Flow Cytometry with Plant Cells

Analysis of Genes, Chromosomes and Genomes

Edited by
Jaroslav Doležel, Johann Greilhuber, and Jan Suda

WILEY-VCH Verlag GmbH & Co. KGaA

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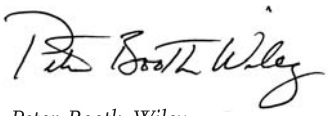

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Preface

This book is being published more than three decades after the publication of the first formal report on flow cytometric analysis of plant material by F. O. Heller in 1973. This pioneering work did not find immediate favor with researchers and it was only after a considerable period of time that the usefulness of the technique was recognized with numerous applications of flow cytometry being developed and applied in plant science and industry. The reason for the growing popularity of flow cytometry is not hard to guess, as the method provides a unique means with which to analyze and manipulate plant cells and subcellular particles. Several optical parameters of particles can be analyzed simultaneously, quantitatively and at high speed. Statistically relevant data are quickly provided and the detection of subpopulations is possible. The ability to purify specific subpopulations of particles by flow sorting then provides a tool for their manipulation and analysis using other methods. As a result, current flow cytometry is now able to provide answers to the once utopian suite of challenging questions on plant growth, development, function and evolution at subcellular, cellular, organismal, and population levels.

Despite significant progress in the development of instrumentation, and the growing number of reported applications, researchers continue to be frustrated when searching for first-hand information on plant flow cytometry. Such information is currently scattered in a number of books and various journals. Due to some fundamental differences between plant, human and animal cells and tissues, and the fact that the scientific targets of those working with these different cell and tissue types only partially overlap, the plethora of biomedical publications cannot provide a substitute. One of the gurus of flow cytometry, Howard Shapiro, pertinently characterized the state of affairs in his fourth edition of *Practical Flow Cytometry* (2003, p. 512): "There are now enough references to justify a book on applications of flow cytometry to plants, but, as far as I know, nobody has written one."

Sharing the same opinion, and stimulated by our long-term experience with plant flow cytometry, we arrived at the conclusion in late 2003 that the time was ripe for the publication of such a treatise. Our intention was to prepare a comprehensive, instructive and stimulating title which would cover virtually all fields of current plant flow cytometric research and offer an easily accessible source of

information. We trust that we have succeeded and look forward to the comments from the readers.

So what is on the menu? We start by describing the origin and evolution of flow cytometry and explaining the principles of flow cytometry and sorting (Chapters 1 and 2). Chapter 3 provides a general overview of plant flow cytometry, setting the stage for the more specialized topics discussed in Chapters 4–17. The first three of which cover the analysis of nuclear DNA content and its applications in the determination of genome size (Chapter 4), ploidy level (Chapter 5) and mode of reproduction (Chapter 6). Chapter 7 then explains the importance of research on nuclear genome size and Chapter 8 discusses the use of flow cytometry to estimate base composition in plant genomes. Focusing on microorganisms, Chapter 9 describes the use of flow cytometry in plant pathology, while Chapter 10 brings us back to plants and explains the analysis and sorting of naked plant cells, or protoplasts. We then move on to Chapter 11, the analysis of chloroplasts. Entering more exotic worlds, non-vascular plants and their DNA content is considered in Chapter 12 and the characterization of phytoplankton provides the subject of Chapter 13. Chapter 14 deals with the analysis of the cell cycle and is logically followed by a discussion on endopolyploidy (Chapter 15). Moving on to genome analysis, Chapter 16 describes the analysis and sorting of mitotic chromosomes and Chapter 17 introduces flow cytometry as a powerful tool for analyzing gene expression. The book closes with Chapter 18, which presents the FLOWER, a plant DNA flow cytometry database, and offers interesting quantitative data retrieved from publications in this area of flow cytometry.

Although the book was written by leading authorities and includes the most recent information, every effort has been made to avoid jargon and to explain all specific terms. Thus the book should be appreciated by users at every level of experience. Indeed, we are very happy with the final outcome and we hope that we have not only filled the gap in the current literature but created a reference volume for plant flow cytometry.

This book would never have materialized without the hard work, encouragement and support of many people. We are particularly indebted to the authors of the individual chapters who joined us on our formidable journey and provided excellent contributions. It is their hard work which makes this book a valuable reference text. We extend our gratitude to them all.

We greatly appreciate the highly professional, efficient, and conscientious work of the team at Wiley-VCH, who made publication of this book possible and who guided us carefully through the whole process. All three of us are excited by the graphical design of the book and the attractive front cover featuring the flower of lotos (*Nelumbo nucifera*). We felt it appropriate to include the nuclear genome size of the cover plant, and our original estimates are 1010 Mbp/1C (Prague) and 1017 Mbp/1C (Vienna).

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We hope that the investment of our time, which was to a significant extent at the expense of our private lives, was justified and that it will promote the use of flow cytometry in plant science and production. We sincerely hope that the readers will enjoy exploring the fascinating world of plant flow cytometry as much as we enjoyed writing and editing this book.

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1
Cytometry and Cytometers: Development and Growth

Howard M. Shapiro

Overview

It took almost 200 years of microscopy, from the mid-1600s until the mid-1800s, before objective data could be derived from specimens under the microscope by photography. The subsequent development of both image and flow cytometry for use by biologists followed the development of photometry, spectrometry, and fluorometry by physicists and chemists. Early cytometers measured cellular characteristics, such as nucleic acid content at the whole cell level; since few reagents were available that could specifically identify different types of cells, higher resolution imaging systems were developed for this task, but were too slow to be practical for many applications. The development of flow cytometry and cell sorting facilitated the development of more specific reagents, such as monoclonal antibodies and nucleic acid probes, which now allow cells to be precisely identified and characterized using simpler, low-resolution imaging systems. Although the most complex cytometers remain expensive, these newer instruments may bring the benefits of cytometry to a much wider community of users, including botanists in the field.

1.1
Origins

If the microscopic structures in cork to which Robert Hooke gave the name "cells" in the mid-17th century may be compared to the surviving stone walls of an ancient city, to what are we to compare the vistas available to 21st-century microscopists, who can follow the movements of individual molecules through living cells?

Between the time Hooke named them and the time that Schleiden, Schwann, and Virchow established cells as fundamental entities in plant and animal structure, function, and pathology, almost two centuries had elapsed. During most of that period, the only record of what could be seen under the microscope was an

observer's drawing, and, even with the aid of a camera lucida, it was difficult if not impossible to eliminate subjective influences on the research product. The development of photography in the 1830s was quickly followed by the marriage of the Daguerrotype camera and the microscope, but it was only in the 1880s that photomicrography became accepted as the definitive objective method in microscopy, due in large measure to Robert Koch's advocacy (Breidbach 2002).

Even by that time, what we would today properly call cytometry, that is, the measurement of cells, was restricted to the quantification of morphologic characteristics, such as the sizes and numbers of cells and their organelles. The visualization of organelles themselves was greatly facilitated by differential staining methods, the development of which accelerated in the late 1800s with the availability of newly synthesized aniline dyes (Baker 1958; Clark and Kasten 1983); Paul Ehrlich's initial researches in this area were to lead directly to the transformation of pharmacology from alchemy to science, and his appreciation of the specificity of antigen–antibody reactions provided an early milestone on the path toward modern immunochemical reagents.

Spectroscopy, a tool of physics adapted to chemistry and astronomy in the 19th century, became a mainstay of cytometry shortly thereafter. Microspectrophotometric measurement, either of intrinsic optical characteristics of cellular constituents or of optical properties of dyes or reagents added to cells, provided objective, quantitative information about cells' chemistry that could be correlated with their functional states.

The subsequent development of both cytometry and cytometers has been characterized by the use of such information, wherever possible in place of the inherently subjective and less quantitative results obtained by human observers.

In the remainder of this chapter, I will consider the history of cytometry from the 20th century onwards. Although much of the material has been covered, sometimes in greater detail, in several of my earlier publications (Shapiro 2003, 2004a, 2004b), this version of the story will pay special attention to one of the principal uses of cytometry in botany, namely, the determination of the genome sizes of plants by measurement of nuclear DNA content (Bennett and Leitch 2005; Doležel and Bartoš 2005; Greilhuber et al. 2005).

1.2
From Absorption to Fluorescence, from Imaging to Flow

It is easy, and probably easier for younger than for older readers, to forget that both Feulgen's staining procedure (Feulgen and Rossenbeck 1924) and Caspersson's ultraviolet (UV) absorption microspectrophotometric method for quantification of nuclear DNA content (Caspersson and Schultz 1938) were developed years before it was established that DNA was the genetic material. The evolution of cytometers from microscopes began in earnest in the 1930s in Torbjörn Caspersson's laboratory at the Karolinska Institute in Stockholm. He developed a series of progressively more sophisticated microspectrophotometers, and confirmed

that, as had been suggested by conventional histologic staining techniques of light microscopy, tumor cells were likely to have abnormalities in DNA and RNA content (Caspersson 1950). In a memoir, which in itself provides useful insights on the development of cytometry, Leonard Ornstein (1987) documents the influence of Caspersson's work in establishing the genetic role of DNA. The first report that DNA contents of haploid, diploid, and tetraploid plant cells were in the ratio of 1:2:4 was published in 1950 by Swift, who made measurements using the Feulgen technique; his paper also introduced the terms C, 2C, and 4C to describe the respective DNA contents for cells of a particular species.

1.2.1
Early Microspectrophotometry and Image Cytometry

Microspectrophotometers were first made by putting a small "pinhole" aperture, more properly called a field stop, in the image plane of a microscope, restricting the field of view to the area of a single cell, and placing a photodetector behind the field stop. Using progressively smaller field stops permits measurement of light transmission through correspondingly smaller areas of the specimen, and, by moving the stage in precise incremental steps in the plane of the slide, and recording the information, it becomes possible to measure the integrated absorption of a cell, and/or to make an image of the cell with each pixel corresponding in intensity to the transmission or absorption value. This was the first, and, until the 1950s, the only approach to scanning cytometry, and, even when measurements were made at the whole cell level, the process was extremely time-consuming, especially since there was no practical way to store data other than by writing down measured values as one went along. Publications were unlikely to contain data from more than a few hundred cells. By the 1960s, Zeiss had commercialized a current version of Caspersson's apparatus, and others had begun to build high-resolution scanning microscopes incorporating a variety of technologies. During the 1950s, what we now call "cytometry" was known as "analytical cytology". The first and second editions of a book with the latter title appeared in 1955 and 1959 (Mellors 1959). The book included chapters on histochemistry, on absorption measurement, on phase, interference, and polarizing microscopy, and on Coons's fluorescent antibody method (Coons et al. 1941).

1.2.2
Fluorescence Microscopy and the Fluorescent Antibody Technique

Fluorescence microscopy was developed around the turn of the 20th century. The earliest instruments used UV light for excitation; later systems could employ excitation at blue and longer wavelengths, but the requirement for relatively high power at relatively short wavelengths made it necessary to use arc lamps, rather than filament lamps, as light sources. Fluorescence microscopy, in principle, allows visualization of bright objects against a dark background. Earlier systems, however, were likely to fall short of achieving this goal because they were essen-

tially transmitted-light microscopes with colored glass filters in both the excitation path, that is, between the light source and the condenser, and the emission path, that is, between the objective and the eyepiece. The combination of stray light transmission through both excitation and emission filters and fluorescence excited in the emission filter often resulted in the background being too bright to permit observation of weakly fluorescent material.

An extremely important application of fluorescence microscopy developed during the 1940s was the fluorescent antibody technique introduced by Coons et al. (1941). Other workers had demonstrated that azo dye-conjugated antisera to bacteria retained their reactivity with the organisms and would agglutinate them to form faintly colored precipitates; however, the absorption of the dye-conjugated sera was not strong enough to permit visual detection of bacterial antigens in tissue preparations.

Albert Coons surmised that it might be easier to detect small concentrations of antibody labeled with fluorescent material against a dark background using fluorescence microscopy. He and his coworkers labelled anti-pneumococcal antibodies with anthracene and could detect both isolated organisms and, more importantly, antibody bound to antigen in tissue specimens, by the UV-excited blue fluorescence of this label, as long as tissue autofluorescence and background were not excessive.

In 1950, Coons and Kaplan reported that fluorescein gave better results as an antibody label than did anthracene, because the blue-excited yellow–green fluorescence of fluorescein was easier to discriminate from autofluorescence. Thereafter, fluorescein became and has remained the most widely used immunofluorescent label.

A significant advance in fluorescence microscopy, epiillumination, was made in 1967 by Ploem (1967), who substituted dichroic mirrors for the half-silvered mirror normally used in an incident light microscope, and added excitation and emission filters to the optical path. Even when color glass filters were still used for excitation and emission wavelength selection, this configuration greatly reduced both stray light transmission and filter fluorescence, yielding much lower backgrounds. Within a short time, it had been reported that, when an epiilluminated apparatus was employed, measurements of nuclei stained by a fluorescent Feulgen procedure using acriflavine yielded results equivalent to those obtained by the standard absorption method (Böhm and Sprenger 1968).

1.2.3 Computers Meet Cytometers: The Birth of Analytical Flow Cytometry

By the mid-1950s, it had become clear that malignant cells often contained more nucleic acid than normal cells, and Mellors and Silver (1951) proposed construction of an automatic scanning instrument for screening cervical cytology (Papanicolaou or ''Pap'' smears). Their prototype measured fluorescence rather than absorption, and anticipated Ploem (1967) in introducing UV epillumination. Tolles (1955) described the ''Cytoanalyzer'' built for cervical cytology. A disc containing a