

## Quantitative Analysis of the Cell Cycle in Imaging Applications



### Introduction

Quantitative analysis of stages in the cell cycle is commonly used in cell biology since many pharmacological interventions aim to modulate the cell cycle in some way. We present here an application that can be used in adherent cells and optionally multiplexed for the use in automated cell signaling analysis and drug-screening applications.

The cell cycle consists of a series of processes involved in the growth, replication, and division of cells and can be divided into two major stages, interphase (the phase between mitotic events) and mitosis. There are three distinct, successive stages within interphase, namely G1, S, and G2 phases. During G1 (first gap), cells monitor their environment and grow (synthesize RNA and proteins). Once conditions are optimal, cells commit to DNA synthesis (S phase) and replicate their chromosomal DNA. The G2 phase (second gap) follows when cells continue to grow and prepare for mitosis (M phase, division). The G2 gap allows time for the cell to complete its DNA replication before initiating mitosis.

During mitosis, the cell cycle is completed giving rise to two daughter cells, each of which contains the same genetic material as the original cell and approximately half of its G2 level of cytoplasm. In addition to these specific stages, a G0 phase has been described for cells that exit from the cell-cycle and enter a quiescent, non-dividing state. In response to external stimuli, cells may undergo reactivation and leave G0 and enter the G1 phase of the cell-cycle. Another consequence of cellular activation may be the induction of programmed cell death (apoptosis).

Understanding the specific phase of a cell is important when cells display heterogeneous responses to drugs as is often the case in high-content applications.

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POB 13 Erembodegem-Dorp 86, B-9320 Erembodegem, Belgium  
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**Introduction** *(continued)*

This four color application demonstrates how fluorescent antibodies can be used in combination to specifically inform on the M and S phases of the cell cycle while it is still possible to “drop in” an additional specificity of interest to the user. For this assay, Hoechst 33342 was used to identify all nuclei and an anti-phospho-Histone 3 antibody was used to identify cells in M phase.<sup>1</sup> Similarly immunofluorescent staining of incorporated bromodeoxyuridine (BrdU) is used to determine the frequency of cells that had synthesized DNA.<sup>2, 3, 4, 5</sup> In this method, BrdU (an analog of the DNA precursor thymidine) is incorporated into newly synthesized DNA by cells entering into and progressing through the S phase of the cell cycle. The “drop in” choice for this assay was a fluorescently labeled anti-Ki-67 antibody. Ki-67 is present in all cycling cells but is not present in quiescent cells (G0). The Ki-67 staining pattern is variable depending on the phase of the cycle, in the G1/S/G2 phases it is punctuate, whereas in late G2/M it appears throughout the entire nucleus (see **Table 1**).<sup>6,7</sup>

The BD Pathway™ Bioimager is well suited for the development of complex assays due to its wide range of acquisition and analysis tools. Using a broad-spectrum illumination source, the system enables the user to select the antibodies based on their compatibility with the assay rather than with specific laser wavelengths. The 16 excitation filter choices of the BD Pathway Bioimager allow the setup of several multi-color assays with minimal bleed-through which enables the system to be used in a wide variety of demanding applications.

**Methods**

HeLa cells (a human cervical cancer cell line, ATCC, #CCL-2) were seeded in BD Falcon™ 96-well black/clear bottom tissue culture plates optimized for automated imaging applications (Cat. No. 353219) at 15,000 cells/well in DMEM (Invitrogen, 11965-084) complete media. Eighteen hours later cells were treated with one of two different metabolic inhibitors, aphidicolin (Sigma-Aldrich, A0781), a DNA topoisomerase alpha inhibitor (G1/S blocker) for two hours or colcemid (Sigma-Aldrich Cat # D7385) a (G2/M blocker for two hours. During drug treatment, cells were co-cultured with 20 µM BrdU (Calbiochem, 203806) (a thymidine analogue that incorporates into DNA) for the final hour of culture.

For image analysis, cells were fixed and permeabilized in BD Cytofix/Cytoperm™ (4% paraformaldehyde with 0.1% saponin, Cat. No. 552598a) for 20min, washed with BD Perm/Wash™ Buffer (PBS with 0.1% saponin and 3% fetal calf serum, FCS, Cat. No. 552598b), and permeabilized again in BD CytoPerm™ Plus Permeabilization Buffer (BD 552598c) for 10 min. Afterwards, they were washed with the Perm/Wash Buffer, and re-fixed for 5 minutes. Cells were then washed with Perm/Wash Buffer, treated with DNase (Sigma-Aldrich, D4513, 15µg/well in PBS at 37°C for 1 hour), washed in Perm/Wash Buffer and stained simultaneously with Alexa Fluor® 488 (Invitrogen, A-20000) conjugated anti-BrdU (Cat. No. 555627), Alexa Fluor® 647 conjugated anti phospho-Histone H3 (Serine 28) (Cat. No. 558217), and Alexa Fluor® 555 (Invitrogen A-20009) conjugated anti-Ki-67 (Cat. No. 610969) in PBS with 3% FCS at room temperature for 1 hour. Cells were washed three times in Perm/Wash Buffer and stained with 2 µg/mL Hoechst 33342 (Invitrogen, H3570), in Perm/Wash Buffer

Images were acquired on a BD Pathway Bioimager and analyzed using Attovision software and BD Image Data Explorer.

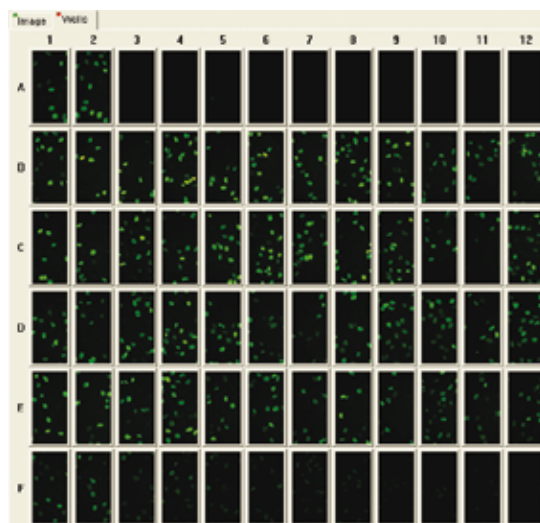
Cells were imaged using a 20x (0.75 NA) objective and analyzed using the built-in tools of the software package. Numerical data was imported into the Microsoft® Excel-based BD Image Data Explorer where the cell population was analyzed for nuclear fluorescence intensity by setting a threshold for the three fluorescence channels. The threshold was set on the nuclear intensity of the BrdU and phospho-Histone 3 negative cells.

**Table 1. Staining patterns for the probes used across the cell cycle phases.** Total refers to a stain that is present throughout the nucleus while punctate refers to visible spots inside the nucleus

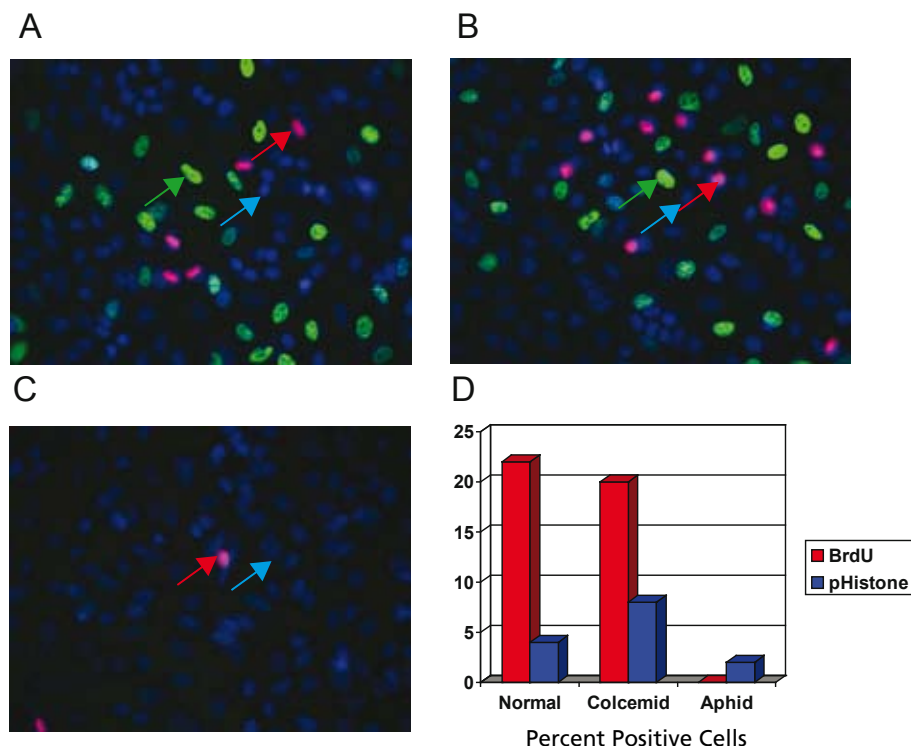
	G0	G1	S	G2	M
BrdU	—	—	Total	—	—
Phospho-Histone 3	—	—	—	—	Total
Ki-67	—	Punctate	Punctate	Punctate/Total	Total

## Results

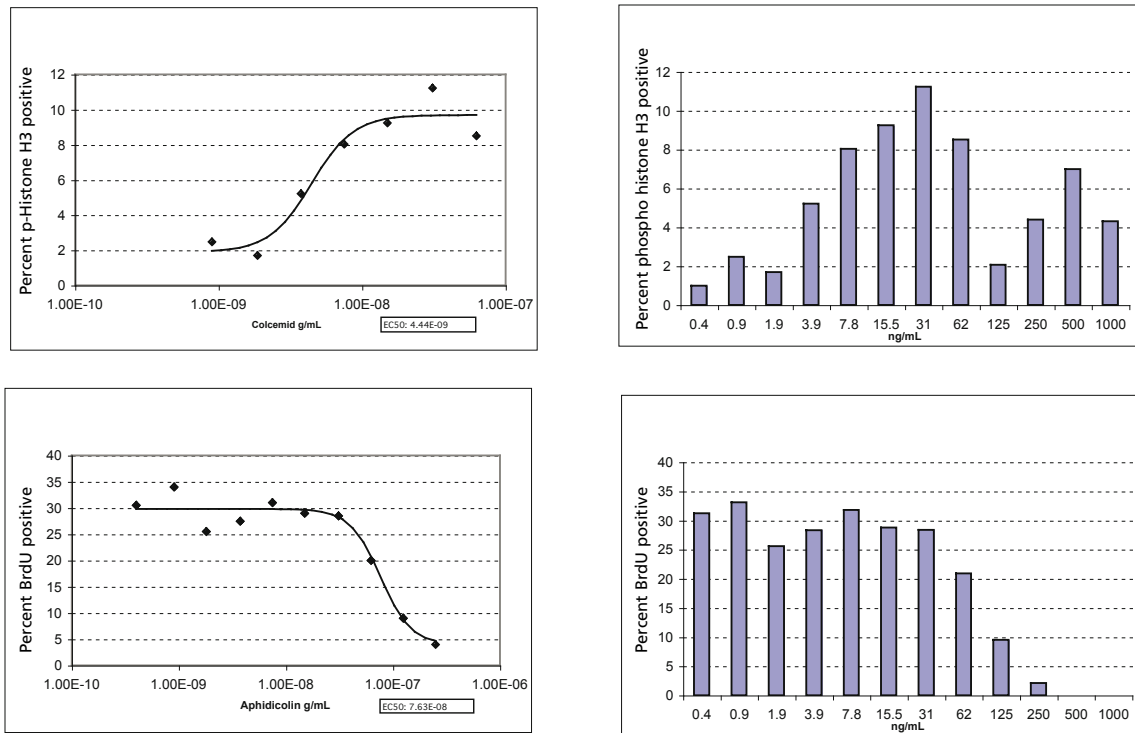
The staining pattern for BrdU and phospho-Histone H3 found in control cells was consistent with a heterogeneous cell population (*Figure 1*). We found that approximately 3% were in M phase based on the positive phospho-Histone 3 staining and approximately 22% were in S phase based on the BrdU results (*Figure 1D*). When cells were treated with either colcemid or aphidicolin changes in cell cycle status were seen (*Figure 1 and Figure 3*). In colcemid treated cells (eg, 500 ng/mL) the percent cells in S phase remained similar (~20%) while the percent cells in M phase doubled to approximately 7% (*Figure 1D*). In aphidicolin treated cells (eg, 500ng/mL), the percent cells in S phase dropped to 0% while the percent cells in M phase dropped slightly to approximately 2%. *Figure 2* shows the dose-response relationship of BrdU incorporation in response to increasing concentrations of colcemid and aphidicolin. BrdU indicates cells in S phase and colcemid had no significant effect at any concentration. Aphidicolin decreased the number of S-phase cells in a dose-dependent manner.



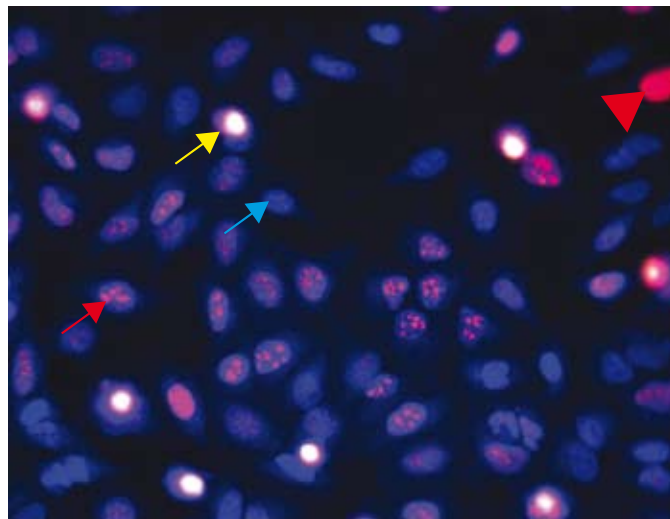
**Figure 2. Screenshot of the thumbnail images.** Shown is the BrdU staining pattern in the green channel. Rows A and B contain control samples. Rows C and D show the response to 12 rising concentrations of colcemid (in duplicates from 0.4 to 1000 ng/mL, left to right), colcemid has no effect on cells entering S phase. Rows E and F show the response to increasing concentrations of aphidicolin (duplicates, from 0.4 to 1000 ng/mL), as the concentration increases, the number of cells entering S phase decreases.



**Figure 1. Effects of colcemid (500 ng/mL) and aphidicolin (500 ng/mL) on cell cycle phase.** Panels A-C are merged pseudo-colored images of the green (BrdU), red (p-Histone H3) and blue (Hoechst 33342) channels. Panel A shows untreated (normal) cells, Panel B colcemid-treated cells and Panel C aphidicolin-treated cells. Panel D shows the numeric data (percent positive cells) generated by the image analysis algorithm. Red arrows denote selected cells positive for phospho-Histone H3 (M phase), Green arrows show representative cells that stain positive for BrdU (S phase) and Blue arrows indicate selected cells negative for both BrdU and phospho-Histone H3. Note that green and red stains are mutually exclusive.



**Figure 3. Dose response curves for colcemid (top panels) and aphidicolin (bottom panels) treated cells.** As colcemid concentration increases, the percent cells blocked in M phase increases. At higher doses (starting at 125 ng/mL), non-specific effects generate a drop in the percent of phospho-Histone H3-positive cells in the bar chart (top right). Initially, increased aphidicolin had no effect on cells progressing through S phase. However, starting at approximately 125 ng/mL the percent cells in S phase declined, eventually reaching 0% for the 500 and 1000 ng/mL concentrations (bottom right).



**Figure 4. Ki-67 staining of HeLa cells.** Merged pseudo-colored image showing Ki-67 positive cells (red channel), phospho-Histone H3 positive cells (green channel) and nuclei stained with Hoechst 33342 (blue channel). Cells were treated with 10ng/ml of colcemid. Yellow arrows denote the co-staining of nuclei with phospho-Histone H3 (green) and Ki-67 (red). The Ki-67 stain in M phase cells always covers the entire nucleus. Cells that have a Ki-67 positive signal covering the entire nucleus but are not positive for phospho-Histone H3 (bold red arrow) are likely in the late G2 phase. The punctate red stain (red arrow) denotes cells in G1, S, and early G2 phases. Quiescent cells, in the G0 phase, are negative for Ki-67 and phospho histone H3 (blue arrow).

## Discussion

To assure homogenous responses in cellular assays, cells are often synchronized using multiple rounds of cell-cycle arrest and –release. This adds complexity and unreliability to the assay in question and is incompatible with many assays especially stem cell or primary cell assays. We therefore developed a versatile application that utilizes standard antibody treatment to identify the cell cycle phases and applied it to automated imaging. As a stand-alone application, the assay quantifies the degree to which cells are arrested in the different cycle phases after drug treatment making it useful in oncology and other fields where drug-responses are suspected to be dependent on the specific cell cycle phase. In addition, the nature of the exchangeable antibodies allows this assay to be multiplexed with other applications. For example, the Ki-67 stain could be substituted with a marker for pathway activation. Therefore this assay can work as a system of building blocks where users can choose their preferred dyes and antibodies in combination to generate higher dimensional data sets.

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