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Analysis of Cell Cycle by Flow Cytometry

Piotr Pozarowski and Zbigniew Darzynkiewicz

Summary

Described are four widely used procedures to analyze the cell cycle by flow cytometry. The first two are based on univariate analysis of cellular DNA content following cell staining with either propidium iodide (PI) or 4',6'-diamidino-2-phenylindole (DAPI) and deconvolution of the cellular DNA content frequency histograms. This approach reveals distribution of cells in three major phases of the cycle (G_1 vs S vs G_2/M) and makes it possible to detect apoptotic cells with fractional DNA content. The third approach is based on the bivariate analysis of DNA content and proliferation-associated proteins. The expression of cyclin D, cyclin E, cyclin A, or cyclin B1 vs DNA content is presented as an example. This approach allows one to distinguish, for example, G_0 from G_1 cells, identify mitotic cells, or relate expression of other intracellular proteins to the cell cycle position. The fourth procedure relies on the detection of 5'-bromo-2'-deoxyuridine (BrdU) incorporation to label the DNA-replicating cells.

Key Words: Flow cytometry; cellular DNA content; cyclins; BrdU incorporation; mitosis.

1. Introduction

A variety of flow or cytometric methods to analyze the cell cycle progression have been developed over the past three decades. These methods can be grouped into three categories:

1. In the first are the methods that rely on a single time point ("snapshot") cell measurement. This analysis may be either univariate, generally based on measurement of cellular DNA content alone ***(1)***, or multivariate (multiparameter), when in addition to DNA content another cell attribute is measured ***(2–4)***. The additional attribute is generally a metabolic or molecular feature that correlates with a rate of cell progression through the cycle or is a marker the cell proliferative potential or quiescence. The single time-point measurement reveals the percentage of cells in G_1 vs. S vs. G_2/M, but it does not provide information on cell cycle kinetics. The duration of each phase, however, can be estimated from the percentage of cells in this phase if the length of the cell cycle (or the doubling time of cells in the culture) is known.

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2. In the second group are the methods that rely on time-lapse measurements of cell populations synchronized in the cycle, or whose progression through the cycle was halted by the agent arresting them at a specific point of the cycle. These methods reveal kinetics of cell progression through the cycle. An example of such methods is the stathmokinetic approach, where cells are arrested in mitosis, e.g., by vinblastine or colcemide, and the rate of cell entrance into mitosis ("cell birth" rate) is estimated from the slope representing a cumulative increase in percentage of mitotic cells as a function of the time of the arrest ***(5)***.
3. Methods of the third category are based on the detection of incorporation of the thymidine analog 5'-bromo-2'-deoxyuridine (BrdU), often combined with DNA content measurements. They may be either single time-point measurements or use the time-lapse strategy. The incorporated BrdU is detected either cytochemically, based on the use of the DNA dyes, such as Hoechst 33258, whose fluorescence is quenched by BrdU ***(6)***, or immunocytochemically, using fluoresceinated BrdU-antibodies ***(7)***. The time-lapse measurement of the cohort of BrdU-labeled cells allows one to estimate their rate of progression through different points of the cell cycle ***(8)***.

Only a few methods are presented in this chapter. In **Subheadings 3.1.** and **3.2.**, two methods are presented that rely on the univariate analysis of cellular DNA content. The first utilizes propidium iodide (PI) as the DNA fluorochrome and requires blue light as the excitation source (e.g., 488 nm argon ion laser). Because PI also stains double-stranded RNA, the latter is removed by the addition of RNase A to the staining solution. In the second protocol, the UV-light-excitable 4',6'-diamidino-2-phenylindole (DAPI) is used as a DNA-specific fluorochrome. Cell staining with DAPI is simpler, as it does not require incubations with RNase, and is generally preferred when a cytometer that has a UV light excitation source is available. In **Subheading 3.3.**, the analysis of cellular DNA content, combined with the expression of proliferation-associated proteins, is described. The method is illustrated by demonstrating the characteristic expression of cyclins D1, E, A, or B1 in normal cells, but this approach may be used to measure the expression of other intracellular antigens in relation to the cell cycle position. The immunocytochemical detection of incorporated BrdU, combined with the analysis of DNA content, is presented in **Subheading 3.4.**. More detailed descriptions of these and other methods, their modifications and applicability to different cell systems, as well as advantages and limitations, are provided in numerous chapters of the methodology books specifically devoted to the cell cycle and cytometry ***(9–15)***.

2. Materials

1. Flow cytometer. A variety of models of flow cytometers of different makers can be used to measure cell fluorescence following staining according to the procedures listed under **Subheading 3.1.**. The manufacturers of the most common flow

cytometers are Becton Dickinson Immunocytometry Systems, Beckman/Coulter Inc., DACO/Cytomation, and PARTEC GmbH.
2. The software used to deconvolute the DNA content frequency histograms, to estimate the proportions of cells in the respective phases of the cycle, is available from Phoenix Flow Systems and Verity Software House (*see* **Note 1**).
3. Centrifuge that can accommodate 5-mL tubes.
4. PI staining solution: 0.1% (v/v) Triton X-100, 10 µg/mL PI (Molecular Probes, Inc.), and 100 µg/mL DNase-free RNase A in PBS.
5. PBS (phosphate buffered saline, e.g. Dulbecco PBS): 136.9 m*M* NaCl, 2.68 m*M* KCl, 8.1 m*M* Na_2HPO_4, 0.9 m*M* $CaCl_2$, 0.49 m*M* $MgCl_2$.
6. DAPI staining solution: 0.1% (v/v) Triton X-100 and 1 µg/mL DAPI (Molecular Probes, Inc.) in PBS.
7. Monoclonal or polyclonal antibodies (Abs) applicable to cell cycle analysis, including cyclin Abs (provided, e.g., by DACO Corporation, Sigma Chemical Co., Upstate Biotechnology Incorporated, B.D. Biosciences/PharMingen, and Santa Cruz Biotechnology, Inc.).
8. Cell permeabilizing solution: 0.25% Triton X-100, 0.01% sodium azide in PBS.
9. Rinsing solution: 1% bovine serum albumin (BSA), 0.01% sodium azide in PBS.
10. DNA denaturation buffer: 0.1 m*M* Na-EDTA in 1 m*M* Na-cacodylate; adjust pH to 6.0. To make 0.2 *M* stock solution of cacodylate buffer, dissolve 42.8 g $Na(CH_3)_2\ As_2{\cdot}3H_2O$ in 100 mL H_2O, take 50 mL of this solution, add to it 29.6 mL of 0.2 *M* HCl, and adjust volume to 200 mL with H_2O.
11. Diluting buffer: 0.1% Triton X-100, 0.5% (w/v) BSA in PBS.
12. 0.2 *M* phosphate buffer, pH 7.4 (mixture of 81 vol of 0.2 *M* Na_2HPO_4 with 19 vol of 0.2 *M* KH_2PO_4).

3. Cellular DNA Content Measurement

3.1. Cell Staining With PI

1. Suspend approx 10^6 cells in 0.5 mL of PBS. Vortex gently (approx 5 s) or gently aspirate several times with a Pasteur pipet to obtain a mono-dispersed cell suspension, with minimal cell aggregation.
2. Fix cells by transferring this suspension, with a Pasteur pipet, into centrifuge tubes containing 4.5 mL of 70% ethanol, on ice. Keep cells in ethanol for at least 2 h at 4°C. Cells may be stored in 70% ethanol at 4°C for weeks.
3. Centrifuge the ethanol-suspended cells for 5 min at 300*g*. Decant ethanol thoroughly.
4. Suspend the cell pellet in 5 mL of PBS, wait approx 30 s and centrifuge at 300*g* for 5 min.
5. Suspend the cell pellet in 1 mL of PI staining solution. Keep in the dark at room temperature for 30 min, or at 37°C for 10 min.
6. Transfer sample to the flow cytometer and measure cell fluorescence. Maximum excitation of PI bound to DNA is at 536 nm, and emission is at 617 nm. Blue (488 nm) or green light lines of lasers are optimal for excitation of PI fluorescence.

Emission is measured using the long-pass 600- or 610-nm filter (for data acquisition, interpretation, and possible pitfalls, *see* **Notes 1–5**).

3.2. Staining With DAPI

1. Suspend the cell pellet in 1 mL of DAPI staining solution. Keep in the dark, at room temperature, for 10 min.
2. Transfer the sample to the flow cytometer and measure cell fluorescence. Maximum excitation of DAPI bound to DNA is at 359 nm, and emission is at 461 nm. For fluorescence excitation, use the available UV light laser line at the wavelength nearest to 359 nm. When a mercury arc lamp serves as the excitation source, use a UG1 excitation filter. A combination of appropriate dichroic mirrors and emission filters should be used to measure cell fluorescence at wavelengths between 450 and 500 nm (for data acquisition, interpretation, and possible pitfalls, *see* **Notes 1–5**).

3.3. Cellular DNA Content and Expression of Proliferation-Associated Proteins

1. Fill 5-ml polypropylene tubes with 4.5 mL of methanol (or 70% ethanol; *see* **Note 6**). Keep tubes on ice.
2. Suspend 1–2 × 10^6 cells in 0.5 mL of PBS. Fix the cells by transferring this suspension with a Pasteur pipet into the ice-cold methanol tube. Keep cells in the fixative at –20°C at least overnight (the cells can be stored in the fixative at – 20°C for days).
3. Centrifuge at 300*g* for 3 min. Resuspend the cell pellet in 5 mL PBS. Keep for 5 min at room temperature. Spin at 200*g* for 5 min.
4. Resuspend the cells in 0.5 mL of the permeabilizing solution. Keep at room temperature for 5 min. Centrifuge as above.
5. Resuspend cell pellet in 100 µL of the rinsing solution that contains the primary Ab. Follow instructions supplied by the vendor regarding the final titer of the supplied antibody (0.5–1.0 µg of the Ab per 10^6 cells suspended in 100 µL is generally optimal). Incubate 60 min at room temperature with gentle agitation or overnight at 4°C (*see* **Notes 6** and **7**).
6. Add 5 ml of the rinsing solution. Centrifuge at 300*g* for 5 min.
7. Use the isotype immunoglobulin as a negative control (*see* **Note 7**). Process as in **steps 5** and **6**.
8. Resuspend cells in 100 µL of rinsing solution that contains the fluoresceinated secondary Ab, generally at a final 1:20 to 1:40 dilution. Incubate at room temperature for 30–60 min, gently agitating.
9. Add 5 mL of the rinsing solution, centrifuge at 300*g* for 5 min.
10. Suspend the cell pellet in 1 mL PBS containing 5 µg of PI and 100 µg of DNase-free RNase A. Keep in the dark at room temperature for 1 h.
11. Transfer the cells to the flow cytometer. Use blue light (488 nm laser line) for fluorescence excitation. Measure cell fluorescence in green (FITC, 530 ± 20 nm) and red (PI, >620 nm) light wavelength.

12. Analyze the data (*see* **Notes 7–9**).
    Note: If the primary Ab is fluorochrome-tagged, skip **steps 8** and **9**.

3.4. Detection of Incorporated BrdU and Cellular DNA Content

3.4.1. Thermal Denaturation of DNA

1. Incubate the cells with 10–50 µ*M* BrdU in the dark (*see* **Note 10**).
3. Fix the cells in suspension in 70% ethanol.
4. Centrifuge the cells ($1–2 \times 10^6$) at 200*g* for 5 min, resuspend cell pellet in 1 mL of diluting buffer containing 100 µg/mL of RNase A, and incubate at 37°C for 30 min.
5. Centrifuge cells (300*g*, 5 min) and suspend cell pellet in 1 mL of ice-cold 0.1 *M* HCl containing 0.1% Triton X-100. After 1 min, centrifuge the cells again. Drain thoroughly and resuspend in 5 mL of DNA denaturation buffer. This step is designed to dissociate histones from DNA in order to increase DNA susceptibility to denaturation.
6. Centrifuge the cells again and resuspend the cell pellet in 1 mL of DNA denaturation buffer.
7. Heat cells at 90 or 95°C for 5 min, then place on ice for 5 min (*see* **Note 11**).
8. Add 5 mL of diluting buffer and centrifuge at 300*g* for 5 min.
9. Drain well and suspend the cells in 100 µL of BrdU Ab dissolved in diluting buffer, for 30 min at room temperature (follow the instructions provided by the supplier regarding the dilution, time, and temperature of incubation with anti-BrdU).
10. Add 5 mL of diluting buffer and centrifuge as above.
11. Suspend the cells in 100 µL of FITC-labeled goat anti-mouse IgG (dissolved in diluting buffer), and incubate for 30 min at room temperature. If the primary BrdU Ab was FITC-labeled, skip this step.
12. Add 5 mL of diluting buffer, centrifuge, drain, and resuspend the cells in 1 mL of this buffer containing 5 µg/mL of PI.
13. Measure the BrdU-associated green fluorescence and DNA-associated red fluorescence as described in **Subheading 3.3., step 12** (*see* Note 10)**.**

3.4.2. Denaturation of DNA by HCl

1. Follow **steps 1–4** as described above for thermal denaturation of DNA (**Subheading 3.4.1.**).
2. Centrifuge the cells (300*g*, 5 min) and resuspend cell pellet in 1 mL of 2 *M* HCl (*see* **Note 11**). After 20 min at room temperature, add 5 mL of PBS, centrifuge, and drain well.
3. Resuspend the cells in 5 mL of 0.2 *M* phosphate buffer at pH 7.4, to neutralize any traces of the remaining HCl.
4. Centrifuge the cells at 300*g* for 5 min.
5. Follow **steps 8–13** as described above for thermal denaturation of DNA (**Subheading 3.4.1.**).

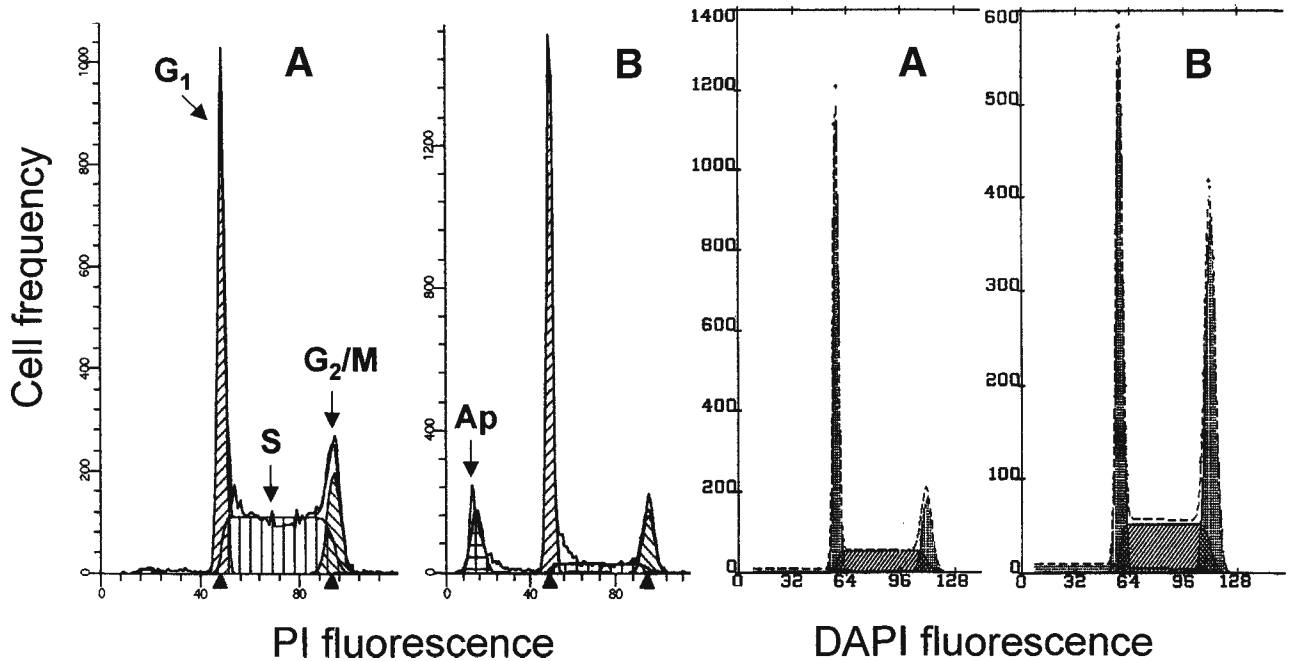

Fig. 1. Typical DNA content frequency histograms representing cells from the untreated cultures **(A)** and from the cultures treated with the drugs that affect the cell cycle distribution and induce apoptosis **(B)**. The cells were stained with PI or DAPI, according to the presented protocols, respectively. Fluorescence of the PI-stained cells was measured using FACScan (Becton Dickinson Immunocytometry Systems) and ModFit deconvolution software. DAPI flourescence was measured using EPICS ELITE cytometer (Beckman/Coulter, Inc.) and the histograms were plotted by the MultiCycle (Phoenix Flow Systems) program. Both software programs provide the estimate of percentage of cells with fractional DNA content (apoptotic cells: Ap) and cells in G_1, S, and G_2/M phases of the cycle.

4. Notes

1. The data acquisition software of most flow cytometers/sorters allows one to record the fluorescence intensities (the integrated area of the electronic pulse signal) of 10^4 or more cells per sample. The data are presented as cellular DNA content frequency histograms **(Fig. 1)**. The data analysis software packages ***(16)*** that deconvolute the frequency histograms to obtain percentage of cells in $G_{0/1}$, S, and G_2 + M are either included with the purchase of the flow cytometer or are commercially available from other vendors, as listed in **Subheading 2.**. For details of the procedures of deconvolution of DNA content frequency histograms, see **ref. *16***.
2. All cells in G_1 have a uniform DNA content, as do cells in G_2/M; the latter have twice as much DNA as G_1 cells. Under ideal conditions of DNA staining, the fluorescence intensities of all G_1 or G_2/M cells are expected to be uniform, and after analog-to-digital conversion of the electronic signal from the photomultiplier (representing their fluorescence intensity), to have uniform numerical values, respectively. In practice, however, the G_1 and G_2 cell populations are represented on frequency histograms by peaks of various width. The coefficient of variation (CV) of the mean value of DNA-associated fluorescence of the G_1 population (width of the peak) is a reflection of the accuracy of DNA content measurement, and should not exceed 6%. Improper staining conditions, instrument mis-adjustment, and the presence of a large number of dead or broken cells, will all result in high CV of the G_1 cell populations.
4. Apoptotic cells often have fractional DNA content due to the fact that the fragmented (low MW) DNA undergoes extraction during the staining procedure. Some cells also lose DNA (chromatin) by shedding apoptotic bodies. Thus. only a fraction of the DNA remains within apoptotic cells. They are then represented on the DNA content frequency histograms by the "sub-G_1" peak **(Fig. 1)**. Commercially available software packages to deconvolute DNA histograms are able to identify and quantity the "sub-G_1" cell population.
5. If the duration of the cell cycle (or the cell doubling time) is known, the length of each phase can be estimated from the percentage (fraction) of cells in that phase. For example, during the exponential phase of cell growth, the duration of G_1 (T_{G1}) can be calculated from the equation:

$$T_{G1} = [T_C \times \ln(f_{G1} + 1)]/\ln 2$$

   where T_C is duration of the cell cycle and f_{G1} is the fraction of cells in G_1. T_C, with some approximation, equals the cell doubling time in cultures that can be estimated from growth curves.
5. The critical steps for immunocytochemical detection of intracellular proteins are cell fixation and permeabilization. The fixative is expected to stabilize the antigen *in situ* and preserve its epitope in a state where it remains reactive with the Ab. The cells have to be permeable to allow access of the Ab to the epitope. The choice of the optimal fixative and permeabilizing agent vary, primarily depending on the intracellular antigen, less on the cell type. General strategies of cell fixation, permeabilization, and stoichiometry of antigen detection are discussed

by Jacobberger ***(17)***. Cold methanol appears to be optimal for the detection of D-type cyclins. For cyclins E, A, and B1, 70% cold ethanol is equally good.

6. Another critical point is the choice of a proper Ab. Often, the Ab applicable to immunoblotting fails in immunocytochemical applications, and vice versa. This may be the result of differences in the *in situ* accessibility of the epitope or differences in the degree of denaturation of the antigen on the immunoblots, compared to that within the cell. Some epitopes may not be accessible *in situ* at all, while the accessibility of others may vary, depending on their functional state—for example, owing to chemical modifications (e.g., phosphorylation or acetylation) or steric hindrance. Since there is strong homology between different cyclin types, cross-reactivity also may be a problem. Because commercially available Abs may differ in specificity, degree of cross-reactivity, and so on, it is essential to provide detailed information (vendor and the hybridoma clone number) of the reagent used in the study. Although the isotypic IgG is commonly used as a negative control, some IgG preparations may show reactivity to various cell constituents. The optimal negative control would be the "knock-out" cell line (if available), where the gene coding for the studied protein has been deleted.
7. Expression of proliferation-associated proteins varies during the cell cycle **(Fig. 2)** and is often different in cycling and quiescent cells. The most widely used markers are the proliferating cell nuclear antigen (PCNA) ***(4)***, the antigen detected by Ki-67 antibody ***(3)***, and cyclins D, E, A, and B ***(2)***.
8. Cyclins are the key components of the cell cycle progression machinery ***(18)***. The timing of expression of several cyclins—in particular cyclins D, E, A, and B—during unperturbed growth of normal cells is discontinuous, occurring as discrete sections of the cell cycle **(Fig. 2)**. This periodicity in cyclin expression provides cell cycle landmarks that can be used to subdivide the cell cycle into several subcompartments, additional to the subdivision into four major phases ***(2)***. Furthermore, bivariate analysis of cyclin expression vs. DNA content makes it possible to discriminate between cells having the same DNA content but residing in different phases of the cycle, such as between G_2 and M cells (based on differences in cyclin A content), or between G_2 diploid and G_1 tetraploid cells (based on differences in expression of cyclins E and/or B1). Also, G_0 cells, lacking expression of D-type cyclins or cyclin E, can be distinguished from G_1 cells, which are cyclin D and/or cyclin E positive. Strategies for the use of cyclins as additional markers of the cell cycle position are discussed elsewhere ***(2)***. It should be noted that some tumor cell lines, or normal cells when their cell cycle progression is perturbed, show unscheduled expression of cyclins D, E, and B1; i.e., the G_1 cyclins (e.g., cyclin E) are expressed during G_2/M and the G_2/M cyclins (cyclin B1) during G_1 ***(2)***.
9. There is often a need to identify mitotic cells in order to estimate the mitotic index, e.g., to assess the effectiveness of the drugs that disrupt microtubules, arresting cells in mitosis. The most convenient immunocytochemical method to detect mitotic cells appears to be the one that utilizes an Ab that is specific to histone H3 phosphorylated on Ser-10 (H3-P) ***(19)***. Because histone H3 is phos-

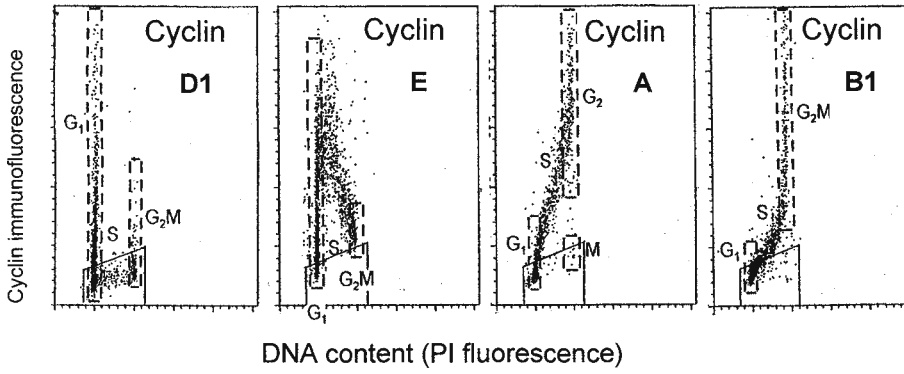

Fig. 2. Bivariate distributions (scatterplots) showing the characteristic pattern of expression of cyclins D1, E, A, and B1 vs DNA content in normal, nontumor cells, processed as described in the protocol ***(2)***. Cyclin D1 was measured in exponentially growing human normal fibroblasts, while cyclins E, A, and B1 were measured in mitogen-stimulated human lymphocytes ***(2)***. The boundaries of G_1 and G_2/M populations are marked by the dashed lines. The trapezoid windows show the level of the unspecific background fluorescence, which is measured separately using the isotypic, irrelevant Ab. It is evident that cyclin D1 is expressed by a fraction of G_1 cells; the cells entering and progressing through S, and most cells in G_2/M are cyclin D1 negative. Cyclin E is maximally expressed by the cells entering S, and its level drops during the progression through S. Cyclin A is expressed by cells in S phase and maximally by G_2 cells; mitotic cells (post-prometaphase) are cyclin A negative. Cyclin B1 is expressed by late S cells, and maximally in G_2 and M.

phorylated during prophase, and dephosphorylated late in telophase, the "time window" of detection of mitosis by this Ab spans these two mitotic stages. Sigma Chemical Co. (monoclonal) and Upstate Biotechnology, Inc. (polyclonal) offer the histone H3-P-specific Abs. The methodology of cell staining and fluorescence measurement is similar to that described above for the analysis of DNA content and proliferation-associated proteins. Optimal cell fixation, however, requires a brief (15 min) pretreatment with 1% formaldehyde (in PBS, on ice) followed by post-fixation in 70% ethanol

10. Different BrdU concentrations and different incubation times may be used depending on the cell type and design/aim of the experiment (pulse, pulse followed by time-lapse analysis, or cumulative BrdU labeling over long periods of time) (**Fig. 3**). Time-lapse analysis of the population of pulse-BrdU-labeled cells as it moves through G_2, M, and G_1 reveals the kinetics of cell cycle progression. Continuous or pulse time-lapse labeling with BrdU allows one to estimate a variety of cell cycle kinetic parameters: applications of this methodology and mathematical analysis of the data are discussed elsewhere ***(8)***. Some cell types/lines may be overly sensitive to BrdU, and their cell cycle may be perturbed at higher concentrations

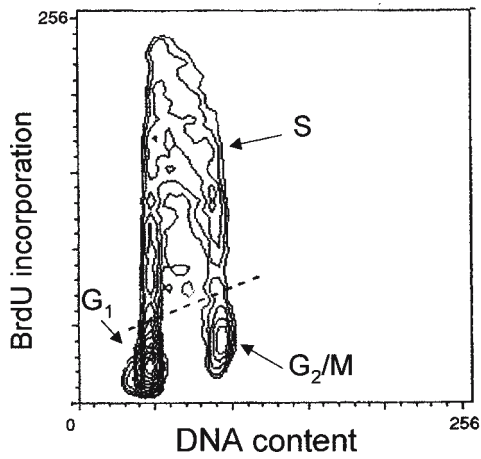

Fig. 3 Bivariate distribution (contour map) of cellular DNA content and BrdU incorporation. The cells were incubated with BrdU for 30 min, fixed, and DNA was denatured by 2 *M* HCl, The incorporated BrdU was detected by BrdU mAb, and DNA was counterstained with PI, as described in the protocol.

and longer exposure times. A control (BrdU-untreated cells) is needed for a comparison to exclude such a possibility and also as a negative control.

11. The critical step in this procedure is induction of partial DNA denaturation by heat or acid, to obtain an optimal balance between denatured (single-stranded, accessible to BrdU Ab) and nondenatured (stainable with PI) sections of DNA. Because DNA susceptibility to denaturation varies depending on chromatin structure (cell type), pilot studies should be done to find optimal conditions for a particular cell type by testing different temperatures of DNA denaturation (80–100°C) or different strengths of HCl to induce DNA denaturation (1–4 *M*). The denaturation step often results in cell damage and may lead to a significant cell loss. Use of silanized tubes during centrifugations may reduce cell loss.

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