Chapter 9

BrdU Staining and Multiparameter Flow Cytometric Analysis of the Cell Cycle

Introduction

Somatic cells proliferate to support tissue and organismal growth and to replace damaged cells. In the case of adaptive immunity, T and B lymphocytes proliferate (clonal expansion) in response to foreign antigenic stimulation. This hallmark response (along with the process of differentiation) ensures that sufficient numbers of antigen-specific effector and memory lymphocytes arise to successfully deal with the offending antigen (eg, pathogenic microbes, viruses, toxins, and other foreign substances). Determination of the frequency and the nature of cells that respond to stimuli (and the type and magnitude of response measured at the single cell level) is crucial for better understanding the cellular basis of immunological and inflammatory responses in health and disease. For this reason, multiparameter flow cytometric analyses of lymphocyte activation and proliferation (cell cycle entry and progression) are featured in many immune function studies.

The eukaryotic Cell Cycle (aka, Cell Division Cycle) consists of a series of events that are involved in the growth, replication, and division of cells.[1] The cell cycle can be subdivided into two major stages, interphase (a phase between mitotic events) and mitosis (***Figure 1***). There are three distinct, successive stages within interphase, called G1, S, and G2 phases. During G1 (first gap), cells "monitor" their environment and upon receipt of requisite signals, they induce growth (synthesize RNA and proteins). If conditions are right, cells "commit" to DNA synthesis (S phase) and replicate their chromosomal DNA. A G2 phase (second gap) follows in which cells continue to grow and prepare for mitosis.
The G2 gap allows time for the cell to ensure DNA replication is complete before initiating mitosis. In mitosis (division), there are four successive phases called prophase, metaphase, anaphase, and telophase that are accompanied by cytoplasmic division (cytokinesis) giving rise to two daughter cells. For the most part, upon completion of the process, each daughter cell contains the same genetic material as the original parent cell and, in the case of lymphocytes, roughly half of its G2 level of cytoplasm.

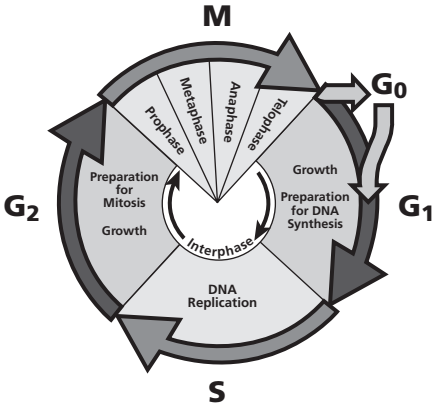

Figure 1. Cell cycle phases.

In addition to these specific stages, the G0 phase has been described for cells that exit from the cell cycle and enter a quiescent, nondividing state. In response to external stimuli, some quiescent cells may undergo reactivation and express early response genes. Resting lymphocytes, for example, can leave G0 and enter the G1 phase of the cell cycle. The G0–G1 transition is marked by cell growth with measurable increases in newly-synthesized RNAs and proteins. This transition is reflected by the increased forward-scattered light signals (blast transformation) and by the expression of early cell-surface activation antigens (eg, CD69 and IL-2Rα/CD25, see ***Chapter 5***) on cells as detected by immunofluorescent staining and flow cytometric analysis. Another consequence of cellular activation may be the induction of programmed cell death (apoptosis), a topic featured in ***BD Biosciences Apoptosis Instruction Manual***. Together, through the counterbalancing processes of cell proliferation and apoptosis, and the establishment of quiescent or nondividing states, appropriate numbers and various types of somatic cells (including cells of the Immune System) are dynamically maintained (homeostasis) throughout the body's lifespan.

Flow Cytometric Analysis of Cycling Cell Populations

A number of fluorescent probes have been developed for the flow cytometric analysis of cycling cells.[1] The prototype for single-color flow cytometric analysis of cycling cells uses propidium iodide staining of the total cellular DNA content expressed by individual cells within activated cell populations. Further discrimination of the cycling status of cells can be achieved using multicolor flow cytometric analyses with two or more fluorescent probes. For example, cells can be analyzed by immunofluorescent staining of incorporated bromodeoxyuridine (BrdU) and staining with a fluorescent DNA-specific dye such as 7-aminoactinomycin D (7-AAD). This method enables determination of the frequency of cells that have synthesized particular levels of DNA (ie, during the time interval that they were exposed to and incorporated the thymidine analog, BrdU) in the context of the G0/G1, S, and G2/M phases defined by total cellular

DNA staining (ie, determined at the point in time when cells were stained for their total DNA levels). In addition, the fluorescent nucleic acid stain, Pyronin Y (PY), can be used to selectively stain RNA in the presence of a DNA specific dye (such as 7-AAD, DAPI, or Hoechst). Two-color flow cytometric analysis of RNA and DNA coexpression patterns permits further discrimination of cells within either G0 or G1 cell cycle phases based on their distinctive RNA contents. Multicolor flow cytometric analyses of cycling cell populations, such as with the combined use of 7-AAD and PY and immunofluorescent staining of incorporated BrdU or other markers (ie, three-color analyses and beyond), provide even higher resolution analyses of cells within different cell cycle compartments.

The combined use of immunofluorescence and fluorescent cell cycle probes with multiparameter flow cytometry provides an extremely important tool for analyzing the complex behaviors of individual cells within cell populations that mediate immunological responses. Detailed information can be obtained concerning the correlated expression patterns of cellular events that lead from cellular activation, growth, proliferation and differentiation to generate cells that play particular roles in immunological and inflammatory responses. Information of this type is crucial for better understanding how the Immune System works and thus how it can be manipulated to promote health.

Flow Cytometric Analysis of Cellular DNA Content

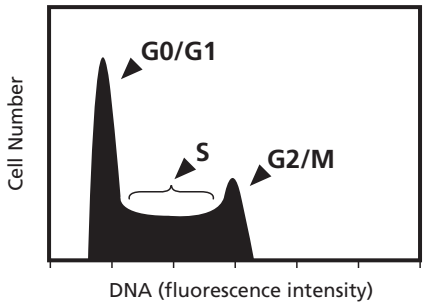

Figure 2. Analysis of relative cellular DNA content using a fluorescent DNA dye and flow cytometry. This illustration depicts a typical data histogram that can be obtained from the relative DNA content analysis of cells within an actively-cycling cell population. The amount of DNA dye that is bound and the strength of the fluorescence signal it gives upon flow cytometric analysis is proportional to each cell's DNA content. The measurement of relative cellular DNA content is useful for identifying cells within G0/G1, S, and G2/M phases of the cell cycle.

Using fluorescent nucleic acid dyes, it is possible to identify the proportions of cells that are in one of the three interphase stages of the cell cycle by using flow cytometry to measure their relative DNA content (see ***Figure 2 and Table 1***). Flow cytometric analyses of activated cell populations that are stained with a DNA stain, such as propidium iodide (PI), lead to the generation of characteristic cellular DNA content profiles as shown in ***Figure 2***. These histograms can be separated into regions that represent cells within G0/G1, S, and G2/M phases of the cell cycle. Cells that are in the G0/G1 phase (before DNA synthesis) have a defined amount (1×) of DNA (ie, a diploid chromosomal DNA content).

During S phase (DNA synthesis), cells contain between 1× and 2× DNA levels. Within the G2 or M phases (G2/M), cells have a 2× amount of DNA (ie, a tetraploid chromosomal DNA content).

Table 1. Commonly-used Fluorescent Dyes that Stain DNA for Cell Cycle Analysis by Flow Cytometry

Dyes	Excitation Wavelength	Compatibility-Viable Cells	Compatibility-Fixed Cells	DNA Profile	Multicolor Analysis
Propidium Iodide	488 nm	No	Yes	Good	Yes*
7-AAD	488 nm	No	Yes	High CV	Yes
DAPI	350 nm	No	Yes	Good	Yes
Hoechst 33342	350 nm	Yes	Yes	Good	Yes

* Propidium Iodide can be combined with FITC conjugates.

Propidium Iodide

Propidium Iodide (PI)[2,3] is the most widely-used fluorescent dye for staining DNA in whole cells (or isolated nuclei). PI intercalates into the DNA helix of fixed and permeabilized cells. Because PI can stain both double-stranded RNA (dsRNA) and DNA (dsDNA), cells must be treated with RNase to ensure that PI staining is DNA specific. BD Biosciences Pharmingen offers PI/RNAse staining buffer suited for this purpose (Cat. No. 550825) PI can be excited with the 488 nm wavelength of light typically generated by single-laser, benchtop flow cytometers. Since PI fluoresces strongly in both the orange and red regions (broad emission centered around 617 nm), it is often limited to use with fluorescein-conjugated antibodies (~525 nm peak emission) in single-laser, two-color flow cytometric analyses.

PI does not cross the intact plasma membrane of viable cells. However, PI can readily enter dead cells (and cells in late stages of apoptosis or that are fixed) that have damaged plasma membranes and can stain their dsRNA and dsDNA. For this reason, PI is also widely used as a discriminator of live and dead cells in experiments using immunofluorescent staining of unfixed cells with flow cytometric analyses. BD Biosciences Pharmingen offers a Propidium Iodide Staining Solution (Cat. No. 556463) that can be used for this purpose.

a. Protocol for Staining DNA with Propidium Iodide for Cell Cycle Analysis[2, 3]

   1. Fix cells with ice-cold 70% ethanol (≥ 1 hr, 4°C). Make sure that the cell suspension is thoroughly resuspended. Ethanol should be added dropwise while vortexing the cells to ensure fixation of all cells and to minimize clumping. Once fixed, cells may be stored for months in 70% ethanol at 4°C prior to PI staining and flow cytometric analysis.

   2. Pellet ~10^6 cells (400 × *g*, 5 min) in tubes and wash 1× in staining buffer [Dulbecco's PBS (DPBS) with 2% FCS and 0.01% NaN_3, 0.2 µm-pore filtered]. Ethanol-fixed cells may require higher centrifugal speeds to be pelleted tightly since they become more buoyant upon fixation than freshly-isolated or cultured cells. Care should be taken when aspirating off supernatants after centrifugation steps (especially after spinning cells out of ethanol) so that the cell pellet is not disturbed and cells are not lost.

3. Treat cells with ribonuclease A (RNase A) (Sigma, Cat. No. R5500; 100 Kunitz units/mg protein). The RNase A can be dissolved in DPBS at a concentration of 1 mg/ml, aliquoted, and stored frozen (–80°C). Add 50–100 µl of RNase A to each cell sample and incubate (30 min, 37°C).

4. Stain cells with 5–20 µg of PI (Sigma, Cat. No. P4170; Stock PI is at 1 mg/ml in distilled H_2O) added to 1 ml of staining buffer. Incubate for ≥ 30 min (room temperature) and then analyze samples by flow cytometry using linear amplification. Store samples protected from light at 4°C until flow cytometric analysis (ie, within 24 hours). When analyzing, keep the flow rate under 400 events/second.

Representative data from the flow cytometric analysis of PI-stained cells is shown in ***Figure 3***.

Note: PI is a potential carcinogen and must be handled with extreme care.

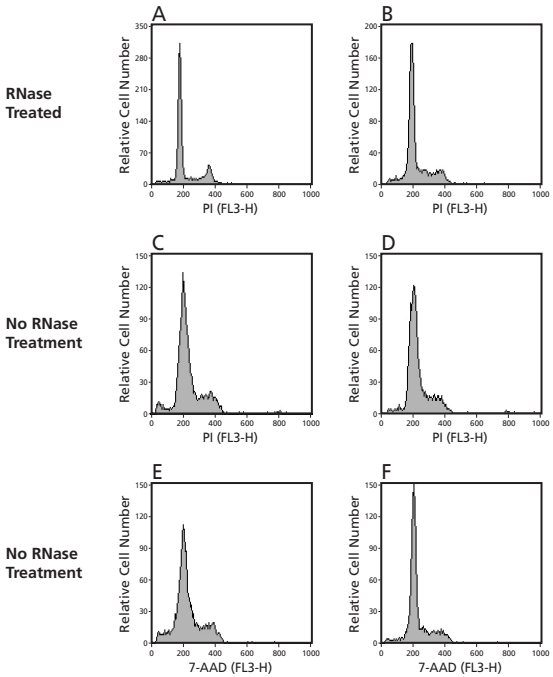

Figure 3. DNA content histograms: PI versus 7-AAD. Cells from two mouse T cell lines, MGG3 (panels A, C, and G) and C20.4 (panels B, D, and F), were harvested, washed 1× with staining buffer, and fixed with ice-cold 70% ethanol (4°C, 1 hr). Cells were then washed to remove the ethanol. Panels A and B: Cells were treated with 100 µg of RNase A (30 min, 37°C) and were stained with 10 µg/ml of PI. Panels C and D: Cells were not treated with RNase A before PI staining. Panels E and F: Cells were stained with 10 µg/ml of 7-AAD. Notice that PI staining with RNase treatment (Panels A and B) gives a very clean DNA profile compared to no RNase treatment (Panels C and D). The DNA profiles obtained by 7-AAD staining of these same cells can be more variable with either higher (Panel E) or similar (Panel F) CV's when compared with the corresponding patterns for RNase-treated, PI-stained cells. Because of this variability, 7-AAD is not recommended as the first choice for single-color DNA content analysis.

Optional Protocol for 2-color Analysis with PI: First stain cells by direct or indirect immunofluorescence using fluorescein-conjugated reagents and then fix and stain cells as directed above. The DNA profiles for the cell subpopulations defined by the differential expression of a cell surface antigen can then be determined. Note that fixation of cells with 70% ethanol may interfere with the detection of some antigens even if they are stained prior to fixation.

Note: An alternative procedure is to incubate cells (30 min, 37°C) with a solution of 5 – 20 µg PI/ml in DPBS containing 50–100 µg/ml of RNase A (Cat. No. 550825).

7-aminoactinomycin D (7-AAD)

7-AAD is a DNA-specific dye that can be used for staining fixed and permeabilized cells to determine the DNA content profiles of cell populations in multicolor flow cytometric analyses.[2,4] It is excited by the 488 nm wavelength (although excited better at 530 nm) of light typically provided by single laser flow cytometers. 7-AAD yields fluorescence emissions (emission peak ~ 650 nm) farther into the red light spectrum than PI and has very little spectral overlap with R-phycoerythrin (PE; emission peak ~578) and fluorescein (emission peak ~ 525).[2] For this reason, 7-AAD can be used in the simultaneous, single-laser analysis of cellular DNA content (cell cycle position) and the coexpressed levels of two other cell-associated molecules detected by fluorescein- and phycoerythrin-conjugated antibodies (three-color fluorescence analyses). Additional parameters can be included in 7-AAD-based, cell cycle studies with the use of an additional laser(s) and fluorescent probes (eg, nucleic acid dyes and antibodies) for flow cytometric analyses of the nature of cells that transit through the cell cycle.

Although 7-AAD is useful for multicolor cell cycle analyses, it's coefficient of variation (CV) of DNA fluorescence is larger (ie, broader G1 peak) than that obtained with PI. This may in part be explained by the fact that 7-AAD staining is more affected by chromatin structure (eg, the decondensation of chromatin upon cellular activation) than is PI or other DNA dyes. It should also be noted that 7-AAD may not give adequately strong fluorescence signals when using low powered (air cooled) 488 nm laser-based flow cytometers. This can result in DNA profiles that are not as well defined as those generated with other DNA stains. Because of this, PI is normally recommended for single-color DNA-content profiling whereas 7-AAD can be used for multicolor staining, (eg, DNA/RNA [7-AAD/PY] and BrdU/DNA [BrdU/7-AAD] staining). 7-AAD, like PI, will not enter live cells but will readily stain dead cells. For this reason, 7-AAD is also used as a live–dead cell discriminator for flow cytometric analyses. A solution of 7-AAD for viability staining is available from BD Biosciences Pharmingen as BD Via-Probe™ (Cat. No. 555815).

Please see the BrdU Flow Kit Staining Protocol (below) for the optional use of 7-AAD as a DNA stain in multicolor flow cytometric analyses. Representative data showing 7-AAD staining and flow cytometric analysis of cells is shown in ***Figure 3***.

DAPI (4′-6-diamidino-phenylindole-2HCl) and Hoechst 33342 (HO33342)

Both of these dyes have a high specificity for DNA and bind preferentially to the A-T base pairs.[2] These dyes can be excited at ~355nm by a UV light source (UV laser beam or a mercury arc-lamp). Since they are specific for binding to DNA, ribonuclease (RNase) treatment is not needed. DNA profiles of fixed cells are very similar to that of RNase-treated, PI-stained cells as shown in ***Figure 4***. An advantage of these dyes is that they can also be used to stain viable cells for cell cycle analyses. Of the two dyes, HO33342 is the preferred dye for maintaining the viability of stained cells because it is less toxic than DAPI. Viable cell staining is performed by directly adding HO33342 to cells in culture and incubating for 30 – 90 minutes depending on the cells being labeled.[2]

a. Protocol for Staining DNA with DAPI or Hoechst 33342 for Cell Cycle Analysis[2]

   1. Fix cells with 70% ice-cold ethanol (≥ 1 hr, 4°C). Make sure that the cell suspension is thoroughly resuspended. Cells may be stored for months in 70% ethanol at 4°C.

   2. Centrifuge cells at 400 × *g* (5 min) and wash 1× in staining buffer (DPBS with 2% FCS and 0.01% NaN_3, 0.2 µm-pore filtered).

   3. Resuspend in staining buffer with 0.5–1 µg/ml DAPI (Sigma Cat. No. D8417) or (0.5 – 1 µg) Hoechst 33342 (Sigma Cat. No. B2261). Incubate for ≥ 30 min (room temperature) and then analyze samples on a flow cytometer. Store samples protected from light at 4°C until flow cytometric analysis (ie, within 24 hours). When analyzing, keep the flow rate under 400 events/second. Stock solutions of DAPI or HO33342 can be made at a concentration of 1 mg/ml in distilled H_20.

Representative data showing DAPI or PI staining and flow cytometric analysis of cells is shown in ***Figure 4***.

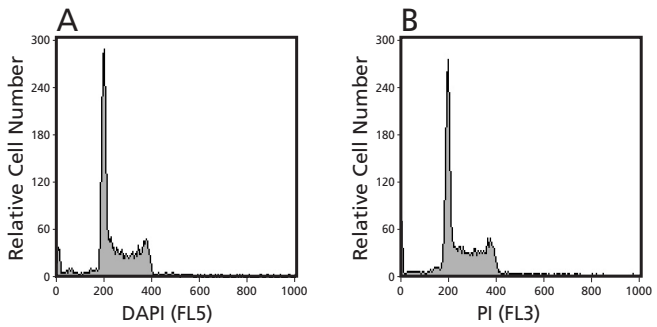

Figure 4. Comparison of DNA staining profiles using DAPI or PI. MGG3 T cells were fixed with ice-cold 70% ethanol for 1 hour at 4°C. The cells were either stained with DAPI (Panel A) or they were RNase A-treated and then stained with PI (Panel B). Cells were then analyzed on a BD LSR cytometer. The DNA profiles of cells stained with DAPI and PI are similar.

Flow Cytometric Analysis of Cellular RNA Content

Based on cellular DNA content alone, flow cytometric analysis can only reveal cells within the broadly-defined G0/G1, S, and G2/M phases. When staining cells for DNA content alone, cells within the G0 and G1 compartments cannot be distinguished. However, cells within G0 and G1 phases (and different stages within G1) can be distinguished by measurements of cellular RNA (and DNA) contents.[2,5,6] The RNA levels measured in cells are mostly attributable to ribosomal RNA contents that can make up as much as ~80% of total cellular RNA content. Cellular RNA content increases as cells progress through G1, S, G2, and M phases of the cell cycle.

G0 cells are defined as resting or quiescent cells that have relatively low RNA content and a diploid chromosomal DNA content. Some quiescent cell types can be activated with an appropriate stimulus to enter the G1 phase and progress through cell cycle. The G1 phase is described as the phase wherein cells of exponentially growing populations increase their RNA and protein content (cell growth) to a level that may ultimately support their "competence" to enter S phase. Early (G1a) and late (G1b) compartments of the G1 phase can be distinguished by the flow cytometric analysis of the DNA and increasing RNA levels coexpressed by activated cell populations. Acridine Orange and PY (in combination with a DNA-specific dye) are dyes that can be used to differentially stain RNA and are described briefly below.

1. Acridine Orange [3,6-bis-(Dimethyamino)acridinium chloride hemi (zinc chloride salt)][2, 5]

   Acridine Orange (AO) is a metachromatic nucleic acid dye that can be efficiently excited with a 488 nm wavelength of light. It can emit strong fluorescence signals at both 530 nm and 640 nm. The unique binding and corresponding fluorescent emission characteristics of AO allow for the distinction of RNA and DNA level measurements in permeabilized cells. When AO is used for differential DNA versus RNA staining, it intercalates into double-stranded DNA as a monomer and fluoresces green (530 nm). In contrast, AO can bind to the phosphate groups of single-stranded RNA in an aggregated or stacking pattern that causes it to fluoresce red (640 nm). To obtain differential staining of DNA and RNA in cells, their RNA must be selectively denatured (ie, cells are treated with EDTA at low pH) to ensure that it is all in a single-stranded form. Under appropriate conditions, AO staining can be used to discriminate cells within different stages of the G1 phase of the cell cycle. A detailed AO staining protocol is found in reference 5.

   A major disadvantage of using this dye is that it sticks to the plastic tubing in cytometers. For this reason, some researchers have found this dye too problematic to work with. If AO-stained samples are run on FACScan or FACSCalibur Flow Cytometers, then bleach must be run through the system for 10 minutes after its use. The completeness of AO removal can be monitored by running unlabeled viable cells through the system to see if there is any evidence of residual AO leaching from the tubing that can

stain cells. If AO remains, the cells will start to fluoresce green. If the lines are clear, ethanol should be run through the lines for 5 minutes. For cell sorters, it is recommended that the sample tubing be replaced.

2. Pyronin Y[2, 6–8]

   Pyronin Y (PY) is the xanthene homologue of acridine orange. PY can be excited with the 488 nm wavelength of light typically generated by single-laser, benchtop flow cytometers and read at ~575 nm. At low concentrations, PY preferentially binds to dsRNA and fluoresces. High concentrations of PY can denature dsRNA; PY's fluorescence is quenched when it is bound to ssRNA. At high concentrations, PY intercalates into dsDNA and fluoresces. Therefore, the dose of PY used for staining cellular RNA is crucial. PY should be used at a concentrations of 2 µM or less to avoid nonspecific staining. PY staining of cellular DNA can be blocked by co-staining cells with a DNA-specific dye. PY can be combined with 7-AAD or any of the UV-excited dyes (DAPI and Hoechst 33342) for the correlated analysis of RNA and DNA levels expressed by cells.[2, 6–8]

   High-resolution, cell cycle analyses are possible with PY and 7-AAD staining and flow cytometry. This method enables the distinction of individual quiescent G0 cells from activated G1 cells based on their RNA content. Cells in early G1, called the G1a phase, have a greater RNA content than G0 cells but a lower RNA content than S phase cells. Cells in the G1b phase have higher total RNA contents than G1a cells. Moreover, G1b cells have RNA contents at the same level or above the RNA levels expressed by S phase cells with the lowest cellular RNA content. Interestingly, even though M phase cells have greater total RNA levels (ie, dsRNA + ssRNA) than G2 phase cells, M phase cells stain less strongly with PY than do G2 phase cells.[6] This may be due to the lower levels of dsRNA that are present in M versus G2 phase cells. It is important to use PY staining with fixed cells since PY can label mitochondria in viable cells.

   Please see the BrdU Flow Kit Staining Protocol (below) for the optional use of PY as an RNA stain. Examples of flow cytometric analyses of PY-stained cells can be found in ***Figures 7 and 8***.

Determination of S Phase Activity using BromodeoxyUridine

The immunofluorescent staining of incorporated bromodeoxyuridine (BrdU) and flow cytometric analysis enable high resolution determinations of the frequency and nature of individual cells that have synthesized DNA in the course of a specific time interval. In this method, BrdU (an analog of the DNA precursor thymidine) is incorporated into newly synthesized DNA by cells entering and progressing through the S (DNA synthesis) phase of the cell cycle.[9, 10]
The incorporated BrdU is stained with specific anti-BrdU fluorescent antibodies (that do not recognize thymidine). The levels of cell-associated BrdU are then measured by flow cytometry. Often, staining with a dye that binds to total DNA, such as 7-AAD, is coupled with immunofluorescent BrdU staining. With this combination, two-color flow cytometric analysis permits the enumeration and characterization of cells that have actively synthesized DNA (BrdU incorporation

for a defined time interval) in terms of their cell cycle position (ie, G0/1, S, or G2/M phases as defined by 7-AAD staining intensities).[11, 12]

Prolonged exposure of cells to BrdU allows for the identification and analysis of actively-cycling, as opposed to non-cycling, cell fractions. Pulse labeling of cells with BrdU at various time points, permits a detailed examination of cell-cycle kinetics. BrdU incorporation studies have been used in a variety of experimental protocols. These include *in vitro* and *in vivo* labeling systems.

An important feature of BD Pharmingen™ BrdU Flow Kit (Cat. No. 559619) is that it provides reagents for immunofluorescent BrdU staining with a protocol that is compatible with the use of additional fluorescent nucleic acid dyes (eg, 7-AAD and PY) and antibodies (eg, phycoerythrin- and/or allophycocyanin-conjugated antibodies) specific for other cellular molecules. These latter molecules may include cell surface antigens or intracellular proteins (eg, cytokines or cyclins) whose expression or activity may be related to the cell's activation, entry and progression through cell cycle or cell death. This is possible because the BrdU Flow Kit staining protocol avoids DNA-denaturing agents such as acid, ethanol, and high temperatures that can change cellular light-scattering characteristics and limit the recognition of antigens by fluorescent antibodies.[13–15]

Fluorescent antibodies that are capable of recognizing cell surface antigens or proteins in cells (eg, cytokines) that have been fixed with paraformaldehyde and permeabilized with saponin can be used with the BrdU Flow Kit. With this combination of reagents, the expressed levels of various surface or intracellular proteins can be measured by flow cytometry relative to the cell's DNA synthetic activity (BrdU incorporation level). The kit ensures consistent results by providing detailed instructions and all critical reagents necessary to implement the staining protocol.

1. Labeling of Cells with BrdU

   a. *In vitro*-labeling of cultured cells and cell lines with BrdU

      Cells can be incubated with BrdU (Mol. Wt. 301.9) at a final concentration of 10–20 µM in cell culture medium (ie 10 – 20 µl of 1 mM BrdU per ml of culture medium).[16] Prolonged exposure of cells to BrdU allows for the identification of actively-cycling cell populations. Pulse labeling of cells by brief BrdU exposures at various time points permits the determination of cell-cycle kinetics (eg, the timepoint for initiation of DNA synthesis).

      To label cells *in vitro*, carefully add 10 µl of BrdU solution (1 mM BrdU in 1× DPBS) directly to each ml of tissue culture media. For this step, it is important to avoid disturbing the cells in any way (eg, by centrifugation steps or temperature changes) that may disrupt their normal cell cycling patterns. The cell culture density should not exceed 2×10^6 cells/ml. The treated cells are then incubated for the desired length of time. For pulse-labeling experiments, the choice of time points and lengths of time for pulsing depend on the test cell population's rate of cell cycle entry and progression. For example, an effective length of

time for pulsing an actively proliferating cell line is 30 – 45 minutes. Cells from the same population that are not BrdU-labeled are the recommended negative staining control for this assay. This will allow determination of background staining levels for the anti-BrdU monoclonal antibody.

b. Methods for *in vivo*-labeling of mouse cells with BrdU

Two common methods for *in vivo* BrdU labeling of cells have been reported. In one method, a BrdU-containing solution can be injected into the peritoneum (i.p.) of each mouse. (A 10 mg/ml solution of BrdU in sterile 1× DPBS is provided in the BrdU Flow Kit for *in vivo* use. Inject mice i.p. with 100 µl [1 mg] of the BrdU solution.)[17, 18] Incorporation of BrdU can be readily detected in the thymus and bone marrow in as little as 1 hr post injection. In a second method, mice can be fed with BrdU by adding it to their drinking water. Dilute BrdU to 0.8 mg/ml in the drinking water. The BrdU mixture should be made up fresh and changed daily.[19] Prolonged feeding of BrdU can have toxic effects for the animal.[17–19] For long-term studies, some researchers have reported that feeding mice with BrdU for 9 consecutive days followed by a changeover to normal water has worked effectively.[20] BrdU incorporation by cells from these animals has been detected past 70 days.[19]

2. BrdU Flow Kit Staining Protocol (Cat. No. 559619)

a. Immunofluorescent staining of cell surface antigens.

1. Add BrdU-pulsed cells (10^6 cells in 50 µl of staining buffer) to flow cytometry tubes. Staining buffer comprises Dulbecco's PBS with 3% Fetal Bovine Serum (heat inactivated) + 0.09% (w/v) sodium azide.
2. Add fluorescent antibodies specific for cell-surface markers in 50 µl of staining buffer per tube and mix well.
3. Incubate cells with antibodies for 15 minutes on ice.
4. Wash cells 1× by adding 1 ml of staining buffer per tube, centrifuge (5 min) at 200 – 300 × *g*, and discard supernatant.

b. Fix and permeabilize cells with BD Cytofix/Cytoperm™ Buffer.

1. Resuspend cells with 100 µl of BD Cytofix/Cytoperm Buffer per tube.
2. Incubate cells for 15–30 minutes at room temperature or on ice.
3. Wash cells 1× with 1 ml of BD Perm/Wash™ Buffer (as in Step 1d).

c. Incubate cells with Cytoperm Plus Buffer.

1. Resuspend cells with 100 µl of Cytoperm Plus Buffer per tube.
2. Incubate cells for 10 minutes on ice.
3. Wash cells 1× by adding 1 ml of BD Perm/Wash Buffer (as in Step 1d).

d. Re-Fixation of cells

1. Resuspend cells with 100 µl of BD Cytofix/Cytoperm Buffer per tube.
2. Incubate cells for 5 minutes at room temperature or on ice.
3. Wash cells 1× by adding 1 ml of BD Perm/Wash Buffer (as in Step 1d).

e. Treatment of cells with DNase to expose incorporated BrdU.[17, 18]

1. Resuspend cells with 100 µl of diluted DNase (diluted to 300 µg/ml in DPBS) per tube (ie, 30 µg of DNase to each tube).
2. Incubate cells for 1 hour at 37°C.
3. Wash cells 1× by adding 1 ml of BD Perm/Wash Buffer (as in Step 1d).

f. Stain BrdU and intracellular antigens with fluorescent antibodies.

1. Resuspend cells with 50 µl of BD Perm/Wash Buffer containing diluted fluorescent anti-BrdU and/or antibodies specific for intracellular antigens.
2. Incubate cells for 20 minutes at room temperature.
3. Wash cells 1× by adding 1 ml of BD Perm/Wash Buffer (as in Step 1d).

Note: Proceed to Step i if the staining of total DNA and/orRNA levels is not desired.

g. *Optional* — Staining of Total DNA for Correlated Cell Cycle Analysis.

1. Resuspend cells with 20 µl of the 7-AAD solution.

   Representative data showing BrdU and 7-AAD staining and flow cytometric analysis of cells is shown in ***Figure 5***.

h. *Optional* — Staining of RNA using Pyronin Y for Correlated Cell Cycle Analysis.

1. Add 20 µl of a 25 µg/ml solution of Pyronin Y (PY) (Sigma Cat. No. P-9172; stock solution is 1 mg/ml in distilled H_2O) after cells have been incubated with 7-AAD for at least 5 minutes. PY will stain DNA if it is not blocked by a DNA-specific stain. Incubate cells for 5 minutes and then analyze the cells by flow cytometry. PY fluorescence data is usually acquired with linear amplification.

i. Resuspension of cells for Flow Cytometric Analysis.

1. Add 1 ml of staining buffer to each tube to resuspend cells.
2. Analyze stained cells with a flow cytometer (run at a rate no greater than 400 events/sec).

Note: Samples may be stored overnight at 4°C, protected from exposure to light, prior to analysis by flow cytometry.

Representative data showing PY, BrdU, and 7-AAD staining and flow cytometric analysis of cells is shown in ***Figure 7***.

It is important to note that all of the different response phases, from cellular activation, to cell cycle entry and mitosis, and to cell death may be accompanied by the differential expression of intracellular and cell surface molecules (see ***Chapters 1 and 4***, respectively). In addition to serving as cell cycle phase markers, analysis of the expression patterns (coexpressed levels) of functional molecules along with DNA and RNA levels permits high-resolution, multiparameter analysis (eg, by multi-color flow cytometric analysis) of the molecular mechanisms that underlie cell cycling (and differentiation) and apoptosis.

Flow Cytometric Analysis of Stained Cell Samples

The flow cytometric data presented in the following examples (***Figures 5 – 7***) were acquired using a BD FACS™ brand flow cytometer equipped with a 488 nm argon laser. This laser permits the excitation of the fluorescent dyes, fluorescein isothiocyanate (FITC) (FL1), phycoerythrin (PE) (FL2) and 7-AAD (FL3), as well as the generation of forward angle (FSC) and side-scattered (SSC) light signals from illuminated cells. Use of other fluorochromes (eg, allophycocyanin) that are excited by light wavelengths outside of the range generated by the argon laser, require flow cytometers such as the BD FACSCalibur™ that have an additional laser light source (***Figure 8***). It should be noted that with the addition of each different fluorochrome used for multicolor staining, the more critical becomes the challenge of properly compensating overlaps in detection of emitted fluorescent signals. Fluorescent signals from the nucleic acid dyes are normally acquired in the linear signal amplification mode, whereas signals generated by fluorescent antibody staining are typically acquired in a logarithmic mode.

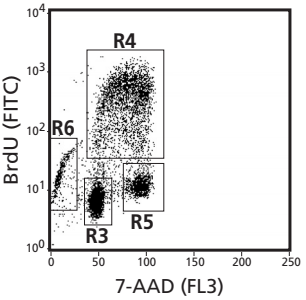

Figure 5. BrdU and 7-AAD coexpression profile for an actively-proliferating cell population. D10.G4.1 T cells were pulsed with 10 µM BrdU for 30 minutes. The cells were then stained for BrdU and 7-AAD using the BrdU Flow Kit and analyzed by flow cytometry. As shown by the boxed region gates, significant proportions of cells are found to occupy distinct cell cycle phases including G0/G1, S, and G2/M. Region 6 identifies apoptotic cells as determined by their sub-G0/G1 levels of DNA (stained by 7-AAD), Region 3 shows cells within the G0/G1 phases (39%) of the cycle, whereas Region 4 includes BrdU$^+$ or S phase cells (39%), with Region 5 showing cells that occupy the G2/M phases (14%).

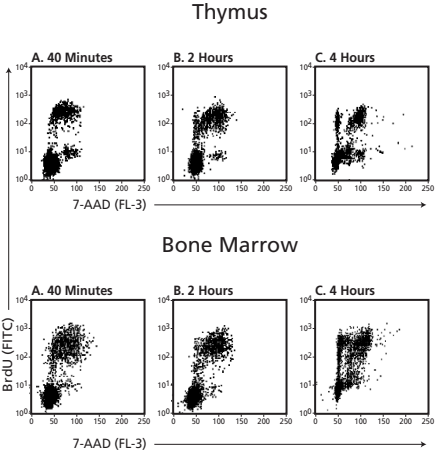

Figure 6. Detection of in vivo-cycling cells. C57BL/6 mice were injected i.p. with 1 mg of BrdU in solution for various time intervals. Animals were sacrificed at 40 minutes, 2 hours, and 4 hours post injection. Thymus and bone marrow cell suspensions were then prepared and stained for incorporated BrdU (FITC-anti-BrdU) and total DNA (7-AAD) levels. The 40–minute timepoint shows the characteristic "horseshoe" pattern that is seen for cell populations that are pulsed with BrdU for a short time. Cells from mice that were pulsed for 2 hours also show the horseshoe pattern. However, another cell population of G0/G1 cells that has incorporated BrdU and has returned to the G0/G1 phase is now detectable. These cells are positive for BrdU but have 1× DNA levels (ie, diploid chromosomal DNA levels) as determined by their cellular DNA content (7-AAD level). The 4–hour timepoint has an even larger population of BrdU$^+$ G0/G1 phase cells.

In vivo pulsing with BrdU can be used as an important tool for determining the status of cycling cell populations within experimental animals. As shown in ***Figure 6***, significant fractions of cell populations obtained from tissues such as bone marrow and thymus (primary lymphoid organs) incorporate *in vivo*-supplied BrdU readily, within 30 minutes of *in vivo*-pulsing.

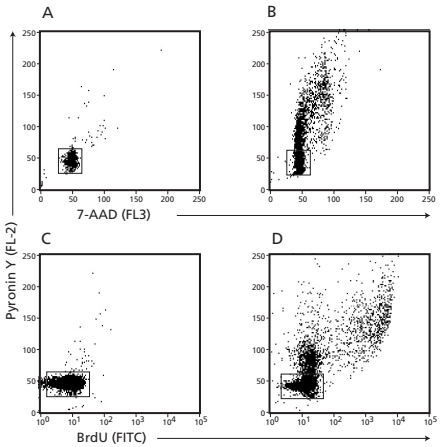

Figure 7. Multiparameter cell cycle analysis of BrdU-pulsed, antigen-stimulated human PBMCs. Human PBMCs were cultured alone (Panels A and C) or with 0.5 µg /ml of tetanus toxoid for 6 days (Panels B and D). 2 cells were pulsed with BrdU (20 µM) for 2 hr prior to harvest. Cells were then stained for their levels of incorporated BrdU (FL1), PY (FL-2), and 7-AAD (FL-3). The results show that the unstimulated cells are primarily in G0 with baseline levels of DNA (7-AAD) and RNA (PY) (Panel A). In contrast, significant proportions of the activated cells either express higher RNA and the same DNA levels (G1 phase) or coexpress higher levels of both RNA and DNA (Panel B). Likewise, nonactivated cells show no significant incorporation of BrdU (and baseline RNA levels) (Panel C) whereas a large proportions of activated cells show higher levels of RNA with or without incorporated BrdU (Panel D) in keeping with the coexpression pattern seen in Panel B.

Multiparameter RNA and DNA analysis of proliferating cell populations. Utilizing the BrdU Flow Kit allows for the simultaneous staining and analysis of cells for their cell surface phenotype, total cellular DNA and RNA contents, and levels of actively-synthesized DNA detected by BrdU that was incorporated for a specific time interval (***Figures 7 and 8***).

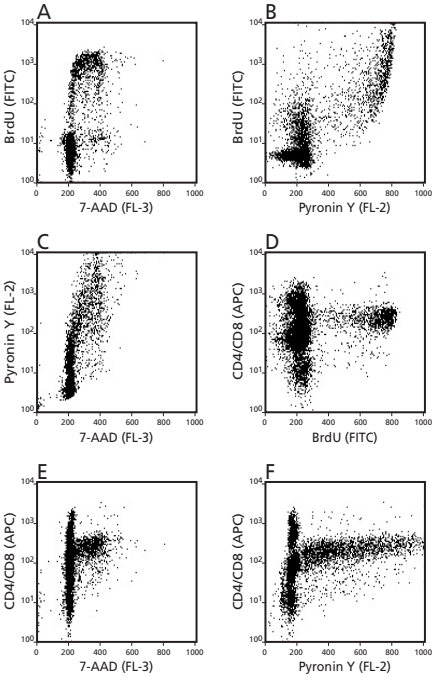

Figure 8. Cell cycle analysis of antigen-activated CD4⁺ and CD8⁺ Human PBMCs. Human PBMCs from a tetanus-vaccinated individual were stimulated with 0.5 µg/ml of Tetanus Toxoid (List Biologicals) for 6 days. During the final 2 hours of culture, the cells were pulsed with 20 µM BrdU. Cells were harvested and then stained using the BrdU Flow Kit for incorporated BrdU (FITC-anti-BrdU) and total DNA (7-AAD). In addition, total RNA (PY), and cell surface CD4 and CD8 levels (ie, using a cocktail of APC-conjugated anti-CD4 and anti-CD8) were assessed with additional reagents. Panel A shows the 7-AAD/BrdU profile for the population. Approximately 12% of the cells are in S phase. Panel B shows the BrdU and PY coexpression profiles of cells. At least 3 major profiles are noted including $BrdU^{-/low}PY^{low}$, $BrdU^{-/low}PY^{+}$ and $BrdU^{+}PY^{bright}$. These patterns suggest that cells must express a certain threshold level of RNA before they can actively synthesize DNA (ie, enter the S phase). Panel C shows the total DNA/total RNA profile (7-AAD/PY). This profile can be used to separate cells within G0 (low RNA) and G1 (intermediate to high levels of RNA) phases of the cell cycle. The data suggests that the activated PBMC population has many cells in the G1 phase of the cell cycle. BrdU (Panel D), DNA (Panel E), and RNA (Panel F) fluorescence profiles for the $CD4^{+}$ (intermediate APC fluorescence) and $CD8^{+}$ (high APC fluorescence) cell subpopulations are shown. The data suggests that the $CD4^{+}$ cells are primarily responding to antigen activation (ie, show cells with increased levels of cellular RNA and DNA).

Carboxy-fluoresceindiacetate Succinimidyl Ester for Tracking Cell Proliferation by Flow Cytometry

Carboxy-fluoresceindiacetate succinimidyl ester (CFDA SE) is a very effective reagent to study the division progress of proliferating cells.[21] It passively crosses the cell membrane and covalently binds to free amine groups of intracellular macromolecules. Endogenous cytoplasmic esterases remove the carboxyl groups, converting non-fluorescent CFDA SE to fluorescent CFSE that remains cell associated. Upon cell division, CFSE is distributed uniformly between daughter cells. Each cell division reduces the CFSE fluorescent intensity of daughter cells by approximately half. Each successive generation can be counted by the number of discreet fluorescent frequency distributions (eg, histogram "peaks" or dot plot "clusters") that are revealed upon flow cytometric analysis. The multipeak histogram (***Figure 9A***) shows several successive divisions that human peripheral blood lymphocytes have undergone when cultured for 72 hr with phytohemaglutinin.

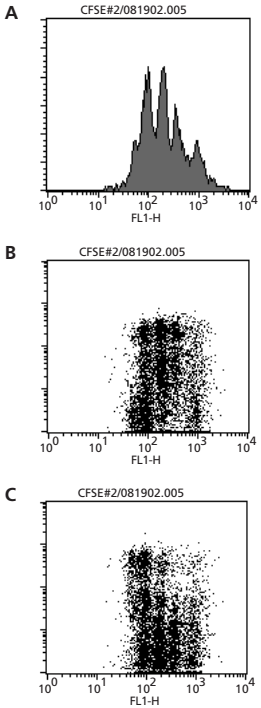

Figure 9. HPBMCs were loaded with 1 µM CFDA SE for 10 minutes at 37°C. Cells were washed twice in 1× PBS then stimulated with 1.5% PHA for 72 hrs. Cells were harvested and then stained with PE anti-human CD4 and allophycocyanin (APC)-anti-human CD8 then analyzed on a BD FACScalibur™. Panel A is the CFSE histogram for the viable cell population. Panel B is the two color dot plot generated by the flow cytometric analysis of cells stained with PE anti-human CD4 and CFSE. Panel C is the two color dot plot obtained for cells stained with allophycocyanin-anti-human CD8 and CFSE.

By using CFSE as a dye for following cell proliferation, one can select additional parameters (eg, CD markers or intracellular cytokines) and perform further flow cytometric analysis to characterize the nature of cells within any cell generation. For example, as shown in ***Figure 9B and 9C***, CFSE staining can be coupled with staining for cell surface CD4 and CD8 to identify the proliferative activities of individual cells within T cell subpopulations. CFSE labeling has also been used to determine the number of divisions required for cells to express new immunoglobulin isotypes[22] or to express cytokines such as Interleukin-4.[23] In addition to its use in experimental culture systems, CFSE-labeling is very useful for determining the proliferative and migratory behavior of cells transferred to adoptive recipient animals.[1]

CFDA SE Labeling Protocol

Dilute CFDA SE in dimethysulfoxide (5 mg/ml is equivalent to 8.8 mM) and store aliquots at –80°C. The working solution of CFDA SE is between 10 nM–5 mM. Researchers should determine the optimal loading concentration for their particular cell type. Normally, a solution of 1 µM CFDA SE in 1× PBS is used to load up to 5×10^7 cells. Cells are loaded at 37°C for approximately 10 minutes. Times can vary depending on how bright or dim you wish to load the cells. CFSE is not highly toxic, but may negatively affect cell function. To stop the reaction, wash the cells twice in 1× PBS. Cells are now ready to be activated or transferred to recipient experimental animals. It is recommended that you confirm the loading of your cells on a flow cytometer prior to proceeding with an experimental protocol.

Summary

In conclusion, a brief overview of reagents and methods for BrdU and nucleic acid staining of cells and the multiparameter flow cytometric analysis of their cell cycle positions has been presented. More detailed information for performing these types of flow cytometric cell cycle analyses is provided by the references listed at the end of this chapter. Kits as well as individual reagents are available from BD Biosciences Pharmingen for staining cells that have been exposed to and incorporated BrdU. Additional reagents, including reagents that utilize propidium iodide and 7-AAD are also listed. The reagents and/or methods referred to in this chapter were presented because they are useful for performing multiparameter flow cytometric analysis of cell populations that are of particular interest in immune function studies.

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BrdU Staining Product List

Description	Clone	Isotype	Apps	Format	Size	Cat. No.
Kits						
FITC BrdU Flow Kit			IC/FCM	FITC	50 tests	559619
APC BrdU Flow Kit			IC/FCM	APC	50 tests	552598
Sets						
BrdU	3D4	Mouse IgG_1, κ	IC/FCM, IHC(Fr)	Purified	0.1 mg	555627
			FCM, IC/FCM	FITC Set	100 tests	556028
			FCM, IC/FCM	PE Set	100 tests	556029
BrdU *In-Situ* Kits						
BrdU *In-Situ* Detection Kit			IHC(F), IHC(Fr), IHC(Zn)		50 tests	550803
BrdU *In-Situ* Detection Kit II			IHC(F), IHC(Fr), IHC(Zn)		200 tests	551321