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Flow Cytometry- A Best Tool for Genome Size Analysis in Plants

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Abstract

Flow cytometry is an efficient tool for estimation of quantity of DNA in nuclei. The method involves preparation of nuclei suspensions, whose DNA is stained using fluorochromes such as Ethidium Bromide, PI and DAPI. Classification of nuclei is based on their relative fluorescence intensity/ DNA content in comparison with a standard. Flow cytometry has gained popularity owing to its rapidness, easy sample preparation and convenient in usage. It has wide application in plant breeding *viz.*, cell cycle analysis, mixoploids and aneuploids detection, ploidy screening, polysomaty studies, reproductive pathway determination and estimation of genome size.

Introduction

C-value is represented as the amount of DNA (picograms) present as an unreplicated haploid chromosome complement (*n*) and also a nucleus at G1 phase with unreplicated DNA has 2C DNA content. However, studies have revealed that there was no correlation between the C-value of DNA and complexity of organism, which was termed as "C value Paradox" by Thomas. Reliable and appropriate methods are necessary for the estimation of C value. Two major approaches for DNA estimation includes, Extraction of DNA from a large number of cells and it analysis; and individual nuclei analysis. Re-association and chemical analysis are the examples of the former approach, however, tissues contain the cells at different phases of cell cycle and with different amounts of DNA and hence the exact representation of 2C DNA amount is not feasible. When compared to the above approaches, single nuclei approach gives higher precision, but it is technically demanding. Single nuclei measurements were initially based on the absorption of UV light by DNA, later Feulgen stained nuclei method. Followed by these techniques, scanning electron micro-spectrophotometry was developed to eliminate the errors due to irregularly shaped nuclei and chromosomes with non-

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homogenously stained nuclei. An alternative method for micro spectrophotometry was DNA image cytometry that is based on the image captured by a video camera.

Flow cytometry – Major components and principle of working

All these above techniques were ruled out with the invention of flow cytometry, which involves the analysis of microscopic particles in suspension, that are constrained to flow in single file within a fluid stream through the focus of intense light. Pulses of the scattered light and fluorescence are collected and converted to electric current pulses by optical sensors and classified. Since the particles are individually analyzed at high speed, large populations can be measured in a short time, precisely. The ease of sample preparation, no need for employing tissues with dividing cells and the ability to analyze DNA quickly in large population in short time made it more attractive than micro-spectrophotometry.

Flow cytometry basically comprises a fluorescence microscope, which analyzes the optical properties of the particles moving in a suspension. These particles, on illumination by a focused source of light in turn produces light-scatter and fluorescence signals, which are selectively detected using a series of filters. These signals are detected into digital values and are displayed in the form of histograms (Fig 1). DNA flow cytometry was initially extensively used for biomedical research to detect aneuploid syndromes, monitor cell cycle and apoptosis. Attempts to apply the same technique for plants were hampered due to the difficulty in preparation of suspension of intact cells that are suitable for flow cytometry. However, Heller in 1973 had prepared a cell suspension of field bean, stained with Ethidium bromide, but it was lost out of track for a decade.

The autoflourescent, irregular and rigid cell wall disturbs the fluid stream, making it unsuitable for flow cytometry analysis. This can be overcome by isolating the protoplast which is spherical and behaves regularly, using hydrolytic enzymes (pectinases and cellulases). Puite and Ten Broeke (1983) showed that the nuclear DNA can be stained in plant protoplasts however; the histograms were unclear which might be due low permeability of plasma membrane and the effect of cytoplasmic autofluorescence, which can be fixed with ethanol–acetic acid permeabilization. Very good histograms can be produced when intact nuclei can be isolated from protoplast by lysis either in a hypotonic solution or in a detergent. Overcoming all these constraints, Galbraith *et al.* (1983) came up with an isolation buffer, into which an intact nuclei can be isolated by chopping a small amount of leaf tissue. This

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technique was rapid, incredibly simple and convenient. The ability to estimate DNA content stimulated a vast array of applications, which ranged from basic research to breeding and seed production, and included estimation of nuclear genome size, ploidy screening, detection of mixoploidy and aneuploidy, assessment of the degree of polysomaty, reproductive pathways and cell cycle kinetics. It is therefore not an exaggeration to conclude that Galbraith's work marked the real beginning of flow cytometry in plants.

Steps involved in Flow cytometry analysis:

The major steps includes

(1) Preparation of nuclei cell suspension,

(2) Staining the nuclei and

(3) Estimation of DNA content.

The steps are shown in brief in Fig. 2.

1) **Prepare of nuclei cell suspension:** Preparation of intact nuclei suspension for the estimation of DNA content has been almost universally performed following the protocol of Galbraith *et al.* (1983), wherein the nuclei are released into an isolation buffer by mechanical homogenization of fresh plant tissue. The composition of the isolation buffer should facilitate the release of the nuclei free of cytoplasm in sufficient quantities, protect their DNA against endonucleases, maintain the integrity of isolated nuclei and facilitate DNA staining. The chemical compositions of six isolation buffers that are popularly used are given below in Table 1.

Table 1: Popular buffers for nuclei isolation (Source: Dolezel and Bartos; 2005)

S.No	Buffer	Compositiom
1.	Galbraith's buffer	45 mM $MgCl_2$; 30 mM sodium citrate; 20 mM MOPS; 0.1 % (w/v) Triton X-100; pH 7.0
2.	LB 01	15 mM TRIS; 2 mM Na_2EDTA; 0.5 mM spermine.4HCl; 80 mM KCl; 20 mM NaCl; 15 mM b-mercaptoethanol; 0.1 % (v/v) Triton X-100; pH 7.5
3.	Arumuganathan and Earle	9.53 mM $MgSO_4.7H_2O$; 47.67 mM KCl; 4.77 mM HEPES; 6.48 mM DTT; 0.25 % (w/v) Triton X-100; pH 8.0
4.	Marie's nuclear isolation buffer	50 mM glucose; 15 mM KCl; 15 mM NaCl; 5 mM Na_2EDTA; 50 mM sodium citrate; 0.5 % (v/v) Tween 20; 50 mM HEPES; 0.5 %

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		(v/v) b-mercaptoethanol; pH 7.2
5.	Otto buffers	Otto I buffer: 100 mM citric acid; 0.5 % (v/v) Tween 20 (pH approx. 2.3) Otto (1990) Otto II buffer: 400 mM $Na_2HPO_4.12H_2O$ (pH approx. 8.9)
6.	Tris-$MgCl_2$	200 mM TRIS; 4 mM $MgCl_2.6H_2O$; 0.5 % (v/v) Triton X-100; pH 7.5

2) **Staining the nuclei:** A wide range of fluorescent dyes *viz.*, Ethidium bromide (Et Br), Propodium Iodide (PI), Mithramycin and Hoechst dyes were used for fluorescent dying of nuclear DNA. Et Br and PI intercalates into the double-stranded DNA. Mithramycin, together with other fluorescent antibiotics *viz.*, chromomycin, olivomycin is highly specific to double-stranded DNA, preferably binding to GC rich regions. Likewise, Hoechst dyes binds to AT-rich segment regions of DNA. Among the Hoechst dyes, 4'6-Diamidino-2-Phenylindole (DAPI), has became most popular, presumably due to its specificity to AT rich region binding without influencing the chromatin structure and many plant scientists preferred arc-lamp-based flow cytometers, with which DAPI fluorescence was particularly easy to excite and measure.

3) **Estimation of DNA content:** Flow cytometry analyses the relative fluorescence intensity, and hence the relative DNA content, the genome size of a test sample can be determined only by comparing with the nuclei of a reference standard, of known genome size. An ideal DNA reference standard should have a genome size close to the species under study. Johnston *et al.* (1999) presented a set of standards consisting of 12 species with 2C-values ranging between 1.08 to 32.97 pg of DNA. The relative fluorescence intensity of the stained nuclei is performed on a linear scale and typically, 5000–20, 000 nuclei have to be analysed for each sample. The absolute amount of DNA in a sample is calculated based on the values of the G_1 peak means:

Sample 2C DNA content = [(sample G1 peak mean)/ (standard G1 peak mean)] X standard 2C DNA Content (pg DNA).

Conclusions:

Flow cytometry had a wide application across biological sciences; however its application in plant sciences has been restricted to certain extent due to the nature of the cell wall. This

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restricted has been uplifted post Galbraith's work and had its application in estimation of nuclear genome size, ploidy screening, detection of mixoploidy and aneuploidy, assessment of the degree of polysomaty, reproductive pathways and cell cycle kinetics.

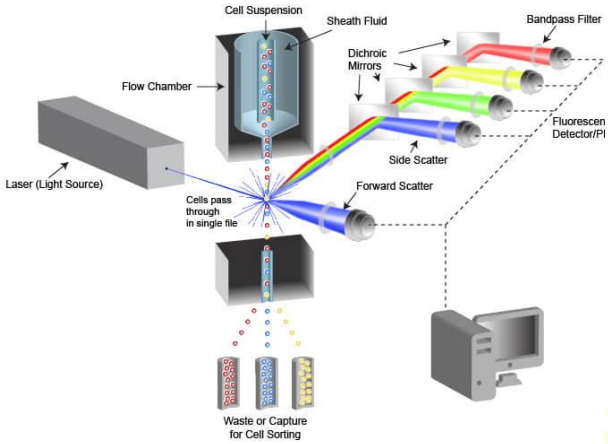

Fig.1. Working principle of Flow cytometer

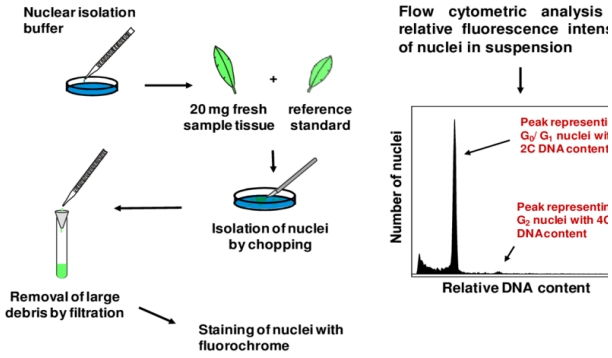

Fig: 2. Procedure of Flow cytometry analysis in plants [Source: Flow cytometric approaches to study plant genomes by Loeruie (2007)]

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