Multicolor Flow Cytometric Analysis of S-phase (BrdU Incorporating) Cells Using the BD FACSArray™ Bioanalyzer

Introduction

Recent advances in both flow cytometric technology and in reagent development for cell-cycle analysis enable researchers to conduct high resolution, multiparameter analyses of normal and abnormal cycling cells. In the past, researchers have used a number of traditional means, such as measuring the incorporation of tritiated thymidine, to examine the DNA-synthesizing capacity of cell populations. Analysis of the DNA-synthetic (S-phase) activity expressed by individual cells was previously accomplished by labor-intensive means including autoradiographic and light microscopic analysis of cells that incorporated radiolabeled DNA precursors.

The development of new multicolor flow cytometers, fluorescent antibodies, and dyes that bind to defined cell surface and intracellular molecules has provided a quantum leap forward in defining the nature of individual cycling cells within mixed-cell populations.
The immunofluorescent staining of cells which have incorporated BrdU and flow cytometric analysis provides a high-resolution technique to determine the frequency and nature of individual cells that have synthesized DNA. 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.1–4 The incorporated BrdU is stained with specific fluorescent anti-BrdU antibodies. The levels of cell-associated BrdU are then measured by flow cytometry. Often, staining with a fluorescent dye that binds to total cellular DNA, such as 7-amino-actinomycin D (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 (ie, incorporated BrdU) during a defined time interval correlating their cell-cycle position at the point of staining.5,6 Cells in G0/G1, S, or G2/M cell-cycle phases are defined by their total cellular DNA levels (ie, determined by 7-AAD staining intensities). For further information, please see the BD Biosciences Application Note entitled Cell-cycle analysis using the BD FACSArray™ Bioanalyzer7 and the BD Biosciences Techniques for Immune Function Analysis Application Handbook.8

This application note provides a concise method for staining cellular DNA, incorporated BrdU, CD8, and intracellular IL-2 so that a detailed analysis of cycling CD8+ and CD8- lymphocytes can be performed using the BD FACSArray bioanalyzer. By including the integrated 96-well plate loader for sample introduction, the BD FACSArray™ bioanalyzer delivers rapid analyses of cell populations containing various proportions of dead/dying, resting, or cycling cells. BD Biosciences has developed a procedure using its BrdU Flow Kits to measure BrdU incorporation utilizing 96-well U-bottom plates, rather than the conventional flow cytometric staining tubes. This application note describes how the BD FACSArray bioanalyzer and BrdU Flow Kit can be used in a rapid manner to characterize the nature of proliferating human CD8+ lymphocytes.

### Fluorescent Reagents to Use with the BD FACSArray™ Bioanalyzer

The BD FACSArray bioanalyzer has a green laser at 532 nm wavelength and a red laser at 635 nm wavelength. Fluorescent emissions from fluorescent dyes or fluorochromes excited by the green laser are detected with the Yellow and the Far Red detectors, while fluorescent emissions excited by the red laser are detected by the Red and NIR detectors. The cytometer setup and fluorescent reagents demonstrated in this application note are presented in Table 1.

### Table 1. BD FACSArray Bioanalyzer Instrument Configuration for this Four-Color Experiment

<table>
<thead>
<tr>
<th>FLUORESCENCE PARAMETER</th>
<th>FLUOROCHROMES OR DYE</th>
<th>ANTIBODY OR PROBE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow</td>
<td>Phycoerythrin (PE)</td>
<td>Anti-Interleukin-2 (IL-2)</td>
</tr>
<tr>
<td>Far Red</td>
<td>7-AAD</td>
<td>DNA-binding Dye</td>
</tr>
<tr>
<td>Red</td>
<td>Allophycocyanin (APC)</td>
<td>Anti-BrdU</td>
</tr>
<tr>
<td>NIR</td>
<td>APC-Cyanine7 (APC-Cy7)</td>
<td>Anti-CD8</td>
</tr>
</tbody>
</table>

### Materials

Components of the APC BrdU Flow Kit and reagents for characterizing the nature of proliferating human peripheral blood lymphocytes are listed in Table 2.

### Table 2. Materials for the Four-Color BrdU Experiment

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>CAT. NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC BrdU Flow Kit</td>
<td>552598 (50 Test Kit)</td>
</tr>
<tr>
<td>BD Cytofix/Cytoperm™ Buffer</td>
<td>557892 (4 × 50 Test Kits)</td>
</tr>
<tr>
<td>BD Perm/Wash™ Buffer (10×)</td>
<td>2 vials</td>
</tr>
<tr>
<td>BD Cytoperm™ Plus Buffer</td>
<td>1 vial</td>
</tr>
<tr>
<td>7-AAD</td>
<td>1 vial</td>
</tr>
<tr>
<td>Kit Manual</td>
<td></td>
</tr>
<tr>
<td>BrdU</td>
<td>5 vials</td>
</tr>
<tr>
<td>DNase</td>
<td>5 vials</td>
</tr>
<tr>
<td>BD GolgiPlug (containing Brefeldin A)</td>
<td>555029</td>
</tr>
<tr>
<td>BD GolgiStop (containing Monensin)</td>
<td>554724</td>
</tr>
<tr>
<td>BD Pharmingen Stain Buffer (FBS)</td>
<td>554656</td>
</tr>
<tr>
<td>BD Cytofix Buffer</td>
<td>554655</td>
</tr>
<tr>
<td>Anti-Human IL-2 PE</td>
<td>554566</td>
</tr>
<tr>
<td>Anti-Human CD8 APC-Cy7</td>
<td>557834</td>
</tr>
<tr>
<td>Anti-Human CD3 (clone UCHT1) Antibody</td>
<td>555329</td>
</tr>
<tr>
<td>Anti-Human CD28 (clone CD28.2) Antibody</td>
<td>555725</td>
</tr>
<tr>
<td>Recombinant Human Interleukin-2 (IL-2)</td>
<td>554603</td>
</tr>
<tr>
<td>Recombinant Human Interleukin-4 (IL-4)</td>
<td>554605</td>
</tr>
<tr>
<td>96-well, U-bottomed, non-tissue-culture treated plates; Falcon brand</td>
<td>353910</td>
</tr>
<tr>
<td>ModFit LT™ Software for PC</td>
<td>349329*</td>
</tr>
</tbody>
</table>

*BD Biosciences Immunocytometry Systems
**Methods**

The following is an abridged procedure for stimulating cells and labeling with BrdU, in the presence of a protein transport inhibitor that improves intracellular cytokine detection. The treated cells are subsequently stained in, and analyzed from, 96-well plates. Detailed procedures are described in the BrdU Flow Kits Manual from the BD APC BrdU Flow Kit (www.bdbiosciences.com/pdfs/manuals/03-8100055-1-A.pdf). Detailed protocols for intracellular cytokine staining are presented in the BD Biosciences Techniques for Immune Function Analysis Application Handbook.

**Outline of Sample Preparation Steps**

1. Stimulate cultured cells and incorporate BrdU in the presence of a protein transport inhibitor
2. Perform immunofluorescent cell surface staining
3. Fix and permeabilize cells
4. Treat cells with DNase to expose incorporated BrdU
5. Perform intracellular immunofluorescent staining of incorporated BrdU and IL-2
6. Stain total cellular DNA with 7-AAD
7. Acquire and analyze cells with the BD FACSArray™ bioanalyzer

**Stimulate Cultured Cells and Incorporate BrdU in the Presence of a Protein Transport Inhibitor**

Human PBMC are stimulated with immobilized anti-CD3 (10 µg/ml for tissue culture plate coating) and soluble anti-CD28 (2 µg/ml) antibodies, with saturating doses of IL-2 (10 ng/ml) and IL-4 (25 ng/ml) for 2 days. The cells are washed and subsequently cultured in medium containing IL-2 and IL-4 for 3 days. Finally, the cells are harvested and restimulated for 4 hrs. with PMA (5 ng/ml) and Ionomycin (500 ng/ml) in the presence of the protein transport inhibitor BD GolgiPlug™. During the final 45 minutes of culture, the cells are labeled with 10 µM BrdU. Cells must be labeled with BrdU for a defined time interval. BrdU incorporated into newly synthesized cellular DNA will be subsequently stained.

**Perform Immunofluorescent Cell Surface Staining**

**Note:** When removing the wash buffer after washing the cells, be careful not to let the pelleted cells dry out completely. Leave a 10 – 20 µl volume of liquid in each well.

1. Transfer BrdU-pulsed cells (2 – 5 × 10⁶ cells in 50 µL of BD Pharmingen™ Stain Buffer) to each well of a 96-well plate.
2. Add appropriate amount of fluorescent antibodies specific for cell-surface markers (anti-CD8 APC-Cy7 in this experiment) diluted in 50 µL of staining buffer [eg, BD Pharmingen™ Stain Buffer (FBS)] into well A4 and A6.
3. Incubate the cells for 15 minutes on ice.
4. Wash the cells by adding 100 µL of BD Pharmingen™ Stain Buffer (FBS) and centrifuge the plate 5 minutes at 300 × g.
5. Remove the buffer.

The samples must be prepared in a specific pattern in the 96-well plate depending on the experiment to be run on the BD FACSArray bioanalyzer. The following procedure will demonstrate a four-color experiment using three fluorescent antibodies (PE, APC and APC-Cy7 fluorochromes) and the fluorescent dye, 7-AAD. With this four-color experiment, specific setup wells will be prepared to set up and optimize the instrument.

**Sample Layout**

Prepare the 96-well sample plate as follows:

- Well A1: Unstained control sample
- Well A2: 7-AAD (Far Red) control sample
- Well A3: IL-2 PE (Yellow) control sample
- Well A4: CD8 APC-Cy7 (NIR) control sample
- Well A5: BrdU APC (Red) control sample
- Well A6: Investigation sample well for complete analysis

**Perform Immunofluorescent Cell Surface Staining**

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3. Incubate the cells for 15 minutes on ice.
4. Wash the cells by adding 100 µL of BD Pharmingen™ Stain Buffer (FBS) and centrifuge the plate 5 minutes at 300 × g.
5. Remove the buffer.
Fix and Permeabilize Cells

The fixation and permeabilization of cells for this experiment is performed in multiple steps. First, the cells are fixed and permeabilized with BD Cytofix/Cytoperm™ Buffer, secondly the cells are further permeabilized with BD Cytoperm™ Plus Buffer, and then finally the cells are refixed with BD Cytofix/Cytoperm Buffer.

1. Suspend the cells using 75 µL of BD Cytofix/Cytoperm Buffer per well and incubate for 15 – 30 minutes at room temperature or on ice.
2. Add 100 µL of 1× BD Perm/Wash™ Buffer and pellet the cells by centrifugation for 5 minutes at 300 × g.
3. Remove the supernatants.
4. Repeat steps 2 and 3.
5. Suspend the cells with 75 µL of BD Cytoperm Plus Buffer and incubate for 10 minutes on ice or at room temperature.
6. Add 100 µL of 1× BD Perm/Wash Buffer and pellet the cells by centrifugation for 5 minutes at 300 × g.
7. Remove the supernatants.
8. Suspend the cell pellet with 75 µL of BD Cytofix/Cytoperm Buffer per well and incubate for 5 minutes at room temperature or on ice.
9. Add 100 µL of 1× BD Perm/Wash Buffer and pellet the cells by centrifugation for 5 minutes at 300 × g.
10. Remove the supernatants.

Perform Intracellular Immunofluorescent Staining of Incorpo rated BrdU and IL-2

1. Suspend the cells in the following wells with 50 µL of BD Perm/Wash Buffer containing the appropriate antibodies at the manufacturer’s suggested concentration:
   • Wells A1 and A2: BD Perm/Wash Buffer alone
   • Well A3: IL-2-PE
   • Well A4: BD Perm/Wash Buffer alone
   • Well A5: BrdU APC
   • Well A6: IL-2 PE and BrdU APC
2. Incubate cells for 20 minutes at room temperature.
3. Add 100 µL of 1× BD Perm/Wash Buffer.
4. Pellet the cells by centrifugation for 5 minutes at 300 × g.
5. Remove the supernatants.
6. Add 200 µL of BD Pharmingen Stain Buffer to wells A1, A3, A4, and A5. Do not add buffer to wells A2 and A6, but proceed directly to the DNA staining step.

Stain Total Cellular DNA with 7-AAD for Cell-Cycle Analysis

The protocol below specifies volumes needed for staining two wells with 7-AAD. Adjust the volumes according to the number of wells in your experiment.

1. Dilute 40 µL of the 7-AAD stock solution from the BrdU Flow Kit in 360 µL of BD Pharmingen Stain Buffer (FBS).
2. Add 200 µL of diluted 7-AAD solution to each of the following wells:
   • A2: 7-AAD control well
   • A6: Investigation well

Acquisition and Analysis with the BD FACSArray™ Bioanalyzer

The BD FACSArray™ bioanalyzer aspirates cell samples from a 96-well plate during flow cytometric analysis; the maximum sample volume that can be aspirated at one time is limited to approximately 100 µL per well. Additional volume should be added to the wells to allow for instrument setup.

It is important to prepare the cell samples according to the above procedure so that the wells are correctly laid out in the plate for easy acquisition by the BD FACSArray bioanalyzer. Before setting up the controls and instrument optimization for spillover (compensation), create a new experiment through the Experiment Wizard.
Creating an Experiment Using the Wizard

The Experiment Wizard allows you to create an experiment by answering a series of questions. Each wizard session can be saved and re-opened for a similar experimental setup at a later time. The following information is saved in each Experiment Wizard session:

- sample and well information
- template used
- fluorophore labels
- custom keywords (if any selected)

Click the Experiment Wizard tool ( ) in the application toolbar (Figure 1).

The Experiment Wizard Welcome dialog appears. The view remains for approximately 2 seconds and then automatically advances to the next view (Figure 2).

1. Choose the Default Wizard Session when creating an experiment session for the first time; click Next.

   - The Template view appears (Figure 3).

Choosing a Template

The Template view in the Wizard session allows you to choose one of the predefined templates provided for cellular applications. The templates contain default loader and instrument settings that serve as a starting point for sample setup and optimization for an immunophenotyping application. Each predefined template contains a specific number of plots for acquisition and analysis of data.

1. Choose the 4 Color template from the Template drop-down menu; click the Next button.

   - The Instrument Setup and Optical Spillover view appears (Figure 4).
**Entering Sample Information**

The software will automatically calculate optical spillover, if setup wells are included. A setup well will be added for each color of this experiment. The names used within this experiment are examples. Customize the names for your own particular experiment.

1. Click the Yes radio button to enable the color selection checkboxes (*Figure 4*).
2. Click in each checkbox for the colors to be used.
   - Click all four colors for this experiment.

*Note*: When adding setup wells to your experiment, an unstained well is also added. The unstained well is run before the other setup wells. For this 4 Color experiment, a total of five setup wells will be added.

3. Click Next.
   - The Number of Samples view appears.
4. Enter the number 1 for the one sample that will be analyzed for this experiment; click Next.
   - The Sample Identification view appears.
5. Leave the default sample name, Sample_001, for the sample in the Sample Identification view.

**Entering Well Information**

1. Click Next.
   - The Number of Wells per Sample view appears.
2. Leave the default 1 well per sample; click Next.
   - The Well Names View appears (*Figure 5*).
3. To identify the investigation well, enter the name of the antibodies that are in this well.
   - In this example, leave the default as the name for Well 1.
   - To enter text in the text field:
     - Double-click in the white area of the text field to highlight the existing entry.
     - Type the new name.
     - Press the Enter key on your computer keyboard.

*TIP*: Click the tab button to easily move between text fields.

4. Click Next.
   - The Fluorophore Labels view appears.
The labels will appear as axes labels in the plots displayed in the Template view (Figure 6).

4. Enter the following names for the four fluorescence parameters:
   - 7-AAD for Far Red
   - IL-2 PE for Yellow
   - CD8 APC-Cy7 for NIR
   - BrdU APC for Red

Choosing Additional Options

1. Click Next.
   - The Custom Keywords view appears. (Do not add keywords in this Wizard session.)

2. Click Next.
   - The Adjusting Plate Layout view appears (Figure 7). In this view, you can choose how to lay out samples on the plate.

3. For this experiment, choose Contiguous Horizontal from the drop-down menu, and 0 as the number of separators.

Specifying Loader and Acquisition Settings

1. Click Next.
   - The Loader Settings view appears (Figure 8).

2. Change the Sample Flow Rate from 2.0 to 0.5 µL/sec and leave the remaining settings as default; click Next.
   - It is important to use the lowest flow rate when acquiring cell-cycle samples to achieve the most accurate measurement.

   - The Acquisition Settings view appears, where you can choose how many events per well to acquire and whether to use a stopping gate.

3. Choose the acquisition settings.

4. Click the arrow next to the Events To Acquire field and choose 20,000 events from the drop-down menu.

   Note: When analyzing cell-cycling data files in a cell-cycle software, such as Verity’s ModFit LT™ software, it is preferable to save a data file with at least 10,000 events, excluding debris and aggregates. Customize the events to acquire for your experiment.

   - Choose All Events as the stopping rule.

5. Click Next.
   - The Experiment Name view appears.
Saving Experiment and Wizard Session

1. Type in BrdU Analysis as the name of the experiment (Figure 9).

2. Click Next.
   • The Saving Wizard Session view appears. Saving the Wizard session allows you to reuse the session for other similar experiments. Here, choose a name and save the Wizard session. You may modify these settings as needed at a later date.

3. Click the Yes radio button and enter the name BrdU Analysis for this wizard session; click Next.
   • The Completing the Experiment Wizard summary view appears (Figure 10).

4. Review the choices that were made.

5. Click Finish.
   • The experiment that has just been created opens in the Prepare workspace.

TIP: Go to the application toolbar and select “Reset Positions” under View to see the workspace.

Review the Experiment in the Prepare Workspace

1. In the Prepare workspace (Figure 11), review your plate layout. Click the Prepare tool ( ) in the toolbar to choose the Prepare workspace.

Note: The first five wells in the plate view have been designated as setup wells. These wells will be used to optimize instrument settings and to calculate automatic optical spillover correction by the software.

2. Click well A1.

3. In the Inspector, choose the Acq. Tab, and then increase the sample volume from 20 to 100 µL (the maximum value) by typing 100; press Enter.
   • Increasing the sample volume for the setup wells allows you to view live events for a longer period of time while optimizing instrument settings.

4. Repeat steps 2 – 3 for the remaining setup wells (A2, A3, A4, and A5).

5. Click the Save button ( ) to save your work.
Preparing for Instrument Optimization

Preparing the Plate

TIP: Use a printout of the plate view as a guide for transferring prepared samples to the 96-well plate.

1. Print the plate view of the experiment by clicking the Print button in the upper right corner of the Prepare workspace.

2. Ensure there is approximately 200 µL of the prepared samples in each of the following wells.
   - Unstained sample in well A1
   - 7-AAD sample in well A2
   - PE sample in well A3
   - APC-Cy7 sample in well A4
   - APC sample in well A5
   - Investigation sample in well A6

Loading the Plate

1. Click the Setup tool in the application toolbar to open the Setup workspace.

   - The wells you selected for acquisition appear in the plate view, and the setup wells are numbered in the acquisition order.

2. Place the plate containing the stained samples on the plate holder.
   - Position the plate such that well A1 is over the A1 mark on the plate holder.

3. Click the Load button in the Plate Control group.

   - If no lid is detected on the plate, the plate is completely retracted into the bioanalyzer.
   - The Status box should display the Instrument Ready Plate Loaded message.

Optimizing Instrument Settings

Next, perform the following steps to optimize settings for your four-color cellular sample.

   - Adjust Forward Scatter (FCS), Side Scatter (SSC), and threshold.
   - Gate the population of interest.
   - Adjust parameter voltages.
   - Save data for all the setup wells.
   - Calculate optical spillover.
Optimizing FCS, SSC, and Threshold Settings

To perform this set of optimization steps, view data only from the first well.

1. In the Select Control group, click None to deselect the wells, and then click well A1 (Figure 12).
2. Click the Setup button in the Acquisition Control group.
   - Events appear in the plots, but data is not being saved in Setup mode.
3. Adjust the FSC and SSC voltages to place the cell population on scale and above the noise level in the FSC vs SSC plot, if needed.
   - Adjust the signal for events displayed in plots by changing voltage settings.
4. Click the Parameters tab in the Instrument frame to display the Parameters pane (Figure 13).
5. Click in the Voltage field to edit this setting. Voltages can be adjusted from 1 – 1,000 by using the up or down arrows, the slider bar, or manually entering a value in the field.
6. Press Enter on your computer keyboard to save the changes once the appropriate scatter profile is achieved.
7. Adjust the threshold to eliminate debris at the lower end of the FSC scale (Figure 14).
   - Click the Threshold tab in the Instrument frame to display the Threshold pane.
   - Click in the Value field to edit the settings.
   - Events below the threshold value are excluded.
   - Press Enter on your computer to save the changes.
   - Press the Set Up button to stop acquisition of sample from the A1 well.
Gating the Population of Interest

Next, adjust the gate to define the cells of interest and exclude debris and aggregates. This ensures that only the cellular population is viewed while optimizing fluorescent settings. You will need to readjust the gate for each of the single-color setup wells.

1. In the Select Control Group, click None to deselect the wells, and then click well A2.
2. Click the Setup button in the Acquisition Control group.

   - Events from well A2 appear in the plots, but data is not being saved.
3. If the gate does not surround the cell population, click on the gate in the FCS vs SSC plot, and drag it to the cell population.

   Note: The histogram plot displays only gated events.

Optimizing Fluorescence Settings

For cell-cycle experiments the goal for optimizing fluorescence settings for the DNA is different than the goal for optimizing fluorescent settings for the intracellular, intranuclear, or cell surface staining. For cell-cycle optimization, the DNA profile must be optimized to adjust the G0/G1 peak to appear around channel 50. Adjusting the G0/G1 population to channel 50 will permit the detection of varying amounts of DNA that may be present in some samples.

Far Red (Cell-Cycle) Parameter Optimization

1. In the Parameters tab of the Instrument frame, deselect the Log checkbox for Far Red.
   - The Far Red parameter will change to a Linear display.
   - DNA measurements must be displayed on a linear scale in order to clearly distinguish between the cell-cycle phases. This will allow the 7-AAD stained cells, measured in the Far Red parameter, to be displayed on a linear scale.
2. While observing the DNA histogram, adjust the Far Red voltage, if necessary, to place the G0/G1 population around channel 50.

   Note: You do not need to adjust the interval gate at this time.
3. Click Setup to stop acquisition.

Yellow, NIR, and Red Parameter Optimization

1. In the Select Control group, click None to deselect the wells, and then click A3 to view the Yellow setup well.
2. Click the Setup button in the Acquisition Control group.

   - Events from well A3 appear in the plots, but data is not being saved.
6. While observing the Yellow histogram, perform the following:
   • Adjust the Yellow voltage, if necessary, to place the brightest population on scale.
   • Verify the dim fluorescent cells remain on scale (Figure 15).
7. Click Setup to stop acquisition.
8. Repeat steps 4 through 7 with well A4 for NIR and well A5 Red samples.
   See the following figures for examples of adjusted populations for the following parameters:
   • NIR (Figure 16)
   • Red (Figure 17)
9. Click the Save button to save your work.

Complete Setup
1. Click the Setup button to stop acquisition.
2. Click the Save button to save your work.

Customizing the Loader Settings for Setup Wells
The instrument settings for the cellular experiment have been adjusted. Before acquiring the setup wells, adjust the loader settings for the sample volume to ensure optimal sample acquisition. Decreasing the sample volume for the setup wells allows you to speed up acquisition and ensure the aspirated sample volume does not exceed the available volume.

1. Click the Prepare tool in the Application Toolbar to open the Prepare workspace.
2. Click well A1 to change the sample volume in the inspector from 100 to 20 µL; press Enter.
3. Repeat step 2 for the remaining setup wells (A2, A3, A4, and A5)

TIP: Always verify you have enough volume in the wells prior to acquisition by unloading the plate and visually inspecting the volume. A minimum of 50 µL is recommended for this experiment.

Acquiring Data to Calculate Optical Spillover
Finally, save the setup well data so the software can use the data to automatically calculate and apply optical spillover correction.

1. Click the Setup tool in the application toolbar to open the Setup workspace.
2. In the plate view, verify setup wells A1 – A5 are selected.
3. Click Acquire.
   • Data will be saved for all five wells. After the last setup well is saved, the software automatically calculates and applies optical spillover correction.
   • If optical spillover correction was successfully calculated, the following message appears.
Ensuring Gates Were Properly Set

1. Click the Analyze tab in the Plate Editor.

2. Click the first set up well in the Plate view. Then examine the plots in the Template view (Figure 18).

3. If needed, perform the following:
   • Click on the gate (P1) in the FCS vs SSC plot, and drag it to the cell population while gating out debris.
   • Adjust interval markers in each histogram to enclose the negative population.

4. Click in each of the remaining setup wells and verify:
   • The gate in the FCS vs SSC plot surrounds the experimental cell population.
   • Interval gates enclose the positive population in each fluorescence parameter.
   • For the 7-AAD positive well, adjust the Interval gate (P2) to encompass the entire cell-cycle population.

5. If you adjusted any of the gates in this section, choose Instrument > Calculate Spillover to recalculate the optical spillover.

6. Click the Acquire tab in the Plate Editor.

7. Click the Save button ( ) to save your work.

Data Acquisition

1. In the Select Control group, click the Auto button.

   The remaining sample well (A6) will become selected and will be labeled as number one. The number in each well indicates the run order.

2. Click the Acquire tool in the application toolbar to open the Acquire workspace.

3. Click the Acquire button in the Acquisition Control group.

   After a short pause, the following sequence occurs:
   • An orange ring appears in the well selected for acquisition, indicating that sample is being acquired.
   • Events appear in the plots.
   • Data is saved.

4. Click the Acquisition Status tool ( ) in the Application workspace to open the Acquisition Status frame.

5. In the Acquisition Status frame, monitor the event rate to ensure that events are being detected and acquired.

6. Click the Plate Control group to eject the plate from the plate sampler.

7. Remove the plate from the Plate Holder. This completes sample acquisition.
Data Analysis Using the BD FACSArray™ Software

The BD FACSArray™ software can be used to analyze the various cell populations within this four-color experiment. The following analysis determined the percentages of each of the populations that were detected by the instrument, which include the DNA compartments with 7-AAD, BrdU incorporation, CD8 cells, and IL-2 producing cells. The analysis performed in this application note is an example of the type of analysis that can be performed. Customize the analysis for your own experiment and applications.

BD FACSArray software can perform cell-cycle analysis by estimating the percentage of cells in each cell-cycle compartment. A more accurate cell-cycle analysis should be performed using a cell cycle modeling software, such as ModFit LT™. Cell-cycle software uses algorithms that model the three cell-cycle compartments and analyze the overlap that occurs between the G0/G1, S, and G2/M phases. In addition, this software provides a more accurate analysis by subtracting out underlying debris and aggregate cells that may interfere with the measurement of the cells in each cell-cycle compartments. Refer to the Application Note entitled Cell-cycle analysis using the BD FACSArray™ Bioanalyzer™ for more detail on performing this procedure.

Use the analysis features in BD FACSArray software to create an estimate of the percentages in each cell-cycle compartment as a starting point for your analysis. Perform further analysis in a third-party cell-cycle modeling software for a more complete analysis.

Create a Gate

1. Click on the Analyze tool ( ) in the Application Toolbar to open the Analysis workspace (Figure 19).
2. Display data from the Investigation well by clicking on the sample well A6.
   In the template, the plots display the stored data from the selected sample well.
3. Click on the Snap-To Auto Polygon gate in the template toolbar.

4. Click on the cell population in the FCS vs SSC plot.
   A Snap-To gate will be automatically created around the population. This gate will be used to gate out the cellular debris and aggregates.

5. To display only gated data in the fluorescent plots, perform the following:
   - To select multiple plots, click on the border of the plots while holding down the Shift key.
   - Right-click on the border of one of the selected plots.
   - Choose Show Populations, and then select P1 from the menu that appears.
   Only the cells that are in gate P1 will now appear in the fluorescence plots.

6. To perform data analysis, confirm the four dot plots display the following parameters:
   - 7-AAD (Far Red-A) vs BrdU APC (Red-A)
   - 7-AAD (Far Red-A) vs CD8 (NIR-A)
   - BrdU APC (Red-A) vs CD8 (NIR-A)
   - IL-2 PE (Yellow-A) vs CD8 (NIR-A)

   To change the parameter display on a given axis, right-click on the axis label and choose the appropriate parameter you would like to display:

7. Create three interval gates on the three cell-cycle compartments; G0/G1 peak, S-phase, and G2/M peak.
• Click on the interval gate tool ( ) to select an interval gate, then click and drag across the population in the Far Red parameter.

• P2 should be placed over the G0/G1 population.

• P3 on the S-phase cells.

• P4 on the G2/M population.

• The statistics for each population will be displayed in the statistics view.

9. Create quadrant markers on the dot plot in the following order:
1. 7-AAD (Far Red-A) vs BrdU APC (Red-A)
2. 7-AAD (Far Red-A) vs CD8 (NIR-A)
3. BrdU APC (Red-A) vs CD8 (NIR-A)
4. IL-2 PE (Yellow-A) vs CD8 (NIR-A)

• Click the quadrant marker tool ( ) to select a quadrant marker, then click and drag across the plot to create the quadrant marker.

• Repeat this for all remaining plots as shown in Figure 20.

Quadrant markers can be used to enumerate the different phenotypic subsets as shown in the example for this application note. Analysis of unstimulated cells (data not shown) is recommended in establishing the approximate location of the quadrants for use in the stimulated sample.

Results
This application note has been designed to guide you through using the BD FACSArray bioanalyzer to characterize the nature of DNA-synthesizing lymphocytes. The following discussion of the results for this particular experiment are an example of BrdU incorporation within stimulated PBMCs. Actual results may vary from lab to lab.

Cell-Cycle Activity of the Total Cell Population (Panels 1–3)

The forward- and side-light scattering characteristics of primed and restimulated human PBMC are shown in Panel 1. Activated PBMC, are defined by their light scattering characteristics, and are colored red. These cells comprise about 91% of the total cell population.

Panel 2 presents the fluorescence frequency distribution for the cells stained with 7-AAD. The analysis approximates 58% of the cells are detected within the G0/G1 phase of the cell-cycle (ie, within the P2 interval marker), 15% are S-phase cells (P3 marker) whereas 20% are G2/M phase cells (P4 marker).

Panel 3 shows the bivariate analysis of cells that have recently synthesized DNA by incorporating BrdU, in terms of their cell-cycle position (total cellular DNA stained by 7-AAD) at the time of harvest. Using quadrant markers, Q3 determined 59% are G0/G1 phase cells that express uniformly low levels of total cellular DNA with no significant BrdU incorporation. To determine the total amount of S-phase cells, combine Q1 and Q2. By combining these two quadrants, 29% of the cells are S-phase cells that coexpress higher levels of cellular DNA (7-AAD fluorescence) and newly synthesized DNA (BrdU immunofluorescence). The G2/M phase cells in Q4 express high DNA levels with little BrdU incorporation and result in 13% of the cell population.
Cell cycle activity of CD8+ and CD8– cells (Panels 4 – 6)

Total cellular DNA levels synthesized by activated CD8+ and CD8– cells are shown in Panel 4. Note that a greater fraction of CD8+ cells (~37%) express higher levels of total cellular DNA (are further in cell-cycle) than CD8– cells (~27%). This pattern correlates with the higher S-phase (DNA-synthesizing) activity of CD8+ cells shown in Panel 5. IL-2 production by restimulated CD8+ and CD8– human PBMC is shown in Panel 6. Approximately 25% of the total cultured cells were CD8+; ~34% of these cells produced IL-2. A greater fraction (~45%) of the CD8– cells produced IL-2 at even higher levels than their CD8+ counterparts. Since the majority of the cultured human PBMC are CD3+ T cells (≥95% CD3+; data not shown), the CD8– cell fraction is comprised primarily of CD4+ T cells. CD4+ T cells characteristically produce higher IL-2 levels than CD8+ cells.

In conclusion, the results from multiparameter BD FACSArray analysis indicate that within this experimental culture system, primed human CD8+ cells can be stimulated to proliferate more than CD8– (presumably CD4+ T cells), although the latter cell population has a greater capacity to synthesize IL-2. Reanalysis of the acquired flow cytometric data with BD FACSArray software provides a great deal of information about the nature of human PBMC that can be activated to produce IL-2 and to enter and progress through the S-phase of the cell-cycle (see panel descriptions).

The 96-well BrdU staining procedure combined with the latest cell-cycle reagents and the BD FACSArray bioanalyzer gives researchers a faster, more efficient way to analyze large numbers of samples in a shorter period of time. In this way, thorough analyses of experimental model systems are well served for multiparameter analyses in a rapid manner.

Tips

Extra Setup Wells:

After an experiment is created with the Experiment Wizard, extra setup or sample wells can be added to the plate layout using the following steps:

1. Click to select the Prepare Workspace in the application toolbar.
2. In the Layout for Plates field of the plate editor, click the Manual button.
3. Click to select the wells in the plate. Use the Shift key for multiple selections.
4. Click Add Sample or Add Setup.
   - Repeat steps 3 – 4 for all the samples you are adding.

Sample Washing:

For all of the wash steps, ensure the cell pellet does not dry out. If the cell pellets dry out completely, even briefly, it can seriously affect the staining of the cells. We recommend flicking, without patting dry, which should leave liquid in each well. If removing the liquid with a pipetter, adjust the pipettor to leave approximately 10 – 20 µl of fluid in each well.

Alternate Procedure for Fixation of Cells:

Cells may be fixed in bulk and then stored at either 4°C or –80°C to be analyzed at a later time. Cells are stable at 4°C for a few days and are stable at –80°C for several months. Bulk fixation of cells is done using either BD Cytofix/Cytoperm™ Buffer of BD Cytofix Buffer.

1. Resuspend 1 × 10⁷ cells/ml of fixation buffer. Incubate on ice for 15 – 30 minutes.
2. Wash cells by adding an equal volume of BD Pharmingen™ Stain Buffer (FBS) to the fixed cell suspension.
3. Pellet the cells by centrifugation for 5 minutes at 300 × g.
4. For storing cells at 4°C
   - Resuspend cells in BD Pharmingen™ Stain Buffer (FBS) at a concentration of 1 × 10⁷ cells/ml; store at 4°C.
   - To resume the staining procedure, add cells to the plate and then pellet the cells by centrifugation.
   - Continue with permeabilization of cells for staining incorporated BrdU by incubating cells with BD Cytoperm™ Plus Buffer (page 4).
5. For storing cells frozen at –80°C
   - Resuspend cells in freezing media (10% DMSO in FCS) at 1 × 10⁷ cells/ml; store at –80°C.
   - To resume the staining procedure, wash thawed cells with BD Perm/Wash Buffer (9 mls of buffer/1 ml of freezing media) to remove the DMSO.
   - Resuspend cells in BD Perm/Wash™ Buffer, add cells to the plate, and pellet the cells by centrifugation.
   - Continue with refixation with BD Cytofix/Cytoperm™ Buffer (page 4).

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References


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