Determining Cell Cycle Stages by Flow Cytometry

The most common approach to determining the cell cycle stage is based on measurement of cellular DNA content. This allows one to discriminate between cells in the $G_{0/1}$ versus the S versus the G_2/M phases of the cell cycle (Fig. 8.4.1). DNA is generally stained with a fluorescent dye and cellular fluorescence is measured by flow, image, or laser scanning cytometry (Crissman and Steinkamp, 1990). A variety of fluorochromes can be used for DNA staining. The spectral properties of the dyes most frequently used for this purpose are presented in Figure 8.4.2. Following cell staining with one of these dyes, the intensity of fluorescence integrated over the analyzed cell is expected to be in stoichiometric relationship to DNA content, and thereby can be used to determine the cell cycle stage. Deconvolution of the DNA content frequency histograms, usually done using special computer software (Rabinovitch, 1994), reveals the percentage of cells residing in the respective phases of the cell cycle.

A plethora of techniques for DNA content measurement utilizing the fluorochromes depicted in Figure 8.4.2 have been published during the past two decades. Description of many methods, which are applicable to different cell systems including eukaryotes and prokaryotes, as well as to clinical samples, can be found in a separate monograph (Darzynkiewicz et al., 1994). The techniques differ primarily according to the mode of cell permeabilization (detergent versus fixation with alcohols) and composition of the stain solution. Four types of procedures are described in this unit. Relatively simple and rather universally applicable methods for staining ethanol-fixed cells are presented in Basic Protocol 1 and Alternate Protocol 1. Because cells may be stored in the fixative for extended periods of time and/or transported while in the fixative, these methods allow one to prepare and collect the cells without regard to the timing of their analysis. The methods presented utilize the two most commonly used DNA fluorochromes, propidium iodide (PI; see Basic Protocol 1) and 4′,6-diamidino-2-phenylindole (DAPI; see Alternate Protocol 1). The DAPI staining procedure, which does not require incubation with RNase A, is simpler and more rapid as compared to the one using PI. However, it requires a flow cytometer equipped with a UV excitation source.

The methods utilizing detergent to permeabilize cells are presented in Basic Protocol 2 and Alternate Protocol 2. These methods provide more accurate estimates of the DNA content, and therefore better discrimination of the cell-cycle phases as compared to measurement of fixed cells. This is due to the fact that exposure of live cells to detergents results in rupture of the plasma membrane and elimination of the cytoplasmic constituents, which contain components that are autofluorescent or that nonspecifically interact with DNA fluorochromes. Isolated nuclei are then stained rather than whole cells. It should be stressed that analysis of the detergent-treated cells may lead to an underestimation of M cells. Lacking a nuclear envelope, these cells may totally disintegrate into chromosomes or chromosome aggregates. In Basic Protocol 2 cells are stained with PI; in Alternate Protocol 2 they are stained with DAPI. As in the case of fixed cells, staining with of detergent-permeabilized cells with DAPI does not require incubations with RNase A. It should be stressed that fluorochromes other than PI or DAPI (Fig. 8.4.2) can be used in these protocols, provided that their fluorescence is excited and measured at appropriate wavelengths, as indicated in the figure.

A different approach is used for cell cycle analysis of live cells (see Basic Protocol 3). The main application of this method is for cell sorting, where cells that have been selected on the basis of their DNA content (cell cycle phase) can be cultured for analysis of their

Contributed by Zbigniew Darzynkiewicz, Gloria Juan, and Elzbieta Bedner
Current Protocols in Cell Biology (1999) 8.4.1-8.4.18

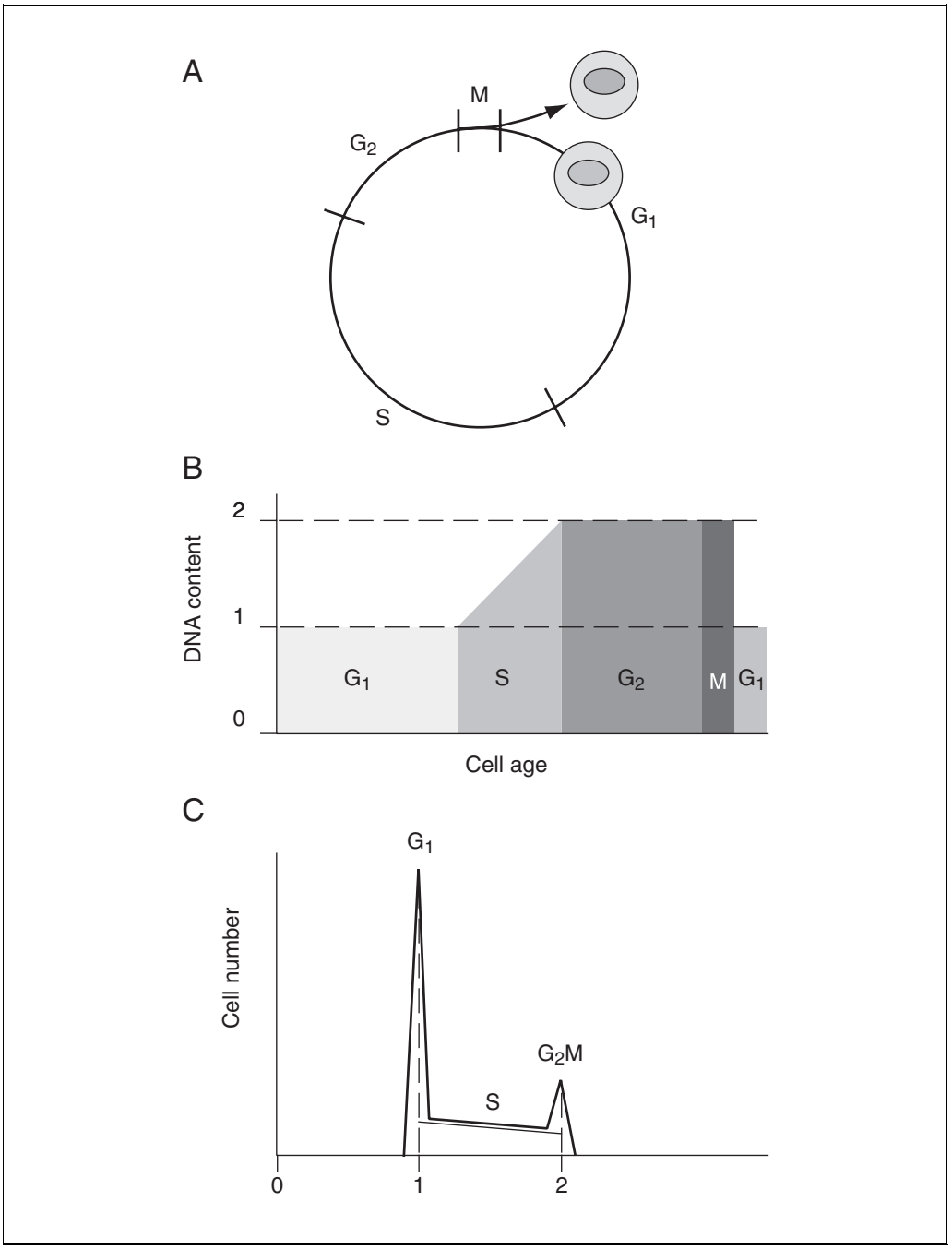

Figure 8.4.1 Relationship between DNA content and the cell cycle. (**A**) Stages of the cell cycle. (**B**) Estimation of cell position in the cell cycle based on DNA content measurement. Content of cellular DNA doubles during S, and therefore the cell age during S can be estimated based on the amount of replicated DNA (increase in DNA content). On the other hand, cells in G_1 and G_2/M are uniform with respect to DNA content, which is equivalent to the DNA ploidy index (DI) 1.0 (for G_1) and 2.0 (for G_2/M). (**C**) If DNA content could be measured with absolute accuracy, based on DNA-specific fluorescence, the G_1 and G_2/M cells would have uniform fluorescence values and be represented on the frequency histograms as the bars of a single channel width (dashed lines). Due to inaccuracy in DNA-content measurement, the actual data are in the form of G_1 and G_2/M peaks. Percentage of cells represented by these peaks, and in the S phase, are estimated by deconvolution of the histograms, using a variety of mathematical techniques (Bagwell, 1993; Rabinovitch, 1994). The interactive software based on these techniques deconvolutes the histograms and is available from several sources (see Background Information).

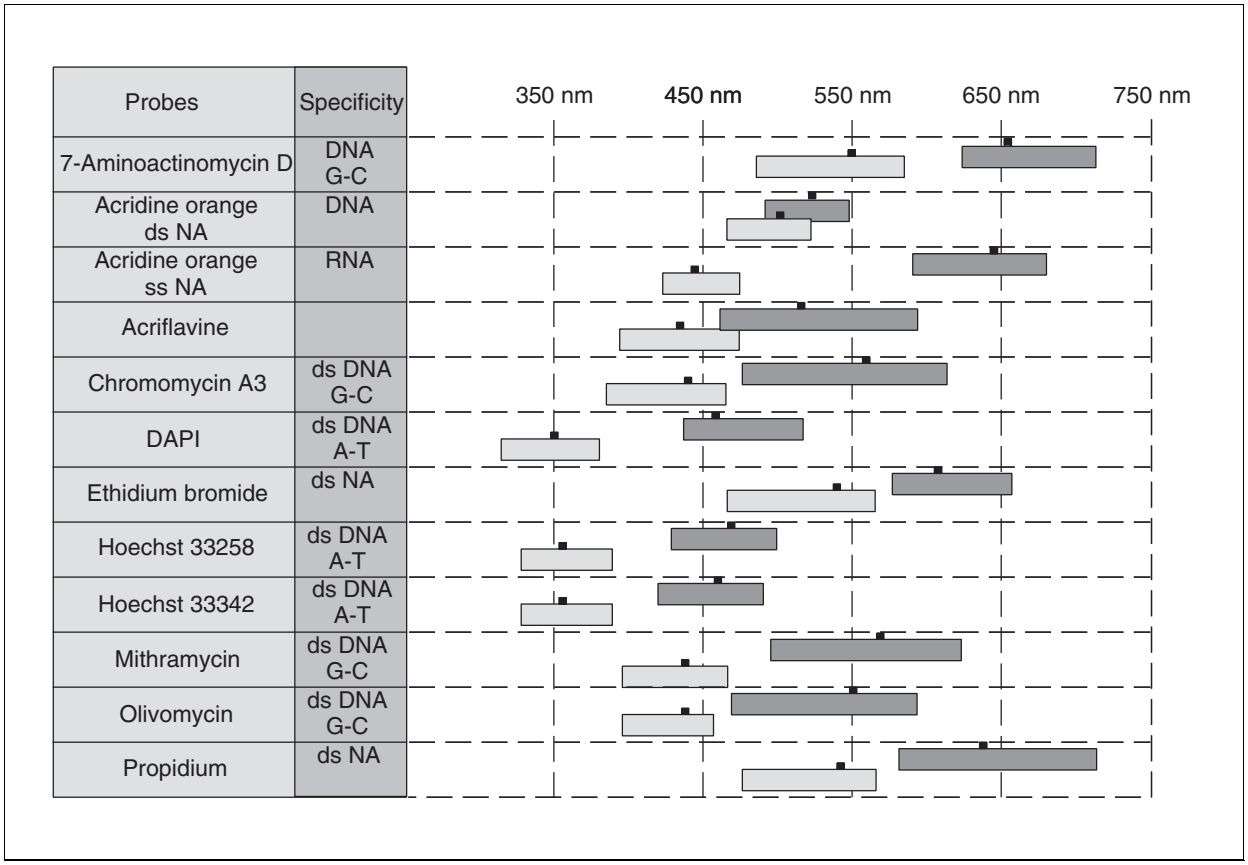

Figure 8.4.2 Fluorescence excitation (shaded bars) and emission (solid black bars) wavelengths of the most commonly used DNA fluorochromes. Abbreviations: ds, double-stranded; ss, single-stranded; NA, nucleic acid.

growth characteristics or sensitivity to drugs or for purposes of cloning or expansion. The method presented in Basic Protocol 3 is simple, based on cell staining with Hoechst 33242 fluorochrome, which, unlike PI or DAPI, is able to penetrate through the plasma membrane and stain DNA in live cells.

The final procedure combines analysis of cellular DNA content with expression of cyclins D, E, A, or B1. Cyclins are the key components of the cell cycle progression machinery and are expressed discontinuously during the cell cycle (see Background Information). Thus, the presence of a particular cyclin detected immunocytochemically within the cell, provides an additional marker of the cell cycle position. Simultaneous bivariate analysis of DNA content and expression of cyclins (Basic Protocol 4) makes it possible to distinguish additional stages of the cell cycle, which cannot be identified by analysis of DNA content alone (Darzynkiewicz et al*.,* 1996).

CELL CYCLE ANALYSIS OF FIXED CELLS STAINED WITH PROPIDIUM IODIDE

BASIC PROTOCOL 1

In this protocol, ethanol is used to fix and permeabilize cells to make them accessible to propidium iodide (PI). As mentioned above, the fixation step makes this protocol applicable in instances when samples have to be stored or transported before analysis. Following fixation, the cells are rinsed with PBS and stained with PI in a solution containing Triton X-100 and RNase A. Triton X-100 additionally permeabilizes the cells, and to some extent decreases cell loss resulting from electrostatic cell attachment to tubes. Because double-stranded sections of RNA also stain with PI, RNase A is included to digest these sections and thereby to increase specificity of DNA staining.

Materials

Cells to be stained
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Fixative: 70% ethanol
Propidium iodide staining solution I (see recipe)

Low-speed centrifuge
12 × 75–mm centrifuge tubes, preferably polypropylene or silanized
Flow cytometer with 488-nm argon-ion laser fluorescence excitation source
Software to deconvolute cellular DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems)

Additional reagents and equipments for counting and trypsinizing cells (*UNIT 1.1*)

Prepare cell suspension for fixation

1a. *For cells growing in suspension or hematologic samples:* Rinse cells once by centrifuging 6 min at 200 × *g*, room temperature, with PBS. Count cells (*UNIT 1.1*) and thoroughly resuspend 1×10^6 to 1×10^7 cells in 0.5 ml of PBS.

1b. *For cells growing attached to tissue culture dishes:* Collect cells from flasks or petri dishes by trypsinization (*UNIT 1.1*) and pool the trypsinized cells with the cells floating in the medium (the latter consist of detached mitotic, apoptotic, and dead cells). Centrifuge the cell suspension 6 min at 200 × *g*, room temperature. Remove the supernatant and resuspend the pellet in medium containing serum (to inactivate trypsin), then centrifuge again and remove the supernatant. Count cells (*UNIT 1.1*) and thoroughly resuspend 1×10^6 to 1×10^7 cells in 0.5 ml of PBS.

Other means of trypsin inactivation, such as addition of protease inhibitors, may also be used.

1c. *For cells isolated from tissues (e.g., tumors):* Rinse free of any enzyme used for cell dissociation using the centrifugation technique described in step 1a, above, and thoroughly resuspend (well dispersed, not in aggregates) 1×10^6 to 1×10^7 cells in 0.5 ml of PBS.

Fix cells in ethanol

2. Prepare for fixation by adding 4.5 ml of 70% ethanol fixative to each of an appropriate number of 12 × 75-mm centrifuge tubes. Keep tubes on ice.

3. Using a Pasteur pipet, transfer 0.5-ml aliquots of cell suspensions prepared as in step 1a, 1b, or 1c into the appropriate tubes containing the cold 70% ethanol fixative and keep cells in fixative ≥2 hr on ice.

It is important to achieve a single-cell suspension. Fixation of cells that are in aggregates while suspended in PBS stabilizes the aggregates, which then become impossible to disperse. It is essential, therefore, to have a monodisperse cell suspension at the time that cells are mixed with ethanol.

Cells suspended in 70% ethanol can be stored at 0° to 4°C for several months if not years.

Stain cells with PI

4. Centrifuge the ethanol-suspended cells 5 min at 200 × *g*. Decant ethanol thoroughly.

5. Suspend the cell pellet in 5 ml PBS, wait 60 sec, then centrifuge again as in step 4. Decant supernatant

6. Resuspend cell pellet in 1 ml of propidium iodide staining solution I. Incubate either 15 min at 37°C or 30 min at room temperature.

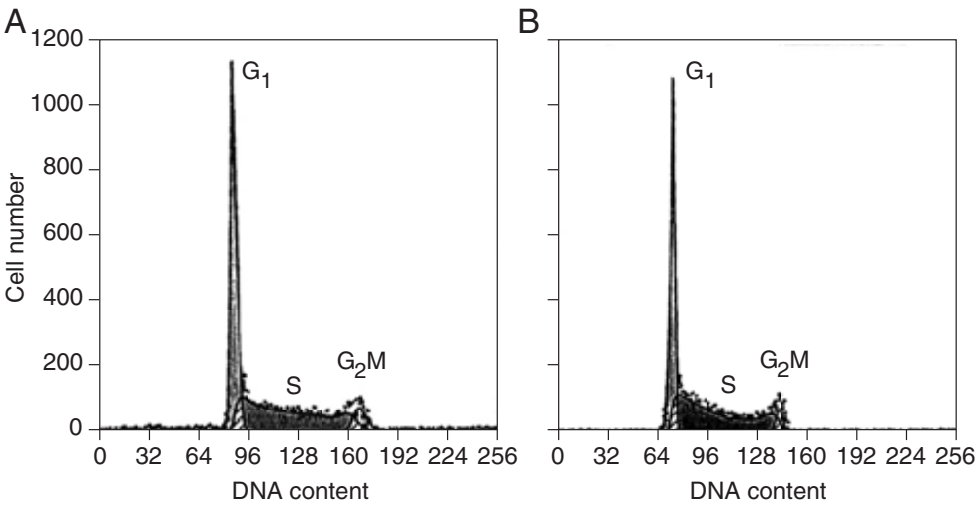

Figure 8.4.3 (**A**) DNA content frequency histograms of fixed HL-60 cells stained with DAPI (Alternate Protocol 1) and (**B**) detergent-permeabilized HL-60 cells stained with DAPI (Alternate Protocol 2). The histograms were deconvoluted using Multicycle software (Phoenix Flow Systems).

Measure cell fluorescence by flow cytometry

7. Set up and adjust flow cytometer for excitation with blue light and detection of PI emission at red wavelengths.

   For excitation, the 488-nm argon-ion laser line may be used. Alternatively use a BG 12 optical filter when the source of illumination is a mercury-arc or xenon lamp. A long-pass (>620-nm) emission filter is recommended.

8. Measure cell fluorescence by flow cytometry. Use pulse-width/pulse-area signal to discriminate between G_2/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using DNA content histogram deconvolution software (e.g., Multicycle from Phoenix Flow Systems).

CELL CYCLE ANALYSIS OF FIXED CELLS STAINED WITH DAPI

ALTERNATE PROTOCOL 1

This protocol is similar to Basic Protocol 1, except the cells are stained with DAPI rather than PI. Because DAPI does not stain RNA, there is no need to treat the cells with RNase A. Excitation of DAPI, however, requires UV light source, which is not universally available. Emission of DAPI is measured at blue wavelengths.

Additional Materials *(also see Basic Protocol 1)*

DAPI staining solution I (see recipe)
Flow cytometer with UV illumination source (e.g., mercury-arc lamp or laser tuned to UV at 340 to 380 nm)

1. Collect cells, fix in 70% ethanol, and wash (see Basic Protocol 1, steps 1 to 5).
2. Resuspend cell pellet in 1 ml DAPI staining solution. Incubate 30 min in the dark at room temperature.
3. Set up and adjust flow cytometer for UV excitation at 340 to 380 nm and detection of DAPI emission at blue wavelengths.

   For excitation, an UG-1 optical filter (short-pass, 390 nm) may be used when the source of excitation is a mercury-arc or xenon lamp. For detection of DAPI emission a band-pass filter at 470 ± 20 nm is recommended.

4. Measure cell fluorescence by flow cytometry. Use the pulse-width/pulse-area signal to discriminate between G_2/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using software that deconvolutes DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems).

BASIC PROTOCOL 2

CELL CYCLE ANALYSIS OF UNFIXED, DETERGENT-PERMEABILIZED CELLS STAINED WITH PI

In this protocol the cells are lysed with detergent to aid in the staining of DNA for flow cytometric analysis. The cells in suspension are mixed with staining solution that contains Triton X-100, PI, and RNase A. DNA content of the stained nuclei is then measured by flow cytometry, using excitation with blue light. Because there is no fixation step, the procedure is simpler and more rapid, and because there are fewer centrifugations, there is less cell loss as compared to Basic Protocol 1

Materials

Cells to be stained: 1×10^6 to 5×10^6 cells/ml suspended in PBS (*APPENDIX 2A*) or culture medium
Propidium iodide staining solution II (see recipe)

Flow cytometer with 488-nm argon-ion laser fluorescence excitation source
Software to deconvolute cellular DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems)

1. Mix 0.2 ml of cell suspension with 2 ml of propidium iodide staining solution II. Incubate 20 min at room temperature.
2. Set up and adjust flow cytometer for excitation with blue light and detection of PI emission at red wavelengths.

   For excitation, the 488-nm argon-ion laser line may be used. Alternatively use a BG 12 optical filter when the source of illumination is a mercury-arc or xenon lamp. A long-pass (>620-nm) emission filter is recommended.

3. Measure cell fluorescence by flow cytometry. Use pulse-width/pulse-area signal to discriminate between G_2/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using DNA content histogram deconvolution software (e.g., Multicycle from Phoenix Flow Systems).

ALTERNATE PROTOCOL 2

CELL CYCLE ANALYSIS OF UNFIXED, DETERGENT-PERMEABILIZED CELLS STAINED WITH DAPI

This protocol is essentially identical to Basic Protocol 2 except that the cells are stained with DAPI. As mentioned previously, there is no need for incubation with RNase A, which simplifies the protocol, but the procedure requires a flow cytometer equipped with UV light excitation source.

Additional Materials (also see Basic Protocol 2)

DAPI staining solution II (see recipe)
Flow cytometer with UV-illumination source (e.g., mercury-arc lamp or laser tuned to UV at 340 to 380 nm)

1. Mix 0.2 ml of cell suspension (1×10^5 to 1×10^6 cells suspended in PBS or culture medium) with 2 ml of DAPI staining solution II. Incubate 10 min at room temperature.
2. Set up and adjust flow cytometer for UV excitation at 340 to 380 nm and detection of DAPI emission at blue wavelengths

For excitation, an UG-1 optical filter (short-pass, 390 nm) may be used when the source of excitation is a mercury-arc or xenon lamp. For detection of DAPI emission a band-pass filter at 470 ± 20 nm is recommended.

3. Measure cell fluorescence by flow cytometry. Use the pulse-width/pulse-area signal to discriminate between G_2/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using software that deconvolutes DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems).

STAINING OF LIVE CELLS WITH HOECHST 33342

BASIC PROTOCOL 3

Supravital staining of DNA offers the possibility of sorting live cells at different phases of the cell cycle, based on differences in their DNA content. The protocol uses Hoechst 33342 fluorochrome, which stains cellular DNA without a need for cell fixation or permeabilization with detergent. The actual procedure for cell staining is simple. Cells are suspended in culture medium or PBS and incubated in the presence of 2.0 to 5.0 μg/ml of Hoechst 33342 for 20 to 90 min. Cell fluorescence is then measured directly, without any additional treatments or centrifugations. Because Hoechst 33342 is excited at UV wavelengths, the procedure requires a flow cytometer with a UV light illumination source. It should be stressed, however, that accuracy of DNA content measurement and therefore the ability to discriminate cells at different phases of the cycle, is much lower with supravital cell staining as compared to staining of ethanol-fixed or detergent-permeabilized cells.

Materials

1 mg/ml Hoechst 33342 in H_2O (store up to several weeks at 4°C in dark or foil-wrapped bottles)

Cells to be stained: 1×10^6 cells/ml suspended in PBS (*APPENDIX 2A*) or culture medium

Flow cytometer with UV light illumination source (e.g., mercury-arc lamp or laser tuned to UV at 340 to 380 nm)

1. Add sufficient 1 mg/ml Hoechst 33342 to cells suspended in PBS or culture medium (1×10^6 cells/ml) to obtain a final fluorophore concentration of 2.0 μg/ml. Incubate 20 min at 37°C.
2. Set up and adjust flow cytometer for UV excitation at 340 to 380 nm, and detection of Hoechst 33342 fluorescence at blue wavelengths.
3. Measure cell fluorescence by flow cytometry. Use the pulse-width/pulse-area signal to discriminate between G_2/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using software that deconvolutes DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems).

When intensity of cell fluorescence or resolution of cells in the cell-cycle phases is inadequate, prolong the staining time (up to 90 min) and/or increase Hoechst 33342 concentration in the medium (up to 5 μg/ml). The same sample may be reanalyzed after prolonged incubation and/addition of more staining solution.

This protocol is predominantly used for sorting live cells. However, because sensitivity of cells to Hoechst 33342 varies depending on the cell type (the dye also sensitizes cells to UV light), it is possible that viability and cell cycle progression of the sorted cells may be affected by the staining procedure.

BASIC PROTOCOL 4

BIVARIATE ANALYSIS OF DNA CONTENT AND EXPRESSION OF CYCLINS D, E, A, OR B1

A special category of methods for cell-cycle analysis combines measurement of DNA content and expression of the proliferation-associated proteins. The latter are detected immunocytochemically, using specific antibodies that are labeled with fluorochrome either directly, or indirectly via a secondary antibody. This protocol is devoted to cyclins, whose analysis provides an insight into the actual components of the cell-cycle progression machinery (see Background Information and *UNIT 8.2*). Because some cyclins are expressed transiently, at very specific time intervals in the cell cycle, their presence in the cell can be considered as a marker of this particular portion of the cycle. The protocol combines measurement of DNA content with expression either of one of the D-type cyclins, or cyclins E, A, or B1. Cells are fixed and labeled with anti-cyclin antibody followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody, and finally stained with PI for DNA measurement. If directly conjugated FITC-anti-cyclin antibody is available, secondary-antibody labeling (steps 8 and 9) can be omitted. This analysis is adapted to the most commonly used flow cytometers, such as those equipped with a single-laser (488-nm) illumination source.

Materials

Cells to be analyzed
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Fixative: 80% ethanol or absolute methanol, −20°C.
0.25% (v/v) Triton X-100 in PBS, pH 7.4 (store at 4°C)
Rinsing buffer: 1% (w/v) bovine serum albumin (BSA) in PBS, pH 7.4 (store at 4°C)
Anti-cyclin IgG1 antibodies: e.g., mouse monoclonal antibodies to cyclin B1 (clone GNS-1), cyclin A (clone BF-683), cyclin D1 (clone G124-326), cyclin D3 (clone G107-565), and cyclin E (clone HE12); all provided by PharMingen; antibodies to cyclin D1 may also be obtained from Immunotech/Coulter
Mouse IgG1 (isotypic control)
FITC-conjugated goat anti–mouse IgG
Propidium iodide staining solution III (see recipe)

15-ml conical tubes, polypropylene or silanized
Low-speed centrifuge
Flow cytometer equipped with 488-nm argon laser fluorescence excitation source

Additional reagents and equipment for collecting and preparing cells for fixation (see Basic Protocol 1, step 1a, b, or c)

Prepare and fix cell suspension

1. Collect cells and resuspend in PBS (see Basic Protocol 1, step 1a, b, or c).
2. Prepare for fixation by adding 10 ml of 80% ethanol or absolute methanol fixative to each of an appropriate number of 15-ml tubes. Keep tubes on ice (0° to 4°C).

   In addition to preparing a tube for each aliquot of cells to be tested with an anti-cyclin antibody, prepare an appropriate number of tubes for isotypic controls .

3. With a Pasteur pipet, transfer 1 ml of the each cell suspension into the appropriate tubes containing cold fixative. Incubate on ice.

   Time of fixation (storage) at 4°C may vary from 4 hr to several days.

   To minimize cell loss, all the subsequent steps should be done in the same tube.

Label cells with anti-cyclin primary antibody

4. Centrifuge fixed cells 5 min at 300 × *g*, room temperature. Remove alcohol, resuspend cells in 5 ml PBS, and centrifuge as before.

5. Remove supernatant and resuspend cell pellet (≤1 × 10^6 cells) in 1 ml of 0.25% Triton in PBS. Keep on ice for 5 min, then add 5 ml of PBS and centrifuge at 300 × *g* for 5 min at room temperature. Remove supernatant.

6. Dissolve each of the anti-cyclin antibodies of interest (primary antibodies) in rinsing buffer at 5 µg/ml. For each sample to be analyzed, take 100 µl of the 5 µg/ml anti-cyclin antibody solution. Also, prepare 100 µl of 5 µg/ml mouse IgG_1 (isotypic control) for each tube of control cells prepared.

7. Resuspend each cell pellet in 100 µl of rinsing buffer containing the appropriate primary antibody or isotypic control. Incubate 60 min at room temperature with gentle agitation or at 4°C overnight.

Label cells with FITC-conjugated secondary antibody

8. Add 5 ml of rinsing buffer to each tube and centrifuge 5 min at 300 × *g*, room temperature. Remove the supernatants.

9. Make a 1:30 dilution of the secondary antibody (FITC-conjugated goat anti–mouse IgG) in rinsing buffer. Resuspend each of the cell pellets in 100 µl of the diluted FITC-conjugated secondary antibody and incubate 30 min in the dark at room temperature with gentle agitation.

   If cyclin antibody that is directly conjugated to FITC is available, this protocol can be simplified by omitting steps 8 and 9.

Stain cells with PI

10. Add 5 ml of rinsing buffer to each tube and centrifuge 5 min at 300 × *g*, room temperature. Remove the supernatants

11. Resuspend each cell pellet in PI staining solution III. Incubate 20 min at room temperature in the dark before measurement.

Measure cell fluorescence by flow cytometry

12. Set up and adjust the flow cytometer for excitation with blue light (488-nm laser line). Use a 530 ± 20 nm band-pass filter for detection of FITC emission and 620-nm long-pass filter for PI emission.

13. Measure the cyclin-associated green fluorescence of FITC and DNA-associated red fluorescence of PI.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

DAPI staining solution I

To 100 ml of 0.1% (v/v) Triton X-100 in PBS (*APPENDIX 2A*) add 0.1 mg 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Store up to 1 month at 4°C in the dark.

DAPI staining solution II

To 100 ml of PIPES buffer (see recipe) add 0.1 mg 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Store up to several weeks at 0° to 4°C in dark or foil-wrapped bottles.

PIPES buffer

3.02 g piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES; Calbiochem; 10 mM final)
5.84 NaCl (0.1 M final)
406 mg $MgCl_2$ (2 mM final)
1 ml Triton X-100 (0.1% final)
H_2O to 1000 ml
Adjust to pH 6.8
Store up to 6 months at 0° to 4°C

Dissolve the dry ingredients and Triton X-100 in ~800 ml water, adjust pH with NaOH or HCl, and add water to 1000 ml.

Propidium iodide (PI) staining solution I

To 100 ml of 0.1% (v/v) Triton X-100 in PBS add 20 mg DNase-free RNase A and 2 mg of propidium iodide (PI; Molecular Probes). Store up to 2 weeks at 4°C in the dark.

If RNase is not DNase-free, boil a stock solution of RNase A (2 mg in 1 ml water) for 5 min, then use it to prepare the PI staining solution.

Propidium iodide (PI) staining solution II

Add 2 mg of propidium iodide (PI; Molecular Probes) to 100 ml of PIPES buffer (see recipe). Store up to several weeks in dark or foil-wrapped bottles at 0° to 4°C. Prior to use add DNase-free RNase A to 200 µg/ml final concentration.

Propidium iodide staining solution III

Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing:
5 µg/ml propidium iodide (PI)
200 µg/ml DNase-free RNase A
Prepare fresh

COMMENTARY

Background Information

Univariate cellular DNA content analysis

Choice of protocol: Choosing a particular protocol from among those presented in this unit depends on the sample type (i.e., determining whether fixed or unfixed cells should be used), availability of UV light illumination in the flow cytometer (which will dictate preference for DAPI over PI), or the need to identify and quantify apoptotic cells in addition to discrimination of the cell cycle phases. In the discussion that follows, characteristics and applicability of each of the methods is presented. Because a variety of different fluorochromes can be used to stain cellular DNA, and occasionally some of them may be preferred over DAPI, PI, or Hoechst 33342, the spectral properties of the most commonly used dyes are presented in Figure 8.4.2.

In Basic Protocol 1 and Alternate Protocol 1, DNA content is measured in prefixed cell samples. The preference for analysis of fixed cells often is dictated by the need to store or transport samples. Extended storage of unfixed cells, unless done at low temperatures following suspension of the cells in cryopreservative medium, leads to cell deterioration and DNA degradation. Fixed cells, on the other hand, often can be stored for months if not years without much deterioration. The fixative essentially has two functions: (1) it preserves the cells by preventing lysis and autolytic degradation, and (2) it makes the cells permeable and hence makes DNA accessible to the fluorochrome. For DNA-content analysis, the precipitating fixatives (ethanol, methanol, and acetone) are preferred over the cross-linking agents (formaldehyde, glutaraldehyde). It should be stressed, however, that damaged DNA, especially DNA having a large number of double-strand breaks (e.g., as are present in apoptotic cells) leaks out from the ethanol-prefixed cells during hydration and subsequent staining. This allows one to identify apoptotic cells as those with fractional DNA content ("sub-G_1 cell population"). Methods for detecting apoptotic cells are reviewed in Darzynkiewicz et al. (1997).

As mentioned, a variety of DNA fluorochromes can be used to stain DNA in the prefixed cells. Staining with dyes that react with DNA and RNA, such as PI, requires incubation of cells with RNase A. The enzyme is included in the staining solution; a 30-min incubation at room temperature in that solution is adequate to remove RNA from the subsequently measured cells. Since most flow cytometers use blue light (488-nm line of the argon ion laser) as the fluorescence excitation source, PI is a useful label.

Alternate Protocols 1 and 2 employ DAPI instead of PI. One advantage of DAPI is its greater specificity towards DNA, which often is reflected in lower coefficient of variation (CV) values of the mean DNA content of G_1 cell populations. Another advantage is that it does not require incubation with RNase A. However, not all flow cytometers are equipped with a UV-light illumination source, which is needed for DAPI excitation.

The major advantage of detergent-based methods (see Basic Protocol 2 and Alternate Protocol 2) is greater accuracy in DNA-content estimates. However, because the cells are lysed by detergents, mitotic cells, which lack a nuclear envelope, may disintegrate to such an extent that individual chromosomes or chromosome clusters are measured. This usually happens when the samples are vigorously pipetted or vortexed. Therefore, one has to be cautious in interpreting the data, because mitotic cells may not be detected by methods utilizing detergents or hypotonic staining solutions. This is of special importance in instances when a large proportion of cells are in mitosis—e.g., during incubations with mitotic blockers. Furthermore, the presence of chromosomes or chromosome aggregates in the sample may contribute to an increased frequency of detection of objects with low fluorescence values, generally classified as debris or apoptotic cells ("sub-G_1" cell population, see Darzynkiewicz et al., 1997). Likewise, the lysis of apoptotic cells, which have fragmented nuclei, releases several fragments from a single cell. Each such fragment may erroneously be identified as an individual apoptotic cell.

DNA staining in live cells (see Basic Protocol 3) is generally performed in combination with cell sorting to obtain cells synchronized at particular cell cycle phases. The fluorochrome for supravital cell staining is expected to be nontoxic and not alter the cell metabolism. Such a probe has yet to be developed. Most DNA fluorochromes are charged molecules that do not adequately penetrate the plasma membrane. Some uncharged Hoechst dyes that can pass through the membrane, though at a limited rate, are exceptions. The most frequently used supravital DNA fluorochrome is Hoechst 33342. The procedure of staining with Hoechst 33342 followed by sorting appears not to induce immediate cytotoxicity (Loken, 1980). Delayed toxicity attributed to Hoechst 33342 has been observed, however, especially when the cells were treated with some antitumor drugs or radiation subsequent to staining. Hoechst dyes photosensitize cells that have BrdU incorporated into their DNA, in particular to UV light at ~300-nm wavelengths. Viability of the sorted BrdU-labeled cells, counterstained with Hoechst dyes and illuminated with UV light laser, is expected to be impaired.

The intensity of supravital cell staining with Hoechst 33342 and the resolution of DNA content (i.e., possibility of discrimination of cells in different cell-cycle phases) varies among different cell types. This variability, to a large degree, is due to a rapid efflux of the dye from the cell generated by the P glycoprotein transport pump. Cells characterized by rapid efflux mechanisms (e.g., multidrug-resistant tumor cells or stem cells) stain poorly with Hoechst 33342. It has been observed, however, that agents that may impair the efflux function (e.g., calcium channel–blocking drugs such as verapamil), at least in some cell types, improve stainability with Hoechst 33342 (Krishan, 1987).

Cell cycle and kinetic parameters: All cells in $G_{0/1}$ have a uniform DNA content, as do cells in G_2/M. Under ideal conditions of DNA staining and measurement, the fluorescence intensities of all $G_{0/1}$ and G_2/M cells are expected to be uniform, and, after digitization of the electronic signal from the photomultipler, to have uniform numerical values with G_2/M cells having twice the fluorescence of cells in $G_{0/1}$. This, however, is never the case, and on frequency histograms the $G_{0/1}$ and G_2/M populations are represented by peaks of various width because of inaccuracy of the measurement. The coefficient of variation (CV) of the mean value of DNA-associated fluorescence of the $G_{0/1}$ population is a measure of the width. The CV value, therefore, is a reflection of the inaccuracy of the DNA estimate. Because of the inaccuracy there is an overlap between early S and $G_{0/1}$ as well as between late S and G_2/M cells on the histograms. Several mathematical methods of deconvolution of the DNA content frequency histograms have been developed to estimate the

percentage of cells in the respective phases of the cell cycle (Bagwell, 1993; Rabinovitch, 1994). The goal of these techniques is to evaluate the extent of the overlap and thereby to correct for the inaccuracy of the measurement. These methods provided the basis for development of software that allows one to estimate percentage of cells in particular phases of the cycle from the histograms, through simple interactions with the computer. Such software is generally provided upon the purchase of instruments or can be obtained separately from other vendors. The most common software programs used to deconvolute DNA content frequency histograms are provided by Phoenix Flow and Verity Software House.

It is often assumed that the cells in S and G_2/M represent the "proliferative" cell fraction and that their frequency reflects the proliferative potential of the cell population. While this indeed may be the case in some cell systems, the DNA content frequency histograms alone do not provide any direct information on cell kinetics. For example, the histogram cannot reveal whether the cell progression through the cycle is slowed down (e.g., compared to control) if the slowdown affects all phases of the cycle proportionally. Likewise, the histogram cannot show whether the cells are "frozen" in the cycle, which may happen during treatment with high doses of certain drugs, hyperthermia, or radiation. However, when the cell-proliferation rate (e.g., the cell-doubling time in culture) is measured in parallel with DNA content, it is possible, with certain assumptions and approximations, to estimate cell kinetics, including the duration of individual phases of the cycle. One of the assumptions is that the cells are in exponential phase of growth. In such a situation there are always twice as many daughter cells as mother cells, and the cell age distribution across the cycle is represented by the diagram shown in Figure 8.4.4B. Duration of a particular phase of the cycle (e.g., G_1) can then be estimated from the equation:

$$\frac{T_{G1}}{T_C} = \frac{\ln(F_{G1} + 1)}{\ln 2}$$

where T_{G1} is duration of G_1 phase, T_C is duration of the cell cycle, and F_{G1} is a fraction of cells residing in G_1. T_C is estimated from the cell growth curves—i.e., when the number of live cells is plotted as a function of time (cell number on exponential scale versus time on linear scale of the x and y coordinates), the exponential growth is reflected by a straight slope. The slope allows one to calculate the cell doubling time in culture. The latter, with generally acceptable approximation, equals T_C. A similar formula, of course, applies for estimating duration of phases of the cell cycle other than G_1. Thus, for example, when the duration of the cell cycle is 24 hr and the fraction of cells in G_1 is 0.5 (50%), the duration of G_1 (T_{G1}) is $1n(1 + 0.5) \times 24/1n\ 2 = (0.405 \times 24)/0.693 =$ 14.0 hr. The graphical approach for measuring duration of the cell-cycle phases from the DNA content frequency histograms is illustrated in Figure 8.4.4.

Bivariate analysis of DNA content and expression of cyclins D, E, A, or B1

Cyclins are the key elements of the cell cycle progression machinery. They combine with particular cyclin-dependent protein kinases (CDKs) forming the holoenzymes that phosphorylate different sets of proteins at consecutive stages of the cell cycle, thereby driving the cell through the cycle (Pines and Hunter, 1991; Draetta, 1994; Hartwell and Kastan, 1994; Sherr, 1994; Cardon-Cardo, 1995; Morgan, 1995; Bartek et al., 1997). The function of cyclins in these holoenzymes is to activate their partner CDKs and to target them to specific protein substrates whose phosphorylation is essential for traversing a particular section of the cell cycle.

Several cyclins, notably D-type cyclins as well as cyclins E, A, and B, are expressed transiently during the cell cycle. Cyclins D and E belong to the family of G_1 cyclins, whereas cyclins A and B are G_2 cyclins (also see Fig. 8.2.1). During unperturbed growth of normal, nontumor cells, the scheduled timing of synthesis and degradation of cyclins takes place at very specific points of the cycle. The periods of expression of these cyclins by the cell, therefore, can be considered landmarks of the cell cycle. These landmarks complement the traditional milestones of the cycle detected by DNA content measurement, namely mitosis and DNA replication.

The development of antibodies to cyclins made it possible to detect them immunocytochemically and to investigate their expression in individual cells by cytometry (Gong et al., 1994; Lukas et al., 1995; Urbani et al., 1995; Juan et al., 1997). Bivariate analysis of DNA content and cyclin expression provides a framework for subdividing the cell cycle into several subcompartments and for defining, with a greater precision than before, the point at which the cell cycle is arrested by some antitumor

drugs (Darzynkiewicz et. al., 1996). Furthermore, the differences in cyclin expression make it possible to discriminate between cells having the same DNA content but residing at different phases, such as in G_2 versus M or G_2/M cells of a lower DNA ploidy versus G_1 cells of a higher DNA ploidy.

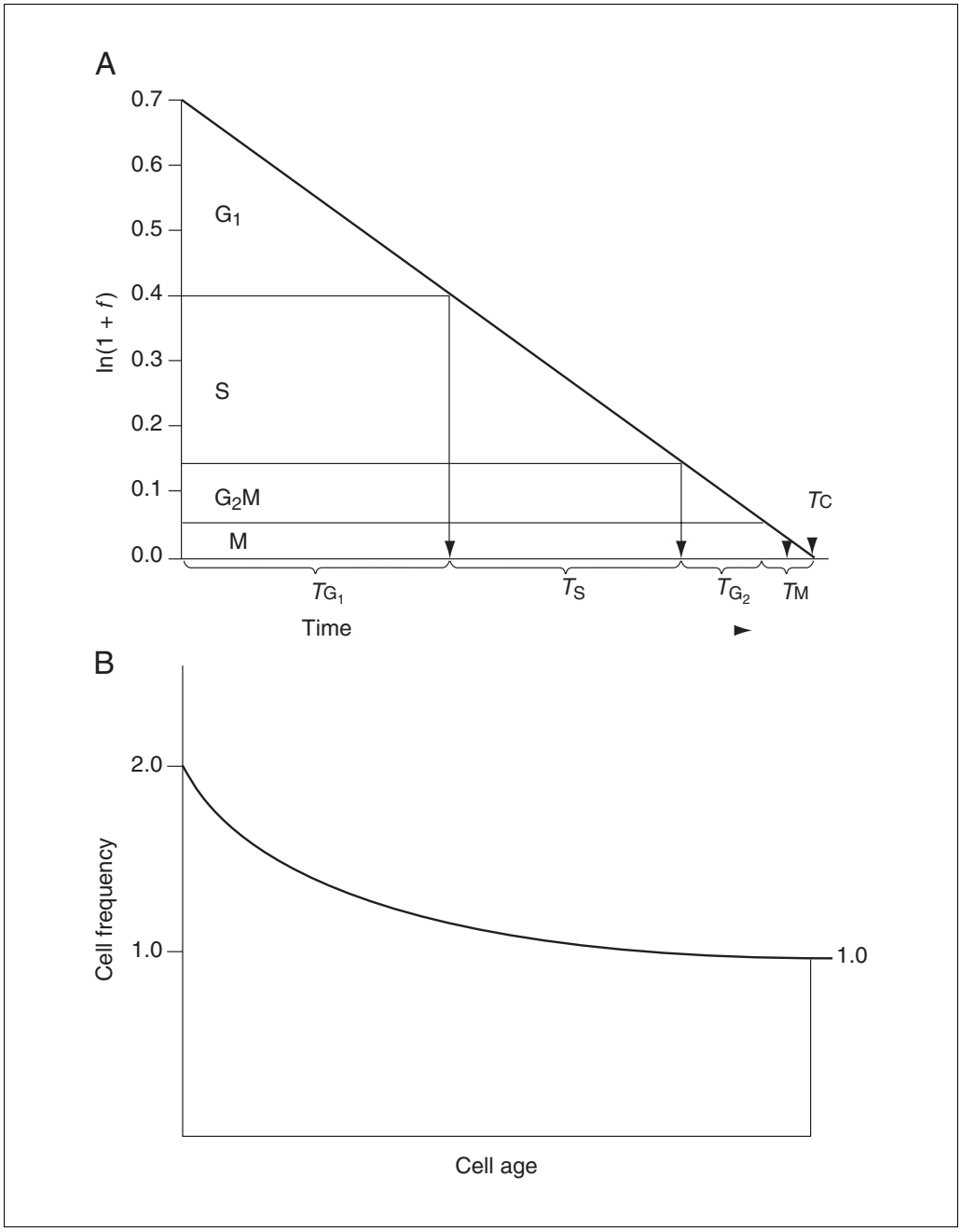

Figure 8.4.4 Graphical method for estimating duration of particular cell cycle phases (**A**) The method is based on the assumption that all cells are in the exponential phase of proliferation—i.e., that there are twice as many daughter as mother cells. (**B**) The proportions of cells in different phase of the cycle are obtained from DNA content frequency histograms (Fig. 8.4.3), and the value of T_C (cell generation, or cell cycle time), which is in turn calculated from the growth curves as the cell-doubling time in culture. T_C equals the cell-doubling time when all cells are in the proliferative cell pool—i.e., when the growth fraction equals 1.0. Fractions of cells in particular phases of the cycle (f) are plotted exponentially as ln(1 + f). T_C is then connected with ln 2 (0.693). The points of intersection of the line connecting ln 2 and T_C with the levels representing frequency of cells in particular phases of the cycle, projected on the time coordinate, represent the duration times of these phases, as shown.

The bivariate analysis of cyclin expression versus DNA content, unlike any other approach, detects the inappropriate ("unscheduled") expression of cyclins—i.e., the presentation of G_1 cyclins by cells in G_2/M and of G_2/M cyclins by G_1 cells—without the need for cell synchronization. Such unscheduled expression of cyclins B1 and A was seen when cell cycle progression was halted, e.g., after synchronization at the G_1/S boundary by inhibitors of DNA replication (Gong et al., 1995; Urbani et al., 1995). The unscheduled expression of cyclins B1 or E, representing a characteristic feature of a particular tumor phenotype, was also observed in some tumor cell lines when their growth was unperturbed (Gong et al., 1994). Likewise, while the expression of cyclins D1 or D3 in nontumor cells was restricted to an early section of G_1, the presentation of these proteins in many tumor cell lines was also seen during S and G_2/M (Juan et al., 1996). As specific markers of cell proliferation, cyclins are expected to reflect proliferative potential of tumors and therefore to be the key prognostic markers in neoplasia.

Critical Parameters

Univariate cellular DNA content analysis

The most critical issue in DNA content analysis is the accuracy of DNA content measurement. The accuracy, as mentioned (see Background Information), is reflected by the extent of variation in cellular fluorescence between the cells with identical DNA content, such as $G_{0/1}$ cells. The CV of the DNA-associated mean fluorescence of G_1 cells, therefore, is considered as an index of accuracy of DNA content measurement. High accuracy is required, in particular, in studies of DNA ploidy, to distinguish between DNA diploid and aneuploid cells, which may differ minimally in DNA content. High accuracy of DNA content measurement is also critical in analysis of the cell cycle distribution. Regardless of the type of the software used to deconvolute DNA frequency histograms, the accuracy in estimation of cell proportions in respective phases of the cell cycle directly correlates with accuracy of DNA content measurement. There is no formal consensus regarding the acceptable maximal CV value of the mean DNA content of $G_{0/1}$ cell population—i.e., maximal error in cellular DNA content estimate. Most researchers, however, would consider the accuracy to be poor and results unacceptable if CV values of $G_{0/1}$ populations of normal, nontumor cells exceed 6%.

A variety of factors can contribute to poor accuracy in DNA content analysis. The most common is inappropriate optical alignment (spatial position of laser beam with respect to sample flow and fluorescent light–collecting lenses) of the flow cytometer. When the instrument is optimally aligned, the measured cell passes precisely through the center of the laser beam, and at the same time, is in the focus of the fluorescence emission collection lenses. A minor adjustment of the sample flow or any of the optical parts leads to a loss in accuracy of DNA content measurement. Proper maintenance of the instrument, and careful adjustment with standard fluorescent beads of known uniformity prior to analysis of the experimental samples, is essential to achieve accurate DNA content measurements. Problems in sample preparation, either resulting in mechanical damage to the cells or involving incorrect composition of buffers and staining solutions, represent another common cause of poor resolution in DNA analysis.

It should be stressed that there may be situations when, in spite of good accuracy in DNA content measurement (in terms of proper instrument adjustments and sample staining), the CV of the mean DNA content of G_1 cell populations is relatively large. This may happen when significant numbers of dead or dying cells are present in the sample or when the cells were treated with drugs interacting with DNA. Many antitumor drugs are known to impair stainability of DNA with the fluorochromes used for flow cytometry. Furthermore, because of the nature of the tumor, which may either be multiclonal or have developed drug resistance by gene amplification mechanisms (e.g., as reflected by the presence of minute chromosomes), the tumor cell populations may have variable DNA content, and therefore intrinsically high CV values for the $G_{0/1}$ cell populations.

Bivariate analysis of DNA content and expression of cyclins D, E, A, or B1

The critical steps for immunocytochemical detection of intracellular proteins are cell fixation and permeabilization. These steps often have to be customized for particular antigens. The fixative is expected to stabilize the antigen in situ and preserve its epitope in a state where it continues to remain reactive with the available antibody. The cell has to be permeable to allow access of the antibody to the epitope.

Most studies on cyclins have employed precipitating fixatives such as 70% to 80% ethanol, absolute methanol, or a 1:1 mixture of methanol and acetone cooled to –20° to –40°C. Brief (15- to 30-min) treatment with 1% paraformaldehyde followed by 70% cold ethanol has been used for fixation of D-type cyclins although this cyclin can also be detected following fixation with cold methanol. The choice of fixative, thus, appears not to be a critical factor for cyclin detection and, although the absolute level of the immunofluorescence may vary, various fixation protocols yield essentially similar cyclin distributions with respect to the cell cycle position. Each fixative has some undesirable effects (e.g., increased cell clumping in the case of the ethanol/acetone mixture or cell autofluorescence and poor DNA stainability when formaldehyde is used), and one often has to compromise between these effects and the optimal detection of a particular cyclin. Fixation in 80% cold ethanol, as presented in this protocol, offers such a compromise.

Much more critical for the detection of cyclins is the choice of a proper antibody. Often an antibody that is applicable to immunoblotting fails in immunocytochemical applications, and vice versa. Likewise, the antibody may show nonspecific reaction with denatured proteins (i.e., reveal several bands on immunoblots), and yet be acceptable in immunocytochemical assays. These discrepancies may be due to differences in accessibility of the epitope or differences in the degree of denaturation of the antigen on the immunoblots as compared to the situation in its in situ location. Some epitopes may not be accessible in situ at all. This especially pertains to epitopes that are the cyclin segments involved in formation of complexes with the partner CDKs, CDK inhibitors, or other molecules such as proliferating cell nuclear antigen (PCNA). Since there is strong homology between different cyclin types, cross-reactivity may also be a problem. Because commercially available monoclonal antibodies may differ, e.g., in specificity and degree of cross-reactivity, it is important to use reagents that have been already tested and referenced in published papers. It is required that the authors provide information (the vendor and hybridoma clone number) on the reagent used in their study.

A very important control is examination of the stained cells by UV light microscopy, or preferably confocal microscopy. All cyclins, with the notable exception of cyclin B1, have nuclear localization. Cyclin B1 has cytoplasmic localization during late S and most of G_2, and is localized in the chromatin only at mitosis. Cyclin B2 is localized to Golgi (Jackman et al., 1995). Inappropriate localization of the fluorescence of the cells stained with the presumed anti-cyclin antibody may be an indication of its nonspecific reactivity with other cell constituents.

The relative cellular content of a particular cyclin plays a role in its detection. The signal-to-noise ratio (ratio of fluorescence intensity of the cyclin-positive cells to the control cells, stained with the isotype immunoglobin), for example, is higher in the case of cyclin B1 than in the case of cyclins E or A, most likely due to the fact that the absolute level of cyclin B1 is higher compared to cyclins E or A. The level of expression of D-type cyclins varies markedly depending on the cell type and the phase of cell growth. High sensitivity of the instrument and low level of cell autofluorescence, therefore, are of greater importance for the detection of cyclins E or A than of cyclin B1 or D-type cyclins.

While isotypic immunoglobin or irrelevant isotypic antibody are generally accepted as appropriate controls, they are not perfect for this purpose, as their fluorescence may also vary depending on the source (vendor) and may not be representative the actual background. Ideal controls represent the cells of the same type and of the same species, but with the gene that codes for the detected protein deleted. Such control cells should be subjected to identical immunocytochemical procedure as the studied cells. Unfortunately, few cell lines with deleted cyclin genes are currently available.

Anticipated Results

Univariate cellular DNA content analysis

Figure 8.4.3 presents DNA frequency histograms of HL-60 cells stained with DAPI according to Alternate Protocol 1 and Alternate Protocol 2, respectively. As is evident based on differences in DNA content, one can identify a population of $G_{0/1}$ cells with uniform low DNA content values, G_2/M cells with DNA content twice that of $G_{0/1}$ cells, and S phase cells with intermediate DNA content. To reveal the percentage of cells in $G_{0/1}$, S, and G_2/M, the DNA frequency histograms in the figure were deconvoluted using the Multicycle software (Phoenix Flow Systems). Similar results are expected when using Basic Protocols 1 and 2. Supravital cell staining with Hoechst 33342, however, yields less accurate DNA measurements, which

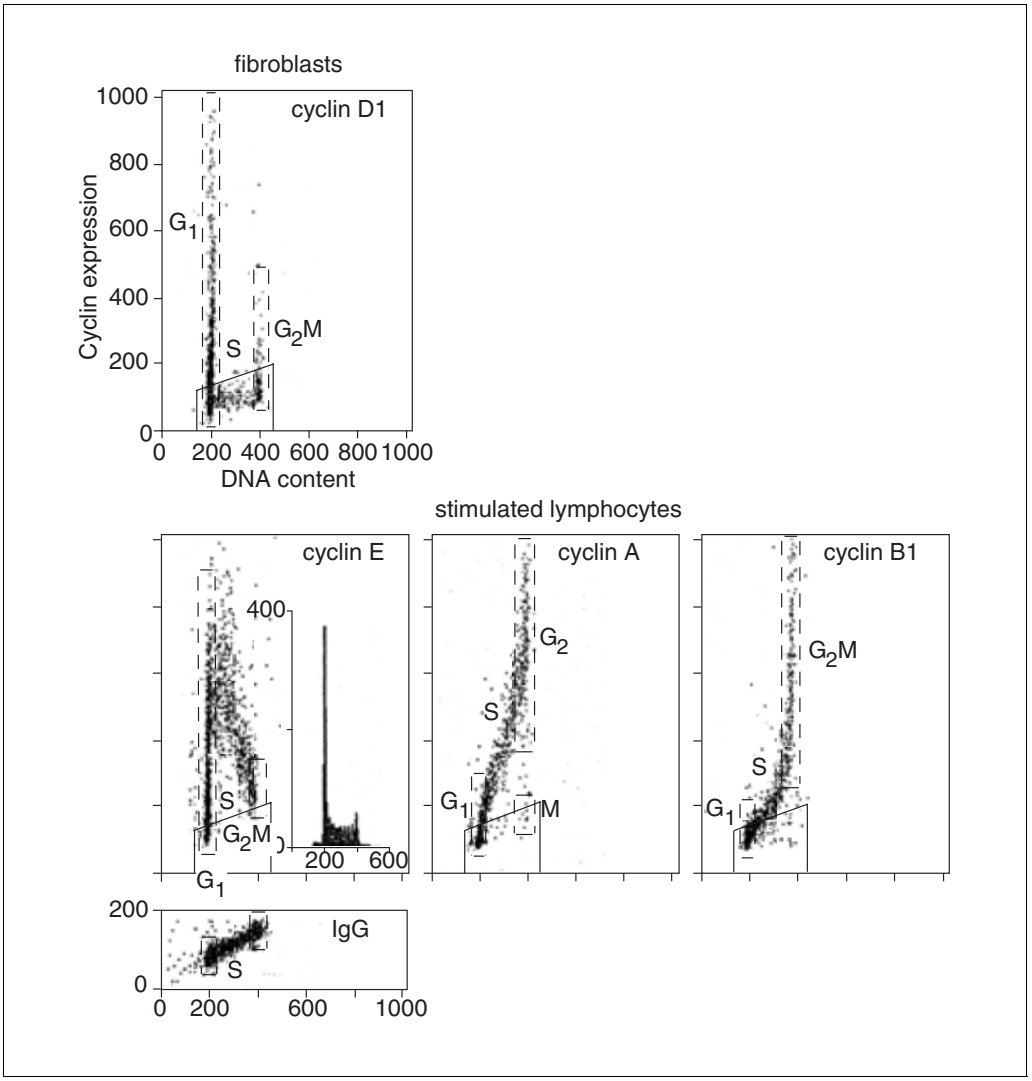

Figure 8.4.5 Bivariate cyclin versus DNA content distributions (scatter plots) showing expression of cyclin D1 in normal human fibroblasts and cyclins E, A, and B1 in mitogen-stimulated human lymphocytes. The trapezoidal windows represent level of fluorescence of the respective control cells stained with the isotype IgG, rather than the respective cyclin-anti-cyclin antibody. The $G_{0/1}$ and G_2/M populations gated based on differences in DNA content are marked by dashed lines.

are reflected by the increased width of the $G_{0/1}$ and G_2/M peaks (i.e., increased CV of the mean fluorescence of $G_{0/1}$ and G_2/M cell populations).

Bivariate analysis of DNA content and cyclin expression

The scheduled timing of expression of cyclins B1, A, E, and D1 in relation to the major phases of the cell cycle is reflected by a very characteristic pattern of the bivariate cyclin–versus–cellular DNA content distributions. These distributions are shown in Figure 8.4.5 for normal human proliferating lymphocytes (cyclins B1, A, and E) and fibroblasts (cyclin D1). As is evident from the cytograms, the expression of cyclin B1 is limited to late S phase cells and the cells with a G_2/M DNA content, while early- and mid–S phase cells show a very low level of this protein. Cells in G_1 phase are essentially cyclin B1–negative.

Similar to cyclin B1, expression of cyclin A is minimal in G_1 cells. It becomes pronounced, however, during S phase where its level progressively increases as the cells advance towards G_2. Maximal expression of cyclin A is seen in cells having a G_2/M DNA content (Fig. 8.4.5). It should be mentioned, however, that because cyclin A is abruptly degraded during prometaphase (Pines and Hunter, 1991), mitotic cells that have advanced past prometa-

phase are essentially cyclin A–negative (not shown).

Expression of cyclin E can be summarized as follows: (1) the maximal level of this protein is detected in the cells undergoing transition from G_1 to S; (2) its level continuously decreases during cell progression through S, with the result that most G_2/M cells are cyclin E–negative; and (3) a distinct threshold in cyclin E expression is apparent at the G_1/S transition. As it is evident from the continuity of the cell clusters on scatter plots (Fig. 8.4.5) the cells have to accumulate cyclin E above the threshold level to enter S phase.

The presence of cyclin D1 in exponentially growing normal fibroblasts is limited to cells in $G_{0/1}$ (Fig. 8.4.5). Most cells in S and G_2/M are cyclin D1–negative, with the exception of a few cells with a G_2/M DNA content. The latter may be G_1 cell doublets, since not all doublets can be identified by analysis of the shape (pulse width) of the electronic signal.

It should be stressed that, as mentioned before (see Background Information), the cyclin distributions as shown in Figure 8.4.5 characterize only those cells that are growing normally, exponentially, and asynchronously. The distributions are very different when the cell-cycle progression is perturbed, or in the case of some tumor cell lines that display unscheduled expression of these cyclins.

Time Considerations

For Basic Protocol 1 (PI staining), cell fixation takes ~10 min, but cells have to be kept in fixative 2 hr; the cell staining procedure takes ~45 min. Alternate Protocol 1 (DAPI staining) requires similar times for cell fixation. However, because there is no need to incubate cells with RNase, the time of staining is shorter. The detergent-based cell-staining procedure of Basic Protocol 2 takes ~60 min. For Alternate Protocol 2, which is simpler, cell staining takes ~15 min. Supravital staining of cells (Basic Protocol 3) requires ~20 min for staining, although extended staining times (up to 90 min) may be needed for some cell types.

For Basic Protocol 4 (DNA and cyclins) cell fixation takes ~10 min, but the cells have to be kept in fixative for ≥2 hr. The cell-staining procedure takes ~1 hr if the antibodies used are directly conjugated with a fluorochrome. The staining may take an additional 40 min if the primary antibody is not fluorochrome-tagged and therefore indirect labeling has to be used. It is sometimes convenient, however, to leave the cells for incubation with the primary antibody overnight, at 4°C.

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