Video Article

Analysis of Cell Cycle Position in Mammalian Cells

Matthew J. Cecchini[1], Mehdi Amiri[1], Frederick A. Dick[2]

[1] Department of Biochemistry, Schulich School of Medicine and Dentistry, University of Western Ontario

[2] London Regional Cancer Program, Children's Health Research Institute, and Department of Biochemistry, Schulich School of Medicine and Dentistry, University of Western Ontario

Abstract

The regulation of cell proliferation is central to tissue morphogenesis during the development of multicellular organisms. Furthermore, loss of control of cell proliferation underlies the pathology of diseases like cancer. As such there is great need to be able to investigate cell proliferation and quantitate the proportion of cells in each phase of the cell cycle. It is also of vital importance to indistinguishably identify cells that are replicating their DNA within a larger population. Since a cell's decision to proliferate is made in the G1 phase immediately before initiating DNA synthesis and progressing through the rest of the cell cycle, detection of DNA synthesis at this stage allows for an unambiguous determination of the status of growth regulation in cell culture experiments.

DNA content in cells can be readily quantitated by flow cytometry of cells stained with propidium iodide, a fluorescent DNA intercalating dye. Similarly, active DNA synthesis can be quantitated by culturing cells in the presence of radioactive thymidine, harvesting the cells, and measuring the incorporation of radioactivity into an acid insoluble fraction. We have considerable expertise with cell cycle analysis and recommend a different approach. We Investigate cell proliferation using bromodeoxyuridine/fluorodeoxyuridine (abbreviated simply as BrdU) staining that detects the incorporation of these thymine analogs into recently synthesized DNA. Labeling and staining cells with BrdU, combined with total DNA staining by propidium iodide and analysis by flow cytometry[1] offers the most accurate measure of cells in the various stages of the cell cycle. It is our preferred method because it combines the detection of active DNA synthesis, through antibody based staining of BrdU, with total DNA content from propidium iodide. This allows for the clear separation of cells in G1 from early S phase, or late S phase from G2/M. Furthermore, this approach can be utilized to investigate the effects of many different cell stimuli and pharmacologic agents on the regulation of progression through these different cell cycle phases.

In this report we describe methods for labeling and staining cultured cells, as well as their analysis by flow cytometry. We also include experimental examples of how this method can be used to measure the effects of growth inhibiting signals from cytokines such as TGF-β1, and proliferative inhibitors such as the cyclin dependent kinase inhibitor, p27KIP1. We also include an alternate protocol that allows for the analysis of cell cycle position in a sub-population of cells within a larger culture[5]. In this case, we demonstrate how to detect a cell cycle arrest in cells transfected with the retinoblastoma gene even when greatly outnumbered by untransfected cells in the same culture. These examples illustrate the many ways that DNA staining and flow cytometry can be utilized and adapted to investigate fundamental questions of mammalian cell cycle control.

Video Link

The video component of this article can be found at http://www.jove.com/video/3491/

Protocol

1. Labeling and fixing of cells

1. Add 1 μL of Cell Proliferation Labeling Reagent (BrdU) per mL of cell culture medium (1 to 1000 dilution) 1 hour before harvesting. The labeling period may need to be lengthened for slower growing cells.
2. To harvest cells, aspirate culture medium and wash thoroughly with phosphate buffered saline (PBS). Repeat to thoroughly remove traces of medium.
3. Wash cultures quickly a third time with PBS containing 3mM EDTA and aspirate thoroughly.
4. Add a small volume of PBS containing 3mM EDTA to each dish, 0.5 mL for a 6 cm dish is ideal. Incubate at 22°C for approximately 5 minutes to detach cells. Transfer to a 15 mL conical tube.
5. Centrifuge cells at 500 x g for 5 minutes to pellet, remove supernatant, and resuspend thoroughly in 100 μL of PBS.
6. Fix cells by adding 5 mL of 95% EtOH, dropwise, while vortexing. At this step, cells can be stored at 4°C for at least a month.

2. Denaturing and staining of BrdU and DNA

1. Centrifuge cells at 500 x g for 5 minutes to pellet cells and remove 95% EtOH. Resuspend in 1 mL of 2N HCl and 0.5% Tx-100 by adding in a drop wise fashion while vortexing. Incubate at room temperature for 30 minutes.
2. Centrifuge as before in section 2.1 and carefully aspirate supernatant since the cells form a very loose pellet at this step. Gently resuspend in 1 mL of 0.1M NaB_4O_7 (pH 8.5) and incubated for at least 30 minutes at room temperature.
3. Pellet cells as above in section 2.1 and resuspend in 0.5 mL of antibody solution (PBS containing 1% BSA and 0.2% Tween-20) with mouse anti-BrdU antibodies diluted 1 to 50. Incubate at room temperature for 30 minutes.
4. Pellet cells again and resuspend in 50 mL of antibody solution containing rabbit anti-mouse secondary antibodies conjugated to FITC diluted 1 to 25. Incubate for 30 minutes on benchtop and protect from light.
5. Centrifuge cells a final time and resuspend in 0.5 mL of propidium iodide and RNase solution (PI-RNAse solution, PBS with 1% BSA, 10 mg/mL propidium iodide, 0.25 mg/mL RNase A) and incubate in the dark at 37°C for 30 minutes. Alternatively RNA can be digested overnight at 4°C in the dark.
6. Pass solution through a cell strainer to remove aggregates and collect single cells in an appropriate tube for sample uptake on your flow cytometer. Again, samples should be protected from direct exposure to light.

3. Analysis by flow cytometry

1. Cells should be analyzed by a standard flow cytometer that is capable of discriminating against doublets (two cells that pass through the flow cell as one) with appropriate detection capability for propidium iodide and FITC. Single cells can be detected in this flow cytometry application by gating for events based on forward scatter and side scatter populations, and by using the properties of DNA staining by propidium iodide. The appearance of a doublet discriminating dot plot varies between flow cytometers and relies on propidium iodide staining properties, see your local flow cytometry facility for advice on setting up a doublet discriminator in your analysis. In general, in an asynchronously proliferating population of cells, single cells are usually the two most abundant peaks (2N and 4N DNA) in the doublet discriminating plot and a gate should be drawn around them. This will select single cell events that are used for all subsequent analysis below.
2. The first sample to be analyzed should be an asynchronously proliferating culture that serves as a positive control for staining and flow cytometer set-up. Adjust the sensitivity of photomultiplier tubes for propidium iodide staining such that the 2N and 4N peaks from singlet cells are centered at 200 and 400 (arbitrary units) on the X-axis. We often stain an abundant supply of these cells to ensure that all parameters of the flow cytometer have been adjusted appropriately without using up this sample. This positive control is also helpful for new users to become more familiar with the operation of the flow cytometer.
3. Adjust the sensitivity of the photomultiplier tube for FITC detection such that G1 and G2/M populations are above background. BrdU positive cells should be approximately 10 times brighter and the Y-axis should be displayed as a logarithmic scale. It is sometimes helpful to include a negative control for BrdU staining (by replacing the anti-BrdU antibodies with non-specific IgG in step 2.3 above) to clearly distinguish positively stained cells.
4. Adjust compensation between propidium iodide and FITC such that single events captured by the flow cytometer form a horse shoe shaped arc from G1 in the lower left up to S-phase and down into the lower right for G2/M. Please see the following reference for a more in depth discussion of the principles and use of flow cytometry and references there in for specific applications[2].

4. Data analysis

1. 5 000 to 10 000 single cell events with the desired range of DNA content should be collected for each sample in order to have confidence that the population of cells in the culture have been thoroughly sampled.
2. Once cells have been measured for propidium iodide and BrdU content they need to be assigned to the G1, S, or G2/M phases. Do this by drawing gates around the two BrdU negative populations centered at 200 and 400 (G1 and G2/M respectively). Everything above these boxes should be included in a single gate that measures S-phase (Fig. 1A). The percentage of cells in each gate represents the relative number of cells in G1, S, and G2/M (Fig. 1B).

5. Alternate protocol for analysis of cell cycle in a mixed population of cells

1. This alternate protocol is most helpful when transfection of expression vectors routinely results in a low percentage of cells expressing the gene product of interest, or when drug treatment to select a pure population of cells is impractical. This results in a relatively small proportion of cells of interest being contaminated with a large population of untransduced cells. In this case, co-transfect plasmids expressing your gene or shRNA of interest along with a plasmid that expresses a marker for detecting transfected cells by flow cytometry, such as a membrane anchored GFP[3], or a foreign cell surface marker such as CD19[4] or CD20[5].
2. For the purposes of this protocol we will use CD20 staining as an example, however, staining for CD19 is essentially identical. In the case of transfection with CD20, there is no need to BrdU label, simply harvest cells as described in steps 1.2 to 1.5 above and stain by adding 20 µL FITC conjugated anti-CD20 antibodies to the cells on ice for 20 minutes in the dark. Fix cells as described in step 1.6. Cells can again be stored for at least a month at 4°C in the dark. For detection of GFP, omit the antibody staining from this step and fix and store cells as described.
3. Re-hydrate the cells by pelleting in the centrifuge at 500 x g for 5 minutes and resuspending in 5 mL of PBS containing 1% BSA.
4. Re-pellet the cells at 500 x g for 5 minutes and resuspend cells in 0.5 mL of propidium iodide and RNase solution as described above in section 2.5.
5. Continue to follow cell preparation instructions in 2.6 and analysis instructions in 3.1 and 3.2.
6. Adjust the sensitivity of the photomultiplier tube for FITC detection such that unstained cells are above background. Ideally, CD20-FITC positive cells will be 10X to 100X more intensely stained than background and the Y-axis of this plot should be displayed as a logarithmic scale (Fig. 2A). CD20 positive cells that are 10X brighter than background (and with propidium iodide staining between 200 and 400) should be selected for display in a propidium iodide versus cell counts histogram as shown in Fig. 2C. For cell lines that express markers that are

readily stained brightly (ie. 10X to 100X above background) the CD20 positive cut-off should be set at 10X background. Inclusion of weaker staining cells increases variability in these experiments, presumably because the gene of interest is also weakly expressed in these cells. For cell lines that only yield weakly stained CD20 positives (ie. less than 10X background), determination of a cut off should be made using a negative control consisting of CD20 stained, untransfected cells.

7. At least 1000 CD20 positive events should be collected for each sample. Experiments with fewer that 1000 events will produce high variability. Software packages such as ModFit LT (Verity Software), Multi Cycle AV (Phoenix Flow Systems), and Flow Jo (Tree Star) use mathematical estimates of the G1, S, and G2/M populations that contribute to the shape of the curve in histograms such as those shown in Fig. 2B and C.

6. Representative Results:

We provide three examples of experimental cell cycle analyses using our approaches. The first uses retroviral expression of the cyclin dependent kinase inhibitor p27KIP1 in mouse embryonic fibroblasts. Twenty four hours after drug selection for viral infection was complete, cells were pulse labeled with BrdU for one hour. In this experiment ectopic expression of the inhibitor is used to arrest proliferation of the cells (Fig. 3A and B). As shown in Fig. 3B little BrdU positive events are evident in the S-phase gate in response to p27. Likewise, the percentage of cells in S-phase for p27 expressing cells is quite low as diagramed in Fig. 3C. This type of analysis has been very effective in characterizing the cell cycle control defects in cells derived from various strains of gene-targeted mice[6, 7, 8].

In the second experiment, untransformed mammary epithelial cells (MCF10A) were treated with the growth inhibitory cytokine, transforming growth factor beta one (TGF-β1) for 24 hours. Cells were labeled with BrdU for four hours immediately prior to harvesting. As shown in Fig. 4 BrdU labeling in S-phase cells is greatly diminished by TGF-β1 signaling, the specificity of our staining is also validated with an IgG negative control (Fig. 4C). Quantification of the different phases of the cell cycle confirms that TGF-β1 primarily inhibits proliferation in the G1 phase of the cell cycle, leading to an accumulation in this phase.

In our last example, pRB deficient SaOS-2 cells are transfected with a CMV-CD20 expression vector and either CMV-RB or CMV-β-Gal as a control. Three days following transfection cells were harvested, stained, and fixed. Flow cytometry analysis of these cells is shown in Fig. 5. This demonstrates the cell cycle distribution of negative control transfected cells (Fig. 5A) in comparison with the distribution following 72 hours of pRB expression (Fig. 5B). Following curve fitting by Multi Cycle software, a direct comparison of the proportion of cell cycle phases is shown in Fig. 5C. This reveals the accumulation of cells in G1 following pRB expression and the relative depletion of cells from the S and G2/M phases. We have used variations on this assay to probe G1 arrest mechanisms extensively[9, 10, 11, 12, 13].

These examples demonstrate how cell cycle control can be measured in response to a variety of stimuli or cell manipulations. This approach can therefore be adapted to many applications that require the measurement of cell cycle position in culture type experiments.
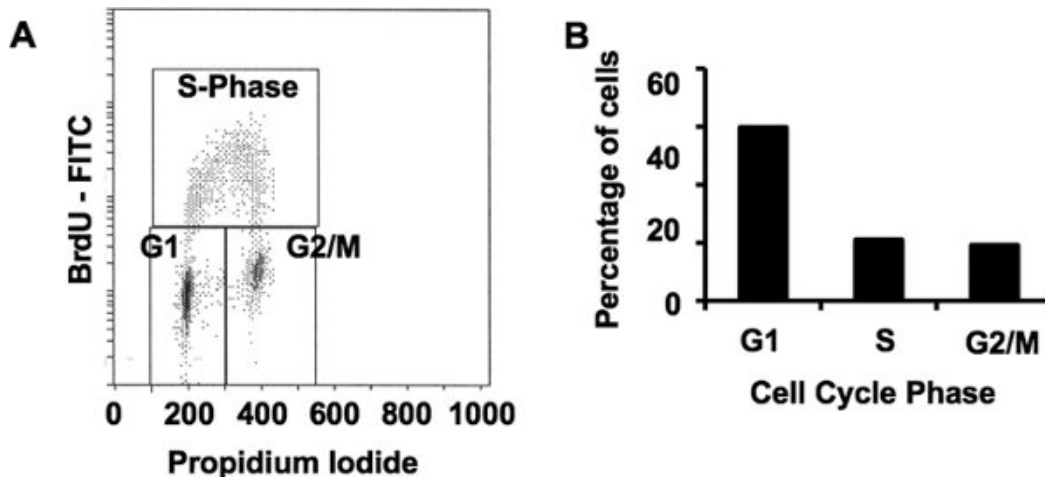

Figure 1. Quantitation of cell cycle phases by combined propidium iodide and BrdU staining (PI-BrdU). (A) This panel displays three dimensional flow cytometry of propidium iodide and BrdU stained cells. Note that cells with 2N and 4N DNA content are centered over the 200 and 400 marks on the X-axis scale for propidium iodide staining intensity. BrdU staining intensity is measured on a logarithmic scale on the Y-axis. Note the position of gates used to quantitate cells in the G1, S, and G2/M phases of the cell cycle. (B) The relative proportion of cells in each of the G1, S, and G2/M gates from A are shown on this graph.
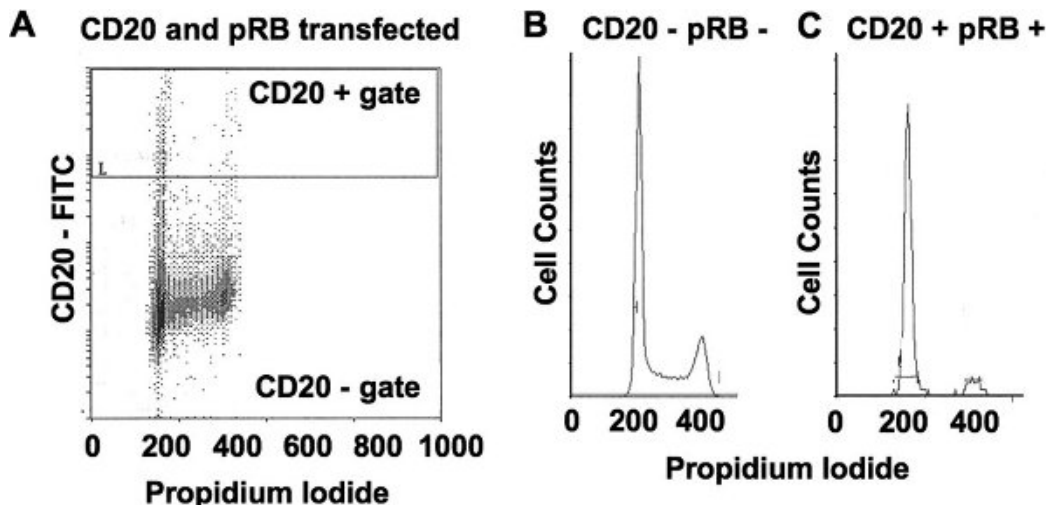
January 2012 | 59 | e3491 | Page 3 of 7

Figure 2. Quantitation of cell cycle phases in selected cells using propidium iodide and the cell surface marker CD20. (A) This panel shows propidium iodide and CD20 staining for a mixture of cells, some of which ectopically express CD20 and pRB. Note that the cells with 2N and 4N DNA (the most abundant populations) are centered over the 200 and 400 marks on the X-axis. The position of the CD20 + gate selects cells that are stained at least 10X more brightly than background. (B) A graph of cell counts versus propidium iodide staining is shown for CD20 negative cells in panel A that are asynchronously proliferating. (C) A similar graph is shown for CD20 positive cells from panel A that have been induced to arrest with pRB expression and contain cells with primarily 2N DNA content. This demonstrates that an arrested sub-population can be distinguished from other cells in this culture using this staining technique.
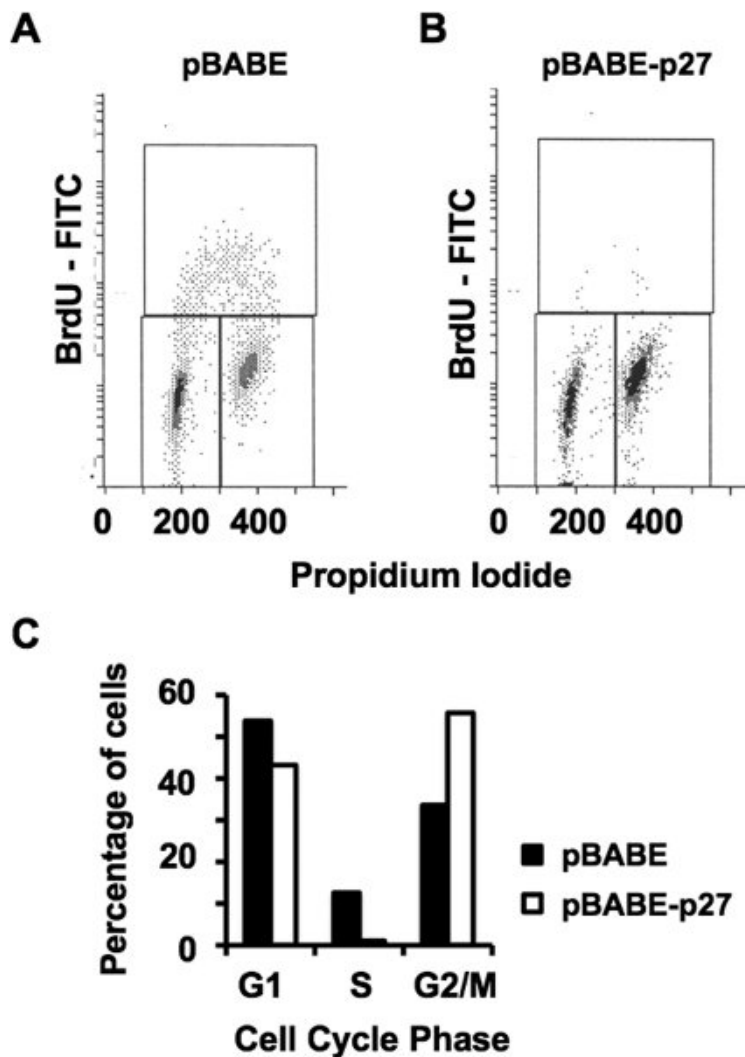
Figure 3. Inhibition of cell proliferation by p27KIP1. (A) PI-BrdU analysis is used to measure the cell cycle phases in an asynchronously proliferating population of cells that were transduced with an empty pBABE retroviral vector. (B) A similar analysis of cells transduced with pBABE-p27. Note the absence of cells in the S-phase and greater intensity of events in G1 and G2/M gates. (C) Quantitation of cells in the respective phases of the cell cycle in asynchronously proliferating control and p27 expressing cells.
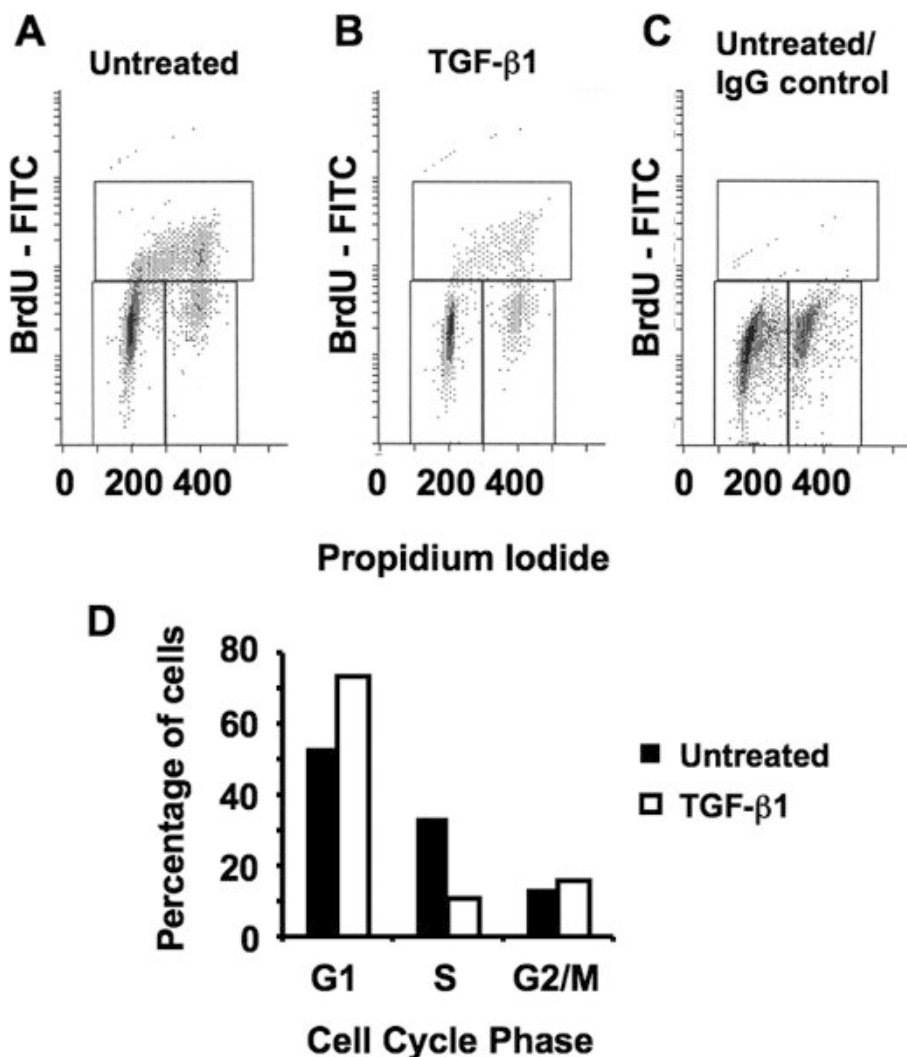

Figure 4. Inhibition of cell proliferation by TGF-β1 (A) PI-BrdU analysis of asynchronously proliferating MCF10A cells. (B) Analysis of cells treated with 100 pM of TGF-β1 for 24 hours. Note the accumulation of cells primarily in the G1 phase of the cell cycle. (C) Validation of BrdU staining by replacing the anti-BrdU primary antibody with a non-specific IgG control in asynchronously proliferating cells. (D) Graphical quantitation of the respective cell cycle phases from A and B.
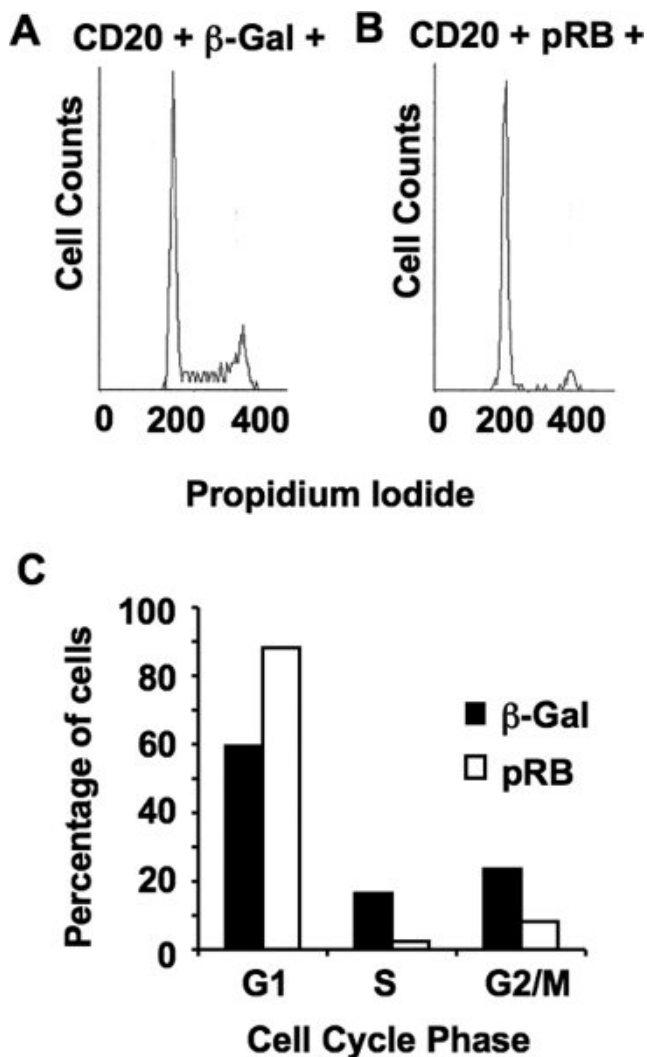
Figure 5. Inhibition of cell proliferation by pRB. (A) Cell counts versus propidium iodide staining of CD20 and ß-gal transfected cells is shown. This is an important control as transfection can partially synchronize cells, rendering the untransfected population (CD20 - cells) as an inappropriate control for the transfected sub-population. Transfected cells need to be compared with other analogously transfected cells. (B) Cell counts versus propidium iodide graph of CD20 and pRB transfected cells. Note the almost exclusive presence of a 2N peak. (C) Graphical representation of cell cycle phase proportions determined from A and B using curve fitting methods in Multi Cycle software.

Discussion

In our experience, success with these techniques is dependent on a few key controls and experimental conditions. One is establishing an asynchronously proliferating control for use in these experiments. This control serves three important purposes. First, it ensures that culture conditions used for all experimental samples are adequate to support continual proliferation in the absence of treatment. This sample also serves the purpose of a positive control for the staining methodology to ensure that BrdU or CD20 positive cells can be detected when they are present. Lastly, this sample is used to calibrate the flow cytometer. This control sample can be used to adjust propidium iodide staining intensity to detect 2N and 4N cells at 200 and 400 respectively. Furthermore, detection sensitivity of BrdU or CD20 can be adjusted so that negative and positive signals are centered as shown in Figures 1A and 2A. When all samples have a similar concentration of cells in PI-RNase solution, then few adjustments to the cytometer are needed as subsequent samples are run. This is important for reasons shown in Fig. 4B and 5B where strong G1 accumulation creates essentially a single G1 peak that could be misinterpreted as G2/M.

The representative experiments also make other important points. First of all, they demonstrate that BrdU uptake and labeling can vary between cell types. For this reason it is important to empirically determine the length of pulse and staining conditions needed to adequately detect cells in S-phase. In general, cell types that double in culture in 24 hours or less can be labeled with BrdU in an hour. Slower growing cell types may require longer pulses and alterations to antibody staining conditions and duration. MCF10A cells are an excellent example in this regard as they were labeled with BrdU for four hours and stained with twice the standard concentration of antibodies for four times as long. Investigators should be careful not to exceed 6 hours of BrdU labeling even with very slowly growing cells as this can erroneously lead to inclusion of G2/M cells in the S-phase population. Secondly, in comparing the effects of p27KIP1 expression with those of TGF-β1, it is clear that p27 can induce an accumulation in either G1 or G2/M phases while TGF-β1 signaling induces a G1 arrest. Traditional means of detecting proliferation such as ^{3}H-Thymidine incorporation and scintillation counting are unable to distinguish these possibilities.

In our alternate protocol we demonstrate the detection of a sub-population of cells in a larger untransfected population. It is important to empirically determine the most appropriate reporter for detecting transfected cells. In our experience some cell types either express cell surface markers poorly, or can't properly traffic them to the plasma membrane, resulting in an inability to detect transfected cells. Likewise, not all cells tolerate the membrane bound form of GFP. Selection of the most appropriate reporter should be made by assessing transfection efficiency of the reporter as well as the relative intensity of expression compared to untransfected controls. Ideally, heterologous expression of these molecules will be well tolerated and this is suggestive that the markers have little to no effect on cell cycle distribution.

One limitation of these types of flow cytometry approaches is that they are only able to establish relative abundances of cell cycle phases compared to one another. For this reason it is ambiguous if expression of p27 in Figure 3 truly induces an arrest in G1 and G2/M, or if it just slows progression through these phases relative to S-phase. These possibilities can be investigated further by treating a parallel sample of cells with a mitotic inhibitor such as nocodazole, or G1/S inhibitor like aphidicolin. Since these drugs create a dominant arrest in M-phase or early S-phase respectively, slowly proliferating cells will accumulate at the drug induced arrest point. For example cells arrested in G1 because of pRB expression will remain in G1 despite nocodazole treatment while control cells will accumulate in M-phase[13].

Taken together, these experimental approaches offer a flexible methodology that can be applied to a wide range of mammalian cell cycle research questions. They can readily detect alterations in cell cycle progression and quantify differences when compared with controls.

Disclosures

The authors have nothing to disclose.

Acknowledgements

References

1. Dolbeare, F., Gratzner, H., Pallavicini, M.G., & Gray, J.W. Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proc. Natl. Acad. Sci. U. S. A.* **80** (18), 5573 (1983).
2. Givan, A.L. Flow cytometry: an introduction. *Methods. Mol. Biol.* **699**, 1 (2011).
3. Kalejta, R.F., Brideau, A.D., Banfield, B.W., & Beavis, A.J. An integral membrane green fluorescent protein marker, Us9-GFP, is quantitatively retained in cells during propidium iodide-based cell cycle analysis by flow cytometry. *J. Exp. Cell. Res.* **248**, 322 (1999).
4. Qin, X.-Q., *et al.* The transcription factor E2F-1 is a downstream target of RB action. *Molecular and Cellular Biology* **15** (2), 742 (1995).
5. van den Heuvel, S. & Harlow, E. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science.* **262**, 2050 (1993).
6. Henley, S.A., *et al.* A cancer derived mutation in the retinoblastoma gene with a distinct defect for LXCXE dependent interactions. *Cancer Cell. Int.* **10**, 8 (2010).
7. Francis, S.M., *et al.* A functional connection between pRB and transforming growth factor beta in growth inhibition and mammary gland development. *Mol. Cell. Biol.* **29** (16), 4455 (2009).
8. Isaac, C.E., *et al.* The retinoblastoma protein regulates pericentric heterochromatin. *Mol. Cell. Biol.* **26** (9), 3659 (2006).
9. Julian, L.M., *et al.* Characterization of an E2F1-specific binding domain in pRB and its implications for apoptotic regulation. *Oncogene.* **27** (11), 1572 (2008).
10. Hirschi, A., *et al.* An overlapping kinase and phosphatase docking site regulates activity of the retinoblastoma protein. *Nat. Struct. Mol. Biol.* **17** (9), 1051 (2010).
11. Dick, F.A. & Dyson, N. pRB contains an E2F1-specific binding domain that allows E2F1-induced apoptosis to be regulated separately from other E2F activities. *Mol. Cell.* **12** (3), 639 (2003).
12. Dick, F.A., Sailhamer, E., & Dyson, N.J. Mutagenesis of the pRB pocket reveals that cell cycle arrest functions are separable from binding to viral oncoproteins. *Mol. Cell. Biol.* **20** (10), 3715 (2000).
13. Dick, F.A. & Dyson, N.J. Three regions of the pRB pocket domain affect its inactivation by human papillomavirus E7 proteins. *J. Virol.* **76** (12), 6224 (2002).