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Critical Aspects in Analysis of Cellular DNA Content

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Abstract

This unit covers general aspects of DNA content analysis and provides introductory or complementary information to the specific protocols of DNA content assessment in this chapter. It describes principles of DNA content analysis and outlines difficulties and pitfalls common to these methods. It also reviews methods of DNA staining in live, permeabilized, and fixed cells, and in cell nuclei isolated from paraffin-embedded tissues, as well as the approaches to stain DNA concurrently with cell immunophenotype. This unit addresses factors affecting accuracy of DNA measurement, such as chromatin features restricting accessibility of fluorochromes to DNA, stoichiometry of interaction with DNA, and "mass action law" characterizing binding to DNA in relation to unbound fluorochrome concentration. It also describes controls to ensure accuracy and quality control of DNA content determination and principles of DNA ploidy assessment. Because many aspects of DNA content analysis are common to protocols in *UNITS 7.3, 7.6, 7.16, 7.20, 7.23, & 7.25*, certain parts of this unit provide information redundant with commentaries in these units.

Keywords

cell cycle; apoptosis; ploidy; DNA index; stoichiometry; fluorochrome; chromatin

INTRODUCTION

DNA content is the most frequently measured cellular constituent. Its quantification serves to assess DNA ploidy level, cell position in the cell cycle, and may reveal the presence of apoptotic cells that are characterized by fractional DNA content. Distribution of cells within the major phases of the cell cycle is based on differences in DNA content between the prereplicative phase cells ($G_{0/1}$) versus the cells that actually replicate DNA (S phase) versus the post-replicative plus mitotic (G_2+ M) cells. DNA content measured by cytometry is defined as "DNA ploidy" or "DNA index" (DI) and for normal (euploid, nontumor) cells in $G_{0/1}$ phase of the cell cycle DI = 1.0. Cells in G_2/M phase have DI = 2.0 and the S-phase cells are characterized by 1.0 < DI < 2.0. Note that "DNA ploidy" should not be confused with the term "ploidy" that refers to number of complete sets of chromosomes in a cell, where the somatic cells containing two complete sets of chromosomes, one set derived from each parent, are diploid and sex cells (sperm and egg) are haploid. Due to extensive DNA fragmentation that occurs during apoptosis, the low-molecular (mono- and oligo-nucleosomal) DNA fragments are extracted during cell preparation and staining, and such cells can be identified as the apoptotic cells with fractional DNA content (DI<1.0). They often are defined as "sub-diploid" or "sub-G_1" cell population (Darzynkiewicz et al., 1997).

By providing the means to measure DNA content of individual cells in large cell populations rapidly and with high accuracy, flow- or laser-scanning cytometry (LSC) have become the methodologies of choice for quantification of DI. The historical progression of development of cytometric methods for DNA content analysis and their applications in different cell types

has been extensively reviewed (Darzynkiewicz et al., 2004). The methods rely on labeling cells with a fluorochrome that is expected to stain DNA stoichiometrically and thus accurately report DNA content. The intensity of DNA-associated fluorescence integrated over the cell or cellular nucleus is measured by photomultipliers, which offer wider dynamic range of fluorescence intensity measurement than the alternative approach, namely fluorescence image analysis (FIA). The methods differ by the mode of cell permeabilization (detergent versus pre-fixation using different fixatives), choice of DNA-specific fluorochrome, composition of the stain solution, and applicability to different cell types and preparations. The most widely used methods of DNA content analysis are presented in this unit.

The results of cellular DNA content measurement are most frequently presented in the form of frequency histograms. Discrimination of cells in particular phases of the cell cycle and their quantification, based on differences in DNA content (deconvolution of the histograms), is helped by computer analysis. The software used for this purpose allows one to estimate the percentage of cells in major phases of the cell cycle (G_1 vs. S vs. G_2/M), as well as the frequency of apoptotic cells with fractional DNA content ("sub-G_1" cells) or "cell debris." Such software is often included with the purchase of the cytometer and is also available commercially from several sources. The most common products are the MultiCycle (Phoenix Flow Systems; Rabinovitch, 1993) and ModFit (Verity Software House; Bagwell, 1993).

SUPRAVITAL CELL STAINING

Cellular DNA can be fluorochrome-stained either in unfixed, usually still live cells, or in the fixed cells. Staining of live cells (supravital staining) requires the use of a plasma membrane–permeant fluorochrome that stoichiometrically stains DNA. The choice of such fluorochromes is limited. The most frequently used is Hoechst 33342 (*UNIT 7.5*), which is excited at UV wavelength (350 nm) and fluoresces in blue light (460 nm). This fluorochrome, especially when used in combination with the membrane potential sensing dye DiOC5(3) offers relatively good resolution in the assessment of DNA content of live cells (Crissman et al., 1990). The presence of DiOC5(3) serves to suppress efflux of Hoechst 33342 by the active efflux pump, which otherwise breaks up equilibrium of the binding/staining reaction. A similar effect can be achieved by using a more specific efflux blocker such as Verapamil (Krishan, 1987). Another fluorochrome used to supravitally stain DNA is the dihydroanthraquinone analog DRAQ5 (Smith et al., 2000). Its emission is detected in far-red wavelength (maximal at 670 nm) while the excitation (maximal at 640 nm) is at wide spectral range, stretching down to 488 nm. More recently, the permeant Vybrant DyeCycle Violet and DyeCycle Orange fluorochromes have become commercially available (Invitrogen), expanding the choice of supravital DNA fluorochromes with different excitation and emission spectra (Telford et al., 2007).

The protocols describing supravital DNA staining are simple. Generally, addition of the fluorochrome into the culture medium for 30 to 60 min during cell culture is followed by cytometric analysis without a need for cell rinsing or centrifugation. However, there are cell type–or cell line–associated differences in the rate of the fluorochrome uptake, and staining optimization sometimes requires testing of several concentrations of the fluorochrome and various incubation periods, different than those in the protocols (e.g., provided by the reagent vendors). The resolution of DNA content analysis in cells supravitally stained, however, is generally worse than that of fixed or detergent-permeabilized cells. Often the cells with their DNA supravitally stained (e.g., the stem cells identified as the "side population") are sorted to be further subcultured for the purpose of analyzing their growth characteristics, sensitivity to drugs, cloning or expanding their number. It should be noted,

however, that exposure of cells to Hoechst 33342 or DRAQ5 induces a distinct DNA damage response and their potential cytotoxic effects cannot be neglected (Zhao et al., 2009). Exposure of cells stained with Hoechst 33342 to light, especially to UV (e.g., during sorting), is particularly harmful, as it causes photolysis of DNA (Huang et al., 2004).

STAINING OF DNA AFTER LYSIS OF PLASMA MEMBRANE

Treatment of live cells with detergents causes lysis of the plasma membrane or leads to nuclear isolation, making DNA easily accessible to fluorochromes. This approach has been introduced to permeabilize cells to acridine orange, the metachromatic dye that differentially stains DNA and RNA (*UNIT 7.3*). Hypotonic salt solutions also cause cell lysis, and DNA within the nuclei isolated this way can be stained with a variety of fluorochromes (Krishan, 1990). Further improvement in the accuracy of DNA content measurement is obtained after controlled proteolysis of detergent-lysed cells. This approach was perfected by Vindeløv and his colleagues who developed a highly accurate method of cellular DNA content measurement, particularly useful for assessment of DNA ploidy in human tumor samples (Vindeløv and Christiansen, 1994; see *UNIT 7.5*). These authors also introduced internal DNA content standards such as nuclei of chicken- and/or trout-erythrocytes, as intrinsic part of the protocol. Their methodology designed for needle biopsy of normal and tumor tissue is being used worldwide.

The accuracy of DNA content measurement for DNA ploidy or cell cycle phase estimate is much greater when isolated nuclei rather than whole cells are analyzed. This is due to the fact that cytoplasmic constituents may be auto-fluorescent, contain DNA (e.g., mitochondria), or nonspecifically stain with DNA-fluorochromes. This background cytoplasmic stainability lowers accuracy of nuclear DNA determination. Furthermore, the proteolytic step in Vindeløv's protocol removes nuclear proteins known to restrict the accessibility of DNA to fluorochromes, which additionally leads to improved stoichiometry of DNA staining (Darzynkiewicz et al., 1984).

It should be warned that the lysis of plasma membrane of mitotic cells lacking a nuclear envelope, which occurs during the detergent or hypotonic-treatment based methods, leads to dispersion of individual chromosomes or chromosome aggregates, which are then suspended in the staining solution. Therefore, these methods may not detect mitotic cells, especially when the cell suspensions are mechanically agitated, pipetted, or vortexed. Furthermore, individual or aggregated chromosomes may be erroneously identified as apoptotic cells with deficit in DNA content ("sub-G_1" cells). Also, lysis of apoptotic cells that have fragmented nuclei releases individual nuclear chromatin (several from single cell) and because each fragment is identified as an individual event (erroneously, as a "cell"), the frequency of "sub-G_1" objects after lysis of apoptotic cells is much greater than the actual number of apoptotic cells in a given cell population. When mitotic cells are in large proportion (e.g., after arrest in the cell cycle by mitotic poisons), the application of cell lysis–based methods for analysis of the frequency of apoptotic cells is therefore not advised (Darzynkiewicz et al., 2001).

DNA STAINING IN FIXED CELLS

Analysis of fixed cells as opposed to the detergent-permeabilized cells is preferred when there is a need to store or transport samples, e.g., in the case of clinical specimens that cannot be immediately analyzed. Their storage, unless done at low temperature in cryopreservative medium, leads to cell autolysis. Fixed cells, on the other hand, can be stored for months or years. Precipitating fixatives (e.g., alcohols, acetone) are preferred over cross-linking ones (e.g., formaldehyde, glutaraldehyde) for analysis of DNA content. The cross-linking of chromatin impairs stoichiometry of DNA staining and thus decreases

accuracy of DNA content measurement (Darzynkiewicz et al., 1984). It should be noted, however, that highly fragmented DNA, such as that present in apoptotic cells, leaks out from the ethanol-fixed cells during their hydration and staining, but is preserved and remains within the cell upon fixation by formaldehyde. Fixation in ethanol, therefore, rather than in formaldehyde, allows one to detect apoptotic ("sub-G_1") cells (Gong et al., 1994). While absolute alcohols or acetone, or a mixture of absolute ethanol and acetone can serve as fixatives (they may be preferred e.g., for immunocytochemical detection of some antigens concurrently with DNA content), they induce more extensive cell aggregation compared with 70% or 80% ethanol, which is most frequently used when analysis is limited to DNA content alone.

A variety of DNA fluorochromes may be used to stain DNA in the fixed cells. The most commonly used are 4′,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), and 7-aminoactinomycin D (7-AAD). Staining with fluorochromes that bind to both DNA and RNA, such as PI, requires either pre-incubation with RNase or inclusion of RNase A (usually at concentration between 10 and 100 μg/ml) into a staining salt solution (e.g., PBS) containing PI. In the latter case, the cells suspended in RNase A solution are maintained for about 30 min or longer at 37°C or room temperature to allow RNase A to completely digest RNA, before subjecting to measurement by cytometry. The RNase A should be free of DNase activity and if such is unavailable, one may heat the solution of RNase at 95° to 100°C for 5 min to selectively destroy DNase while preserving RNase activity. PI is excited in blue light, which is conveniently provided by the 488-nm line of the argon ion laser available on most flow cytometers, while DAPI requires UV or near UV excitation.

ANALYSIS OF DNA CONTENT IN CELL NUCLEI ISOLATED FROM PARAFFIN-EMBEDDED TISSUES

To retrieve archival pathology samples for flow cytometric analysis Hedley and his collaborators developed the method of isolating cell nuclei from paraffin-embedded tissues (Hedley et al., 1983), which was later modified by Heiden et al. (1991). This methodology enables for retrospective studies to determine the prognostic significance of DNA ploidy or cell cycle distribution in tumor progression. The method can also be applied for prospective studies when fresh material cannot be used. One virtue of this methodology stems from the possibility to examine by microscopy the tissue sections and select the adjacent tumor area of interest to be processed by flow cytometry. This is done by trimming the paraffin block to exclude areas of non-involved tissue in order to diminish the proportion of stromal cells, or of necrotic and hemorrhagic areas to decrease the quantity of debris. The areas of non-involved tissue can be similarly selected as internal DNA content standard. The accuracy of DNA content analysis of cells from paraffin blocks is generally inferior compared to the methods that rely on ethanol fixation or detergent/hypotonic treatment of fresh tissues. This is due to the fact that the cells embedded in paraffin frequently are usually prefixed in formaldehyde. As mentioned, by cross-linking DNA and proteins, such fixation impairs stoichiometry of DNA detection. Because cross-linking with formaldehyde is to some extent reversible by hydrolysis of the formaldehyde-induced bonds, prolonged (1 to 2 days, at 4°C) incubation of the rehydrated nuclei suspended in aqueous salt solutions (e.g., PBS) after their isolation from the paraffin blocks improves resolution of DNA analysis. In nuclei isolated from paraffin blocks, DAPI is the preferable fluorochrome since it is the least affected, in terms of stoichiometry of DNA staining, by the chromatin structure and thus by protein-DNA cross-linking (Darzynkiewicz et al., 1984).

The presence of cell/tissue debris is another factor that lowers accuracy of DNA content analysis in samples of nuclei isolated from paraffin blocks. Most debris is due to the presence of transected nuclei with incomplete, partial DNA content. Because the probability

of transecting a nucleus is inversely proportional to the thickness of the section, and varies with nuclear size, preparation of thicker sections (≥50 nm) is advisable, particularly for tumors with large nuclei such as tetraploid and larger stemlines.

CONCURRENT ANALYSIS OF DNA CONTENT AND CELL SURFACE ANTIGEN

It is often desirable to define DNA ploidy or cell cycle distribution for a particular cell subpopulation identified by its surface immunophenotype. The most common approach, in such a case, is to carry on standard immunocytochemical labeling of live cells with the fluorochrome (most frequently FITC or Alexa Fluor 488)–conjugated antibody, which is then followed by short (10 to 30 min) fixing of the cells in 0.5% to 1.0% methanol-free formaldehyde ("paraformaldehyde") dissolved in PBS. Because formaldehyde fixation does not adequately permeabilize the cells, it is essential to subsequently include detergent (e.g., 0.1% Triton X-100 or Nonidet) in the staining solution to make the DNA accessible to DNA-fluorochromes such as PI, DAPI, or 7-AAD. Post-fixation in alcohol (methanol or ethanol) following formaldehyde additionally permeabilizes cells. A gentle fixation with formaldehyde (0.25%) followed by permeabilization in Tween 20 detergent is another procedure designed to preserve both external and internal antigens that can be detected immunocytochemically concurrently with analysis of DNA content (Schmid et al., 1991). Cellular green (FITC or Alexa Fluor 488), red (PI), or blue (DAPI) fluorescence is then measured by flow cytometry. During analysis, the cell subpopulation of interest is gated based on its immunophenotype (green fluorescence), and DNA content of this selected subpopulation is then presented generally as a frequency histogram. It is also possible to combine analysis of DNA content with both a cell immunophenotype marker and an intracellualar marker such as telomere length (Schmid et al., 2002).

A more straightforward approach to concurrently measure DNA content and cell surface immunofluorescence is to combine the staining of DNA in live cells (e.g., with Hoechst 33342) with surface immunophenotyping (Loken, 1980). As mentioned, however, in some cell types it is difficult to obtain high resolution of DNA content analysis after supravital staining of DNA either with Hoechst 33342 or DRAQ5.

ACCURACY OF DNA CONTENT MEASUREMENT

The accuracy of DNA content measurement is represented by variation in fluorescence intensity between individual cells with identical DNA content, such as for a population of G_0/G_1 cells. This variation is being assessed by the value of coefficient of variation (CV) of the mean value of DNA content of this cell population. The CV of the DNA-associated mean fluorescence of $G_{0/1}$ cells is thus considered to be the index of accuracy of DNA content measurements. High accuracy is particularly required in assessing DNA ploidy to distinguish between DNA diploid and aneuploid cells, which may differ minimally in DNA content. Accurate DNA content measurement is also critical in analysis of cell cycle distributions. There is no formal consensus regarding the maximal CV of $G_{0/1}$ cells (maximal error in cellular DNA content estimate) that is acceptable. Most researchers, however, would consider the accuracy to be poor and results unacceptable if CV values of normal, nontumor cells exceed 6%. Optimal resolution is achieved when CV is below 3%. An exception is analysis of the DNA content of cell nuclei isolated from paraffin blocks, where by the nature of the sample (formaldehyde fixation) such good accuracy is difficult to achieve.

Many factors can contribute to poor accuracy in DNA content analysis. Inappropriate sample flow and optical adjustment of the flow cytometer are the most common causes.

Proper maintenance of the cytometer and its careful adjustment (e.g., using calibrated standard beads, to maximize the electronic signal intensity and minimize variability of the measurement of the beads) are required to optimize fluidic and optical settings of the instrument. Problems in sample preparation, either resulting in mechanical damage to the cells or involving incorrect composition of buffers and staining solutions, are another potential cause of poor resolution in DNA analysis. An excessively large number of cells in the sample, which leads to significant depletion of the unbound fluorochrome in the solution and alters the staining equilibrium (see below), may be still another source of the problems. Adjusting samples to achieve proper number of cells (DNA content) in the sample (proper dye:DNA ratio) improves the results.

It should be noted that despite the attempts to achieve good accuracy of DNA content measurements (in terms of proper instrument adjustments and sample staining) the CV of G_1 cell populations might still remain high. This may occur when dead or dying cells in significant numbers are present in the sample. If this is the case, the dead (necrotic) cells and cell debris can be removed by incubating the cell suspension in a solution of PBS containing a mixture of DNase I (100 μg/ml) and 0.1% trypsin (freshly mixed) for 30 min at 37°C. Under these conditions, all necrotic cells become entirely dissolved (digested) with only live cells having preserved plasma membrane integrity remaining in suspension (Darzynkiewicz et al., 1994). Activity of trypsin can then be stopped by addition of serum (10%) or bovine serum albumin (1% w/v). Then, upon centrifugation, the population of live cells not contaminated by necrotic cells and debris can be subjected to DNA content analysis. It should be noted that G_0/G_1 cell populations might have intrinsically variable DNA content and therefore high CV in tumors that are polyclonal or have developed drug resistance by gene amplification (e.g., presence of minute chromosomes). Also, poor accuracy in assessing DNA content is expected if the cells were previously treated with DNA-interacting and/or fluorescing drugs that impair DNA stoichiometric stainability.

ROLE OF CHROMATIN STRUCTURE IN ACCESSIBILITY OF DNA TO FLUOROCHROMES

Chromosomal proteins, predominantly histones, restrict accessibility of DNA in situ to fluorochromes and thus affect stoichiometry of DNA staining. The extent of restriction may vary depending on cell type. The maximal restriction is seen in cells undergoing differentiation such as in the course of spermatogenesis or erythropoiesis, when DNA stainability (per unit of DNA) is distinctly diminished compared to nondifferentiated cells (Darzynkiewicz et al., 1984; Evenson et al., 1986). This creates potential bias in assessment of DNA ploidy in differentiating cells. The degree of restriction is different for different fluorochromes. While binding of DAPI is relatively little influenced by differences in chromatin structure, binding of 7-AAD, an intercalating and bulky fluorochrome, is affected to a much greater extent. One may expect, therefore, intercellular variation in DNA stainability when cells with different chromatin structure are combined in the same sample, reflected as the presence of pseudoaneuploid populations, or widening of the G_1 peak (increased CV value) on DNA content histograms. For example, under certain conditions of staining, monocytes show higher DNA stainability with PI than lymphocytes or granulocytes, and form a typical pseudohyperdiploid peak on DNA frequency histograms (Bedner et al., 1997). Treatment of detergent-permeabilized cells with cold 0.1 M HCl to dissociate histones (Darzynkiewicz, 1990; see *UNIT 7.3*) or with detergent and proteolytic enzymes (Vindeløv and Christiansen, 1994) increases accessibility of DNA and improves stoichiometry of DNA staining.

There are several ways to assess stoichiometry of DNA staining. Fluorescence intensity of the cell populations represented on DNA histograms by the G_2/M peaks is expected to be

twice higher than that of the $G_{0/1}$ cells, and deviation from this ratio indicates problems in DNA quantification. Normal hepatocytes or megakaryocytes grow at different DNA ploidy levels and may also serve as markers of linearity in DNA measurement. Inclusion of internal standards such as chicken or trout erythrocytes provides still another marker of the stoichiometry of DNA measurement, and it is highly recommended when DNA ploidy is estimated (Vindeløv and Christiansen, 1994). It should be stressed that to demonstrate stoichiometry of DNA staining, one has to use linear and not exponential scale for plotting intensity of DNA-associated fluorescence (*x*-coordinate) and include the origin (point zero) of this coordinate, on the published DNA content frequency histogram.

In some instances, however, stoichiometry in DNA staining with fluorochromes content analysis cannot be attained. This can be seen when cells were treated with antitumor drugs that modify DNA and/or chromatin structure. Intercalating drugs that interact with DNA fluorochromes by fluorescence resonance energy transfer (FRET), or drugs damaging DNA structure, or cross-linking chromatin, all can alter staining properties of in situ DNA, often in unpredictable ways. The possibility of stoichiometric measurement of DNA content may also be hampered when cells differing markedly in chromatin structure are being compared.

CHEMICAL MASS ACTION LAW IN FLUOROCHROME BINDING TO DNA

Staining of cellular DNA is usually carried on at equilibrium between the ligand (fluorochrome) and the ligand-binding sites in the DNA within the cells; therefore, it follows the chemical law of mass action. Stable level of staining is achieved when there is large excess of the free ligand per binding site, so minor variation in cell number per sample (total binding sites) has no significant effect on the equilibrium. Because it is difficult to have an identical number of cells per each sample, the variation is inevitable. However, one may calculate an approximate concentration of the free fluorochrome and relate to the cell number to find out the range within which a decrease in free ligand concentration (due to its binding to DNA) may not significantly affect the equilibrium. There are 3×10^9 DNA base pairs per cell (diploid cell in G_1). Most intercalators such as DNA-binding fluorochromes like PI, reacting with free (naked) DNA at saturation, bind every second base pair. Thus, potentially, in a single diploid G_1 cell there are ~1.5×10^9 binding sites. However, because a large portion of nuclear DNA within the cell is inaccessible to the intercalators (Darzynkiewicz et al., 1984), only a fraction of the potential binding sites (10% to 70%, depending on the fluorochrome) can actually bind the fluorochrome. Thus, between 1.5–10 $\times 10^8$ sites that actually bind the ligand are present in a single cell and therefore in 10^6 diploid cells, which is approximately a size of average sample subjected to staining, there are 1.5–10 $\times 10^{14}$ binding sites. Assuming the average molecular weight (MW) of most DNA fluorochromes to be about 300 daltons (Da), one can estimate (from Avogadro number) that at a concentration of 100 μM (30 μg/ml) there are 6×10^{16} molecules of the ligand in 1 ml volume of the stain solution. Given the above, there is nearly 100-fold excess of the ligand per binding site when 10^6 cells are stained in 1 ml volume at 100 μM dye concentration. Under these conditions, a change in cell number from 1 to 2 million (which alters a concentration of the free, unbound ligand by 1%) should not be reflected by greater than 1% change in stainability of DNA. However, at a lower dye concentration (e.g., below 20 μM) or when the cell number is drastically increased (e.g., from 1 to 5 or 10 million), the change in DNA stainability becomes noticeable. Furthermore, if cells have higher DNA content (tetraploid, arrested in G_2/M), the equilibrium is shifted even more towards the lower concentration of free dye, which leads to further decrease in DNA stainability. The above estimates should be considered when samples with different cell number are stained to compare DNA ploidy.

ASSESSMENT OF DNA PLOIDY

As mentioned previously, cellular DNA content provides the yardstick to estimate frequency of cells in the main phases of the cell cycle, as well as to assess DNA ploidy. The presence of an aneuploid cell population is by itself a definitive marker of a tumor and is often considered to be a prognostic indicator of tumor progression and outcome of the treatment. To assess DNA ploidy of the tumor one has to compare DNA content of the $G_{0/1}$ cell population of the presumed tumor cells with that of normal (control) cells. Towards this end, the peak value of the integrated fluorescence intensity (peak channel number) of $G_{0/1}$ population of normal cells is often being considered to have a DNA index (DI) of 1.0. DNA ploidy of the tumor cells is expressed as a ratio of the peak value (channel number) of fluorescence intensity of these cells with respect to that of the normal $G_{0/1}$ cells. It is also common to express DI of the tumor as a ratio of modal value of fluorescence intensity representing DNA content of $G_{0/1}$ population tumor cells to modal value of $G_{0/1}$ population of normal cells. Some authors still prefer to use the mean values of fluorescence intensity of $G_{0/1}$ population rather than the peak or modal values to obtain this ratio. In essence, when DNA measurement is done correctly and accurately (low CV), either of these approaches yield a similar estimate of DI of aneuploid cells.

Normal lymphocytes or fibroblasts often serve as standard of DI = 1.0. Since there are minor differences in DNA content between individuals, optimally, lymphocytes from the same patient whose tumor is being analyzed should be used, both as external and internal control standards. When used as external control they have to be subjected to identical processing and staining procedure, and measured by cytometry under identical laser and detector settings as the tumor sample. The external control cells should be measured twice, prior to and also after measurement of tumor sample. This allows one to detect the possible drift in fluorescence readout (e.g., due to misadjustment in instrument settings, flow rate, etc.) in the course of the sequential measurements. Normal cells should also be admixed (e.g., in 1:1 proportion) with the tumor sample cells to serve as an internal control in another set of measurements. Normal stromalor infiltrating cells that are present in the tumor sample can additionally be used as an internal control of DNA ploidy. When DNA ploidy is assessed by measurement of nuclei isolated from paraffin blocks, the internal control (DI = 1.0) provided by the presence of stromal and infiltrating normal cells isolated from the same paraffin block is the only acceptable standard to assess DNA ploidy of the tumor. This is due to the fact that DNA stainability after formaldehyde fixation and paraffin embedding is markedly altered, and often different in different paraffin blocks, making external standards or other blocks useless at the standard.

Chicken and trout erythrocytes have been proposed as internal standards for analysis of DNA content (Vindeløv and Christiansen, 1994; see *UNIT 7.5*). Their use is helpful to maintain and ascertain consistency of the staining and measurement procedures. However, one has to be cautious using them as absolute standard for DNA content. Trout, like other fish species, are known to vary in their DNA ploidy level (most species are tetraploid) and it is therefore important to know ploidy of these cells when used as a standard.

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