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Flow Cytometry Protocols

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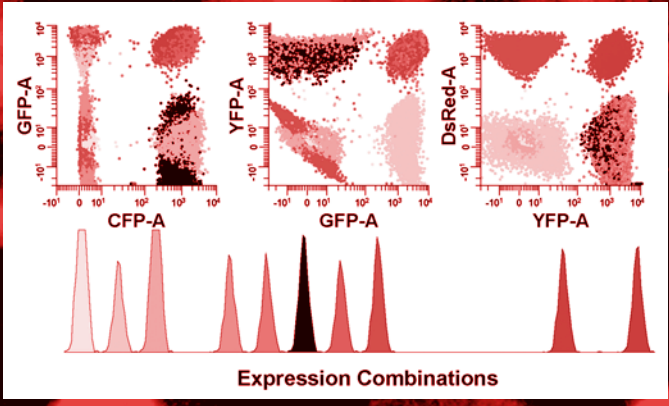

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Cell-Cycle Analysis of Asynchronous Populations

Michael G. Ormerod

Summary

Cells are incubated continuously in bromodeoxyuridine (BrdUrd), which is incorporated into cells synthesizing DNA. At intervals, cells are harvested and nuclei are prepared and stained with a *bis*-benzimidazole, Hoechst 33258, and propidium iodide. In the flow cytometer, the dyes are excited by UV and blue and red fluorescences recorded. BrdUrd quenches the blue fluorescence of the Hoechst dye. The degree of quenching records the progress of the cell through S phase(s); the red (PI) fluorescence yields the cell cycle phases. By this means, the progress of cells around the cell cycle can be followed and the effects of cytotoxic drugs, radiation, and other treatments observed.

Key Words

Bromodeoxyuridine, flow cytometry, Hoechst 33258, proliferation.

1. Introduction

Measurement of a DNA histogram can be achieved by fixing or permeabilizing cells and staining them with a DNA-binding dye, such as propidium iodide (PI). The histogram will yield the percentage of cells in the G_1, S, and G_2/M phases of the cell cycle ***(1)***. Although some inferences about the movement of cells through the cycle may be drawn, the information gained is essentially static. For example, it is not known whether a cell with S-phase DNA content is actually synthesizing DNA; also, the presence of subpopulations with different cycle times cannot be detected.

Dynamic information about cell cycle progression can be obtained by labeling cells with 5′-bromodeoxyuridine (BrdUrd) which is incorporated into DNA

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in place of thymidine. Detection of BrdUrd in the DNA allows the fraction of cells in S-phase to be enumerated and, if samples are taken at different time intervals, also gives information about the cell cycle kinetics.

Three methods have been used. After the application of a pulse-label, monoclonal antibodies that react specifically with BrdUrd reveal those cells that are in S-phase. Counterstaining with PI shows the cell cycle. If cells are harvested at times after the application of the pulse-label, the movement of labeled cells through the cell cycle can be followed. This method has been applied in vitro ***(2)*** and in vivo ***(3,4)***. The other two methods exploit the observation of Latt that the fluorescence of *bis*-benzimidazoles (Hoechst 33342 and 33258) bound to DNA is quenched by BrdUrd ***(5)***. One method, in which the cells are pulse-labeled and then stained with a combination of Hoechst 33258 and mithramycin, requires a dual-laser flow cytometer and a complex analysis in which fluorescence signals are subtracted in real time ***(6)***. In the method described in this chapter, cells are continuously labeled with BrdUrd, permeabilized, and stained with Hoechst 33258 and PI. The quenching of Hoechst/DNA fluorescence reveals whether the cells have incorporated BrdUrd; the PI/DNA fluorescence (unaffected by BrdUrd) gives the cell cycle phase. This method was originally applied to quiescent cells that had been stimulated into the cell cycle ***(7)***. It can also be applied to asynchronous populations of cells ***(8)***. Although the data analysis is more complex, a wealth of information can be derived. In particular, cell cycle specific effects of toxic treatments can be observed without resorting to artificial cell synchronization ***(8–16)***.

It should be noted that, in all three methods, the cells have to be fixed or permeabilized to allow access of reagents to the DNA. Analysis of viable cells is not possible. In addition, for the antibody method, the DNA of the cells has to be partially denatured to allow access of the antibody to the BrdUrd incorporated into the DNA.

In the method described here, BrdUrd is added and cells are harvested at fixed time intervals, typically, every 4 h for 36 h. Samples may be collected and the cells frozen prior to analysis. For analysis, cells are suspended in a buffer containing Hoechst 33258 and a detergent, which releases the nuclei, and PI is then added. Hoechst 33258 is exited in the UV and the analysis requires a flow cytometer equipped with a source of UV light.

2. Materials

1. 5′-Bromo-2′-deoxyuridine (BrdUrd) (Sigma [St. Louis, MO], cat. no. B5002): Make up a stock solution of 10 m*M*. Store frozen.
2. Hoechst 33258 (Sigma, cat. no. B2883 or Molecular Probes [Eugene, OR], cat. no. H-1398).

3. Propidium iodide (Sigma, cat. no. P4170 or Molecular Probes, cat. no. P-1304): Make up a stock solution of 100 µg/mL in distilled water. Store in the dark at 4°C. Stable for at least 6 mo.
4. Staining solution: 100 m*M* Tris-HCl, pH 7.4, 154 m*M* NaCl, 1 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 0.1% (v/v) Nonidet-P40, 0.2% (w/v) bovine serum albumin (BSA), and 1.2 µg/mL of Hoechst 33258. Make up at 10X final strength and store in the dark at 4°C. The solution is stable for at least 6 mo. Batches of staining buffer can be prepared weekly from the 10X concentrated stock solution in distilled water.

3. Methods

3.1. Analysis of Cells

1. If desired, treat the cells (radiation, drug, heat, and so on).
2. Immediately after the treatment, add a suitable concentration of BrdUrd to the cell culture (*see* **Notes 1–3**).
3. At fixed time intervals (3–8 h, depending on the cell cycle time), harvest an aliquot of cells (*see* **Notes 4–6**).
4. Centrifuge the cells and resuspend in 500 µL of ice-cold staining buffer. Briefly vortex mix. Stand on ice for 15 min.
5. Add 10 µL of PI solution. Briefly vortex mix. Store on ice (*see* **Notes 7** and **8**).
6. Analyze on the flow cytometer recording red (PI/DNA) and blue (Hoechst/DNA) fluorescences. Adjust the flow rate to about 500 particles/s. If possible, perform a pulse shape analysis on the red fluorescence signal and gate to exclude any clumped nuclei *(1)* (*see* **Fig. 1**). Display a cytogram of red vs blue fluorescence (*see* **Notes 9–12**).

3.2. Data Analysis

Figure 2 shows cytograms obtained from an untreated human embryonic fibroblast cell line (MRC5/34). At time 0, G_1, S, and G_2/M phases of the cell cycle could be identified from both the red (PI) and blue (Hoechst) fluorescence. When the cells were incubated in 40 µ*M* of BrdUrd, as the cells progressed through S-phase, their red fluorescence increased but their blue fluorescence (which was partially quenched by BrdUrd) did not change. After 4 h in BrdUrd, there had been a small progression giving the S-phase a slight bow shape on a plot of red vs blue fluorescence. At 8 h, the bow shape was more pronounced; also many of the cells originally in G_2/M had divided and moved into G_1 (unlabeled). At 16 h, all the cells in S-phase at time 0 h had reached G_2 (labeled G_2*) and a few had divided (G_1*). Some of the cells which had been in G_1 at time 0 h were now in S-phase (Sf); some had progressed as far as G_2/M (G_2f). By 28 h, some cells that began the experiment in G_2 had completed a cell cycle and returned to G_1 again (labeled G_1').

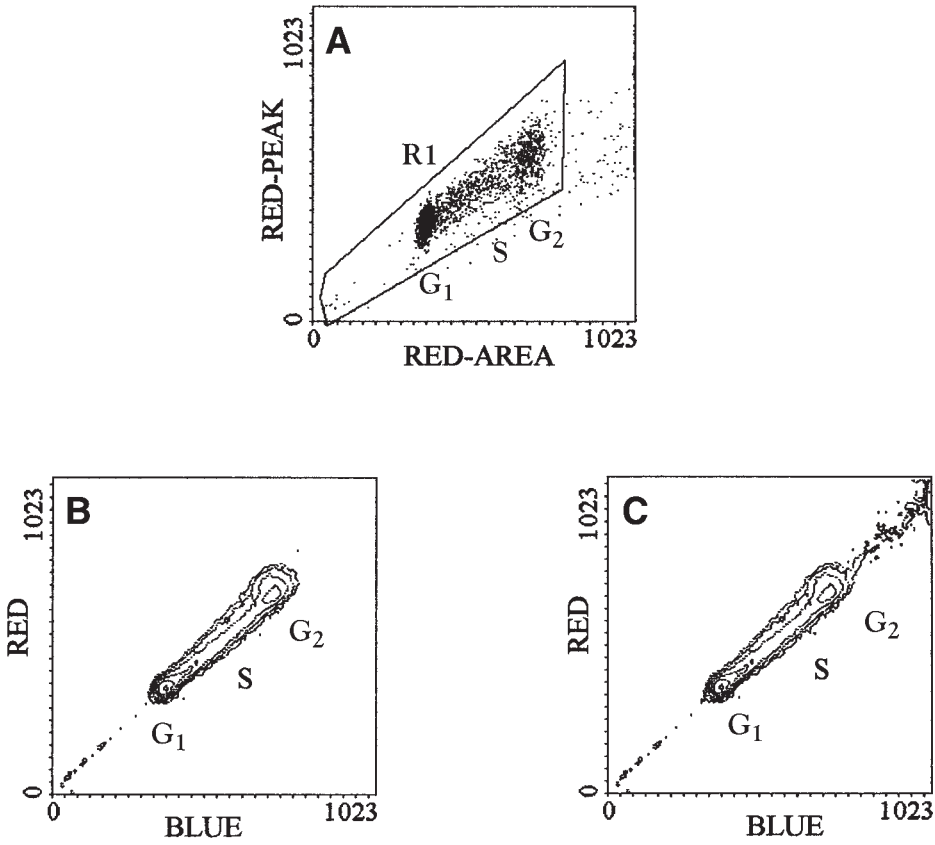

Fig. 1. Hoechst 33258-PI analysis of nuclei from a human embryonic fibroblast cell (MRC5/34) without incubation in BrdUrd. **(A)** PI/DNA fluorescence (red) showing a cytogram of the peak of the red fluorescence signal vs the integrated area. A gate has been set to include single nuclei and to exclude clumps. The cytogram is displayed as a "dot-plot." (**B**,**C**) Hoechst/DNA (blue) vs PI/DNA (red) fluorescence. (cytograms displayed as contour plots). The cytogram in **(C)** shows ungated data; that in **(B)** shows the effect of gating on region, R1, in cytogram **(A)**. Cells prepared by Dr. David Gilligan and data recorded by Mrs. Jenny Titley on a Coulter Elite ESP using a Spectra-Physics argon-ion laser tuned to produce 100 mW in the UV. Red (>630 nm) and blue (460 nm) fluorescence were measured. Data were acquired on an IBM-PC compatible computer and the figure was prepared using the WINMDI program supplied by Dr. Joe Trotter (Salk Institute, and BD Biosciences, USA).

Figures 3–5 illustrate typical effects of cell cycle perturbation. **Figure 3** shows MRC5/34 cells that had been given 5 gy γ-radiation immediately prior to addition of the BrdUrd. The cells suffered a G_2 block. Cells that were irradiated

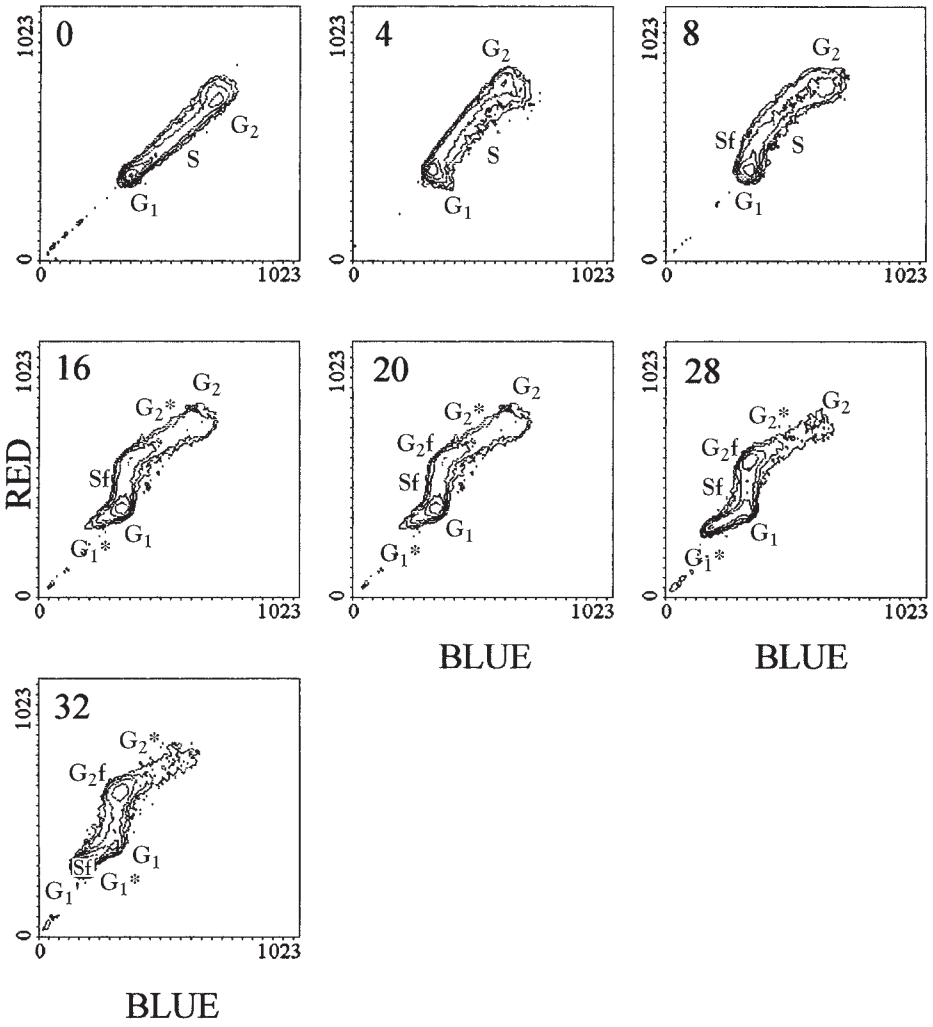

Fig. 2. Cytograms of PI-DNA (red) vs Hoechst 33258-DNA fluorescence of nuclei from MRC5/34 cells incubated in 40 μ*M* BrdUrd and 40 μ*M* deoxycytidine BrdUrd for the times shown on the panels. Other details as in **Fig. 1**. For a description of the data, *see* the main text.

in all phases of the cell cycle had become arrested in G_2. (Compare **Fig. 3** to **Fig. 2**, 32 h time point). **Figure 4** shows W1L2 cells (human lymphoblastoid cell line) that had been incubated with cisplatin for 2 h before adding BrdUrd. Only cells treated with drug in G_1 and early S-phase were arrested in G_2. The other cells had divided. **Figure 5** shows a human medulloblastoma cell line

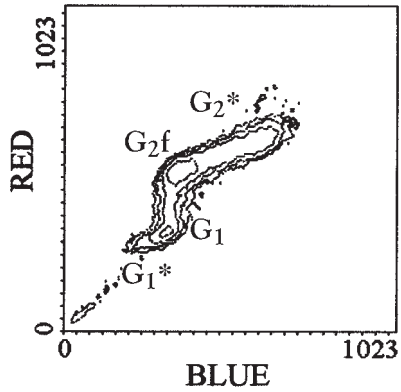

Fig. 3. A cytogram of PI-DNA (red) vs Hoechst 33258-DNA fluorescence of nuclei from MRC5/34 cells given 5 gy γ-radiation and then incubated in 40 μ*M* BrdUrd and 40 μ*M* deoxycytidine BrdUrd for 32 h. Other details as in **Fig. 1**. For a description of the data, *see* the main text.

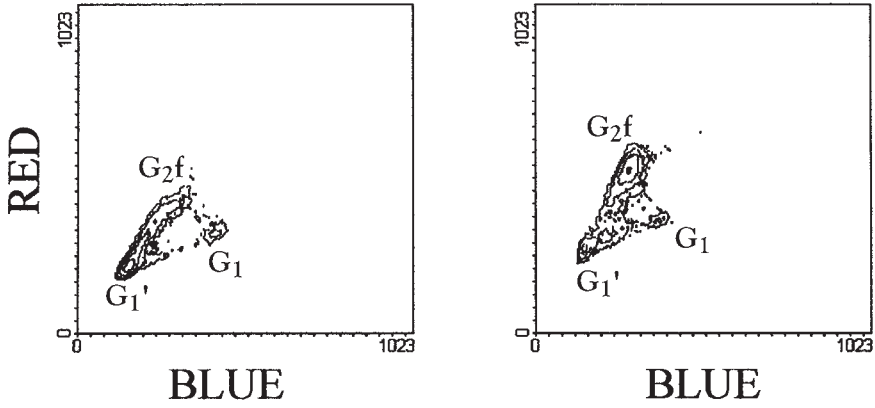

Fig. 4. Cytograms of PI-DNA (red) vs Hoechst 33258-DNA fluorescence of nuclei from W1L2 (human lymphoblastoid cell line) cells either untreated **(left)** or incubated for 2 h with 20 μ*M* cisplatin **(right)** and then incubated in 40 μ*M* BrdUrd for 24 h. Experiment run by the author. Other details as in **Fig. 1**.

(D283) that underwent a G_1 block after γ-radiation. Only a proportion of the cells in G_1 became blocked in G_1, some of the G_1 cells progressed through the cycle and divided (in compartment G_1'). Presumably these were cells irradiated in the late G_1 phase.

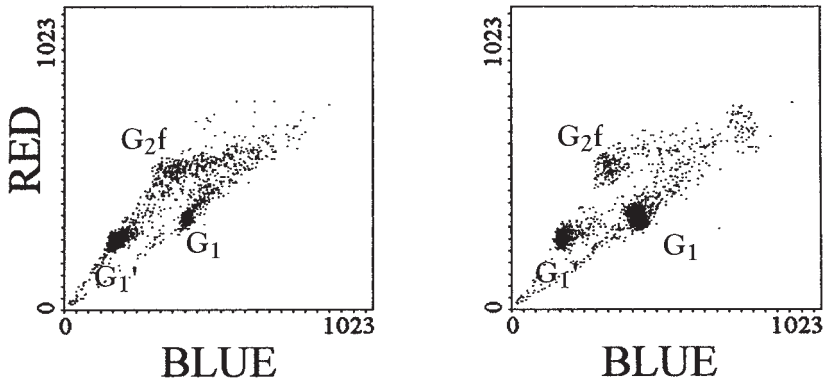

Fig. 5. Cytograms of PI-DNA (red) vs Hoechst 33258-DNA fluorescence of nuclei from a medulloblastoma cell line (D283) either untreated **(left)** or given 8 gy γ-radiation **(right)** and then incubated in 40 μ*M* BrdUrd and 40 μ*M* deoxycytidine BrdUrd for 32 h. Cells prepared by Mrs. Cyd Bush. Other details as in **Fig. 1**.

4. Notes

1. The correct concentration of BrdUrd must be determined by a preliminary experiment. The concentration should be such that cells in G_1 after one round of replication have about half the blue fluorescence of unlabeled cells in G_1.
2. Concentrations of BrdUrd vary from 10 to 100 μ*M*. If the concentration of BrdUrd is >20 μ*M*, the BrdUrd may become exhausted by 24 h. The remedy is to either work at a lower cell density or to replenish the BrdUrd every 12 h.
3. A trial experiment should be performed to check that the incorporation of BrdUrd into the DNA is not inhibiting the progression of the cells through the cycle. After different times of incubation with BrdUrd, fix cells in 70% ethanol, centrifuge and resuspend in phosphate-buffered saline containing 20 μg/mL of PI and 0.1 mg/mL of RNase. Incubate at 37°C for 1 h and record the DNA histogram. Addition of deoxycytidine (equimolar with the BrdUrd) can reduce any deleterious effect of BrdUrd.
4. For suspension cultures, shake to resuspend cells and remove 3 mL. For adherent cell cultures, use a separate flask for each time point. Harvest the cells by a short incubation with trypsin.
5. After harvesting, cells can be frozen and stored for later analysis.
6. The detergent in the staining buffer releases nuclei. At this stage, the samples are stable for several hours on ice.
7. The cell concentration is important and should be between 5×10^5 and 2×10^6. If the concentration is too high, the nuclei will be understained and may give distorted cytograms.

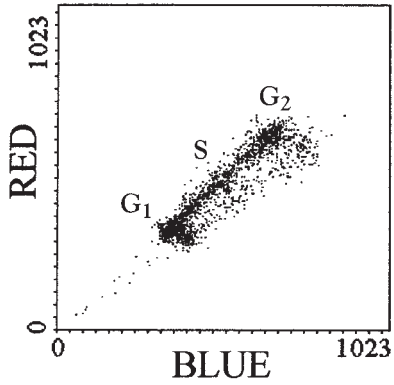

Fig. 6. A cytogram of PI-DNA (red) vs Hoechst 33258-DNA fluorescence of nuclei from MRC5/34 cells. The cells had not lysed completely. The nuclei and the partially lysed cells stained slightly differently, creating a "shadow" in the cytogram. Experimental details as in **Fig. 1**.

8. Either PI, ethidium bromide (EB), or 7-aminoactinomycin D (7-AAD) may be used as a counterstain for DNA. 7-AAD has to be excited at 488 nm using a second argon-ion laser tuned to this wavelength.
9. When setting a gate on a plot of DNA-peak signal vs DNA-area, be careful to include material of DNA content less than that of cells in G_1.
10. If it is not possible to perform a pulse shape analysis, clumps may usually be differentiated on a cytogram of forward light scatter vs red fluorescence.
11. A PI/DNA complex is excited by UV. It also absorbs blue light and will be excited by energy transfer from the Hoechst dye. When the Hoechst fluorescence is quenched, there will be a consequent reduction in PI fluorescence. If a helium–cadmium (He–Cd) laser is used as a source of UV (325 nm) (rather than an argon-ion laser, 360–390 nm), direct absorption will predominate and the secondary quenching will be reduced. For this reason, it is preferable to use a He–Cd laser ***(17)***. Alternatively, if a dual laser instrument is being used, 7-AAD can be used as the DNA stain ***(16)***.
12. If the pattern produced from the sample at 0 h has a "shadow" (*see* **Fig. 6**), the cells have not lysed properly. Incubate the samples at 37°C for 5 min and re-run.

Acknowledgments

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