

## Epithelial Cell Death Analysis of Cell Cycle by Flow Cytometry

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# Epithelial Cell Death

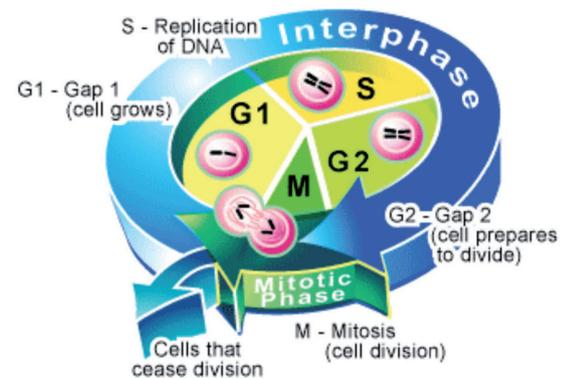
## Analysis of Cell Cycle by Flow Cytometry

### PRINCIPAL OF TECHNIQUE

#### Background:

Cell cycle, or cell-division cycle, is the series of events that takes place in a cell leading to its division and duplication (replication). In cells without a nucleus (prokaryotic), cell cycle occurs via a process termed binary fission. In cells with a nucleus (eukaryotes), cell cycle can be divided in two brief periods: interphase—during which the cell grows, accumulating nutrients needed for mitosis and duplicating its DNA—and the mitosis (M) phase, during which the cell splits itself into two distinct cells, often called «daughter cells». Cell-division cycle is a vital process by which a single-celled fertilized egg develops into a mature organism, as well as the process by which hair, skin, blood cells, and some internal organs are renewed.

Cell cycle consists of four distinct phases: G<sub>1</sub> phase, S phase (synthesis), G<sub>2</sub> phase (collectively known as interphase) and M phase (mitosis). M phase is itself composed of two tightly coupled processes: mitosis, in which the cell's chromosomes are divided between the two daughter cells, and cytokinesis, in which the cell's cytoplasm divides in half forming distinct cells. Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G<sub>0</sub> phase.



### Resting ( $G_0$ phase)

The term «post-mitotic» is sometimes used to refer to both quiescent and senescent cells. Non-proliferative cells in multicellular eukaryotes generally enter the quiescent  $G_0$  state from  $G_1$  and may remain quiescent for long periods of time, possibly indefinitely (as is often the case for neurons). This is very common for cells that are fully differentiated. Cellular senescence is a state that occurs in response to DNA damage or degradation that would make a cell's progeny nonviable; it is often a biochemical alternative to the self-destruction of such a damaged cell by apoptosis.

### Interphase

Before a cell can enter cell division, it needs to take in nutrients. All of the preparations are done during the interphase. Interphase proceeds in three stages,  $G_1$ , S, and  $G_2$ . Cell division operates in a cycle. Therefore, interphase is preceded by the previous cycle of mitosis and cytokinesis.

### $G_1$ phase

The first phase within interphase, from the end of the previous M phase until the beginning of DNA synthesis is called  $G_1$  (G indicating gap). It is also called the growth phase. During this phase the biosynthetic activities of the cell, which had been considerably slowed down during M phase, resume at a high rate. This phase is marked by synthesis of various enzymes that are required in S phase, mainly those needed for DNA replication. Duration of  $G_1$  is highly variable, even among different cells of the same species <sup>[1]</sup>.

### S phase

The ensuing S phase starts when DNA synthesis commences; when it is complete, all of the chromosomes have been replicated, i.e., each chromosome has two (sister) chromatids. Thus, during this phase, the amount of DNA in the cell has effectively doubled, though the ploidy of the cell remains the same. Rates of RNA transcription and protein synthesis are very low during this phase. An exception to this is histone production, most of which occurs during the S phase <sup>[2] [3] [4]</sup>.

### $G_2$ phase

The cell then enters the  $G_2$  phase, which lasts until the cell enters mitosis. Again, significant protein synthesis occurs during this phase, mainly involving the production of microtubules, which are required during the process of mitosis. Inhibition of protein synthesis during  $G_2$  phase prevents the cell from undergoing mitosis..

## Research Applications

### Introduction:

Cells undergoing apoptosis display typical changes in their morphological and physical properties (cell shrinkage, condensation of chromatin and cytoplasm) which are well measurable by flow cytometry. Thus, a series of methods have been proposed for measuring apoptotic cell death through evaluation of light scattering parameters of cells. Interaction of a particle with the laser beam produces a light scatter in a forward direction (FSC that correlates with cell size) and a lateral direction (SSC that correlates with granularity and/or cell density). While necrotic death is characterized by a reduction in both FSC and SSC (probably due to a rupture of plasma membrane and leakage of the cell's content), during apoptosis there is an initial increase in SSC (probably due to the chromatin condensation) with a reduction in FSC (due to the cell shrinkage). This pattern can be easily observed in some models of apoptosis, such as the apoptotic death induced by glucocorticoids in murine thymocytes but it is much less clear in other cell models (i.e. the majority of tumor cell lines).

### Standard Procedure:

Cells were treated with agents that induce cell cycle arrest or cell death. Cells were stained with propidium iodide (PI) followed by cell cycle analysis as follows:

Cells were plated at a confluency of 70-80% on the day before the treatment. The cells were treated with various agents including PMA (100 ng/mL)/Ionomycin (0.5  $\mu$ M), Etoposide (50  $\mu$ M) and CD437 (0.5  $\mu$ M) for a period of 48 h. At the end of the treatment period, both the floating as well as adherent cell population were collected and stained with PI as described in [5].

PMA-12-O-Tetradecanoylphorbol-13-acetate (TPA), also commonly known as phorbol 12-myristate 13-acetate (PMA), is a diester of phorbol and a potent tumor promoter often employed in biomedical research to activate the signal transduction enzyme protein kinase C (PKC). The effects of TPA on PKC result from its similarity to one of the natural activators of classic PKC isoforms, diacylglycerol. TPA is also being studied as a drug in the treatment of hematologic cancer. TPA is not to be confused with tPA; the former is a small molecule drug, whereas the latter is an enzyme. TPA was first found in the croton plant, a shrub found in Southeast Asia, exposure to which provokes a poison ivy-like rash. It is currently undergoing phase 1 clinical trials. Treatment with PMA causes dephosphorylation, which then makes the cells inactivated. In this step the PMA blocks the progression of cell cycle at the G<sub>1</sub>/S transitional phase of mitosis.

Etoposide- Etoposide phosphate (brand names: Eposin, Etopophos, Vepesid, VP-16) is an inhibitor of the enzyme topoisomerase II. It is used as a form of chemotherapy for malignancies such as Ewing's sarcoma, lung cancer, testicular cancer, lymphoma, non-lymphocytic leukemia, and glioblastoma multiforme. It is often given in combination with other drugs. It is also sometimes used in a conditioning regimen prior to a bone marrow or blood stem cell transplant. This chemotherapy drug is effective in that it halts cell division. This particular drug is made from a plant alkaloid, which means it is cell cycle specific. Cell cycle specific drugs, unlike other chemotherapy drugs, attack cells during various phases of division.

CD437/AHPN- First described as a RAR selective retinoid, this molecule has been found to inhibit growth and promote apoptosis in an extremely wide variety of tumor cell lines. Compared with retinoic acid and other RAR selective synthetic retinoids, CD437 is characterized by unique properties. It is active not only in retinoic acid-sensitive cell lines but also in retinoic acid-resistant cells and in RAR-negative cells. Moreover, its antiproliferative and apoptogenic activities in cancer cells are not inhibited by either RAR $\alpha$ , or RAR $\beta$  or by pan-RAR selective antagonists. CD437 arrests cells in G<sub>0</sub>-G<sub>1</sub> by increasing the cyclin-dependent kinase inhibitor p21cip1/waf1. This increase has been related to a posttranscriptional stabilization of p21cip1/waf1 mRNA. CD437 induces both p53-dependent and -independent apoptosis in human cancer cells. [6]

*In conclusion, with using all three of these products in combination with Flow Cytometry, cell cycle analysis can be detected by the fluorescence of the cell cycle progression. This method would be beneficial for finding out the apoptosis percentage, the G<sub>1</sub>/S percentage, as well as if the different experimental treatments affect the cell death/cell division in various cells.*

*The most commonly used dye for DNA content/cell cycle analysis is Propidium Iodide. The PI intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission centered around 600 nm. Since PI can also bind to double-stranded RNA, it is necessary to treat the cells with RNAase for optimal DNA resolution. The excitation of PI at 488 nm facilitates its use on the benchtop cytometers. [PI can also be excited in the U.V. (351-364 nm line from the argon laser) which should be considered when performing multicolor analysis on the multi-laser cell sorters.]*

### **Analysis of DNA content of apoptotic cells by flow cytometry:**

#### **a. Quick method (direct DNA staining in fluorochrome solution)**

- Wash cells ( $1 \times 10^6$ ) in PBS and centrifuge at 200 g for 5 min
- Aspirate off the PBS and gently resuspend the cell pellet in 1.5 mL fluorochrome solution (Propidium iodide 50 mg/liter in 0.1% Sodium Citrate plus 0.1% Triton X-100). Place the tubes in the dark at 4°C at least 60 min before analysis by flow cytometry.

#### **b. Standard Method**

- Wash cells ( $1 \times 10^6$ ) in PBS and centrifuge at 200 g for 5 min
- Resuspend the cell pellet in 100  $\mu$ L PBS and fix in 70% (vol/vol) ethanol by adding on ice 900  $\mu$ L of cold (-20°C) ethanol. Incubate cells on ice for at least 30 min.
- Centrifuge cells, decant ethanol and resuspend cells in 1.5 mL PBS at room temperature for 10 min. Centrifuge at 200 g for 5 min.
- Resuspend the cell pellet in 1 mL of DNA staining solution (in 10 mL of PBS add 200 mg Propidium iodide plus 2 mg RNase A) and incubate for 30 min at room temperature in the dark.
- Analyze cells by flow cytometry.
- Fixed Cells can be analyzed up to 5 days later. However, fresh cells should be analyzed within 4 hours.<sup>[5]</sup>

#### **c. Instrument Set-Up**

The following parameters were used on a Gallios\* flow cytometer. The following tubes were prepared as stated above with the previously listed agent amounts:

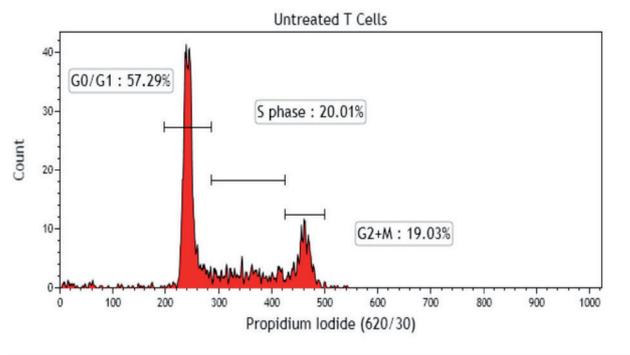
1. Untreated cultured T cells "Control" Sample
  2. Untreated Epithelial cells
  3. Epithelial cells with PMA
  4. Epithelial cells with Etoposide
  5. Epithelial cells with CD437/AHPN
- In addition, PI beads were used for confirmation of  $G_0/G_1$ , S phase, and  $G_2/M$  peaks (graph not shown).
  - A dot plot was created with FL3 PEAK on the x-axis and FL3 INT on the y-axis.
  - A histogram plot was created with FL3 INT on the x-axis and counts displayed on the y-axis.
  - The untreated cultured T cells were acquired at a setting of LOW with the following settings:
    1. Volts = 308 (determined by placement of  $G_0/G_1$  peak at the 200 marker of FL3 linear scale).
    2. Discriminator FL3 = 30 (remove low-end noise; should still be able to visualize  $G_0/G_1$  peak clearly).
    3. 10,000 Total Events

Gates were set according to previous PI cell cycle analysis gating strategies<sup>[5]</sup>, and confirmed by observing the Mean Fluorescent Intensities (MFI) of the  $G_0/G_1$  and  $G_2/M$  peaks and determining ratio to be approximately 2.0.

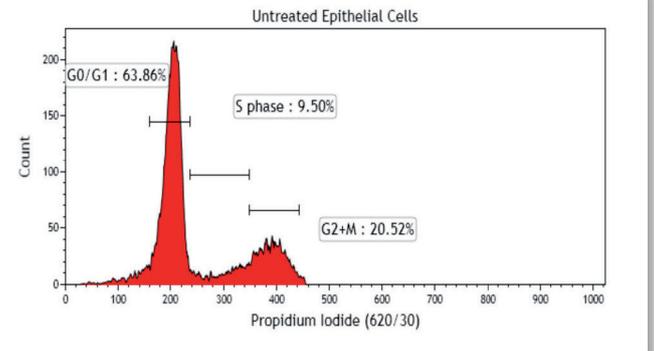
## Results

Cell cycle analysis should reveal distinct populations at various stages of cell cycle, namely  $G_0$ ,  $G_1$ , S and  $G_2$  phase.

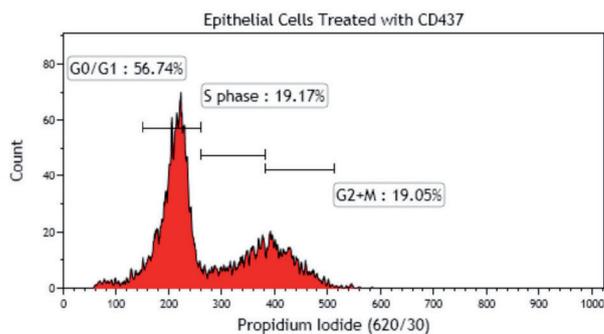
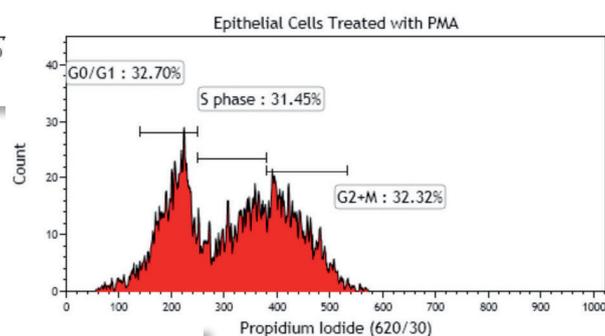
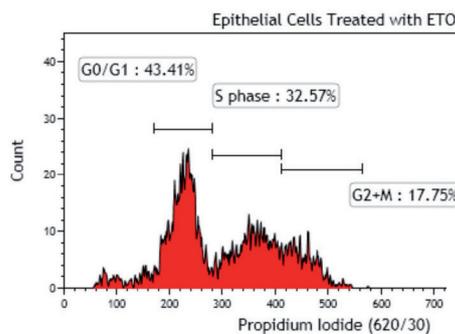
### Untreated Cultured T cells:



Untreated Cultured T cells:  
 $G_0/G_1$  MFI = 243  
 $G_2/M$  MFI = 460  
Ratio = 1.89



## Treated Epithelial cells:



| Results   | G <sub>0</sub> /G <sub>1</sub> MFI | G <sub>2</sub> /M MFI | Ratio |
|-----------|------------------------------------|-----------------------|-------|
| Untreated | 203                                | 392                   | 1.93  |
| ETO       | 210                                | 396                   | 1.89  |
| PMA       | 228                                | 459                   | 2.01  |
| CD437     | 207                                | 422                   | 2.04  |

## Conclusions

All three agents showed differences from the untreated sample. Most notable was the S phase of both the PMA and ETO samples. The agents pushed the cells into a division stage without DNA profile change. However, the results did not indicate G<sub>0</sub>/G<sub>1</sub> arrest and subsequent halting of cell division.

Further studies will look at the apoptotic effect of these agents on both "normal" cells and tumor cells.

### Helpful Hints

- Acquire “Control” sample at a high rate until G<sub>0</sub>/G<sub>1</sub> peak found and placed at 200 marker position, then acquire and save 10,000 total events on LOW speed.
- PI is excited and emitted across a broad spectrum [excitation 495 nm (460 nm-520 nm)/ emission 610 nm (570 nm-700 nm)]. Therefore, cell cycle can be analyzed using numerous filter sets (either FL2 or FL3 on a standard Gallios “Boulevard”).
- Check G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M MFI and subsequent ratio to ensure proper gate placement. Ratio should be between 1.5 and 2.5 to ensure accuracy of gate positions.
- S phase gate is placed between the G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M gates.
- Software such as ModFit LT Flow Cytometry Modeling Software (Verity Software House) can be very helpful in cell cycle analysis.

### References

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5. Riccardi, C. and Nicoletti, I., (2006). «Analysis of apoptosis by propidium iodide staining and flow cytometry». Nat Protoc., 1(3): p. 1458-61.
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### Notes

*The results demonstrated in this application sheet represent those generated on the Beckman Coulter Gallios Flow Cytometer.*

*As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow Cytometers. Quality control and assurance were performed to meet manufacturer's specifications using Flow Check Pro (Beckman Coulter) and 8-peak Rainbow Beads (Spherotech).*