezel J, Lambert G, Macas J. DNA and ploidy analyses in higher plants. In: Robinson JP, et al., editors. *Current protocols in cytometry* New York: Wiley; 1998. p 7.6.1-7.6.22.
 102. Galbraith DW, Herzenberg LA, Anderson M. Flow cytometric analysis of transgene expression in higher plants: green fluorescent protein. *Methods Enzymol* 1999;320:296-315.
 103. Lucretti S, Dolezel J. Cell cycle synchronization, chromosome isolation, and flow-sorting in plants. *Methods Cell Biol* 1995;50:61-83.
 104. Dubelaar GBJ, Gerritzen PL. CytoBuoy: a step forward towards using flow cytometry in operational oceanography. *Science* 2000;64:255-265.