DNA, or deoxyribonucleic acid, is the molecule that carries the genetic information for all living organisms and is the main constituent of chromosomes. DNA is made from a discrete number of chemical building blocks, or bases, which combine to form two strands arranged as a double helix. Existing mainly in the cell nucleus, DNA is important in cell reproduction, cell life and cell death. Consequently, DNA has proved to be of interest to flow cytometrists, both in research and clinical fields.

**DNA Probes**

In order to measure DNA by flow cytometry, it is first necessary to stain or label the DNA with a fluorescent probe. Unlike those fluorochromes used on labeled antibodies, DNA probes fluoresce more when bound to their target molecule. The base-probe bond is not as strong as that of antibody to antigen, and the DNA probe is, consequently, in equilibrium with the free probe in solution. Changes in probe concentration, by dilution of the sample, for example, can therefore, influence the fluorescence intensity of the DNA due to the change in equilibrium. For this reason, DNA preparations are not washed to remove unbound probe; otherwise the equilibrium will be upset. Fortunately, the unbound probe does not fluoresce and, hence, demonstrates low background fluorescence.

The DNA labeling fluorochrome usually intercalates between the bases in double stranded nucleic acids. However, such fluorochromes can also stain double stranded RNA, which should be eliminated by addition of RNAase to the cell preparation in order to obtain good results. Single stranded nucleic acids do not stain.

The key feature of DNA probes is that they are stoichiometric. This means that the number of molecules of probe bound to DNA is equivalent to the number of molecules of DNA present. Consequently, the amount of emitted light is proportional to the amount of bound probe and, therefore, the amount of DNA.

Generally, the DNA probes belong to the family of chemicals broadly known as the phenanthridiniums (includes propidium iodide and ethidium bromide). They generally excite in the ultraviolet or blue part of the spectrum and show red spectral emission. Table 1 demonstrates the properties of some of the commonly used DNA probes. A more comprehensive list may be found in *Flow Cytometry: First Principles* by A.L.Givan.³

<table>
<thead>
<tr>
<th>Probe</th>
<th>Excitation</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propidium Iodide</td>
<td>536 nm (488 nm laser)</td>
<td>623 nm</td>
</tr>
<tr>
<td>DAPI</td>
<td>359 nm (UV laser)</td>
<td>461 nm</td>
</tr>
<tr>
<td>DRAQ5</td>
<td>650 nm (488 nm or 633 nm laser)</td>
<td>680 nm</td>
</tr>
<tr>
<td>Hoescht</td>
<td>346 nm (UV laser)</td>
<td>460 nm</td>
</tr>
</tbody>
</table>
Guide to Flow Cytometry

**Terminology**

In order to effectively measure DNA, it is first necessary to become familiar with some of the terms employed in DNA analysis.

**Cell Cycle**

Most cells regenerate to replace dead or damaged cells or to grow. In order to grow, the cell doubles its DNA content. The DNA forms two sets of chromosomes, which line up on spindles within the cell before the cell divides into two equal halves. At one time point in this process, prior to cell division, or mitosis, the cell will contain twice the normal amount of DNA. The normal amount of DNA is referred to as diploid or 2n.

The cell cycle is generally split into three identifiable component parts by the flow cytometrist (Figure 1). These are the G₀G₁, S and G₂M phases of the cell cycle. More correctly, G₀ is a phase where cells are quiescent and not taking part in cell division. G₁ is the phase where cells are gearing up to move through cell division. As both of these components of the cell cycle have 2n DNA, these phases are not distinguishable from each other using solely a DNA probe. S phase is that part of the cell cycle where synthesis of DNA occurs and where DNA staining increases. G₂ and M phases of the cell cycle are where 4n DNA is present, just prior to and during mitosis, respectively. Again, with just a DNA probe, these two phases are not distinguishable from each other by the flow cytometer.

![Figure 1. The cell cycle is generally split into three identifiable component parts by the flow cytometrist: G₀G₁, S and G₂M phases of the cell cycle.](image)

This single-parameter histogram (Figure 2) shows fluorescence on a linear scale along the x-axis with number of events up the y-axis. Linear is chosen for fluorescence when examining DNA. This allows the ready determination of DNA index values, or ratios between peaks. Also, in cases where euploid and aneuploid cells are mixed, it aids identification of which G₀G₁ belongs to which G₂M.
Ploidy

The ploidy of a cell is an indication of the number of chromosomes in that cell. Each species has a different ploidy value. There can also be variations within an individual population due to mutations, natural multiploidy (plants), certain diseases (including cancer) and apoptosis. The flow cytometrist tries to define these different ploidy levels and may use a series of definitions and terms.

1. **Diploid**: The normal (euploid) 2n number of chromosomes. This is the number of chromosomes in a somatic cell for a particular species.

2. **Haploid**: Half the normal 2n number of chromosomes, or 1n. This is the number of chromosomes in a gamete or germ cell (sperm/egg). Again, this is species-dependent.

3. **Hyperdiploid**: Greater than the normal 2n number of chromosomes.

4. **Hypodiploid**: Less than the normal 2n number of chromosomes.

5. **Tetraploid**: Double the normal 2n number of chromosomes, or 4n.

6. **Aneuploid**: An abnormal number of chromosomes.

Sample Preparation

In order to successfully measure DNA by flow cytometry, the following issues must be addressed.

Single-Cell Suspension

Certain cell types naturally lend themselves to flow cytometric analysis, coming in a pre-made solution as a single-cell suspension (e.g. blood). However, this does not preclude the use of flow cytometry to determine DNA measurement from solid tissues. It merely lengthens the procedure necessary in order to obtain a single-cell suspension.
Making single-cell suspensions from solid tissues is usually facilitated by one of two methods — mechanical disaggregation (e.g. Dako Medimachine) or enzymatic disaggregation (collagenase, hyaluronidase). Both methods have their advantages and disadvantages. Mechanical methods are usually quicker and tend not to be so harsh that they strip antigens from the cell membrane. Furthermore, mechanical techniques mean that the cell can be kept cold and, therefore, better preserved. For enzymatic methods to work effectively, temperatures must normally be around 37°C, often for several hours. During this time, cell viability may decrease. Conversely, there may be more shear damage from mechanical techniques. However, mechanical techniques usually process the entire tissue, whereas enzymatic techniques may leave behind undigested tissue, which can potentially contain cells of interest.

**DNA Probes**

An important point is to check the excitation and emission spectra of the chosen probe (Table 1) and make sure the flow cytometer has the correct laser to excite the probe and the correct filters to detect the emission spectra. Most bench-top instruments and some cell sorters tend not to have powerful UV lasers, effectively reducing the number of probes available for use as DNA probes.

**Cell Permeabilization**

Also, as most DNA is located in the cell nucleus, it is necessary to get the DNA probe into the nucleus. This may be done in several ways, with varying degrees of harshness, depending on what the final measurement aims are. The treatments usually involve addition of detergents (Saponin, Triton X-100, Nonidet) or alcohol (ethanol, methanol) to the cells. Permeabilizing the cell membrane with, for example, low levels of saponin, enables DNA measurement while maintaining cell surface labelling. Use of Triton X-100 can strip away the cell membrane and cytoplasm, leaving bare nuclei but giving tight DNA peaks. Alcohol permeabilization is also a popular technique, particularly when measuring nuclear antigens in combination with a DNA probe. Some DNA probes actually enter the cell without need for permeabilization (DRAQ5 and Hoescht). Cells have the ability to remove chemicals from their interior by membrane pumps, which are believed to be the cause of multi-drug resistance in cancer patients. For more in-depth discussions on permeabilization, see M. Ormerod’s book, *Flow Cytometry*.3

**DNA Stability**

Introducing detergents or alcohol can lead to loss of antigens and more rapid DNA degradation. Introduction of a fixative (e.g. acetone, formaldehyde) helps stabilize the cell. However, fixation can also lead to protein conformational changes and DNA condensation, resulting in reduced antigen and DNA fluorescence profiles.4
Doublet Discrimination

One problem that must be overcome when obtaining results for DNA analysis is the exclusion of clumps of cells. On a flow cytometer, two cells stuck together may register as a single event, known as a doublet. If each of those two cells is diploid (2n), seen as one event, they have 4n DNA. In other words, they have the same amount of DNA as a tetraploid cell (G_2M), or the same amount as a normal cell that is about to divide (G_2M). To add to the confusion, further peaks may exist for three or more cells stuck together.

The doublet problem is resolved by employing a doublet discrimination gate based on the characteristics of fluorescence height, fluorescence area and signal width. Fluorescence height is the maximum fluorescence given out by each cell as it travels through the laser beam; fluorescence area is the total amount of fluorescence emitted during the same journey; and signal width is the time a cell takes to pass through the laser beam. These characteristics are different for a cell that is about to divide when compared to two cells that are stuck together (doublets shown in Figure 3). A dividing cell does not double its membrane and cytoplasmic size and, therefore, passes through the laser beam more quickly than two cells stuck together. In other words, it has a smaller width signal or a bigger height signal but the same area as two cells stuck together. Also, all of the DNA in the dividing cell is grouped together in one nucleus and consequently gives off a greater intensity of emitted fluorescence, compared with the DNA in two cells that are stuck together. Thus, a doublet, which has two nuclei separated by cytoplasm, emits a lower intensity signal over a longer period. This appears as a greater width signal, lower height signal and the same area. These differences can be seen on histograms of FL2-Area vs. FL2-Width or FL2-Area vs FL2 Height, allowing the generation of gates to exclude doublets from sample analysis for both diploid and aneuploid cells.

Figure 3. Doublet discrimination is possible based on fluorescence height, fluorescence area and signal width.
Guide to Flow Cytometry

Instrument Setup

Instrument setup varies with manufacturer. However, there are some general principles to observe.

1. Select the LIN channel that is most appropriate for the DNA probe.
2. Set the trigger on the channel detecting the DNA probe, as light scatter parameters are not usually of much use for triggering in the case of DNA.
3. Select parameters to enable doublet discrimination.
4. Set a gate to exclude doublets and apply it to the histogram that will display the DNA profile. Displaying an ungated plot of the same may data may also be useful.
5. Make sure the sheath tank is full, as it may help with stability.
6. Make sure the cytometer is clean. Stream disruption will increase the CV.
7. Set a low flow rate and dilute cells to a concentration that is appropriate for the DNA probe solution.
8. Make sure the instrument has been optimized by running routine calibration particles.

Standardization

Running daily alignment checks and passing standard quality control procedures should mean the instrument is capable of good DNA profiles. Further DNA standards are available and include trout and chicken erythrocyte nuclei as indicators of DNA ploidy. Tumor samples normally contain diploid cells that can be used as an internal standard. For aneuploid cell lines, spiking samples with known diploid cells can help with DNA index determination.5-6

Data Analysis and Reporting

Attempts have been made to standardize the generation of DNA flow cytometry results, both in reporting for clinical use and for publication of research data. These attempts have resulted in several articles, based on cell type.7-11 However, some general guidelines are as follows.

1. Determine the cell ploidy and report the DNA index of all ploidy populations.
2. Report the CV of the main G0,G1 peak. Generally, less than 3 is good; greater than 8 is poor.
3. When measuring the S-phase fraction (SPF) of a diploid tumor, make a statement as to whether the S-phase was measured on the whole sample, including normal cells, or on the tumor cells alone, gated by tumor-specific antibody.
4. Add a brief comment if necessary to cover any other information that may be helpful to someone looking at the result (e.g. inadequate number of cells, high debris levels, high CV, % background, aggregates and debris).

Not all attempts at DNA measurement will be successful. Methodological, sample and instrument issues may mean some of the DNA information is not suitable for interpretation and use in publications. Generally, a result should be rejected if any of the following apply:

1. CV of the G0G1 peak is greater than 8%.
2. Sample contains less than 10,000 to 20,000 nuclei.
3. Data contains more than 30% debris.
4. Flow rate was too high, as indicated by a broad CV or curved populations on two-parameter plots.
5. G0G1 peak isn't in channel 200 of 1024 or 100 of 512 (i.e. on a suitable scale in a known channel).
6. Fewer than 200 cells in S-phase (if SPF is to be reported).
7. G0G1 to G2M ratio is not between 1.95 and 2.05.

**DNA and Beyond**

Having mastered simple DNA applications, the cytometrist may incorporate them into more complex protocols. Combinations of surface, cytoplasmic and nuclear markers allow the cytometrist to explore chromosomes, apoptosis, cell doubling times and beyond.

**References**