

Determination of Cell Cycle Time

Using protein and DNA staining to monitor cell stage in synchronized cells



Author

Paul Held, PhD
Agilent Technologies, Inc.

Abstract

Growing and dividing cells progress through a pattern of gene expression, nucleic acid duplication, and cell division referred to as the cell cycle. Uncontrolled proliferation is the hallmark of malignancy; therefore, the determination of the length of the cell cycle is a key element in elucidating the parameters of cell growth kinetics. This application note describes the use of immune-fluorescent-stained PC-3 cells synchronized by thymidine block or mitotically labeled asynchronous cells as a means to calculate cell cycle time.

Introduction

In proliferating cells, the cell cycle consists of four phases. Gap 1 (G1) is the interval between mitosis and DNA replication and is characterized by cell growth. The transition that occurs at the restriction point (R) in G1 commits the cell to the proliferative cycle. If the conditions that signal this transition are not present, the cell exits the cell cycle and enters G₀, a nonproliferative phase during which growth, differentiation, and apoptosis occur. Replication of DNA occurs during the synthesis (S) phase, which is followed by a second gap phase (G2) during which growth and preparation for cell division occurs. Mitosis and the production of two daughter cells occur in the M phase (Figure 1).¹ Dysfunction at any step in this regulatory process can result in abnormal cellular proliferation, which underlies many pathological conditions, most notably cancer and aging. One crucial measurement parameter that is part of understanding cell cycle progression is the length of time required to complete a full growth cycle.

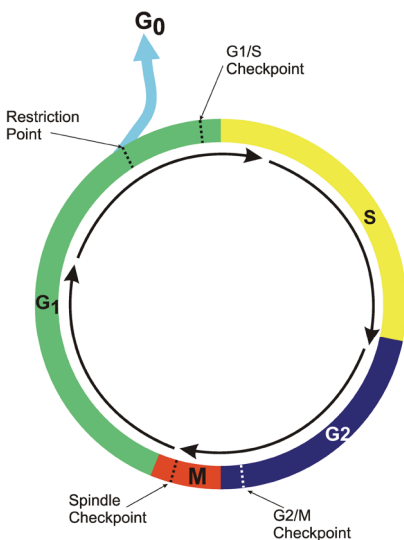


Figure 1. Regulation of the cell cycle.

The length of the cell cycle for cell cultures can be determined using either asynchronous or synchronized cell models. Asynchronous determination uses physically observable cell processes as a means to calculate the total cell cycle time. Synchronization methods use either cellular physical properties or the transient disruption of cell cycle progression as a means to isolate cells in specific phases of the cell

cycle. While asynchronous methods do not perturb the cells, it offers little information other than time-length in regards to cell cycle progression with treated cells. Synchronous methods can isolate cells in specific portions of the cell cycle, but have the potential to perturb normal cellular processes through drug treatments or physical trauma.

Mitotic index, which is the percentage of cells undergoing mitosis at any time, is the most common marker used for asynchronous determination. By knowing the time-length for mitosis, one can use the ratio of the length of mitosis to the entire the cell cycle time as a means to calculate doubling time. The probability of observing mitotic cells is, theoretically, the ratio of the duration of mitosis to the entire cell cycle. Assuming that the duration of mitosis is relatively constant; the extreme hypothetical cases are 0 or 1, in which there are no mitotic cells or no interphase cells, respectively. In reality, the ratio is in-between these extremes. When mitotic duration occupies half of the cell cycle, the probability of observing mitotic cells is 0.5.

Synchronization methods isolate cells by their physical characteristics or through chemical means. Methods based on the physical characteristics, such as centrifugal elutriation⁵, which separates cells based on cell size or mitotic shake, which isolates based on cell attachment⁶, do not expose cells to chemical agents, but can require expensive specialized instrumentation or can be labor-intensive. Several chemical agents have been shown to reversibly stall cells in specific stages of the cell cycle. The HMG-co A reductase inhibitor, lovastatin has been shown to block cells in G₁.⁷ Likewise nocodazol, which prevents microtubule assembly⁸, and RO3306, which inhibits CDK1 activity⁹ block cells in G₂.

While there are a number of chemical means to synchronize mammalian cells in culture, one of the most widely used method is thymidine block. Excess thymidine generates negative feedback on the production of deoxycytidine triphosphate (dCTP) from cytidine-5'-phosphate. The lack of dCTP results in cell cycle arrest in S-phase. Because cells will be arrested throughout S-phase when the inhibitor is applied, a second round of thymidine, with a release in between, is used to induce more cells to arrest in early S-phase.

The basis for thymidine block involves the enzyme ribonucleotide reductase (RNR). Under normal conditions the RNR complex reduces purine (ADP and GDP) and pyrimidine (CDP and UDP) ribonucleotide diphosphates to their deoxy nucleotide forms, which then contribute to the

deoxytrinulceotide (dNTP) pools, dATP, dGTP, dTTP, and dCTP respectively (Figure 2). This enzyme complex is regulated in a bimodal fashion. Binding to the ATP cone region in the α -site regulates overall activity by turning the enzyme on or off. Binding of ATP to this site results in the enzyme being switched on, while binding of dATP will inhibit enzyme activity. Thus the cellular concentration ratio of ATP to dATP regulates overall activity. Secondly, the presence of high concentrations of dTTP and dGTP act as modulators for the production of specific nucleotides. Most significant is the allosteric inhibition by dTTP to the conversion of the purines UDP and CDP to dUDP and dCDP respectively. With thymidine blockade, large amounts of thymidine (dT) are present, resulting in the production of dTTP by the enzyme thymidine kinase, which is part of an independent salvage pathway (Figure 2). Unlike thymidine, there is no salvage pathway for deoxycytidine (dC) for the production of deoxycytidine triphosphate (dCTP); in the presence of high dTTP levels the allosteric inhibition of the RNR enzyme results in low levels of dCTP, leading to the cessation of DNA replication.

This study used both synchronized as well as asynchronous cells to calculate cell cycle time in PC-3 cells.

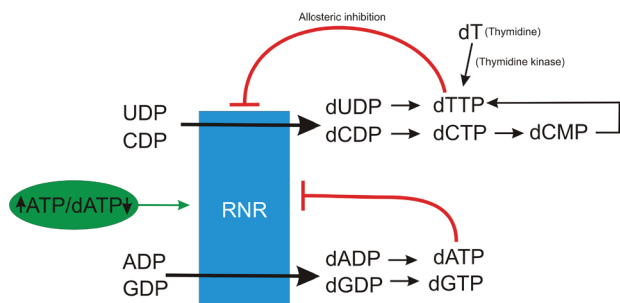


Figure 2. Schematic regulation of ribonucleotide reductase by dNTP pools.

Materials and methods

Cell culture

PC-3 cells were cultured in Hams F12K media supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO₂. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluence. For experiments, cells were plated into Corning 3904 black-sided clear-bottom 96-well microplates.

Thymidine double block

PC-3 cells were seeded into Corning 3904 plates at a density of 4,000 cells per well in a volume of 100 μ L and allowed to attach overnight at 37 °C, 5% CO₂ in a humidified environment. The following day, thymidine was added to all the wells for a final concentration of 2 mM. Cells were treated for 16 hours then released by washing 1x with fresh media, followed by 100 μ L of media without thymidine. Cells were allowed to grow for 9 hours, after which thymidine was added to a final concentration of 2 mM for a second time. Cells were thymidine-treated for 16 hours and released as described above. Plates were immediately loaded into an Agilent BioTek BioSpa 8 automated incubator for cell cycle progression.

Cell cycle progression

Following the final thymidine block release, a BioSpa 8 system was used to control experimental timing and maintain the necessary environmental control. Individual strips of two plates were fixed with 4% PFA for 10 minutes at 1-hour intervals using an Agilent BioTek MultiFlo FX multimode dispenser attached to the system. Fixed cells were maintained with 200 μ L of PBS until the completion of the experiment.

Staining

All cell washes and reagent additions for cell fixation and fluorescent staining were carried out using an Agilent BioTek EL406 washer dispenser. Cells were washed 2x with 200 μ L of PBS (NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM, KH₂PO₄ 7.4 mM). Cells were permeabilized and nonspecific antibody binding blocked for 120 minutes with 50 μ L of PBS with 3% goat serum and 0.3% Triton X-100. After blocking, rabbit antigeminin (ab195047, Abcam) or a rabbit anti-phospho-histone H3 antibody (53348S, Cell Signaling Technologies) were added (1:200 dilution in PBS, 1% BSA, 0.3% Triton X-100) and the plate incubated at 5 °C overnight. The plate was washed 2x with PBS and the secondary Alexa Fluor 488 labeled fluorescent antirabbit IgG antibody (part number A11008, Life Technologies) (1:200 dilution in PBS, 1% BSA, 0.3% Triton X-100) was added and allowed to incubate for 120 minutes at room temperature followed by 3x wash with PBS. Cells were counterstained for 15 minutes with 50 μ L of working solution of Hoechst 33342 (10 μ M) followed by 2 washes with 200 μ L of DPBS. After aspiration, 250 μ L of PBS was added to all wells and the plates sealed using an optically clear adhesive plate sealer.

Imaging

Montage (6 × 6) images of each well were obtained with an Agilent BioTek Cytation 5 cell imaging multimode reader configured with DAPI and GFP LED cubes using a 10x objective. The DAPI cube is configured with a 377/50 excitation filter and a 447/60 emission filter in conjunction with a 409 nm cut-off dichroic mirror. The GFP cube was configured with a 469/35 excitation filter and a 525/39 emission filter with a 497 nm cut-off dichroic mirror. The imaging parameters, set using Agilent BioTek Gen5 microplate reader and imager software, used a LED intensity setting of 5, an integration time of 72 ms, and a gain of 0 for DAPI and a LED intensity setting of 5, an integration time of 72 ms, and a gain of 0 for GFP.

Images were automatically stitched into a single file using Gen5 software. After stitching, montage images were preprocessed to subtract background fluorescence before analysis (Table 1). Primary mask analysis identified cellular nuclei using a threshold of 5,000 and a lower and upper size limitation of 5 and 50 μm , respectively. Gen5 histogram analysis relating total nuclear fluorescence to object count were used to set upper and lower fluorescent signal thresholds, identifying G1 and G2 subpopulations. The intervening region between the G1 and G2 was used to identify S-phase cells. In addition to cell cycle phase subpopulation based on DAPI nuclear staining, p-Histone H3 and geminin-positive nuclei were identified using a mean GFP fluorescence threshold of 10,000.

Analysis

Table 1. Image processing and image analysis parameters for protein determination.

Parameter	Value
Stitching	
Channel	DAPI
Size	6,731 × 4,843 (62.18 Mb)
Crop Edges	Yes
Reduce Image	40%
Preprocessing	
Background	Dark
Flattening	Auto
Rolling Ball Diameter	746 μm 462 pixels
Image Smoothing	0
Object Counting	
Channel	Ts[Stitched[DAPI 377,447]
Threshold	
Value	5,000
Background	Dark
Split Touching Objects	Yes
Fill Holes in Masks	Yes
Advanced Options	
Smoothing	0
Background	5% lowest pixels
Object Selection	
Minimum Object Size	5 μm
Maximum Object Size	50 μm
Include Edge Objects	No
Entire Image	Yes
Subpopulations	
(DNA)	Obj Integral (DAPI)
G1	6.91×10^5 to 1.8×10^6
S	1.8×10^6 to 2.3×10^6
G2	2.3×10^6 to 3.41×10^6
(Protein)	Obj Mean (GFP)
	Geminin >10,000
	p-Histone H3 >10,000

Results and discussion

Cell cycle progression in synchronized cells was monitored by DNA content and protein expression. Cell nuclei stained with Hoechst 33342 fluoresce in relationship to the amount of nucleic acid present. Cells in the G2-phase of the cell cycle have duplicated their DNA before mitosis and have twice the nucleic acid as cells in G1, while cells in the process of DNA synthesis (S-phase) exhibit fluorescence in between that of G1 and G2. This cycling can be observed in Figure 3, where the percentage of cells in specific G1, S, and G2 subpopulations oscillates with time. Peaks in G1 cell percentages can be seen at 6 and 26 hours post thymidine release, while G2 cells peak at 16 and 36 hours. S-phase cell subpopulations peak at 11 and 31 hours after release. These data suggest that the cell cycle time of PC-3 cells following thymidine release is approximately 20 hours.

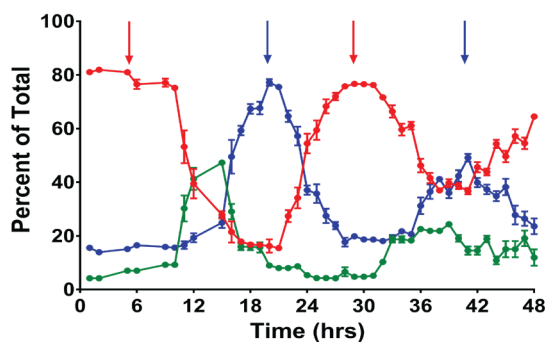


Figure 3. Cell cycle progression of synchronized PC-3 cells. PC-3 cells synchronized with double thymidine block were fixed and stained at timed intervals after release. The percentages of cells in G1, S, and G2 determined by nuclear staining analysis were plotted versus time. Red and blue arrows denote G1 and G2 percentage peaks, respectively. Data points represent the mean of eight determinations.

Protein expression also fluctuates with the cell cycle. Using specific antibodies, the presence or absence of geminin and p-histone H3 proteins in the nuclei can be monitored (Figure 4). Figure 5 demonstrates the marked difference in protein levels of these proteins at 4 and 24 hours after thymidine release. Image analysis of samples made over 48 hours shows the temporal and cyclical relationship of geminin and p-histone H3. Geminin can be detected in high percentages of nuclei during G1 and G2 phases of the cell cycle, while almost absent during mitosis. Phospho-histone H3 is detected almost exclusively during mitosis (Figure 5).

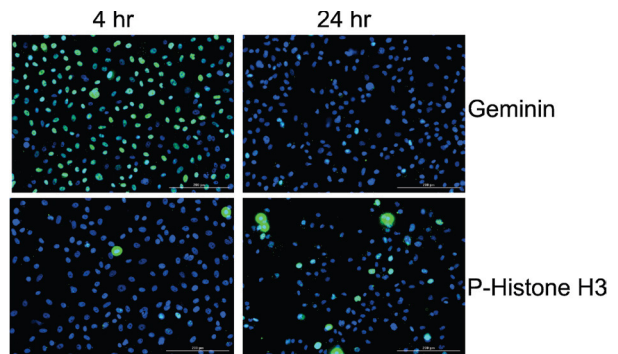


Figure 4. Geminin and p-histone staining in synchronized PC-3 cells. Synchronized PC-3 cells were fixed at 4 and 24 hours after thymidine release. Cells were treated with rabbit antigeminin and rabbit anti p-histone H3 primary antibodies in parallel reaction wells. Specific targets were visualized by staining with Alexa Fluor 488 labeled goat antirabbit IgG secondary antibodies. Cells were counterstained with Hoechst 33342 to identify nuclei. Images were captured using a 20x objective with a 2 x 2 stitched montage.

As with DNA content, protein expression peaks suggest a cell-cycle time of 20 hours with synchronized PC-3 cells. Geminin levels show elevated levels at approximately 14 and 34 hours after thymidine release, while p-histone H3 levels peak at 24 and 44 hours after release (Figure 5).

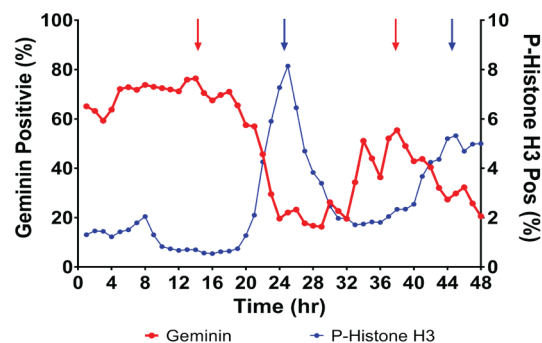


Figure 5. Temporal relationship between geminin and p-histone H3 in synchronized PC-3 cells. PC-3 cells synchronized with double thymidine block were fixed at timed intervals after release. Fixed cells were stained with rabbit monoclonal antibodies specific for geminin and p-histone H3 in parallel wells. A fluorescently tagged antirabbit IgG antibody was used to visualize the specific target. Cells were counterstained with Hoechst 33342 and to identify nuclei. Data analysis was performed on 6 x 6 montage images made using a 10x objective. Red and blue arrows denote geminin and p-histone H3 percentage peaks, respectively. Data points represent the mean of four determinations.

The specificity of p-histone H3 (ser 10) towards mitotic cells makes it a good candidate to assess the mitotic index of a cell population through immunostaining.³ Only cells in mitosis have the phosphorylated modification of serine 10 on histone H3 present. Located in the cell nucleus, identification of cells with this protein modification are easy to identify with immunostaining in conjunction with a nuclear counter stain (Figure 6). As demonstrated in Figure 7, the percentage of mitotic cells increases dramatically after release from thymidine block. Interestingly, the timing delay between release and peak in the percentage of cells in mitosis can vary by as much as 3 to 4 hours in different experiments. In both experiments, the degree of synchrony appears to be similar. Additionally, a second mitotic index peak is observed as the cells pass through a second round of mitosis; this peak has a similar temporal relationship, with a time between mitotic peak levels of 20 hours.

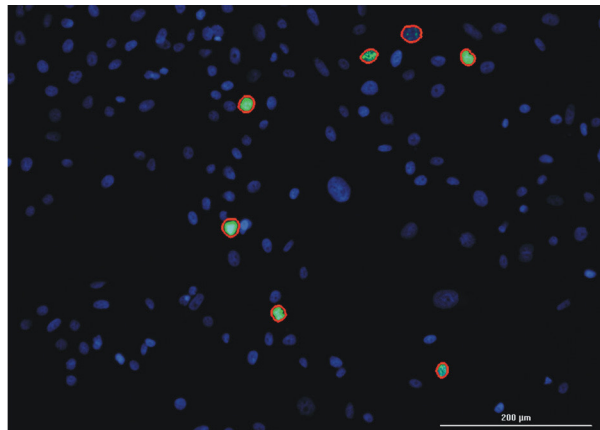


Figure 6. Antibody staining of p-histone H3 PC-3 cells. PC-3 cells undergoing mitosis were with an anti-p-histone H3 antibody and counterstained with Hoechst 33342 to identify nuclei. Positive nuclei are denoted with a red tracing.

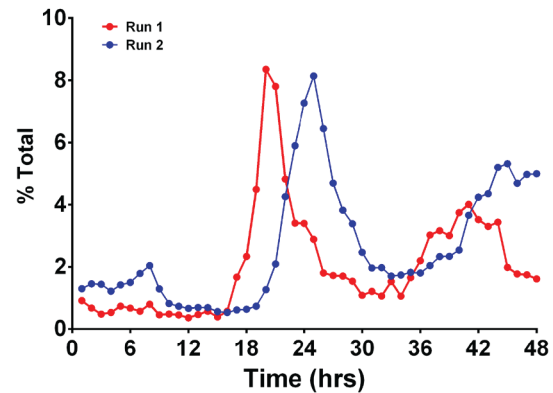


Figure 7. Comparison of PC-3 cell mitotic index from separate synchronization experiments. PC-3 cells synchronized with double thymidine block were fixed at timed intervals after release in two separate experiments. Fixed cells were stained with an AlexaFluor 488 labeled rabbit monoclonal antibody specific p-histone H3 and counterstained with Hoechst 33342 and to identify nuclei. Data analysis was performed on 6 × 6 montage images made using a 10x objective. Positive nuclei had a mean GFP signal greater than 10,000 after blank subtraction. Each data point represents the mean of four determinations.

The mitotic index of asynchronous populations can be used to determine cell cycle time. Using p-histone H3 as an indicator of mitosis, Figure 8 demonstrates the effect of serum concentration on the mitotic index of asynchronous populations of PC-3 cells. With a decrease from the normal 10% serum to 0.1%, the mitotic index drops from 5.7% to 3.6% after 24 hours. Above 10% serum, the mitotic index no longer increased (data not shown). The typical length of time for mitosis in mammalian cells in tissue culture has been reported to be 60 minutes.⁴ Based on the determined mitotic index and the length of mitosis, a cell cycle length of 17.5 hours (Table 2) is calculated from the probability of observing mitosis. This is in close agreement with the values determined using DNA content or protein expression.

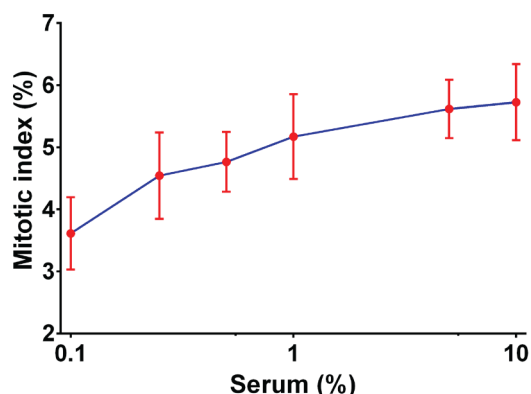


Figure 8. Effect of serum concentration on mitotic index. PC-3 cells were washed with serum free media twice and treated with media with various concentrations of fetal bovine serum for 24 hours. Cells were fixed and treated with a rabbit anti-p-histone H3 primary antibody. The primary antibody was visualized with an AlexaFluor 488 labeled goat anti-rabbit IgG secondary antibody. Cells were counterstained with Hoechst 33342 to identify cell nuclei. Data points represent the mean and standard deviation of 16 determinations.

Table 2. Effect of serum on PC-3 cell cycle time in asynchronous cultures.

Cell Cycle Time		
FBS Conc. (%)	Mitotic Index	Cycle Time (hrs)
0.1	3.6 ± 0.6	27.7
0.25	4.5 ± 0.7	22.1
0.5	4.8 ± 0.5	21.0
1.0	5.2 ± 0.7	19.3
5.0	5.6 ± 0.5	17.81
10	5.7 ± 0.6	17.5

The concentration of serum present in asynchronous cultures influences the length of the cell cycle for PC-3 cells in culture. A 10-fold reduction of serum concentration from 10% to 1% increased the calculated cell cycle time by nearly 2 hours (11%). Further reduction of the serum concentration from 1% to 0.1% increased the cell cycle time by 8 hours.

Conclusion

The progression of cellular growth through the cell cycle is a highly regulated process. To investigate the different processes at different stages of the cell cycle, various means have been developed to synchronize the passage of cells through the cell cycle as a means to enrich cells in each stage. In these experiments, double thymidine block was used to enrich cells at the G1/S border phase. Upon release, they transition to S-phase and begin replicating nuclear DNA, which is observed as an increase in fluorescence from a dsDNA-specific dye. With complete DNA replication, the cell contains twice the total DNA it had in G1-phase, which is reflected in a two-fold increase in fluorescence. With mitosis and cytokinesis, the DNA content is halved and each daughter cell returns to G1. The use of PC-3 cells can cloud the degree of synchronization due to its polyploidy. Cells with extra chromosomes will have a greater degree of fluorescence than cells having a normal chromosome number. With enough chromosome duplication, cells in G1 (pre S-phase) can contain enough excess DNA as to be interpreted as being in G2, based on nuclear content. This can be observed in Figure 2, where almost 20% of the cells released from thymidine block are denoted as being in G2, based on nuclear content.

These data indicate that image-based detection of DNA or specific proteins can be used to monitor cell cycle progression. Subpopulation analysis of total fluorescence of fluorescently stained nuclei can partition cells into G1, S, and G2 phases of the cell cycle based on DNA content. Likewise, cells in mitosis can be differentiated from G1, S, and G2 by differential expression of p-histone H3. Regardless of the metric used, the cell cycle time for PC-3 cells was approximately 20 hours. It is interesting to note that the use of thymidine block lengthens the cell cycle. Despite the cells being cultured in 10% FBS during and after release from the block, the calculated cell cycle time was similar to asynchronous cells grown in 1% serum.

The Agilent BioTek BioSpa live cell analysis system with a liquid handler in conjunction with an Agilent BioTek Cytation 5 cell imaging multimode reader and Agilent BioTek Gen5 microplate reader and imager software are an ideal combination of value and performance. The BioSpa system maintains environmental control of temperature, CO₂, and humidity levels during the experimental run. At various time intervals, the system transports a plate to a liquid handler to remove media, add fixative, and wash with PBS to capture a snapshot of the cells through fluorescent staining. At the completion of the experiment, multiple plates are stained as a group and imaged using a Cytation 5. The Cytation 5 is capable of making multiple images to form a montage, allowing a larger cell sampling for each well. The multiple tiles are stitched into a single image file before analysis. Preprocessing of the image subtracts background, eliminating any well-to-well differences before object identification. Gen5 histogram analysis of object information allows the researcher to visually apply threshold gates to define G1 and G2 populations based on nuclear staining. A quick check of multiple wells can be used to confirm goodness of the fit. The percentages of each subpopulation are then calculated and reported. Identification of specific proteins through antibody staining can be analyzed concurrently with DNA assessment.

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