

## Cell Cycle Overview

In flow cytometry, the discrimination of cells in the different phases of the cell cycle is generally done by measuring cellular DNA content. Univariate analysis, which is the simplest and most commonly used, allows discrimination between cells that differ in deoxyribonucleic acid (DNA) content, namely cells in G<sub>0</sub>/1 versus those in G<sub>2</sub>/M that have replicated (doubled) their DNA, and cells in S phase that have intermediate content of DNA.

In most assays, the cells are fixed/permeabilized with ethanol or detergent and stained with a fluorescent dye specific for nucleic acids. The most frequently used are: **Propidium iodide** (PI) and 4,6-diamidino-2-phenylindole (**DAPI**). The DNA-bound PI emits red fluorescence when excited with blue light (488 nm). However, because PI also stains ribonucleic acid (RNA), the staining procedure includes incubation with RNase to remove RNA. The complex DAPI-DNA is excited by ultraviolet (UV) light (355nm) and the emission occurs in the blue region (1).

The use of fixatives allows the storage of samples before their analysis. Cells could be collecting at different time points, stored in fixative for extended periods and then stained and analyzed in the cytometer all at once. The fixation step has two functions: (a) preserves the cells by preventing their lysis and autolytic degradation, and (b) makes the cells permeable and their DNA accessible to the fluorescent dye. Fixatives like ethanol, methanol and acetone are preferred over cross-linking agents (formaldehyde, glutaraldehyde). This is because cross-linking of chromatin has deleterious effects on the stoichiometry of DNA staining (2).

An alternative option is the use of detergents and/or hypotonic solutions to permeabilize cells; this method generally provide more accurate estimates of DNA content compared to measurements of fixed cells.

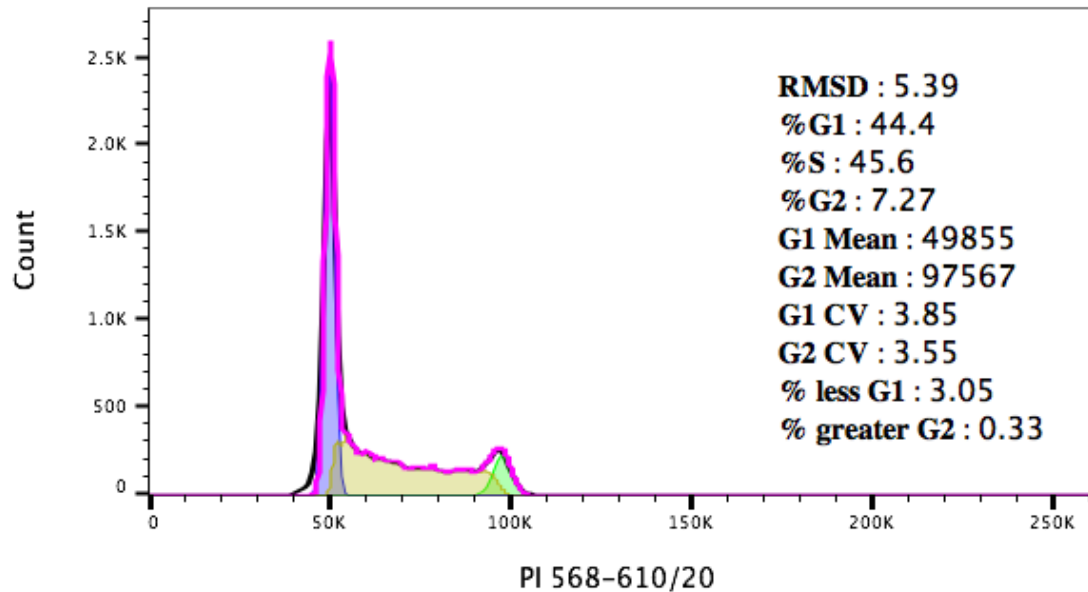
A variety of DNA dyes can be used to stained DNA, the binding characteristics of some of them are described in the following table.

Dye name – Binding Mode	Target molecule	Base pair specificity	Comments
<b>Minor groove-binding</b>			
DAPI	DNA	AT	Impermeant
Hoechst 33342	dsDNA	AT	Permeant-cell cycle analysis in live cells
<b>Interbase-Intercalating</b>			
7AAD	DNA (RNA)	GC Weak	Weakly permeant
PI	DNA (RNA)	No	Impermeant
DRAQ5	DNA (RNA)	No	Permeant
<b>Dual-binding</b>			
SYTOX Green	DNA (RNA)	No	Impermeant
TO-PRO3	DNA (RNA)	No	Impermeant

### Staining of DNA in live cells

In live cells, DNA can be stained (supravivally) with the fluorescent dye **Hoechst 33342**, a cell permeable dye that is excited with UV light. Determination of DNA content using Hoechst 33342 offers the possibility of sorting live cells on the basis of differences in their DNA content. When using Hoechst, the intensity of cell staining and resolution of DNA content vary among different cell types. This variability is due to a rapid efflux of the dye from the cell mediated by the P glycoprotein pump. To improve the staining, the use of inhibitors of the P glycoprotein pump is highly recommended (2).

The results of DNA staining are displayed as cellular DNA content frequency histograms that reveals the percentage of cells in the respective phases of cell cycle. The percentage of cells in every phase is obtained by deconvolution of the histograms using a variety of mathematical approaches. The accuracy of the DNA content measurement is presented as a coefficient of variation (CV) of the mean value of intensity of cells with a uniform DNA content such as, for example, G0/G1 cells. (1). Figure 1



**Figure 1. Cellular DNA content**

### **Identifying cell in a particular cell cycle phase**

There are several methods for cell cycle analysis by flow cytometry and each provides different insights into the cell cycle phase (Reviewed in 1).

Cell in S phase could be identified based on the **analysis of DNA content versus BrdU incorporation**. The thymidine analog BrdU is incorporated into the DNA during cell progression through the S phase. Live cells therefore have to be exposed to BrdU *in vitro* or *in vivo*. The incorporated precursor is then detected with anti-BrdU antibodies (1).

Since cells in G2 and M have the same DNA content, they cannot be distinguished from each other by univariate DNA content analysis. The best manner to identify M cells is by the **analysis of cellular DNA content combined with detection of histone 3 phosphorylation**. Histone 3 (H3) is phosphorylated on serine 10 only during mitosis from profase to telofase. Antibodies against phospho histone 3 are used to discriminate between G2 and M populations.

Cyclin-related proteins are the main regulators of proliferation and cell cycle progression. The oscillations in the cellular level of cyclins through the cell cycle may serve as new landmarks of the cell cycle, additional to the changes in cellular DNA content.

## Synchronizing Cells

Cell populations divide asynchronously. Thus, at any given moment, a dish of tissue culture cells will comprise individual cells that are in each phase of the cell cycle. In some cases, the study of the cell cycle related molecules requires the enrichment of the cell population in a specific phase. This can be achieved by synchronizing cells at a particular point in the cell cycle. Synchronization methods fall into two categories: physical methods, such as mitotic shake off or cell sorting; and chemical methods, where drug-like inhibitors prevent cells from progressing beyond a given point in the cycle.

While nonchemical methods including serum and amino acid starvation are sufficient for synchronizing cells in G<sub>0</sub>/G<sub>1</sub>, the synchronization of cells in S phase is achieved by a procedure known as **“Thymidine Block”**. The procedure consists in the addition of high concentration of thymidine, which interrupts the deoxynucleotide metabolism pathway, thereby blocking DNA replication. As treatment with thymidine arrests cells throughout S phase, a double thymidine block procedure (which involves releasing cells from a first thymidine block before trapping them with a second thymidine block) is generally used to induce a more synchronized early S phase blockade.

Finally, cells in G<sub>2</sub>/M could be enriched by mitotic shake off, which simply involves shaking the culture dish so that the rounded mitotic cells, which have reduced cell contacts with the culture dish, float off into the medium. A more specific manner to synchronize cells in G<sub>2</sub>/M phase is by the addition of compounds like colchicine, colcemid or nocodazole that depolymerize mitotic spindles, arresting the cells in mitosis (Reviewed in 3).

### References

1. Darzynkiewicz, Zbigniew; and Zhao, Hong (February 2014) Cell Cycle Analysis by Flow Cytometry. In: eLS. John Wiley & Sons, Ltd: Chichester.
2. Currents Protocols in Cytometry (1997) 7.5.1-7.5.24 Copyright ©1997 by John Wiley & Sons, Inc.
3. Tate, Sharon and Ko Ferrigno, Paul (2015) Cell Cycle: Synchronization at Various Stages. In: eLS. John Wiley & Sons, Ltd: Chichester.