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Ploidy determination using flow cytometry

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Introduction

Measuring ploidy level is of highest importance in the final stages of haploid induction programs. For breeding purposes, a large number of haploids usually need to be tested, so an efficient ploidy analysis is a prerequisite for successful application. Methods to determine ploidy level may be direct (chromosome counting) or indirect (flow cytometry, stomatal size, chloroplast number of the guard cells and morphological observations). Various opinions about the usefulness of the mentioned techniques can be found. Sari et al. (1999) for instance, comparing various ploidy measurement methods in haploid watermelons concluded that “counting chromosomes is cumbersome, producing plants for morphological observations requires a long time and flow cytometry is expensive and labour intensive.” They proposed that “measurement of stomata and chloroplast counting methods are simple to use and less labor intensive, and hence can be considered a practical alternative to the others.” The author’s experiences are based on flow cytometric measurements of a large number of haploid regenerants obtained mainly by androgenesis (cabbage), gynogenesis (onion) or distant fertilization (potato), as well as on genome size analysis of various species. Several points will be made to explain why, in the author’s opinion, for ploidy analysis of haploid regenerants, flow cytometry is of much higher relevance than any other proposed method. The main aim of this article is to explain the basic features of flow cytometry and to propose optimized protocols for rapid and simple flow cytometric evaluations using either a HBO lamp or laser equipped flow cytometers.
**Principles of flow cytometry**

A flow cytometer is a device that measures light fluoresced or scattered from particles. For ploidy analysis, the particles used are interphase nuclei obtained from somatic tissues. Excitation light can be emitted from either a high pressure mercury lamp (HBO lamp) with broad spectral characteristic or laser light of very narrow wavelength. Nuclei are stained with DNA specific fluorochromes that after absorption of excitation light emit fluorescence of longer wavelength. The emitted light passes through specific optical filters and dichroic mirrors (which reflect some wavelengths and allow others to pass through) enabling only emitted light to reach the appropriate photomultiplier.

On photomultipliers, signals are detected and measured for each particle (nucleus) separately. More than 1000 nuclei per second can be measured. Acquired data are processed by computer and results are displayed on screen in real time distributed to linear or logarithmic scales, usually divided into 1024 channels. The position of signals is achieved by adjustment of “gain” which is related to the voltage on the photomultiplier. After acquisition, data can be stored and, if necessary, further analyzed using specialized software, normally provided by the producer of the flow cytometer. For ploidy analysis, only a single parameter, the fluorescence excited by the DNA specific fluorochrome, is usually measured, although a number of parameters can be measured for each particle simultaneously. This means that, for instance, only blue or red light signals can be measured, or up to 5 different color emissions including scattered light and time can be measured for each particle in a multiparameter function.

The term flow cytometry is based on the fluidic flow of particles passing a quartz chamber (flow cuvette). Particles from a sample are pumped through the flow cuvette in a very narrow stream flowing inside a larger “sheath” stream of water or saline, which is used to focus the sample stream into the center.

When nuclei are measured, undesired small particles are also often stained, representing «debris» consisting of broken nuclei or other smaller particles. It is useful to set a left threshold level to exclude such particles from analysis, allowing higher precision of measurement of nuclei. A similar improvement can be achieved using a side or forward scatter function on laser based flow cytometers. In such case, two parameter dot plot analysis and setting a gating region are used, allowing elimination of both small particles and nuclear doublets or similar larger particles that represent noise in the measured interval.

**How is ploidy interpreted in flow cytometry**

In eukaryotes, cell growth and division is a cyclical process including mitosis (M) followed by an interphase period starting with the first “gap” (G1), a synthetic (S) phase and a final “gap” (G2). Non-proliferating cells may leave the cell cycle either in G1 or G2. In somatic diploid tissues, nuclei arrested in both G1 and G2 stages are usually found representing 2n and 4n nuclei. The terms used to describe ploidy are C-values rather than chromosome number n values, 1C value representing the quantity of DNA corresponding to the haploid complement. C value may be presented in arbitrary units or expressed as picograms/nucleus or number of nucleic base pairs - Mbp/nucleus.

For determination of genome size, a standard species with known genome size is mixed with the studied species, and the mean values of histograms of G1 of both species are used for calculation of the genome size of the measured plant. Typically, such analysis requires staining of nuclei with intercalating stain (mainly propidium iodide), slow measurement (about 20 nuclei/second) resulting in low variation, 10,000 nuclei measured per sample and several repetitions of measurement. Detailed explanations have been given elsewhere (Dolezel, 1991; Marie and Brown, 1993).

For determination and interpretation of the haploid status of regenerants, a greatly simplified procedure can be used. Firstly, tissue of known ploidy of the studied species,