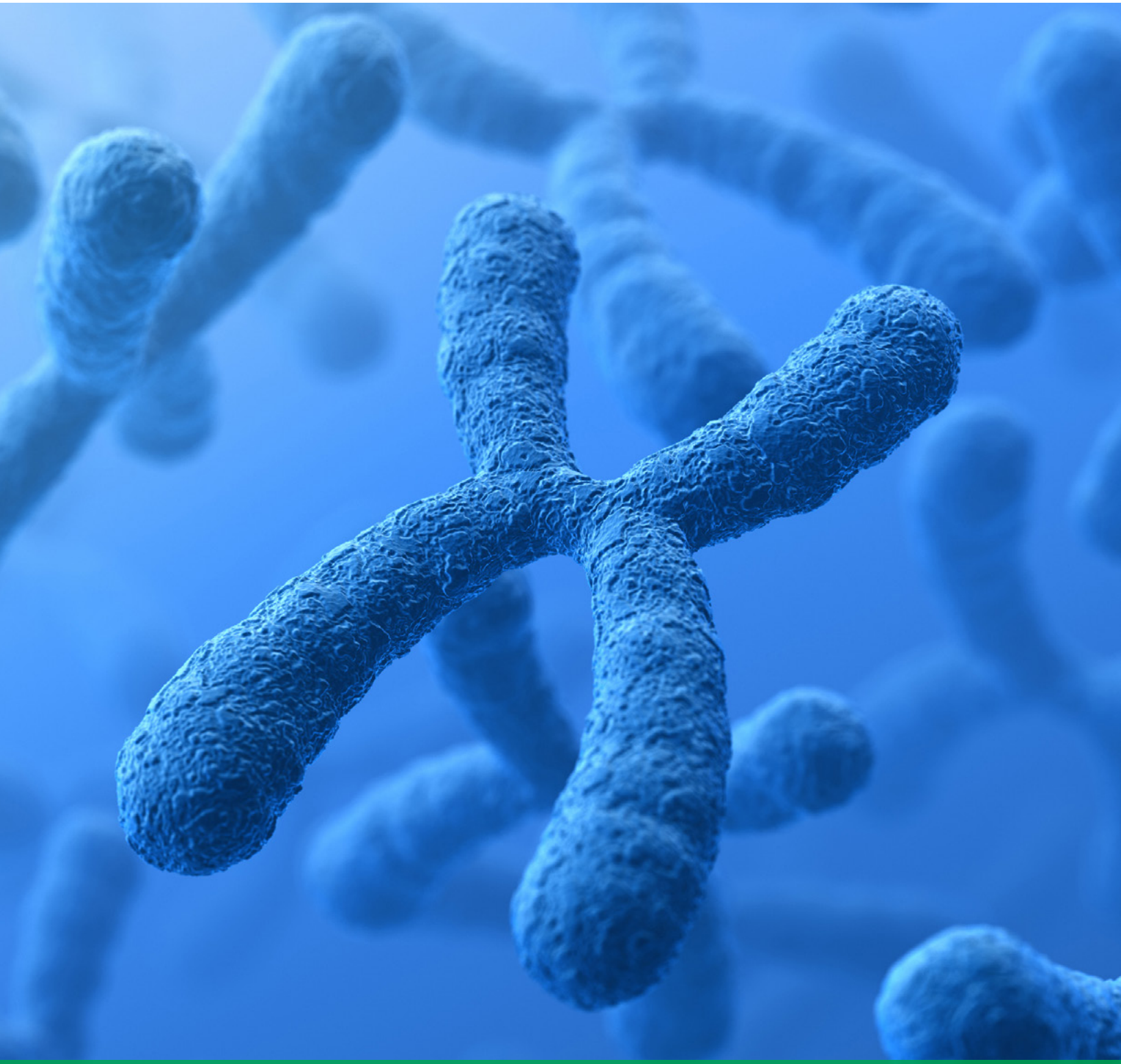
Introduction to Ploidy



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Introduction to Ploidy

Ploidy represents the number of complete chromosome sets in the nucleus of a cell. Typically, chromosomes exist in pairs, a condition described as diploidy. The generation of gametes, or germ cells by meiosis, results in haploidy, with half of the normal or somatic number of chromosomes. When two germ cells (e.g. egg and sperm) unite in fertilization, then the diploid formation is restored.

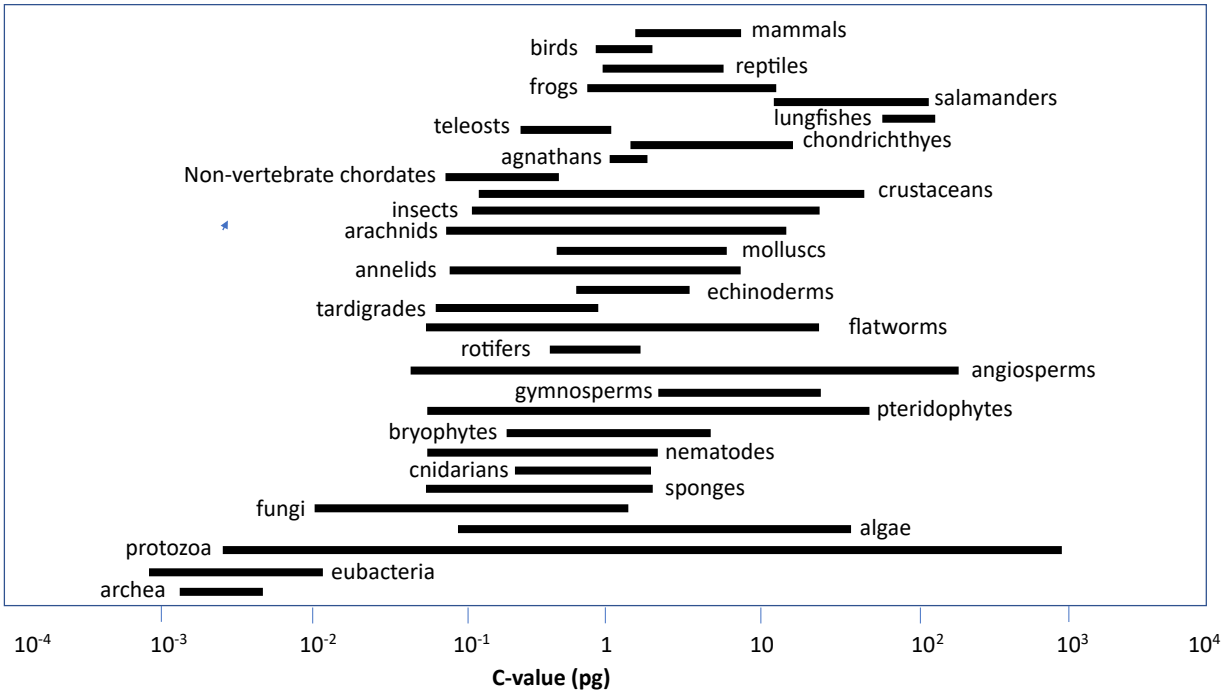
A condition called aneuploidy can arise in cells following an unequal distribution of chromosomes to the daughter cells, resulting in an abnormal number of chromosomes i.e. not as multiple of the haploid number. Organisms with aneuploidy frequently have noticeable differences in appearance and behavior.

Polyploidy describes a condition where the nuclei carries 3 or more times the number of chromosomes found in haploid cells. This may arise through the combining of two diploid germ cells, or through the duplication of chromosomes without the division of the cell. Within the animal kingdom, polyploidy is typically lethal, although there are some examples such as triploid trout, and the red viscacha rat which is tetraploid. However, polyploidy is particularly common on plants, and it thought to provide hybrid vigor and protect against the effect of potentially harmful recessive mutations. Over the course of evolution, polyploidy has enabled gene function to diversify and disrupt self-incompatibility systems, allowing self-fertilization and asexual reproduction both of which would strengthen fitness within a relatively static environment.

There are however disadvantages in polyploidy also, including issues with mitosis and meiosis from spindle irregularities and the improper segregation of chromatids, as well as epigenetic instability. There is also the potential for disruption of nuclear membrane functions due to the increased size of the nucleus in proportion to cellular volume.

The size of an organism's genome is referenced by the DNA content of the monoploid chromosomes set (in polyploids, this value is averaged), whereas the so called C-value is referenced by the DNA content in the unreplicated haploid nucleus, and represents the genome size is diploids. Both genome size and C value may be expressed in either picograms of DNA or megabase pairs (for reference, 1pg = 978Mbp).

Fig 1. Below is a summary of C-values across different taxa, demonstrating the enormous range of genome 1C values across the plant and animal kingdoms. Positive correlations have been identified between genome size and cell volumes, mitotic cycle and meiosis cycle durations.



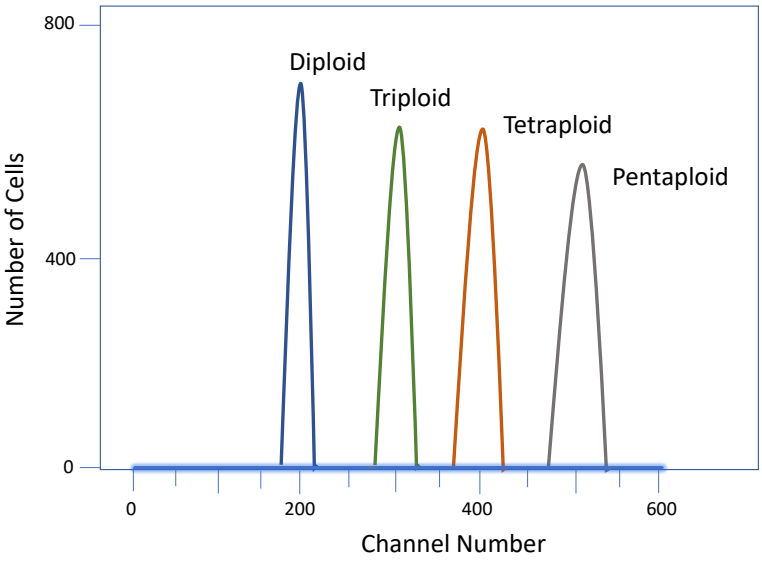


Fig. 2. Example of flow cytometry analysis showing polyploidy patterns. It is key to note that the peaks are tight and narrow, and the channel signal doubles from diploid to tetraploid, demonstrating the linearity of the assay.

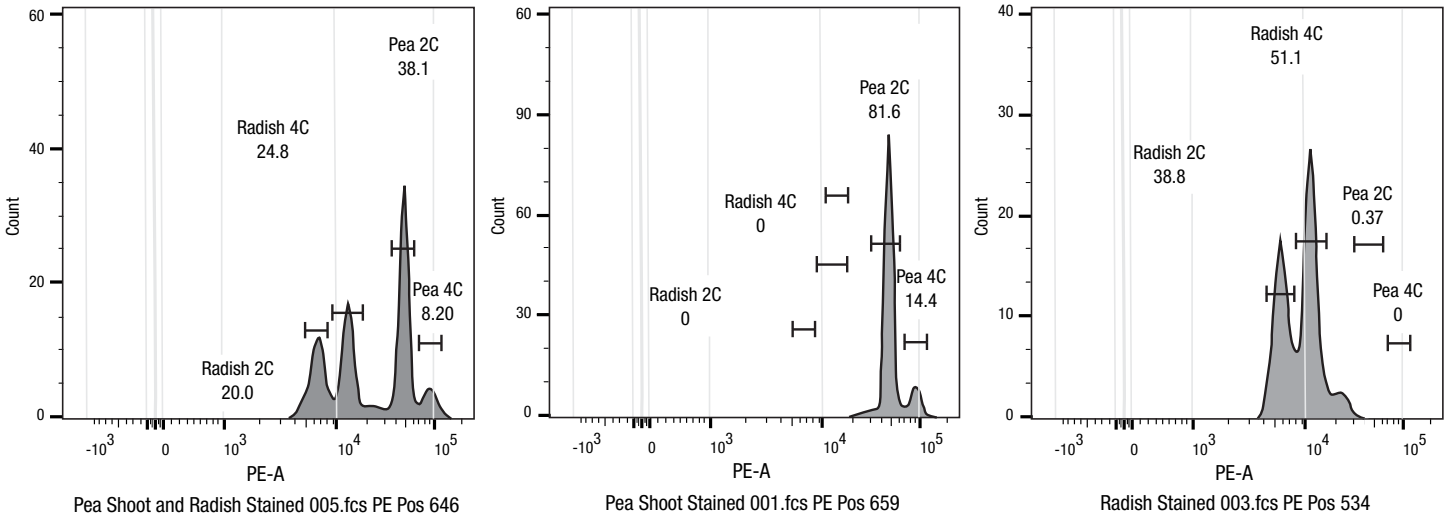


Fig. 3. Flow cytometry analysis of ploidy from pea shoot and radish. This analysis distinguishes the 2C and 4C nuclei from each plant, either run together (a) or separately (b & c). In both instances, the 2C:4C ratio is determined to be 0.5.

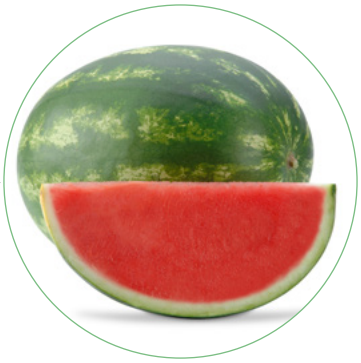
Fig. 4. Ploidy manipulation has been applied for strawberry cultivation. Strawberry species and hybrids can be diploid, tetraploid, pentaploid, hexaploidy, heptaploid, octoploid or decaploid (i.e. having 2, 4, 5, 6, 7, 8, or 10 sets of the seven chromosomes respectively). The larger size of polyploid strawberries has made them popular in modern agriculture, however, the quality of the fruit product is vastly different from that of the wild strawberry.

Fig. 5. Seedless watermelons are now commonplace. These fruits are produced by triploid plants generated by crossing diploid and tetraploid lines of watermelon. Since the triploid plants are sterile, they are grown alongside a diploid strain of watermelon that produces the pollen required to trigger pollination and fruit production.

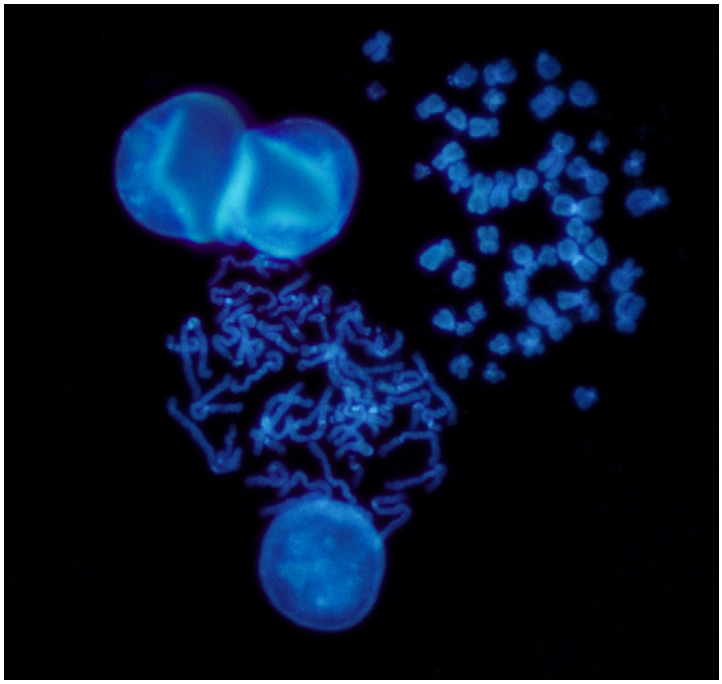
Fig. 4: **Wild Strawberries**

Fig. 4: **Cultivated Strawberries**

Fig. 5: **Seedless Watermelon**

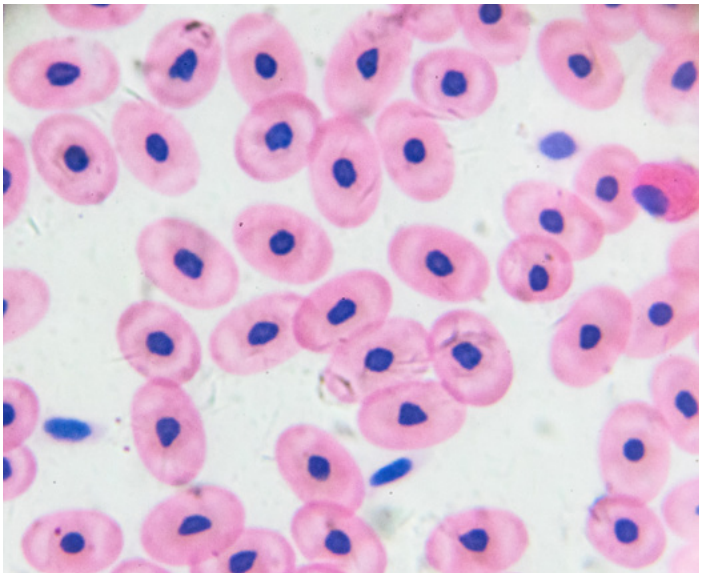


The Determination of Ploidy by Flow Cytometry



Ploidy determination is carried out either through laborious chromosome counting by microscopy, or more commonly, using flow cytometry. Sample is generally straight forward, involving chopping of the tissue in ice-cold hypotonic buffer supplemented with a non-ionic detergent (such as Triton X-100), RNase A (to remove background RNA) and proteinase K (to maximize the signal: noise ratio), followed by filtration and centrifugation to remove debris. However, some tissues can be a challenge to process based on the presence of tannins or high levels of organic acids, which can interfere with fluorochrome binding to DNA. The inclusion of 2-mercaptoethanol may help prevent polyphenol oxidation that can exacerbate the problem, or Polyvinylpyrrolidone may be used to help remove these compounds from the nuclear preparation. The presence of mucilaginous compounds and starch granules can also be problematic in the separation of nuclei from the cell remnants.

Techniques used differ primarily in the method of cell permeabilization (detergent or prefixation with alcohols) and the fluorescent dye used. The DNA is quantified in the nucleus based on the stoichiometric binding of fluorochromes to nucleic acids, examples of these include Propidium Iodide (PI), Ethidium Bromide (EB), DAPI (A-T specific), Hoechst 33258 (A-T specific), mithramycin, chromomycin A3 and olivomycin (G-C specific). Intercalating dyes (PI, EB) are needed to estimate nuclear DNA content in absolute units (picograms or megabase pairs) and are used at 50-70µg/mL at pH 7.2-7.4. Both dyes are excited by a 488nm argon laser, however, this suboptimal excitation condition may impact signal resolution. Ploidy level estimation is typically achieved using A-T specific fluorochromes such as DAPI and Hoechst 33258, used at ~4µg/mL. These both emit blue light when excited by a UV laser, although DAPI is generally regarded to produce higher fluorescence intensity and better resolution, and significantly DAPI has been reported to be less affected by the state of chromatin condensation than other fluorochromes. GC-specific fluorescent stains are less widely used due to the low resolution of the histograms they generate.



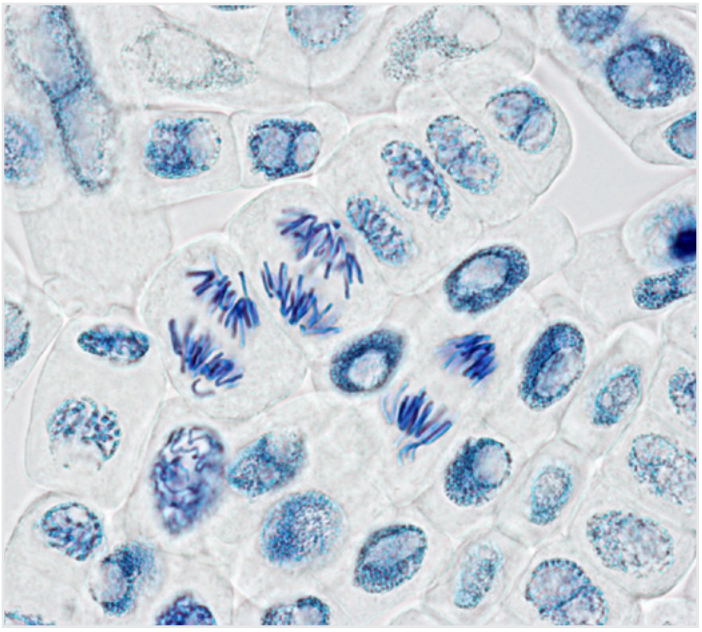
Supravital staining offers the ability to sort live cells on the basis of differences in DNA content. This process uses DRAQ5, one of the Vybrant DyeCycle compounds, or more commonly, Hoechst 33342 to measure the DNA content of live cells, typically from tissue culture. Staining simply involves the addition of 2µg/mL of Hoechst 33342 to the cell suspension for 20-90 mins at 37˚C. The dye is excited by the UV laser and detected at blue wavelengths (band-pass filter 470± 20nm).

The acquisition process can require some optimization. Excitation of DAPI requires a UV light source (typically a short pass, 390nm filter), and emission is measured at blue wavelengths (band-pass filter 470± 20nm). Often internal controls are used as standards, these include trout or chicken erythrocytes; these are nucleated and the DNA content of these two species is less than that of plant (or human) cells. Alternatively, UV Teflon beads can be used, or if the analysis is for a plant breeding program, the mother/ parental plant nuclei can help identify ploidy more easily. Two haploid nuclei stuck together will have the same DNA content as a single diploid nuclei, it is therefore important to exclude all clumps from the analysis. This is typically achieved by gating singlets on the DNA peak versus DNA area plot. Cytometric one parameter analysis such as DAPI staining is typically visualized in the form of a 2-D histogram: horizontal axis describes the intensity of a (gated) recorded signal, and the vertical axis in the number of particles with a given intensity range. Thresholds should also be adjusted during acquisition to remove unwanted background noise whilst maintaining the signal from the particles of interest. The speed of acquisition also may impact the quality of the data; too fast and the histogram peaks become broader, whereas if acquisition is too slow, then sedimentation effects can influence counts.

The quality of a DNA histogram is estimated from the width of the peak

Coefficient of variation = 100 x SD/(peak channel) %

The smaller the CV of the peaks, the more accurate the measure of ploidy. The key elements in obtaining high quality histograms are samples preparation, instrument alignment and the analysis of the data.



Cell Cycle and Genome Size Analysis

Aside from ploidy, flow cytometry is also used for cell cycle analysis, examining the nuclear DNA content of each cell undergoing cyclic changes. Resting cells (G0 of the cell cycle) and cells in the primary growth period (G1 phase) have 2C nuclear DNA content, reflective of their two copies of each chromosome. When transitioning into the secondary period of cell growth (G2 phase), the DNA is replicated to 4C levels. Daughter cells, each with a 2C nuclear content, are subsequently formed following mitosis (M phase). Typically, this type of cell cycle analysis reveals prominent peaks corresponding to G0/G1 nuclei, and smaller peaks for G2/M, with some S nuclei spanning the range between these peaks. The CV of nuclei in G0/G1 phase can vary from 1-10% depending upon the source material (species with lower genome sizes generally have higher CV), the staining fluorochrome (DAPI generally gives tighter histogram profiles) and the method of sample preparation. Calibration standards are useful in ensuring instrument set up and operational conditions are optimal to yield maximum signal amplitude, minimal CV and minimal background. Reference standards of known DNA content may be run within samples, or at the beginning and end of an experiment. If the reference standard in run within the sample, it is important that the reference signal and sample signals are well separated, that the relative signal amplitudes are close and ideally that the sample and reference are from the taxa. In addition, control beads should be used to demonstrate linearity of measurements; this will help ensure that the modal position of G2/M is exactly twice that of G0/G1.

An extension of this type of cell cycle analysis can be used to measure the frequency of cell division or 'mitotic index' MI.

$MI = 4 \times 4C/\Sigma 2C + 4C$

C values for different species can be found in literature.

If the C value is unknown, then MI can be determined by simply counting the number of cells in mitosis and divide this number by the total number of cells.

Typical healthy MI range from 2000-3000. Elevated MI is indicative of increased cell division associated with tissue growth, cellular repair or disease.

Summary

Plant DNA flow cytometry analysis is now a very popular method in both agricultural and environmental research. This analytical platform has been instrumental in the way that plant hybrids are generated and their potential in farming determined.

New applications continue to emerge in the fields of taxonomy, population biology, and ecology. The success of these initiatives will rely on careful sample preparation and flow cytometry analysis that employs optimal controls and instrument settings.

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