

Simultaneous three-color analysis of the surface phenotype and DNA-RNA quantitation using 7-Amino-actinomycin-D and Pyronin Y

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Abbreviations: FITC, fluorescein isothiocyanate; 7AAD, 7-amino-actinomycin D; PY, pyronin Y.

Abstract We developed an improved technique that permits simultaneous DNA and RNA quantitation by a flow cytometry using 7-amino-actinomycin D (7AAD) and pyronin Y (PY), respectively. Detailed cell cycle analyses based upon the cellular DNA/RNA levels were performed using cells suspended in a buffer containing 0.004 % saponin. This method preserved the light scattering properties of human peripheral blood cells, thus lymphocyte, monocyte and granulocyte populations could be evaluated. In addition, since 7AAD and PY exhibit red (> 650 nm) and orange fluorescence (570 nm) respectively, the green fluorescence channel of the flowcytometer was reserved for surface phenotyping using FITC-conjugated antibodies. The 7AAD/PY method is applicable to the simultaneous three-color analysis of the surface phenotype and DNA-RNA quantitation when combined with FITC-conjugated surface markers in heterogeneous samples.

To demonstrate the three color analysis, PHA-activated human peripheral blood lymphocytes were stained for cell surface markers with monoclonal antibodies. The cells were suspended in buffer containing 0.004% saponin, then stained with 7AAD and PY. The DNA and RNA were analyzed in individual CD4+, CD8+ and CD20+ cells, and the characteristic cell cycle status was found. Cell activation was further analyzed using antibodies against IL-2 receptors (CD25), transferrin receptors (CD71) or HLA-DR molecules. Transferrin receptors were expressed in late G1 phase (G1B) just before the initiation of DNA synthesis, whereas IL-2 receptors and HLA-DR were expressed very early in the G1 phase (G1T). Since this technique preserves both light scatter properties as well as cell surface proteins, it is ideally suited for detailed cell cycle analyses of heterogeneous samples such as peripheral blood or bone marrow cells.

Key words: cell kinetics; DNA/RNA quantitation; 7-amino-actinomycin D; pyronin Y; saponin; Flow cytometry.

1. Introduction

Detailed analysis of the cell cycle is essential for understanding the growth status of cells and the mechanism of bioactive growth factors. For example, growth factors have been classified as competence factors ($G_0 \rightarrow G_{1A}$), progression factors ($G_{1A} \rightarrow G_{1B}$) and differentiation factors ($G_{1A} \rightarrow G_{1D}$) based upon their effects on the cell cycle (Darzynkiewicz et al., 1980). The ability to correlate the effect of a growth factor with the cell cycle will help to elucidate the complex biological events induced by one or more growth factors.

Cellular DNA quantitation using Propidium iodide (PI) or its analogue provides only indirect and imprecise information regarding cell cycle events. The detection of BrdUrd incorporation in combination with DNA quantitation permits the analysis of dynamic cell cycle parameters such as cell cycle time (Dolbear et al., 1983;

Terry et al., 1991; Toba et al., 1992). Simultaneous staining of DNA and RNA using acridine orange (AO) has led to new and important concepts regarding the cell cycle (Darzynkiewicz et al., 1980). For example, mitogens are competence factors and interleukin-2 is a progression factor for lymphocytes (Walker et al., 1983; Bettens et al., 1984).

AO methods require that cells be extracted with detergent. Furthermore, the emission spectrum of AO is sufficiently broad to make simultaneous staining with another fluorochrome for surface phenotyping impossible. Thus, AO methods are only applicable to homogeneous samples as cell lines and leukemias with a high population of leukemic cells. Another major problem of AO methods arises from the fact that these techniques are based on the equilibrium of AO with DNA versus RNA (Traganos et al.,

1976). This problem results in practical difficulties in applying these techniques.

The simultaneous analysis of DNA and RNA content using Hoechst 33342 and Pyronin Y has been described. Cells can be simultaneously stained with fluorochrome-conjugated antibodies. However, HO33342/PY methods require a dual laser flowcytometer (Shapiro, 1981).

We reported an improved means of simultaneously analyzing the DNA content, namely BrdUrd incorporation and the surface phenotype (Toba et al., 1992). We analyzed the levels of DNA and BrdUrd incorporation in individual CD4+, CD8+ and CD20+ subsets of PHA-activated human peripheral blood lymphocytes. However, we could not analyze subsets of the G1 population such as G0 (G1q), G1T, G1A and G1B, which are essential for understanding the detailed mechanisms of cell growth.

In this paper, we describe the development of a new and convenient methodology for measuring DNA and RNA using a single 488 nm argon laser excitation system. To demonstrate the potential utility of this methodology, we used PHA-activated human peripheral blood lymphocytes, and simultaneously analyzed the surface phenotype and quantified the DNA/RNA.

2. Materials and methods

2.1. DNA/RNA staining reagents and monoclonal antibodies.

Propidium iodide (PI), 7-amino-actinomycin-D (7AAD) (Zelenin et al., 1984; Rabinovitch et al., 1986) and Hoechst No. 33342 (HO342) were purchased from Sigma Chemical Company, St. Louis, MO. Acridine orange (AO) (Rigler, 1966), pyronin Y (PY) (Müller et al., 1975) and methyl green (MG) (Scott, 1967) were purchased from Polysciences, Warrington, PA. Thiazole orange (TO) (Lee et al., 1986) was purchased from Molecular Probes, Inc., Eugene, OR. Each DNA/RNA dye was dissolved in PBS at 20 times the final concentration, and 50 µl of dye solution was added to 1×10^6 cells in a 1 ml volume.

FITC-conjugated anti-Leu3a (CD4), Leu2a (CD8), Leu16 (CD20), transferrin receptor (CD71), control IgG1 and control IgG2a were purchased from Becton Dickinson Immunocytometry Systems, Mountain View, CA. FITC-conjugated anti-IL-2 receptor (CD25) and anti-HLA-DR were purchased from Coulter Immunology, Hialeah, FL.

2.2. Cells and culture conditions.

KG-1, K562 and KY821 (Saito et al., 1992) cells were maintained in RPMI-1640 (Gibco Laboratories, Grand Island, NY) medium supplemented with 100 U/ml Penicillin G sodium (PCG; Gibco), 100 µg/ml Streptomycin sulfate (SM; Gibco), and 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) at 37°C in 5% CO₂ in humidified air. Exponentially growing cells were harvested, seeded at 1.0×10^6 cells/ml in RPMI-1640 supplemented with 10% FBS, cultured for 24 hours, then harvested for cell cycle analysis.

Heparinized human peripheral blood was obtained from healthy donors. For direct analysis, erythrocytes were lysed using Gey's solution for 5 min on ice (Mishell and Shiigi, 1980), washed twice with RPMI-1640 and analyzed. To demonstrate the application of the method to the analysis of activated lymphocytes, human peripheral blood lymphocytes (PBL) were isolated by Histopaque-1077 (Sigma, St. Louis, MO) density centrifugation. Light density mononuclear cells were washed with medium and resuspended in RPMI-1640 supplemented with PCG, SM and 10% FBS at 2×10^6 cells/ml. Culture flasks (25 cm²; 25100; Corning Glass Works, Corning, NY) were seeded with 5 ml of the above cell suspension. Phytohemagglutinin (M form; Gibco) was added at a final concentration of 1% (v/v) as a polyclonal T cell mitogen, then cells were incubated at 37°C in a humidified CO₂-incubator for 24, 48, 72 or 96 hours and harvested.

2.3. Comparison and titration of DNA/RNA dye

To compare five DNA/RNA dyes (PI, 7AAD, AO, PY, and TO), exponentially growing KG-1 cells were harvested, washed with medium, and fixed with absolute methanol for 10 min at -20°C. Fixed cells were washed twice with IFA buffer (10 mM/l Hepes buffer in 0.15 M NaCl supplemented with 0.1% NaN₃ and 4% FBS), suspended in 1 ml of PBS containing 1,000 U/ml RNase (Calbiochem, La Jolla, CA) or 1,000 U/ml DNase-I (Sigma Chemical, St. Louis, MO), incubated at 37 °C for 30 min, pelleted and washed once with IFA buffer. The cells were suspended in 1 ml of nucleic acid staining solution (NASS; 0.15 M NaCl in 0.1 M phosphate-citrate buffer containing 5 mM sodium EDTA (Sigma) and 0.5% bovine serum albumin (Sigma; BSA, fraction V), pH 6.0). Thereafter, 50 µl of DNA/RNA dye was added, and the cell suspension incubated at room

temperature (RT) for 30 min. After an incubation with DNA/RNA dye, the cells were cooled on ice for at least 5 min then analyzed.

To titrate MG, 7AAD and HO342, fixed KG-1 cells were washed with IFA buffer, suspended in 1 ml of NASS, to which 50 μ l of MG or 7AAD or HO342 solution was added, and the cell suspension was incubated at RT 30 min. The cells were cooled on ice for at least 5 min, 50 μ l of PY was added, then incubated for an additional 10 min on ice and analyzed.

2.4. Saponin titration assay for permeabilization

KG-1 cells, human peripheral white blood cells after Gey's lysis and PHA-activated PBL were harvested, washed with medium and resuspended in NASS buffer supplemented with varying concentrations of saponin (Sigma; final concentration at 0.001 to 0.010 %), and stained DNA/RNA using following 7AAD/PY method. Light scattering properties, the resolution of the DNA/RNA dot plots and G1CV of DNA histograms were compared for each sample.

2.5. 7AAD/PY method

Unfixed cells were suspended in NASS containing 0.004% saponin (Sigma), then 50 μ l of 400 μ M 7AAD (final concentration, 20 μ M) was added and the cells were incubated at RT for 30 min. Then cells were cooled on ice for at least 5 min after which, 50 μ l of 100 μ M PY (final concentration, 5 μ M) was added, then the cell suspension was incubated for an additional 10 min on ice and analyzed.

To calculate the DNA (D.I.) and RNA indices (R.I.), 0.5×10^6 normal human PBL were added to 0.5×10^6 K562 cells as an internal standard, and the mixed sample was stained and analyzed with 7AAD/PY.

2.6. Three color staining

PHA-activated lymphocytes were harvested and washed with RPMI-1640. Cells, 1×10^6 , were pelleted, incubated with FITC-conjugated monoclonal antibody for 30 min on ice, and washed twice with IFA buffer (10 mM/l Hepes buffer in 0.15 M NaCl supplemented with 0.1% NaN₃ and 4% FBS). Cells were then stained for DNA/RNA using 7AAD/PY.

2.7. Flow cytometry

Cell fluorescence was analyzed with a FACScanTM Flow Cytometer (Becton-Dickinson, Mountain View, CA). Cells were excited with a single, 488 nm argon laser, analyzed for simultaneous green (FL1; 525 nm), orange (FL2; 570 nm), and red (FL3; >650 nm)

fluorescence emission, and 20,000 events were collected. The data were analyzed on FACScan ResearchTM, Lysis IITM and CellFitTM software (Becton-Dickinson). Detector and compensation settings for data collection were as following : FL2 = 600 (linear), FL3 = 400 (linear), FL2 - %FL3 = 0.0, FL3 - %FL2 = 9.0 for cell lines and 4.5 for resting human peripheral blood white cells, or FL1 - %FL2 = 3.3, FL2 - %FL1 = 5.0, FL2 - %FL3 = 0.0, and FL3 - %FL2 = 9.0 for the three color analysis of FITC/DNA/RNA. To quantify the DNA and RNA levels of K562 cells using 7AAD/PY, D.I. and R.I. were calculated using the relevant histograms as follows :

D.I. = (peak channel number of 2N K562 cells on a DNA histogram) / (peak channel number of resting lymphocytes on the same DNA histogram)

R.I. = (mean channel number of a subset of K562 cells on an RNA histogram after DNA gating) / (the mean channel number of resting lymphocytes on an RNA histogram)

In some studies of activated lymphocytes, the R.I. of discrete subsets were calculated using RNA histograms as follows :

R.I. = (peak channel number of a subset) / (peak channel number of G0)

3. Results

3.1. Comparison of the emission spectra and specificity of nucleic acid binding dyes

To determine which combination of DNA and RNA dyes could be used simultaneously with a single argon laser system, we initially compared the emission spectra and nucleic acid binding characteristics of five dyes. Exponentially growing KG-1 cells were fixed with methanol and stained with 30 μ M PI, 20 μ M 7AAD, 1 μ M AO, 5 μ M PY, or 0.25 μ M TO. DNA and RNA binding was selectively eliminated using 1,000 U/ml DNase-I and 1,000 U/ml RNase respectively. PI had a relatively wide emission spectrum ranging from orange (FL2) into the red (FL3), while 7AAD exhibited predominantly red emission (FL3). AO and TO had wide emission spectra ranging from green (FL1) to red, while PY had a relatively narrow emission spectrum in the orange (FL2) and very low fluorescence in the green (FL1). The very low green emission of PY made it useful in multiparametric analysis combined with FITC (Table 1).

The fluorescence of all 5 dyes was markedly reduced by DNase. In contrast, PI and 7AAD fluorescence was only minimally reduced, while nearly one-half of the fluorescence of AO, PY and TO was eliminated by RNase (Table 2).

3.2. 7AAD blocked the PY staining of DNA

Initially, we observed that DNA stained with 7AAD produced an emission predominantly in the red range, whereas PY stained DNA and RNA with emission mostly in the orange range. Based upon these findings, we selected 7AAD as the DNA dye and PY for the RNA dye. HO342 and MG reportedly block the interaction of PY with DNA (Shapiro, 1981; Scott, 1967). To compare the blocking effect of 7AAD, MG or HO342 on the PY interaction with DNA, fixed cells were serially stained with varying concentrations of 7AAD, MG or HO342 and 5 μ M PY. FL2 (orange) fluorescence intensity was measured, and the relative fluorescence intensity was compared to a sample stained with PY alone. Among those samples that were stained with 7AAD first, FL2 was measured with the compensation set using a blank sample (no dye) and a 7AAD stained sample at each concentration. Incubating the fixed cells with 7AAD blocked subsequent PY binding to DNA in a manner equivalent to that of MG and HO342 (Figure 1). Thus, cellular DNA and RNA can be simultaneously quantified by red (7AAD, 20 μ M) and orange emission (PY, 5 μ M) respectively.

3.3. Saponin titration and application of 7AAD/PY method

Saponin, a detergent like substance, permeabilizes cell membranes without fixation at a concentration of 0.1% (Sander et al., 1991). Initially we had to determine the concentration of saponin that would result in acceptable DNA/RNA staining while preserving light scatter properties. DNA/RNA two dimensional dot plots and the G1CV(%) of DNA histograms were measured as parameters of permeability in the presence of various concentrations of saponin in buffer, and the light scatter properties of human peripheral white blood cells were observed. The 7AAD/PY method worked well with saponin in the buffer at concentrations above 0.003% (Figure 2). Light scattering properties of human peripheral blood leukocytes were preserved at saponin concentrations below 0.005% (data not shown). Thus, we selected a saponin concentration of 0.004%.

Unfixed KY821 cells were stained sequentially with 7AAD and PY in NASS buffer supplemented with 0.004% saponin. Figure 3 shows the DNA/RNA two dimensional analysis and calculations of each population of cell cycle stages of exponentially growing KY821 cells. These data were comparable to those previously

reported using cell lines and AO (Darzynkiewicz et al., 1980) or HO342/PY (Shapiro, 1981).

Since cell cycle analysis using 7AAD/PY does not present the absolute quantity of DNA nor RNA, internal standardization using resting normal lymphocytes is necessary, especially in samples of aneuploid cells. To demonstrate this standardization, a mixed sample of K562 cells and normal resting lymphocytes was stained and analyzed using 7AAD/PY (Figure 4). The DNA index (D.I.) was calculated from the peak channel numbers of resting lymphocytes and G1 K562 cells using a DNA histogram. On the other hand, to calculate the RNA indices, the populations of resting lymphocytes (R1), G1, S and G2+M subsets of K562 cells (R2, R3, and R4) were gated using DNA histograms, then mean RNA channel numbers of discrete subsets were measured.

Figure 5 shows both light scattering and a DNA/RNA plot of resting peripheral leukocytes. The mean RNA fluorescence of monocytes was about 1.4 times greater than that of lymphocytes, while that of granulocytes was about 1.1 times greater.

3.4. Three color analysis with CD4/CD8/CD20-FITC, DNA and RNA

To demonstrate the convenience of using 7AAD/PY, we monitored lymphocyte subset markers, as well as the cellular DNA and RNA content in PHA-activated peripheral blood lymphocytes. Figure 6 shows the cycling status of PHA-activated lymphocytes determined using 7AAD/PY on days 1 through 4 (24 - 96h). On day 1, most lymphocytes were in G0 and G1, with a small portion in the early S phase. Most of the cells entered S and G2+M phase on day 2, advanced from S and G2+M into G1 on day 3, and a portion of G1 cycling cells re-entered S on day 4. The DNA cell cycle was calculated using the RFIT program on CellFitTM software. Cell aggregates and dead cells were eliminated using FL3-width versus FL3-area gating.

Using 7AAD/PY, the cell cycle of a specific lymphocyte subset can be analyzed as shown in Figure 7, which displays the results from a representative three-color analysis using Leu2a-FITC, 7AAD, and PY on PHA stimulated cells after 48 hours of culture. The detailed cell cycle of CD4+, CD8+, and CD20+ populations on each day after PHA activation was analyzed. A specific gate was set on G0+G1 cells using an FL3-width versus FL3-area plot, and RNA histograms of the G0+G1 population were analyzed as shown in Figure 8. The majority of CD4+ cells were in the early stage of G1 (R.I.

= 1.3) on day 1, progressed to late G1 (G1B, R.I. = 2.0) on day 2, stopped in mid G1 (G1A, R.I. = 1.7) and appeared to wait to re-enter G1B for the second cell cycle on days 3 and 4. CD20+ cells showed a similar cell cycle to the CD4+ population on each day. About half of the 2N CD8+ cells were in G0 (R.I. = 1.0) from days 1 through 4, and the remaining CD8+ cells were in G1B during the same period (Figure 8).

3.5. Three color analysis of HLA-DR/IL2R/TfR-FITC, DNA and RNA

Lymphocytes were analyzed to correlate the DNA and RNA content with the expression of activation markers following a 24 hr activation with PHA. Data were collected after gating with activation markers, and RNA histograms were analyzed as shown in Figure 9. IL2R was expressed in early G1 (G1T). HLA-DR was expressed at the very beginning of G1 without a detectable increase in the RNA level. TfR was expressed at late G1 (G1B) just before the initiation of DNA synthesis.

4. Discussion

The goal of this study was to establish a new and convenient method using a single argon laser to simultaneously accomplish surface phenotyping and quantify cellular DNA and RNA level while preserving light scatter properties.

Darzynkiewicz and his group have established an Acridine orange (AO) staining method for the simultaneous two-color analysis of DNA and RNA content. They proposed a new and important concept of proliferation and the cell cycle which classified cell cycle stages into G1Q (G0), G1T, G1A, G1B, G1D (terminal differentiated G0), SQ, ST, S, G2Q, G2T, G2, and M. Using this classification, they showed that competence factors work on G0 cells inducing them to enter G1A, whereas progression factors work on G1A cells inducing them to enter G1B (Darzynkiewicz et al., 1980). The use of this procedure revealed that mitogens are competence factors, and that interleukin-2 (IL-2) is a progression factor for lymphocytes (Walker et al., 1983; Bettens et al., 1984).

AO simultaneously stains DNA and RNA with green and red fluorescence respectively using 488 nm argon laser excitation. However, the AO staining procedure is complex, because it is based on the balanced competition of AO reactivity between single and double stranded nucleic acids. Moreover, AO has a wide emission spectrum from green to red, such that a third parameter cannot be added to the analysis on most single laser systems. The advantage

of AO staining is that it helps to measure the absolute amount of RNA which varies according to cell type and maturation stage. However, the use of internal standardization such as normal human lymphocytes added to samples is still necessary to measure the DNA index from which the quantity can be calculated when the cell cycle of aneuploid or cancer cells is analyzed in clinical samples and cell lines. Our 7AAD/PY method described here also requires internal standardization against which to quantify DNA and RNA in growing cells. The DNA index of a 2N subset and the RNA indices of G0, G1, S, and G2+M subsets must be chosen as parameters to correlate the amounts of DNA and RNA of each subset with those of resting normal human lymphocytes.

To determine the optimal distribution of FL2 (RNA) and FL3 (DNA), we used fluorescence compensation at about 5 to 10% of FL3 - %FL2 and at 0 % of FL2 - %FL3. The orange (FL2) fluorescence emission of 7AAD is at a 2.6 % intensity of the dominant red (FL3) fluorescence, while PY has red fluorescence emission at a 40 % intensity of the dominant orange (Table 1). Thus, to quantify the DNA as well as to produce a low coefficient of variation of the G1 peak (G1CV) on DNA histograms, an optimal setting of FL3 - %FL2 is necessary. Moreover, standardization using resting normal blood lymphocytes helps to quantify the DNA in a sample. In actual examinations, FL3 - %FL2 was set upward from 0 % until the long axis of the 2N (G0+G1) subset becomes parallel to that of FL2 on a DNA/RNA dot plot, and the G1CV becomes the smallest. On the other hand, the FL2 - %FL3 was set at 0 %. The influence of 7AAD upon FL2 detectors in comparison with PY fluorescence is very small. Cells which have the same amount of DNA such as the G0+G1 population are influenced by fluorescence on the FL2 detector from 7AAD at the same intensity. Moreover, internal standardization is used for DNA/RNA analysis, so the influence of 7AAD emission on FL2 can be ignored to obtain the relative quantity of RNA of each subset.

PY is an analogue of AO that stains DNA and RNA with emission within the same range. PY has maximal absorption between 547 and 563 nm and it fluoresces with maximum emission between 565 and 574 nm in the intercalated form (Darzynkiewicz, 1994). PY is widely used in absorption microscopy as well as in flow cytometry as a fluorochrome for RNA (Tanke et al., 1980). PY binds by intercalation to double stranded (ds) nucleic acids. Its binding affinity is several fold higher to dsRNA

than to dsDNA. Therefore, when PY is used as a fluorochrome, it mainly detects dsRNA. Furthermore, RNA stainability and specificity is assured at relatively narrow concentrations of this dye. High concentrations of PY cause RNA condensation, and PY fluorescence in the condensed complex is quenched (Kapuscinsky et al., 1987; Darzynkiewicz, 1994). Thus, the PY concentration required for optimal RNA staining is critical. Shapiro used PY at a concentration of 4 μ M for HO342/PY (Shapiro, 1981), whereas we used 5 μ M for 7AAD/PY. In live cells, PY also stains mitochondria. It is not known whether saponin permeabilization, like that with Triton X-100 or fixation with ethanol, eliminates the stainability of mitochondria. Other studies have showed that PY stains only RNA when DNA binding is blocked with the non-fluorescent DNA intercalator, methyl green (Scott, 1967; Pollack et al., 1982). PY is also useful for DNA/RNA double staining when combined with the A-T specific DNA dye, Hoechst 33342, which emits in the blue spectrum, requiring the use of a dual laser system (Shapiro, 1981; Darzynkiewicz, 1987). In contrast, 7AAD was applied in DNA/RNA double staining in combination with PY, since 7AAD blocks DNA and emits fluorescence predominantly red (Tables 1, 2 and Figure 1). Given the emission spectra of 7AAD and PY, another green emitting dye such as FITC may also be used. In fact, we found that when clinical samples such as blood leukocytes and bone marrow cells which contain heterogeneous subpopulations were concurrently stained with FITC-conjugated antibodies, 7AAD and PY, the DNA/RNA cell cycle could be analyzed in individual subpopulations such as erythroblasts, or early myeloid cells (data not shown).

The compound 7AAD (Rabinovitch et al., 1986; Toba et al., 1992), which is also abbreviated as 7-amino-AMD (Zelenin et al., 1984) or 7-A-AMD (Gill et al., 1975) or 7-AMD (Stokke et al., 1991; Stokke et al., 1988), is a red fluorescent analogue of actinomycin D. The dye intercalates into G-C regions in DNA, has maximal absorption at 555 nm and fluoresces with maximum emission at 655 nm. It is also a nucleic acid stain that can be excited by an argon laser and detected as a third color along with fluorescein and phycoerythrin (Toba et al., 1992; Haugland, 1994). We chose 7AAD as the "third color" with which to simultaneously stain surface markers, DNA, and RNA.

Here, we studied the cell cycle progression of subsets of activated lymphocytes. CD4+ and

CD20+ cells entered early G1 (G1T) on day 1, progressed into late G1 (G1B), S and G2+M on day 2, and appeared to be waiting to re-enter G1B on days 3 and 4. The reason for this delay is not known. In contrast, almost half of the CD8+ cells quickly entered G1B on day 1, and progressed in the cell cycle through day 4. It appeared that a portion of the CD8+ cells had not entered the cell cycle by day 4.

We were also able to observe the expression of activation markers in conjunction with the cell cycle. The transferrin receptor (TfR) is a proliferation related antigen (Larrick and Cresswell, 1979; Judd et al., 1980; Ho et al., 1989), HLA-DR is an activation marker of T lymphocytes (Hansen et al., 1978), and the IL-2 receptor (IL2R) the trigger for the progression factor (IL-2), is expressed on lymphocytes during or before the G1A phase (Greene, 1986; Urdal et al., 1984; Fox et al., 1984). IL2R was expressed in early G1 (G1T), HLA-DR was expressed at very early G1 without a detectable increase in the RNA level, and TfR was expressed in late G1 (G1B) just before the initiation of DNA synthesis. K562 and KY-821 cells have a relatively narrow range of RNA quantities in S-phase fractions (Figure 3 and 4), while activated peripheral lymphocytes have a wide range of RNA levels (Figure 6). The reason for this difference is not known. We suggest that the wide range of RNA in activated lymphocytes is due to the heterogeneity of the lymphocyte subsets, such as T- and B-cells, and the characteristic cell cycle status of lymphocyte subpopulations (Figure 8).

In summary, 7AAD/PY staining offers the following advantages over existing methodology: 1) detailed analysis of the cell cycle by simultaneous RNA/DNA quantitation; 2) preservation of light scattering properties; 3) simple and reproducible procedure; 4) applicable to three-color analysis on single laser systems in combination with surface phenotype staining (this feature is essential for analyzing the cell cycle status of individual subsets in heterogeneous cell suspensions such as bone marrow, peripheral blood and other complex tissues); 5) virtually no cell loss when compared to surface phenotypic analysis of an unfixed cell sample (this minimizes selective loss of specific subsets of the cell cycle); 6) low coefficient of variation of the G1 peak, usually less than 3.0, which is essential for accurately measuring the cell cycle.

Acknowledgments

We thank Dr. Robert A. Bray for his important suggestion about flow cytometry.

We thank Ms. Pat Jacobs, Mrs. Susan Rozmiarek, and Ms. Linda Stempora and Ms. Arlene Glass of the Winship Cancer Center, Flow Cytometry Facility for their technical assistance. We also thank Mrs. Julie Cook for her assistance in the preparation of the manuscript.

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Table 1. Emission fluorescence spectra of five DNA/RNA dyes.

Dye ^a	fluorescence (relative % to dominant color) ^b		
	green (FL1)	orange (FL2)	red (FL3)
PI	0 (0.0) ^c	55 (12.4)	442 (100.0)
7AAD	0 (0.0)	13 (2.6)	496 (100.0)
AO	494 (100.0)	142 (28.7)	35 (7.1)
PY	8 (1.7)	465 (100.0)	188 (40.4)
TO	448 (100.0)	236 (52.7)	112 (25.0)

^a DNA/RNA were dyed with propidium iodide (PI; 30 μ M), 7-amino-actinomycin D (7AAD; 20 μ M), acridine orange (AO; 1 μ M), pyronin Y (PY; 5 μ M) and thiazole orange (TO; 0.25 μ M).

^b Green (525 nm), orange (570 nm) and red (> 650 nm) fluorescence was measured on a linear scale using same gain for each dye.

^c The mean fluorescence channel was measured and the relative level of the fluorescence to the dominant color was calculated.

Table 2. Effects of DNase-I and RNase on DNA/RNA dye incorporation.

Dye ^a	Emission ^b	relative fluorescence (%) ^c			
		(-)	+DNase	+RNase	+DNase +RNase
PI	Red	100	6 ^d	84	12
7AAD	Red	100	20	85	42
AO	Green	100	19	56	25
PY	Orange	100	33	55	36
TO	Green	100	17	60	15

^a DNA/RNA were stained with propidium iodide (PI; 30 μ M), 7-amino-actinomycin D (7AAD; 20 μ M), acridine orange (AO; 1 μ M), pyronin Y (PY; 5 μ M) and thiazole orange (TO; 0.25 μ M).

^b Green (525 nm), orange (570 nm) and red (> 650 nm) fluorescence were measured on a linear scale using same gain in each dye.

^c Mean fluorescence intensity was measured using the same gain setting for each sample. (-), without prior digestion; +DNase, prior digestion with 1,000 U/ml DNase-I; +RNase, prior digestion with 1,000 U/ml RNase; +DNase+RNase, prior digestion with both nucleases.

^d Relative fluorescence intensity compared with samples that were not pretreated.

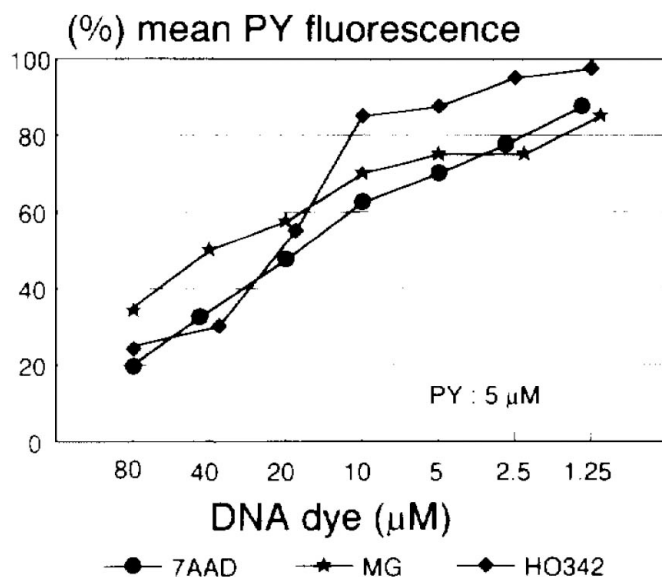


Figure 1. Blocking the PY stain with 7AAD, MG and HO342. DNA binding with PY (5 μ M) was blocked using a titrating concentration of 7AAD, MG and HO342 (1.25 - 80 μ M), then the mean PY fluorescence was measured. Control FL2 intensity was measured in samples stained with only PY, and the mean fluorescence of each titration sample was calculated as a percent of the control.

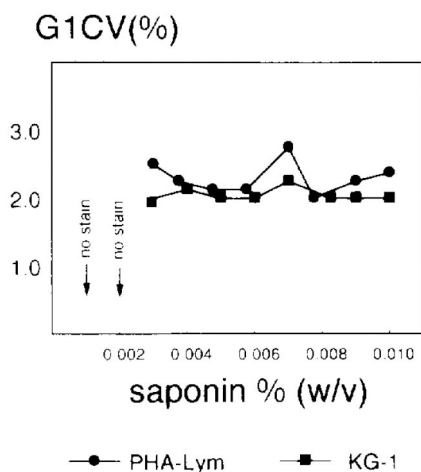


Figure 2. Saponin titration. KG-1 cells and human peripheral lymphocytes activated for 72 hr with PHA were stained sequentially with 7AAD and PY in buffer containing a titrating concentration of saponin. Measured G1CV levels were acceptable when cells were stained with saponin in buffer at concentrations 0.003% or higher. The resolution of the DNA/RNA two dimension dot plot graph was acceptable at 0.004% or higher.

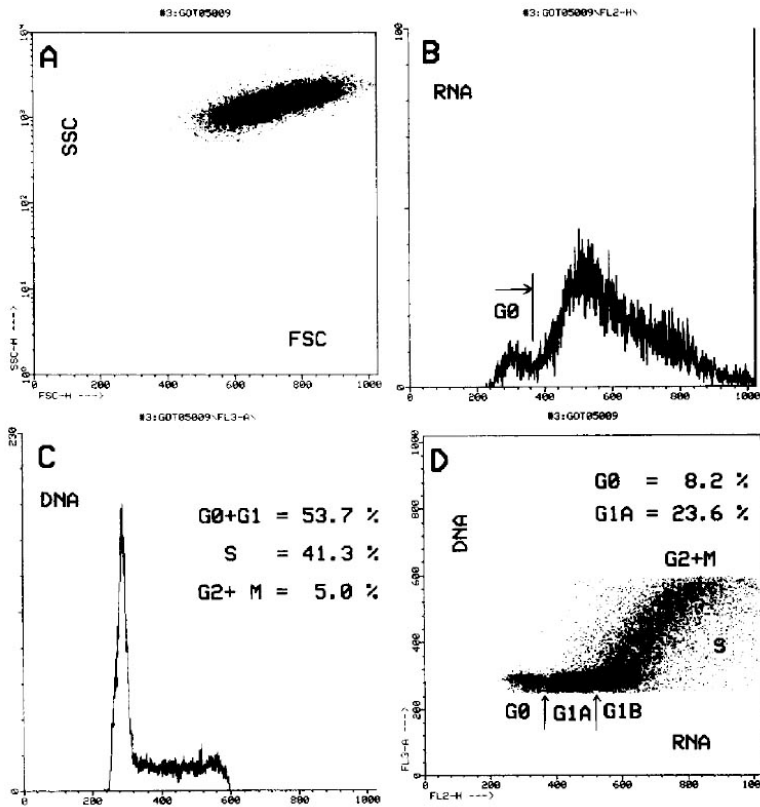


Figure 3. Cell cycle analysis of exponentially growing KY821 cells using 7AAD(DNA)/PY(RNA) staining. A gate was set to remove cell aggregates and dead cells using dot plots of the pulse width versus the area of the FL3 signal (DNA). Light scatter (panel A), histograms of RNA (panel B) and DNA (panel C), and the DNA/RNA dot plot (panel D) of the gated data are shown. The G0 population is demonstrated as separated 2N cells with minimal RNA levels.

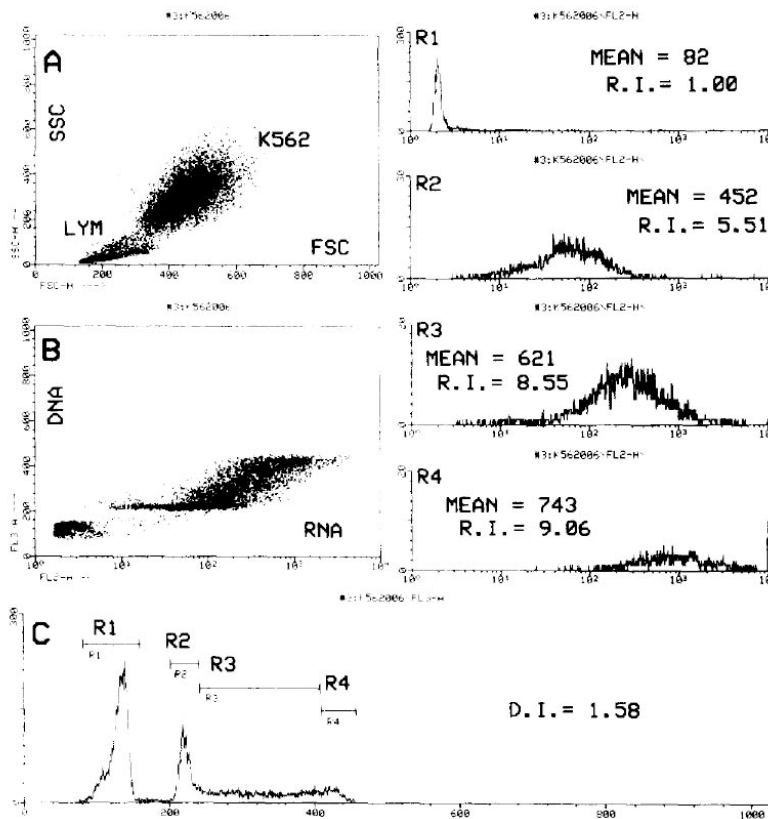


Figure 4. K562 cell cycle analysis during exponential growth using 7AAD(DNA)/PY(RNA) staining. Human peripheral lymphocytes from a healthy donor were added as an internal standard, and a mixed sample of K562 cells and normal lymphocytes was analyzed using 7AAD/PY. Light scatter (panel A), DNA/RNA dot plot (panel B), DNA histogram (panel C), and RNA histograms of the gated population in panel C (panels R1, R2, R3, and R4) are shown. Populations of 2N lymphocytes (R1), 2N (R2), S-phase (R3), and 4N (R4) of K562 cells were gated using the

DNA histogram (panel C). The relative DNA index (D.I.) of K562 cells was calculated as the relative peak channel number of 2N to that of normal lymphocytes. RNA indices (R.I.) of 2N, S-phase, and 4N populations of K562 were calculated as the relative mean channels of RNA fluorescence to that of normal lymphocytes after DNA gating.

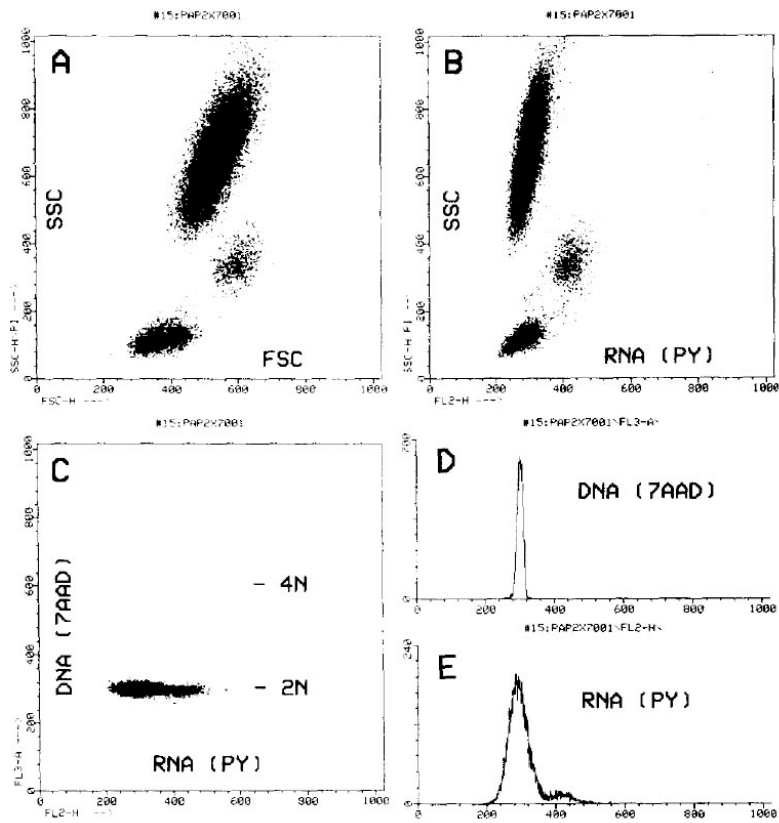


Figure 5. Cell cycle analysis of resting human peripheral white blood cells using 7AAD(DNA)/PY(RNA) staining. Two dimensional analysis of light scattering (panel A), RNA quantity v.s. side scatter (panel B), DNA v.s. RNA (panel C), histograms of DNA (panel D) and RNA (panel E) are shown. Light scattering properties were preserved. The relative RNA indices of granulocytes and monocytes to G0 lymphocytes were 1.1 and 1.4 respectively.

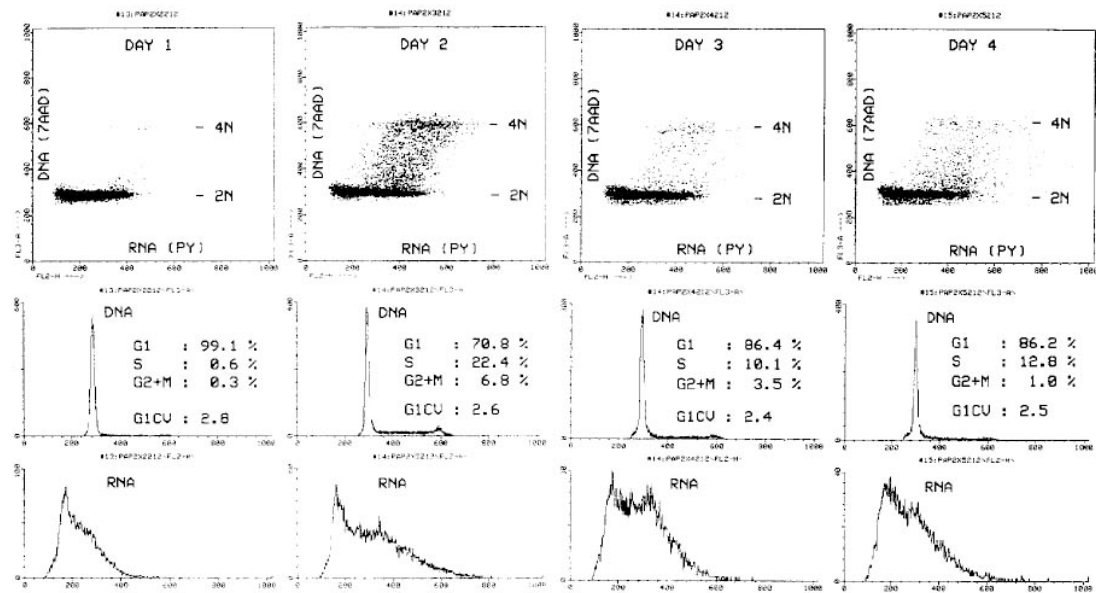


Figure 6. DNA/RNA two-color analysis of PHA-activated (24, 48, 72 and 96 h) human peripheral lymphocytes. DNA/RNA two dimensional, DNA histogram, and RNA histogram analyses are shown.

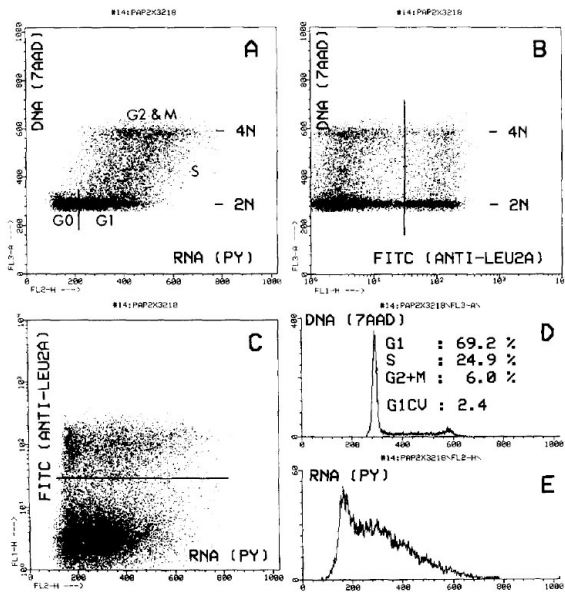


Figure 7. Three-color analysis of PHA-activated (48 h) human peripheral lymphocytes stained with anti-Leu2a (FITC), 7AAD (DNA), and PY (RNA). Two dimensional analysis of RNA/DNA (Panel A), Leu2a/DNA (Panel B), RNA/Leu2a (Panel C), and a histogram analysis of DNA (Panel D) and RNA (Panel E) are shown.

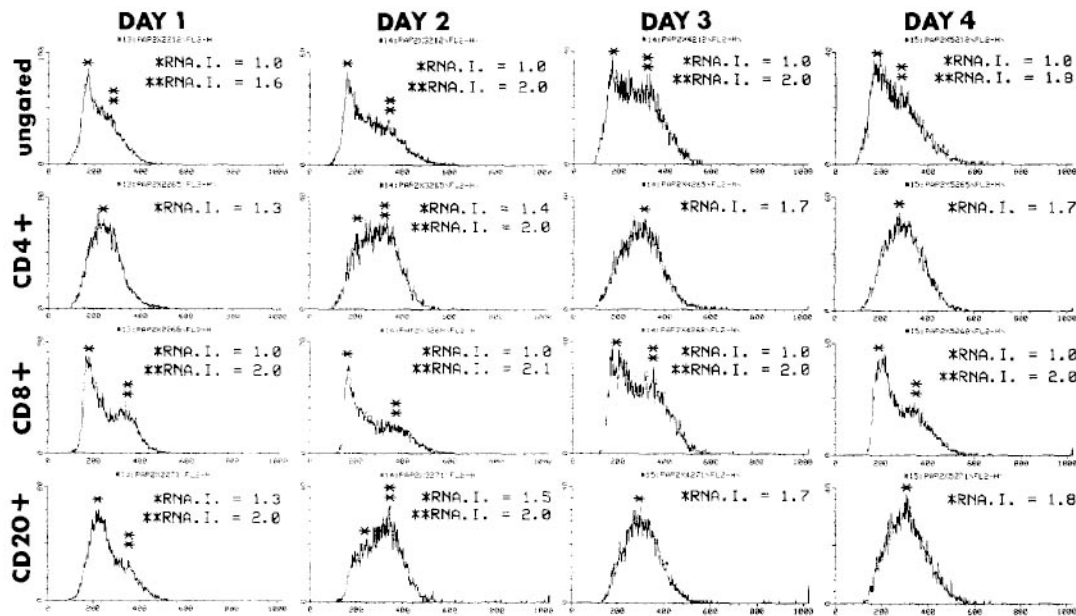


Figure 8. RNA histograms of G0+G1 (2N) cells after PHA activation (24 h, 48 h, 72 h and 96 h). The cells were stained with a surface marker (FITC), 7AAD (DNA), and PY (RNA). Data from the CD4+, CD8+, and CD20+ subsets were collected, and RNA histograms of G0+G1 cells were analyzed using analytical gate on the FL3-width versus the FL3-area. The RNA index was calculated as the relative RNA quantity to G0 cells. Only the CD8+ cells had a persistent G0 population.

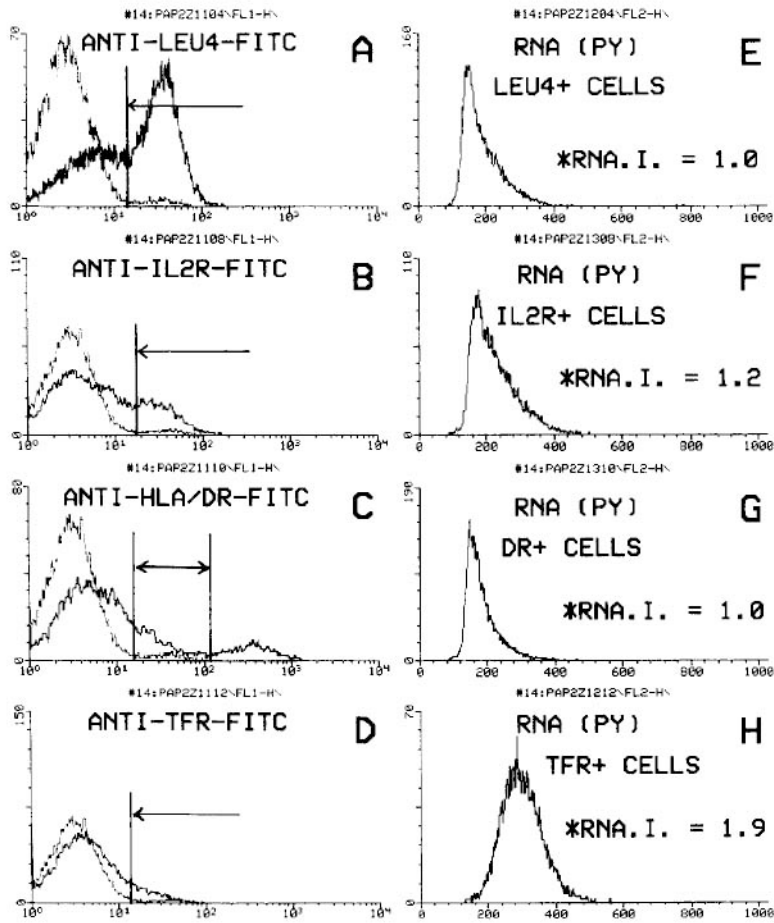


Figure 9. Three-color analysis of PHA-activated (24 h) human peripheral lymphocytes stained with anti-Leu4 or activation markers (FITC), 7AAD (DNA), and PY (RNA). Data were collected after the gate was set on the FITC histogram (panel A - D) of monoclonal antibodies (black lines) and controls (gray lines). Cell aggregates and dead cells were gated out using the FL3-width versus the FL3-area graph, and RNA histograms were analyzed (panel E - H). The RNA index was calculated as the relative RNA quantity to G0 cells. Low-density HLA-DR and transferrin receptors were expressed in T-cells as activation markers on day 1, whereas high density HLA-DR was present in B-cells before culture (panel C).