

FLOW CYTOMETRY CORE

Cell Cycle Analysis

G0 phase: quiescent and senescent cells..... **Lowest DNA content**

- This is the typical state for fully differentiated cells.

Interphase: preparatory phase

- Lasts for at least 90% of the total time required for the cell cycle.
- The cell is preparing for division (it takes up nutrients, produces proteins etc).
- It includes three sub-phases: G1, S, and G2.

G1 phase (G for gap) is defined by cell growth **Lowest DNA content**

- Biosynthetic activities of the cell, which are slowed down during M phase, resume at a high rate.
- The amount of organelles increases, cell becomes larger.
- Proteins required in S phase are produced, including enzymes needed for DNA replication.

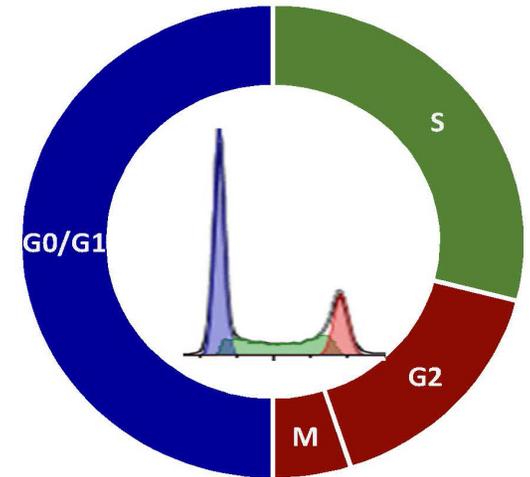
S phase (S for synthesis) is characterized by DNA synthesis..... **Intermediate DNA content**

- The S phase starts when DNA replication commences; it is complete when all chromosomes have been replicated. At the end of this phase the amount of DNA in the cell is effectively doubled.

G2 phase = the cell has doubled its DNA content and prepares to divide **Double DNA content**

- The G2 checkpoint control mechanism ensures that cell is able to enter mitosis.

M phase = Mitosis is defined by nuclear division (karyokinesis) **Double DNA content**



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- Relatively brief, complex and highly regulated.
- The sequence of events is divided into phases:
 - **Prophase:** chromatin condensation; sister chromatids are attached to each other at centromere
 - **Metaphase:** condensed and highly coiled chromosomes align in the middle of the cell
 - **Anaphase:** The centromeres are split, and the new daughter chromosomes are pulled toward the poles.
 - **Telophase:** Two daughter nuclei form in each daughter cell

Cytokinesis (the division of the cell cytoplasm) follows mitosis and results formation of two daughter cells.

Experimental Procedure for Cell Cycle Assay

Reagents

- 95% ethanol
- DNA dye
 - Many DNA-binding dyes may be used. Propidium iodide (PI) and 2-(4-amidinophenyl)-1H-indole-6-carboxamide (DAPI) are very common.
 - **PI**
 - **PI/RNase Staining Solution** (Cell Signaling Cat# 4087S) - ready to use.
 - Alternatively, prepare **PI stock solution** (50 µg/ml) in 3.8 mM sodium citrate and **Ribonuclease I stock solution** (100 µg/ml).
 - **DAPI**
 - **DAPI Stain Solution** (DAPI 1µg/ml, Triton X100 0.1%) in PBS: prepare by adding 1 ml Triton X100 stock solution (1%) and 10 µl DAPI stock solution (1 mg/ml) to 9 ml PBS. Make fresh. Do not store.

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- *DAPI stock solution* (1 mg/ml): dissolve 1 mg DAPI in 1 ml distilled water (Do not use PBS. Sonicate if necessary until a clear solution is obtained. Store at -20°C indefinitely.)
- *Triton X 100 stock solution* (1%): dilute 100 µl Triton X100 (this is a very viscous liquid) in 10 ml PBS.

Method

- Harvest cells and wash them with PBS (Ca²⁺-free and Mg²⁺-free) to remove serum proteins.
- Resuspend the cell pellet (up to 3 million cells) in 1.1 ml PBS.
- Add 3.0 ml ice cold 95% ethanol dropwise while vortexing to crosslink proteins. Do not rush! Adding ethanol too fast cells will result in formation of cell aggregates (clumps). Final concentration of ethanol will be 70%. Incubate at 4°C for at least 30 minutes to completely fix the cells.
- Cells can be stored in the freezer in this solution for up to one week.
- Dilute the cell suspension with 12 ml PBS (for a total volume of 15 ml). Spin at 850 - 1,500 g for 10 min. (Cells are hard to pellet when suspended in ethanol. If the pellet is not visible, transfer a drop of supernatant on a microscope slide and confirm that the supernatant does not contain cells. Spin again the cells if necessary.)
- Remove supernatant. Wash cells again in 15 ml PBS and discard supernatant.
- Resuspend the pellet in 0.5 - 1 ml of solution containing DNA dye.
 - Because PI binds to DNA and RNA, RNase has to be included in the staining solution (add 20 -50µl of stock 100 µg/ml RNase to each sample). Incubate at room temperature for ~40 mins or at 37 °C for ~20 min for RNA digestion. Incubation with RNase will ensure that only DNA, not RNA, is stained.
 - PI can be analyzed on all cytometers existing in the lab.
 - DAPI does not bind to RNA, and for this reason it is not necessary to include RNase in the staining solution when using DAPI. However, samples stained with DAPI have to be analyzed only on instruments equipped with an UV laser.
- Do NOT wash cells prior to flow analysis. The dye must be present in the medium. It is important to have identical concentrations of cells and dye in each tube, otherwise the intensity of staining will be variable and the histograms will be shifted.

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Cell Cycle Analysis

FACS analysis

Cells stained with PI can be analyzed on any cytometer in our lab. Samples stained with DAPI should be analyzed on LSRFortessa or LSR II. Check with the staff to make sure that the instrument that you chose is well calibrated for DNA analysis.

1. Set flow rate on slow and record at least 20,000 events. Slow flow rates are necessary for ensuring precise measurements (low CV values)
2. Plot all events using forward scatter (FSC) and side scatter (SSC) parameters to distinguish intact cells from debris.
3. Use a doublet discrimination gate to exclude doublets from analysis (e.g, pulse height vs. pulse width and/or pulse area vs. pulse height in the channel used to detect the DNA dye).
4. Preliminary analysis using the acquisition software may be performed by manually setting gates on the histogram. However, a cell cycle modeling software (FlowJo or ModFit) should be used after exporting the fcs files.

Watson (pragmatic) approach and Dean-Jett-Fox models can be used in FlowJo. Both Watson and Dean-Jett-Fox models fit Gaussian curves to G1 and G2/M phases. The Dean-Jett-Fox model also fits a polynomial curve to the S phase that can be used to model cell distribution in S phase for synchronous cell populations. The Watson model does not fit a curve to S phase, it simply takes the S phase curve as the difference between the measured curve and the curves fitted to G1 and G2/M phases.

DRAQ5, Vybrant DyeCycle compounds (Life Technologies) or Hoechst 33342 may be used for cell cycle measurements in live, unfixed cells.

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Cell Cycle Analysis

References:

1. Darzynkiewicz ZI, Juan G. **DNA content measurement for DNA ploidy and cell cycle analysis.** Curr Protoc Cytom. 2001 May; Chapter 7: Unit 7.5. doi: 10.1002/0471142956.cy0705s00.
2. Watson JV, Chambers SH, Smith PJ. **A pragmatic approach to the analysis of DNA histograms with a definable G1 peak.** Cytometry. 1987 Jan;8(1):1-8.
3. Dean PN, Jett JH. **Mathematical analysis of DNA distributions derived from flow microfluorometry.** J Cell Biol. 1974 Feb;60(2):523-7.
4. Fox MH. **A model for the computer analysis of synchronous DNA distributions obtained by flow cytometry.** Cytometry. 1980 Jul;1(1):71-7.