A High Throughput, High Content Assay for Cell Cycle Analysis

Wayne Bowen and Paul Wylie
TTP LabTech Ltd, UK

Background

The cell cycle is a target for many anti-cancer drugs; making the ability to monitor the effects of such agents on the cell cycle an important part of the drug development process. Traditionally, flow cytometry has been used to sort cells into G1, S and G2/M phases of the cell cycle by determining their DNA content following staining with fluorescent dye. The main disadvantages of this technique are low throughput, use of large numbers of cells and an inability to analyse adherent cell lines in situ. To improve screening efficiency, we have developed a cell cycle analysis method that uses fluorescence microplate cytometry to measure the DNA content of cells in microplates. This method offers a high content and high throughput approach to cell cycle analysis.

Key Assay Benefits:

- **rapid *in situ* cell cycle analysis of adherent cells.**
- **cells classified as G1, S or G2/M phase.**
- **high content and high throughput approach to cell cycle analysis.**

Introduction

The cell cycle represents one of the most fundamental and important processes in eukaryotic cells, culminating in cell growth and division into two daughter cells. The process is strictly controlled by proteins in the cytoplasm, principally cyclins and cyclin dependent kinases, causing the cell to move from G1, S, G2 and M phases of the cycle following an ordered set of events (Figure 1). Defects in cell cycle regulation are a characteristic feature of tumour cells and mutations in the genes involved in controlling the cell cycle are extremely common in cancer. Monitoring dysfunctional cell cycle regulation is thus the focus of intense interest, since it provides an opportunity to discover new targets for anti-cancer drugs and improved therapeutics.

Standard methods measure changes in DNA content by staining the nuclei of fixed cells with fluorescent dye. The cells are then sorted by flow cytometry into G1, S and G2/M phases according to fluorescent intensity (3,4). Cells in G2 and M phases contain identical amounts of DNA, therefore DNA staining alone cannot resolve them. The most commonly used DNA dye is propidium iodide (PI), which intercalates in the DNA helix and fluoresces strongly red. It has the advantage that it is excited by 488 nm light so can be used on most flow and microplate cytometers. PI is not able to penetrate an intact membrane, therefore cells must first be permeabilised and are consequently nonviable. PI also stains double-stranded RNA, necessitating its removal with ribonuclease prior to analysis.

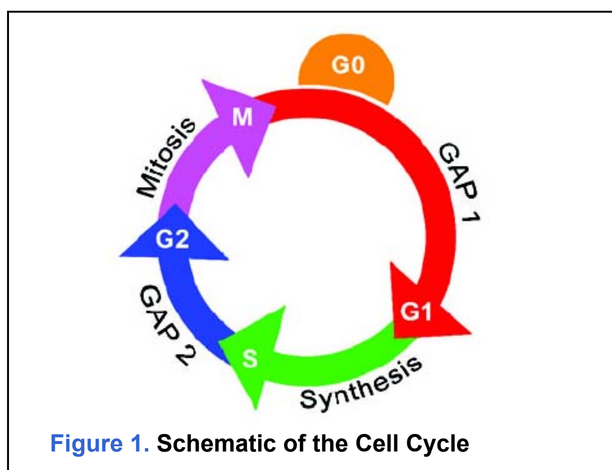


Figure 1. Schematic of the Cell Cycle

Acumen Explorer

The Acumen Explorer™ combines the object recognition capability of image-based systems with the high read speeds of traditional bulk readers. At the heart of the system is a proprietary, non-confocal optic system which permits focus-free, area-based scanning, collecting up to 4 channels of data in a single scan. To prevent problems of variable cell number, patchy stimulation and edge effects, Acumen Explorer allows the researcher to define the scan area within each well. Scan options include the whole well area, centre well or user-defined strips.

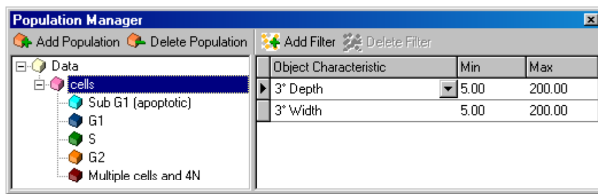
Materials and Methods

Cell Culture

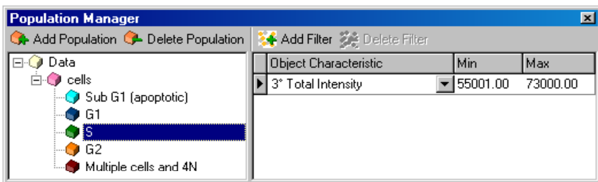
CHO cells were routinely passaged and plated out at 2,000 cells per well into a 96 well plate. Vinblastine and paclitaxel solutions were prepared in complete growth medium. After aspiration of the serum-free medium, a sample of each compound solution (100 µL) was added. Control wells received an equal volume of complete growth medium.

Following incubation for 22 hours @ 37°C / 5% CO2, the growth medium was removed and cells fixed using cold ethanol (100%, 100 µL, -20°C). The cells were washed with PBS and incubated with RNase in PBS (0.2 mg/mL, DNase free) for 4 hours at room temperature. Subsequently, nuclei were stained by addition of propidium iodide at a final concentration of 3 µM. After a further 15 minute incubation at room temperature, the plate was scanned on an Acumen Explorer fluorescence microplate cytometer.

Figure 2a: Classification of Cell Populations in the Acumen Explorer Software



Classification of cells using width and depth measurements.



Classification of cells population according to total fluorescent intensity (S phase shown).

Figure 2b: Total Intensity Histograms of Control and G2/M Arrested CHO Cells

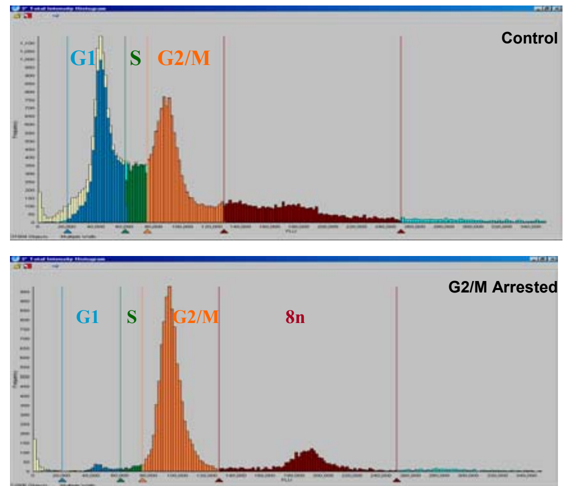
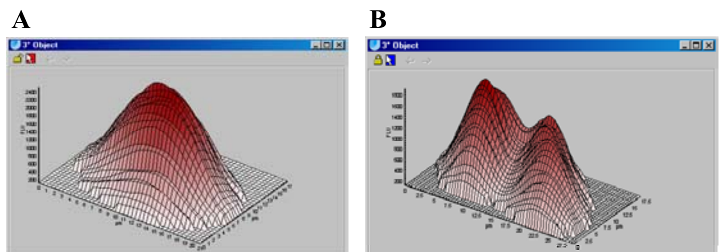


Figure 3: 3D Objects views of:

(**A**) a nucleus in G1 phase (42,006 FLU)

(**B**) a nucleus in G2/M phase (86,937 FLU)



Scanning with the Acumen Explorer

Due to the additions and wash steps, the whole well area was scanned to increase the quality of the data obtained. Possessing a large field of view (400 mm2), the Acumen Explorer is ideal for enumerating DNA content in all cells within each well and offers new possibilities for cell cycle analysis. The protocol was capable of reading an entire 96 well microplate in under 10 minutes. Precision optics and photomultiplier tube detection enable rapid scanning and analysis of fluorescently-labelled cells in microplate wells.

Results and Discussion

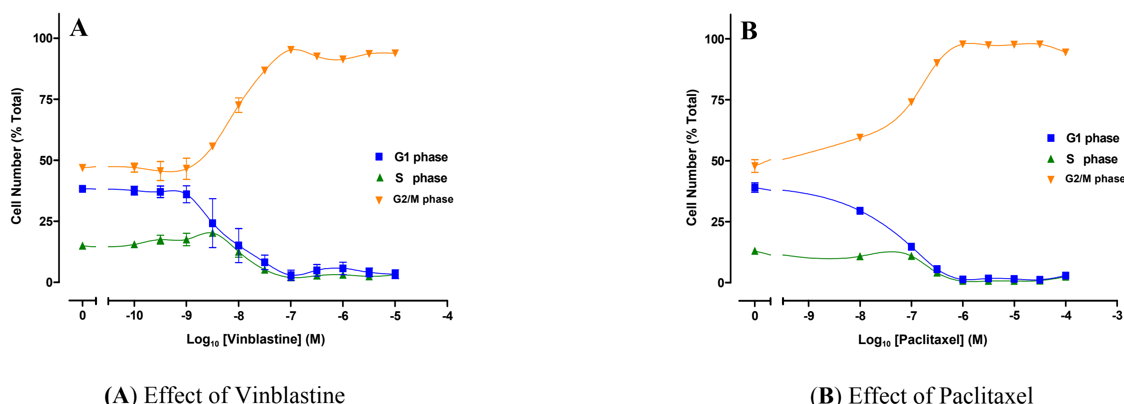
Standard methods measure changes in DNA content by staining the nuclei of fixed cells with fluorescent dye.

The cells are then sorted by flow cytometry into G1, S and G2/M phases according to fluorescent intensity (3,4). Cells in G2 and M phases contain identical amounts of DNA, therefore DNA staining alone cannot resolve them.

The most commonly used DNA dye is propidium iodide (PI), which intercalates in the DNA helix and fluoresces strongly red. It has the advantage that it is excited by 488 nm light so can be used on most flow and microplate cytometers.

PI is not able to penetrate an intact membrane, therefore cells must first be permeabilised and are consequently nonviable. PI also stains double-stranded RNA, necessitating its removal with ribonuclease prior to analysis.

Figure 4: **Concentration dependence curve of anti cancer drugs on the cell cycle in CHO cells**



(**A**) Effect of Vinblastine (**B**) Effect of Paclitaxel

One advantage of laser scanning is that it is possible to generate three-dimensional models of fluorescent objects, including nuclei. This permits visual determination of the number of nuclei present in each cell. The multiple sampling also means integration of fluorescence intensity is not affected by the presence of multiple nuclei, leading to well defined peaks for cells in G1 and G2/M phases (Figure 2a+b). Mathematical models have been developed to calculate the percentages of cells occupying the different phases of the cell cycle from a single DNA histogram into G1, S and G2/M populations and are applicable to data from microplate cytometry data.

Of major significance is the ability to analyse adherent cells in situ, unlike flow cytometry which requires their suspension for processing. *In situ* reading preserves morphological changes that may have occurred during drug treatment thus giving valuable secondary information, especially when analysing highly differentiated cells such as neurones. It also permits analysis of the shape and determination of the location of the nucleus or nuclei within the cell (Figure 3a+b). This information can permit 'visualisation' of multiple nuclei in the later stages of mitosis allowing their resolution from cells in G2 phase. In addition, it may identify necrotic or apoptotic cells within the normal cycling population. Finally, in multiplex assays where cell cycle analysis is combined with other read-outs such as receptor or pathway signalling, analysis within microplates eliminates the secondary effects associated with the harsh process of resuspending the cells for flow cytometry.

The utility of microplate cytometry for compound profiling has been demonstrated using the standard agents, such as vinblastine (6), which arrested CHO cells in the expected phase of the cell cycle (Figure 4).

Conclusion

For screening purposes, the throughput of a microplate cytometer for cell cycle analysis is unparalleled, being able to analyse in a few hours what normally takes a week on a flow cytometer. Since all the cell processing is performed with microplates, it is also more amenable to automation. The novel design features of the Acumen Explorer microplate cytometer used in these studies permit multiplex, whole well analysis at high read times compatible with primary screening campaigns, with daily throughputs of 30,000 compounds per day being reported for some assays (5). For cell cycle analysis, automated throughput of 100 samples in 10 minutes has been achieved.

References

1. Crissman, H.A. & Steinkamp, J.A. (1973) *J. Cell Biol.*, **59**,766-771.
2. Krishan A. (1975) *J. Cell Biol.*, **66**, 188-193.
3. Darzynkiewicz, *et al.*, (1975) *Exp. Cell Res.*, **95**, 143-153.
4. Darzynkiewicz, Z., *et al.*, (1976) *Proc. Natl. Acad. Sci. USA.*, **76**, 358 362
5. Shah, A. (2004). Drug Discovery and Development, **7,** 52-57
6. Jordan, M.A & Wilson, L. (1999) *Methods Cell Biol.*, **61**, 267-295.

TTP LABTECH

Melbourn Science Park
Cambridge Road
Melbourn, Royston
Herts SG8 6 EE, UK

Tel: +44 1763 262626
Fax: +44 1763 261964

www.ttplabtech.com/explorer

sales@ttplabtech.com

We gratefully acknowledge the assistance of Dr Walter Bordogna (Argenta Discovery, Harlow, UK) during the development of the cell cycle analysis protocol.
www.argentadiscovery.com