Cell Cycle Analysis

UWCCC Flow Cytometry Laboratory
https://cancer.wisc.edu/research/resources/flow/

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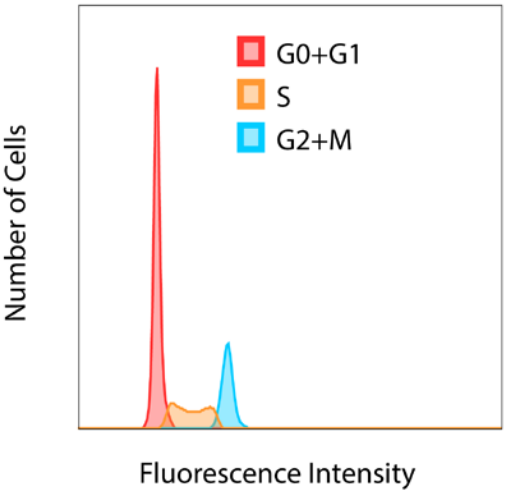
Overview

The cell cycle profile of a sample can be determined by staining the DNA with a fluorescent dye and measuring its intensity. The dye stains DNA stoichiometrically, allowing differentiation of cells in G0/G1, S phase, and G2/M, as well as identification of aneuploid populations. A variety of staining protocols can be adapted for different sample types, but the general analysis remains the same.



Common Dyes

Some DNA dyes do not stain live cells. The sample must be fixed and permeabilized to allow the dye to enter the cells.

- Propidium Iodide (PI) – Blue/Green lasers, also stains RNA
- DAPI – UV/Violet lasers

Some DNA dyes are membrane-permeable and can be used to stain live, intact cells.

- Hoechst 33342 – UV/Violet lasers
- DRAQ5 – Red lasers

Useful References

Darzynkiewicz, Z. 2011. Critical Aspects in Analysis of Cellular DNA Content. Current Protocols in Cytometry. 56:7.2:7.2.1–7.2.8.

Darzynkiewicz, Z. and Juan, G. 2001. DNA Content Measurement for DNA Ploidy and Cell Cycle Analysis. Current Protocols in Cytometry. 00:7.5:7.5.1–7.5.24.

Example Protocol

1. Harvest cells
2. Resuspend in PBS
3. Add cold ethanol, dropwise, to a final concentration of 70%
4. Fix on ice for at least two hours
5. Wash in PBS
6. Resuspend in staining buffer (PBS with 100 µg/mL RNase A, 50 µg/mL Propidium Iodide, and optionally 0.1% Triton X-100)
7. Wrap in foil to protect from light
8. Incubate overnight at 4^{O}C
9. Acquire data on a flow cytometer

Tips for Consistent Staining

- Count the cells and calculate the cell concentration
- Use the same number of cells in each sample
- Use an excess of dye
- Stain overnight to achieve equilibrium
- Get a good single cell suspension before adding fixative; once they are fixed, aggregates can't be dissociated

Sample Considerations

The most straightforward method for cell cycle analysis is to fix the cells with ethanol, treat with RNase, and stain with PI. However, different staining protocols may be necessary for some experiments.

What about RNA? Propidium Iodide stains RNA in addition to DNA, so cells must be treated with RNase to analyze cell cycle accurately. RNase treatment is not required when using DAPI.

Can cells be alive? Dyes that cross membranes work without permeabilizing cells. Additional reagents may be required to inhibit efflux pumps that actively export the dye. Be aware that this staining and subsequent exposure to laser light may induce a DNA damage response in the cells.

Which fixative is best? Alcohols typically result in better staining than crosslinking fixatives. However, the highly fragmented DNA in apoptotic cells may be lost without crosslinking.

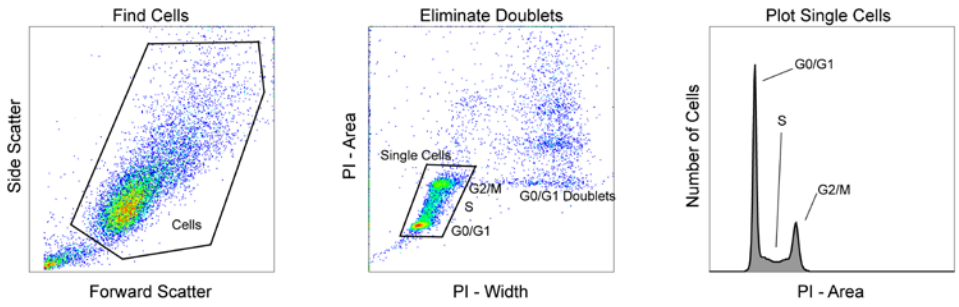
Whole cells or nuclei? Some protocols call for lysing the plasma membrane. This decreases measurement variability that can come from autofluorescence or staining of cytoplasmic components. Keep in mind that the nuclear membrane breaks down during mitosis, so lysing the plasma membrane allows those chromosomes to disperse.

Is co-staining possible? It is possible to label cells with fluorescent antibodies and also analyze DNA content. One option is to add a membrane-permeable DNA dye to an existing surface staining protocol. Alternatively, after surface staining, do a gentle crosslinking fixation, permeabilize the cells, and add a DNA dye.

Data Acquisition

There is at most a two-fold difference in fluorescence intensity between a cell in G0/G1 and a cell in G2/M. Therefore, it is best to view the DNA staining on a linear scale. Collect the Area, Height, and Width parameters for the DNA channel in addition to Forward Scatter and Side Scatter.

Run the samples at a low flow rate for the best results.



Internal Standards

It can be useful to spike in a known standard, such as Chicken Erythrocyte Nuclei, when determining the ploidy of a new sample. Analysis software such as ModFit LT can incorporate these standards when fitting a model to the data.

Assessing Data Quality

The coefficient of variation (CV) of the G0/G1 peak provides an indication of the accuracy of measurements.

There is no universal benchmark for this assessment. However, CVs under 3% are ideal. Anything over 6% is generally considered poor.

Sources of Variability

Sample conditions affect the accuracy of measurements. These factors include:

- Cell damage during sample preparation
- Too many cells or too little dye
- Too many dead/dying cells

Despite all efforts to achieve optimal staining, some samples don't produce data with good CVs. Factors may include:

- Intrinsic variability of DNA content in tumors
- Drug treatments that modify DNA structure by damaging DNA, intercalating into helices, etc.
- Treatment with chromophores that interact with DNA dyes by FRET
- Isolation of nuclei from paraffin blocks