Biosynthesis of Macromolecules

Practical Lessons

2013-2014

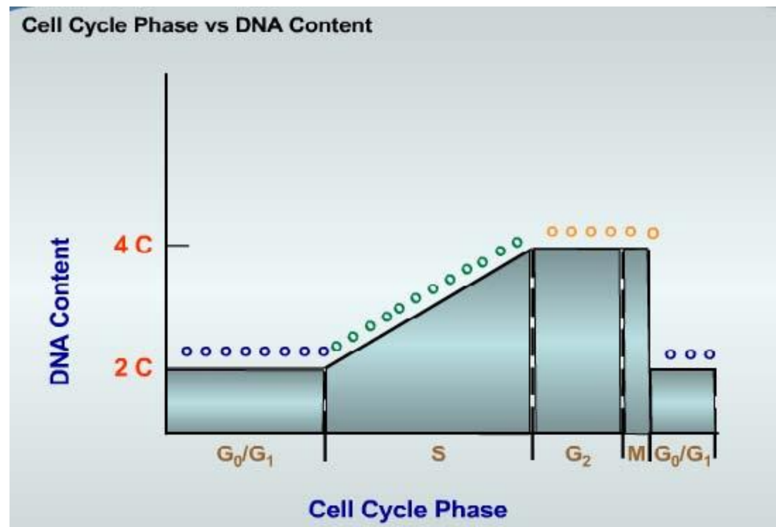
OBJECTIVE:

This approach reveals distribution of cells in the three major phases of the cycle (G1 vs S vs G2/M).

BACKGROUND

Flow cytometry or FACS (Fluorescence Activated Cell Sorting) is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. Fluorescence activated cell sorting (FACS) analysis has become a standard tool to analyze cell cycle distributions in populations of cells although it provides only a snapshot of the cell cycle distribution at any given point in time.

Fluorochromes are essentially dyes, which accept light energy (e.g. from a laser) at a given wavelength and re-emit it at a longer wavelength. The DNA labeling fluorochrome that we are going to use in this practical lesson is Propidium Iodide (PI). It intercalates between the bases in double-stranded nucleic acids. However, it can also stain double stranded RNA, which should be eliminated by addition of RNAase to the cell preparation in order to obtain good results. 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.**



The normal amount of DNA is referred to as diploid or 2n. Cells have to divide. During this process, prior to cell division, or mitosis, the cells will contain twice the normal amount of DNA (4n). During the S phase, cells will contain an intermediate amount of DNA (2n-4n) whether they have just started the replication process or they are finishing it. As PI fluorescence is proportional to the amount of DNA present in the cell, we can determine in which of the cell cycle phases each cell has been fixed.

PROTOCOL

1. Preparation of cells:

1.1. Wash cell culture twice with PBS

1.2. Harvest cells by incubating them three minutes with 0.5ml of Trypsin/EDTA solution. Detachment of the cells can be checked under microscope.

1.3. Transfer cells to an Eppendorf tube.

1.4. Centrifuge at 300 g for 3 minutes at 4°C.

1.5. Totally discard the supernatant.

2. Cell fixation:

2.1. Resuspend pellet in 0.2ml of cold PBS and 0.2ml of 2% paraformaldehyde in PBS.

2.2. Vortex cells to mix and incubate for 15 minutes.

3. Cell permeabilization:

3.1. Centrifuge at 300 g for 3 minutes at 4°C.

3.2. Totally discard the supernatant.

3.3. Resuspend pellet carefully in 0.3ml of cold PBS

3.4. Add carefully over the vortex 0.7ml of cold (kept at -20°C) Ethanol (drop by drop) to avoid clumping of cells.

3.5. Store at 4°C until next day

4. Single-Cell Suspension

4.1. Pass cells several times through an insulin syringe at 4°C.

5. DNA staining

5.1. Centrifuge at 800 g for 3 minutes at 4°C.

5.2. Totally discard the supernatant.

5.3. Resuspend cells in 0.5ml of PI solution (200µg/ml Ribonuclease A y 5µg/ml of PI in PBS.

5.4. Stain in dark for 30 minutes

5.5. FACS analysis.