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Assaying cell cycle status using flow cytometry

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Abstract

In this unit, we describe two protocols for analyzing cell cycle status using flow cytometry. The first is based on the simultaneous analysis of proliferation specific marker (Ki-67) and cellular DNA content, which discriminates resting/quiescent cell populations (G0 cell) and quantifies cell cycle distribution (G1, S or G2/M, respectively). The second is based on differential staining of DNA and RNA through co-staining of Hoechst 33342 and Pyronin Y, which is also useful to identify G0 cells from G1 cells. Along with these methods for analyzing cell cycle status, we outline the basics of two additional methods for cell proliferation assays and recent updates of newly-developed fluorophores, which allows multiplex analysis of cell cycle status, cell proliferation and a gene of interest using flow cytometry.

Keywords

Cell Cycle; Flow Cytometry; Ki-67; Propidium Iodide; Pyronin Y; Hoechst 33342

UNIT INTRODUCTION

Assessing cell cycle distribution and cell proliferation is important for studying cell growth differentiation, senescence and apoptosis. This enables one to investigate underlying basic mechanisms as well as to evaluate therapeutic efficacies of anti-cancer drugs. During cell cycle progression, proliferating cells sequentially undergo a transition of G1→S→G2→M phases for synthesis of DNA, preparation of cell division and subsequent mitosis process (Malumbres and Barbacid, 2009). However, under certain circumstances, cells can enter G0 phase, where the cells are neither dividing nor preparing for proliferation. These resting cells are characterized by having minimal cell cycle machinery and maintaining specialized

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Conflict of Interest

The Authors declare no conflict of interest.

INTERNET RESOURCES

<http://www.lifetechnologies.com/us/en/home/life-science/cell-analysis/flow-cytometry/cell-health-and-viability-assays-for-flow-cytometry/cell-proliferation-assays-for-flow-cytometry/click-it-edu-cell-proliferation-assay-kits-for-flow-cytometry.html>

Web site for the introduction of Click-iT® Plus Assay Kits

cellular functions rather than by proceeding to cell proliferation. This resting state is also referred as quiescent (Zetterberg et al., 1995).

The earliest and simplest approach to analyze cell cycle status is to measure cellular DNA content at a single time point (Darzynkiewicz and Huang, 2004). This reveals a snapshot of cell cycle status among 3 distinct groups (*i.e.*, G0/G1 (2n), S (2n~4n), and G2/M (4n) phase, respectively). In 1969, the feulgen-DNA staining method was first described to analyze cell cycle distribution (Van Dilla et al., 1969). Since then, many fluorescent DNA dyes have been developed for multiplex analysis of cellular DNA content and other proliferation-related markers (Table 1). However, this method is insufficient to understand detailed cell cycle status because DNA content alone cannot distinguish resting/quiescent cells (G0) from G1 phase cells.

To overcome this limitation, some alternative methods have been developed. First, resting/quiescent and proliferating cell fractions can be identified by proliferation-associated proteins and/or nuclear proliferation antigens such as Ki-67 and proliferating cell nuclear antigen (PCNA). Ki-67 antigen is rarely detected in G0 phase, highly expressed in the nuclear region of proliferating cells (maximum in G2 and early M phases) and rapidly degraded during anaphase and telophase of mitosis processes (Gerdes et al., 1984). Likewise, PCNA is a good marker for proliferating cells and is concentrated in S phase (Kurki et al., 1986), which is useful to separate S phase cells. Second, quantification of intracellular RNA by Hoechst 33342/Pyronin Y double staining can be an alternative way to study cell cycle status because highly proliferating cells usually contain higher levels of RNA compared to resting/quiescent cells. Historically, Pyronin Y has been widely used for microscopic observation of cellular RNA in combination with methyl green (Scott, 1967). Its application was extended to flow cytometry by Howard Shapiro in 1981 and further defined by Zibgniew Darzynkiewicz in 2004 (Darzynkiewicz et al., 2004; Shapiro, 1981). In this unit, two basic flow cytometric techniques are described for assessing cell cycle status through costaining of Ki-67/DNA (Basic Protocol 1) and quantification of intracellular RNA (Basic Protocol 2).

BASIC PROTOCOL 1

Title

Flow cytometric analysis of Ki-67 and DNA content for analyzing cell cycle status.

Introduction

The Ki-67 antibody was first described to recognize a nuclear protein only present in proliferating cells (Gerdes et al., 1983). Later, the nuclear antigen detected by Ki-67 antibody was identified as two isoforms of 320 kDa and 359 kDa Ki-67 protein, which may be necessary for maintenance of cell proliferation (Schluter et al., 1993). The Ki-67-positive population is mainly limited to proliferating cells in many cell types during active phases of the cell cycle (G1, S, G2 and M phases), whereas it is absent from resting/quiescent cells (Gerdes et al., 1984; Schwarting et al., 1986). Thus, Ki-67 has been extensively used to predict the growth rate of many cancer samples from human patients. This protocol provides

a detailed procedure for determining cell cycle status of tissue culture cells through double staining of Ki-67 and PI using flow cytometry.

Materials

Solutions and reagents—1X Phosphate buffered saline (PBS)

70% Cold ethanol (-20°C)

FACS buffer (see recipe)

PI staining solution (see recipe)

FITC-conjugated Ki-67 antibody

NOTE: Alternatively, various fluorescent dyes such as PE and APC can be used for Ki-67 in combination with other DNA-binding fluorescent dyes (Table 1) in order to avoid significant spectral overlap.

Special equipment—Flow cytometer equipped with 488 nm blue laser and appropriate filter sets detecting FITC and PI fluorescence.

Steps and Annotations

Harvest, fix and permeabilize cells

1. Plate cells at proper density so that cells should not be confluent at the time of cell harvest (See *Critical Parameters*).
2. Harvest and pellet cells (1×10^6) after washing with 10 ml PBS by centrifuging 5 min at $200 \times g$.
3. Remove supernatant and resuspend cells in 0.5 ml PBS
4. Add 4.5 ml pre-chilled 70% cold ethanol (-20°C) in a drop wise manner to the cell suspension while vortexing.
In this step, cell clumping should be minimized (see Troubleshooting).
5. Incubate the fixed cells at least 2 hour at -20°C . Cells may be stored in ethanol fixative for several weeks at -20°C prior to antibody staining.

Stain cells with Ki-67 antibody and fluorescent DNA dyes

6. Centrifuge 3 min at $300 \times g$ and remove ethanol.
7. Rinse cells with 5 ml FACS buffer twice by centrifuging 5 min at $200 \times g$.
8. Remove supernatant and resuspend cells in 100 μl FACS buffer (1×10^6 cells/100 μl).
9. Add 10 μl pre-diluted Ki-67-FITC antibody and incubate 30 min at room temperature.

Refer to manufacturer's instruction for optimal antibody dilution. For the best quality of positive cell discrimination from negative cells, titration of Ki-67-FITC antibody is required if nothing is specified.

After this step, the rest of the procedure should be performed in the dark.

10. Wash with 5 ml FACS buffer twice by centrifuging 5min at $200 \times g$.

11. Remove supernatant. Add 500 μ l PI staining solution and resuspend pellet gently.

12. Incubate 20 min at room temperature.

Washing is not necessary.

Perform flow cytometry

13. Set up and adjust flow cytometer with a blue laser (488 nm) and detection filters (530/30 nm band pass for FITC and 610/20 nm band pass for PI).

Ki-67-FITC signal in logarithmic mode and PI signal in linear mode. PI fluorescence can be detected in 585/42 nm band pass, 670 nm long pass filters or something capable of detecting PI fluorescence.

14. Set a low flow rate (less than 400 events/second) for optimal resolution of PI fluorescence.

15. Exclude doublets by creating a combination of same-channel bivariate plots utilizing Area vs Height or Area vs Width (*i.e.*, FSC, SSC and PI fluorescence).

Singlet events are presented in a diagonal pattern. Doublets have lower Height and higher Width values.

16. Acquire the fluorescence and analyze cell cycle stages of each sample (*See Anticipated Results*).

Appropriate Compensation procedures between fluorophores should be utilized.

ALTERNATE PROTOCOL 1

Title

Simultaneous staining of cell surface antigens with Ki-67 and PI.

Introduction

This method can be used for multiplex staining of surface proteins and Ki-67/DNA, which enables analysis of surrogate markers for resting/quiescent cells. After first staining of surface antigen, both fixation and permeabilization are subsequently required. Here, we describe classical procedures of surface antigen staining followed by fixation (4% formaldehyde) and permeabilization (1% saponin). The saponin-based permeabilization is a reversible process so the remainder of the procedure should be performed in a presence of saponin. This method usually preserves cell shape as well as structure of cellular components but takes longer time for surface antigen staining, fixation and permeabilization processes.

Material List

Solutions and reagents—1X Phosphate buffered saline (PBS)

FACS buffer (see recipe)

Fluorophore-conjugated antibody against surface antigen

Fixation solution (4% paraformaldehyde)

Permeabilization solution (see recipe)

Saponin wash buffer (see recipe)

FITC-conjugated Ki-67 antibody

PI/saponin staining solution (see recipe)

NOTE: Other fixatives and permeabilization buffers are commercially available (FIX & PERM® Cell Fixation & Cell Permeabilization Kit (#GAS003/GAS004) from Life Technologies; Cytotfix/Cytoperm™ Fixation/Permeabilization Solution Kit (#554714) from BD Biosciences, etc). For detailed procedures, refer to manufacturer's instructions.

NOTE: Optimization of paraformaldehyde (1~4%) and saponin (0.1~1%) concentrations are needed.

Special equipment—Flow cytometer equipped with 488 nm blue laser and appropriate filter sets for detecting FITC and PI fluorescence. Depending on the fluorophore for surface antigens, additional laser and filter sets are needed.

Steps and Annotations

Stain cell surface antigen with fluorescent-conjugated antibody

1. Harvest cells (1×10^6) and wash with 10 ml PBS by centrifuging 5 min at $200 \times g$.
2. Remove supernatant and resuspend cells in 100 μ l FACS buffer.
3. Add non-fixation sensitive fluorophore-conjugated primary antibody detecting cell surface antigen and incubate 20 min in the dark at 4°C.

The detection wavelengths for the fluorescent dyes for surface antigens should not overlap with those for FITC and PI. For optimal antibody dilution, refer to the manufacturer's instructions or perform a titration. After this step, the rest of the procedure should be done in the dark.

4. Wash with 5 ml FACS buffer twice by centrifuging 5 min at $200 \times g$.

Fix and permeabilize cells for intracellular staining

5. Resuspend cells in 200 μ l Fixation solution (4% paraformaldehyde).
6. Incubate 20 min at room temperature.

7. Add 5 ml PBS and centrifuge 5 min at $200 \times g$ to remove fixative.
 8. Resuspend cells in 200 μ l Permeabilization solution and incubate 20 min at room temperature.
- After this step, 0.5% saponin should be present in all buffers used in this protocol.
9. Wash cells with 5 ml Saponin wash buffer and centrifuge 5 min at $200 \times g$.

Stain with Ki-67 and PI

10. Resuspend cells in 100 μ l Saponin wash buffer and add 10 μ l pre-diluted Ki-67-FITC antibody.

Refer to manufacturer's instruction for optimal antibody dilution. For the best quality of positive cell discrimination from negative cells, titration of Ki-67-FITC antibody is required

11. Incubate 30 min at room temperature.
12. Wash cells with 5 ml Saponin wash buffer twice by centrifuging 5 min at $200 \times g$.
13. Add 500 μ l PI/saponin staining solution and resuspend pellet gently.
14. Incubate 20 min at room temperature.

Washing is not necessary.

Perform flow cytometry

15. Set up and adjust flow cytometer with proper laser and filter sets

For detecting FITC and PI, a blue laser (488 nm), detection filters of 530/30 nm band pass (for FITC) and 610/20 nm band pass (for PI) are used. Alternatively, 585/42 nm band pass, 670 nm long pass filters or something capable of detecting PI fluorescence can be used. For detecting surface staining, use proper laser and filter sets according to the selected fluorophore. Ki-67-FITC signal in logarithmic mode and PI signal in linear mode.

16. Set a low flow rate (less than 400 events/second) for optimal resolution of PI fluorescence.

17. Exclude doublets by creating a combination of same-channel bivariate plots utilizing Area vs Height or Area vs Width (*i.e.*, FSC, SSC and PI fluorescence).

Singlet events are presented in a diagonal pattern. Doublets have lower Height and higher Width values.

18. Acquire the fluorescence and analyze cell cycle stages of each sample.

Appropriate Compensation procedures between fluorophores should be utilized.

BASIC PROTOCOL 2

Title

Pyronin Y and Hoechst 33342 staining for analyzing cell cycle status.

Introduction

The other way to identify the resting cells (G0 cells) from proliferating cell is to determine the total RNA content inside the cells. Generally, resting/quiescent cells at G0 phase have lower levels of RNA compared with proliferating interphase cells (G1-S-G2-M phase). To address this, double staining of Hoechst 33342 and Pyronin Y is widely used. Pyronin Y intercalates both double stranded DNA and double stranded RNA, which can be used for visualization of RNA as an orange-red band during electrophoresis. In the presence of DNA-chelating fluorescent dye such as Hoechst 33342, interactions of Pyronin Y and DNA complex are disrupted and Pyronin Y mainly stains RNA (Shapiro, 1981), allowing the quantification of RNA amount in a single cell level. Here, we describe a basic protocol for double staining of cells with Pyronin Y and Hoechst 33342 to dissect resting and proliferating cells.

Material List

Solutions and reagents—1× Phosphate buffered saline (PBS)

70% Cold ethanol (−20°C)

FACS buffer (see recipe)

Hoechst/PY staining solution (see recipe)

Special equipment—Flow cytometer equipped with both 355 nm UV and 488 nm blue laser to activate Hoechst 33342 and Pyronin Y. 488 nm laser can be replaced by 532 nm green or 561 nm yellow-green lasers. Appropriate filter sets are needed.

Steps and Annotations

1. Harvest cells (1×10^6) and wash with 10 ml PBS by centrifuging 5 min at $200 \times g$.
2. Resuspend cells in 0.5 ml PBS.
3. Fix cells by adding 4.5 ml pre-chilled 70% ethanol (−20°C) drop wise while vortexing
4. Incubate at least 2 hour at −20°C.

In this step, cell clumping should be minimized (see Troubleshooting). Ethanol-fixed cells can be stored several weeks at −20°C.

5. Eliminate residual ethanol by centrifuging 3 min at $300 \times g$. Remove and discard supernatant.
6. Wash cells with 5 ml FACS buffer twice by centrifuging 5 min at $200 \times g$.
7. Stain cells using 0.5 ml Hoechst/PY staining solution.

The sample should be kept in the dark. A washing step is not necessary.

8. Incubate 20 min at room temperature and analyze the fluorescences in flow cytometry.

Perform flow cytometry

9. Set up and adjust flow cytometer with UV (355 nm) and blue (488 nm) lasers as well as proper filter sets (450/50 nm band pass for Hoechst and 575/26 nm band pass for Pyronin Y)

Pyronin Y is excited by 488 nm blue laser and 532 or 561 nm lasers are also available. Maximal emission wavelength of Hoechst 33342 is 461 nm and that of Pyronin Y is 575 nm. Both Hoechst 33342 and Pyronin Y signal in a linear mode.

10. Exclude doublets by creating a combination of same-channel bivariate plots utilizing Area vs Height or Area vs Width (*i.e.*, FSC, SSC and Hoechst fluorescence).

Singlet events are presented in a diagonal pattern. Doublets have lower Height and higher Width values.

11. Acquire the fluorescence and analyze cell cycle stages of each sample (*See Anticipated Results*).

Appropriate Compensation procedures between fluorophores should be utilized.

REAGENTS AND SOLUTIONS

FACS buffer

1X PBS supplemented with:

2% (v/v) heat-inactivated, sterile-filtered fetal bovine serum (10 ml FBS per 500 ml)

1 mM EDTA (1 ml of 0.5 M EDTA stock per 500 ml)

Store at 4°C for up to 6 months. 2% (v/v) FBS can be replaced by 0.2~0.5% (w/v) bovine serum albumin (BSA). Sodium azide (NaN₃, 0.1%) can be added to prevent microbial contamination.

PI staining solution

1X PBS supplemented with:

50 µg/ml propidium iodide (50 µl of 1 mg/ml PI stock per 1 ml)

100 µg/ml RNase (10 µl of 10 mg/ml RNase stock per 1ml)

2 mM MgCl₂ (2 µl of 1 M MgCl₂ stock per 1 ml)

Prepare freshly and keep in the dark at 4°C before use.

Permeabilization solution

10 mM HEPES buffer, pH 7.2 (10 µl of 1 M HEPES buffer stock per 1 ml)

1% (w/v) saponin (100 µl of 10% (w/v) saponin stock per 1 ml)

Prepare freshly and store at 4°C before use.

Saponin wash buffer

10 mM HEPES buffer, pH 7.2 (10 μ l of 1 M HEPES buffer stock per 1 ml)

0.5% (w/v) saponin (50 μ l of 10% (w/v) saponin stock per 1 ml)

Prepare freshly and store at 4°C before use.

PI/saponin staining solution

Saponin wash buffer (see recipe) supplemented with:

50 μ g/ml propidium iodide (50 μ l of 1 mg/ml PI stock per 1 ml)

100 μ g/ml RNase (10 μ l of 10 mg/ml RNase stock per 1ml)

2 mM MgCl₂ (2 μ l of 1 M MgCl₂ stock per 1 ml)

Prepare freshly and keep in the dark at 4°C before use.

Hoechst/PY staining solution

FACS buffer (see recipe) supplemented with:

2 μ g/ml Hoechst 33342 (2 μ l of 1 mg/ml Hoechst 33342 stock per 1 ml)

4 μ g/ml Pyronin Y (4 μ l of 1 mg/ml Pyronin Y stock per 1 ml)

Prepare freshly and keep in the dark at 4°C before use. Pyronin Y concentration may vary (see Critical Parameters and Troubleshooting)

COMMENTARY**Background Information**

Flow cytometric analysis of Ki-67 was described to determine the growth fraction of lymphoma cell lines (Schwartz et al., 1986) and further applied to a cell cycle and cell proliferation analysis on many cancer cells and hematopoietic stem cells. Pyronin Y was first synthesized in 1889 and it has been used as a convenient histological/cytochemical dye to stain RNA in combination with methyl green (for DNA staining). Later, double staining of Pyronin Y and Hoechst 33342 was developed for flow cytometric analysis to estimate DNA and RNA content in intact cells (Shapiro, 1981). These methods have been widely used for analyzing cell cycle status.

Along with these techniques, the kinetics of cell cycle status can be assessed by cell proliferation assays based on measuring newly-synthesized DNA content and cellular metabolism parameters. For flow cytometric analysis of cell proliferation, genomic DNA in replicating cells can be labeled by exposing cells to thymidine analog, 5'-bromo-2'-deoxyuridine (BrdU) during the S phase of cell cycle. Incorporated BrdU is further stained with fluoresceinated anti-BrdU antibodies and fluorescent DNA dye (e.g., propidium iodide, PI; 7-Aminoactinomycin D, 7-AAD) to separate the cells according to the cell cycle of each

phase (i.e., G1, S, G2/M phases) (Rothausler and Baumgarth, 2007). A disadvantage of BrdU incorporation method is that both membrane permeabilization and harsh DNA denaturation processes are required for antibody penetration to the incorporated BrdU. As an alternative of BrdU, 5-ethynyl-2'-deoxyuridine (EdU) has been developed to overcome the limitations of BrdU method (Cappella et al., 2008; Cavanagh et al., 2011; Salic and Mitchison, 2008). After EdU treatment during cell proliferation, incorporation of EdU can be subsequently detected by a fluorescent azide molecule through a copper (I) catalyzed reaction which results in a stable triazole ring formation between EdU and fluorescent dye (called "Click reaction"). Since the small-sized fluorescent dye readily penetrates the cell and it easily reacts with EdU even in intact DNA double strand, EdU method is highly sensitive and much faster than a classical BrdU incorporation method. Also, EdU incorporation assay can be combined with multiplex cell surface/intracellular staining, which is very useful for many applications (Cappella et al., 2008; Diermeier-Daucher and Brockhoff, 2010). The original version of the Click reaction cannot be used for multiplex detection of some fluorophores such as GFP and R-PE which are easily damaged by high concentration of copper and reactive oxygen species. Recently, chemical modification of Click reaction enables to preserve GFP and R-PE fluorescence and to obtain a bright EdU signal. This is extended to cover at least three different fluorophores (Alexa Fluor® 488, Alexa Fluor® 647 and Pacific Blue™, see the INTERNET RESOURCES below).

Dye dilution assays using membrane-permeable fluorescent dyes are currently used to assess cell proliferation as well. Carboxyfluorescein succinimidyl ester (CFSE or CFDA-SE, carboxyfluorescein diacetate succinimidyl ester) is one of the widely-used fluorescent dyes that enters the cytoplasm and covalently couples to intracellular amino acids (Lyons, 2000; Lyons et al., 2013). Because this reaction results in extremely long-term retention of fluorescent dye within the original cell, it was originally used to track immune cells. Assuming that cells have homogenous cell size and undergo symmetric division, each daughter cell has half of the parental cell volume and cellular components, as well as labeled CFSE dyes. Thus, CFSE labeling can be applied to estimate the number of the generation after rapid cell proliferations. Usually, CFSE dye may be traced through 6-8 generations by flow cytometry. Similar to CFSE dye, other fluorophores for dye dilution proliferation assay have been developed for encompassing broad range of excitation/emission spectrum (Table 2). These fluorescent dyes are better suited for multicolor applications where GFP derivatives or FITC or similar fluorescent-conjugated antibody is used. Further, some of dilution dyes emit in channels where cells have less natural autofluorescence that can be detected up to 10 generations during cell proliferation.

Critical Parameters

Proper cell density—Cell density should be optimized because confluent cultures may cause growth arrest by contact inhibition, which leads to G0/G1 arrest of the cell cycle. Excessive confluency also affects nutrient availability as well as media acidity, which may distort experimental results. Generally, cells are harvested during the time window of exponential growth (usually 50~70% confluency).

Titration of antibody and fluorescent DNA dye concentration—For the first use, antibodies against Ki-67 or surface antigen need to be titrated (usually 1:500-1:50 for flow cytometry) to maximize the detection of signal-positive populations. Fluorescent DNA dye concentrations may vary among cell lines and/or specific conditions, which have to be determined empirically.

Determination of Pyronin Y concentration—Proper PY concentration is critical. Low PY concentration does not ensure stoichiometric correlation between actual RNA level and PY fluorescence. High PY concentration also results in condensation (precipitation) of PY-nucleic acid complex, which interferes with PY fluorescence. Thus, optimal PY concentration will vary among cell line, cell density and condition. Generally, titration is required for the first use (starting from 1 µg/ml to 5 µg/ml).

Fluorescent protein analysis—To utilize BASIC PROTOCOL 1 or 2 in combination with fluorescent proteins such as GFP and RFP, it is the best to use the ALTERNATIVE PROTOCOL 1 as the PFA fixation followed by saponin permeabilization helps retain structure of these proteins. Changes to BASIC PROTOCOL 1 may need to be made as GFP and FITC spectra overlap. So, consider another non-overlapping probe for detecting Ki-67. Some RFP derivatives also overlap with PI and, in some cases, alternative DNA probes should be utilized.

Troubleshooting

Poor positive signal or high background fluorescence—Check appropriate laser and filter combinations. Adjust concentration and incubation time of antibodies and fluorescent DNA/RNA dyes. For high background fluorescence, increase FBS/BSA concentration in the FACS buffer. In some instances (*e.g.*, ALTERNATIVE PROTOCOL 1), PFA concentrations and incubation times may need to be adjusted to reduce background signals. In cases where the signal is poor or non-existent with regard to surface staining, check the manufacturer's instructions if the conjugated antibody is fixation sensitive (*e.g.*, prolonged exposure to paraformaldehyde affects emission spectra of some fluorophores such as APC-CyTM7, PE-CyTM7).

Cell clumping and extensive cell loss during fixation/washing process—Improper fixation procedure may result in cell clumping and significant cell loss. To avoid this, inject the cell suspension directly into the cold ethanol using a Pasteur pipette and mix well immediately. Alternatively, use non-alcohol fixatives such as 4% paraformaldehyde (see ALTERNATIVE PROTOCOL 1). The stained sample should be passed through a cell strainer before analysis.

High Coefficient of Variation (CV) or wide peaks for DNA cell cycle probes—Ensure that the samples are run in the lowest sample pressure setting possible to allow for best interrogation of sample. Acquiring the sample in the linear setting/range of the flow cytometer is also important. Additionally, proper cell and dye concentration is critical for consistent histograms giving better CVs and decreasing variation between samples.

Anticipated Results

On the basis of differences in Ki-67 expression level (Figure 1A) and RNA content (Figure 1B) of G0 cells, Basic Protocol 1 and 2 allow discrimination of resting/quiescent (G0) population from other proliferating cells (G1, S, G2/M phases). Generally, the G0 cells have lower levels of Ki-67 and RNA levels, so these cells may be distinguishable from other proliferating cells. To quantify cell cycle distribution of proliferating cells precisely, fitting software such as ModFit LT (Verity Software) and MultiCycle AV (Phenix Flow Systems) can be used (Darzynkiewicz and Huang, 2004).

Previous studies have demonstrated that a small portion of cells showed a significant increase of Ki-67 level in G2/M cells (Figure 1A, asterisk) (Landberg et al., 1990). These cells were regarded as early mitotic cells but this has not yet been firmly established. To further separate M phase cells, other markers such as Cyclins, MPM-2 and phospho-Ser10-histone H3 (to detect M phase) need to be combined (Juan and Darzynkiewicz, 2001; Landberg et al., 1990; Vignon et al., 2013).

Time Considerations

Preparation and staining of Ki-67 and fluorescent DNA dye staining should take 4 hours. The staining of Pylonin Y and Hoechst 33342 will take 3 hours. Analysis the samples through flow cytometry will take from 1 to 10 minutes per sample depending on concentrations. Total time is dependent upon total sample numbers.

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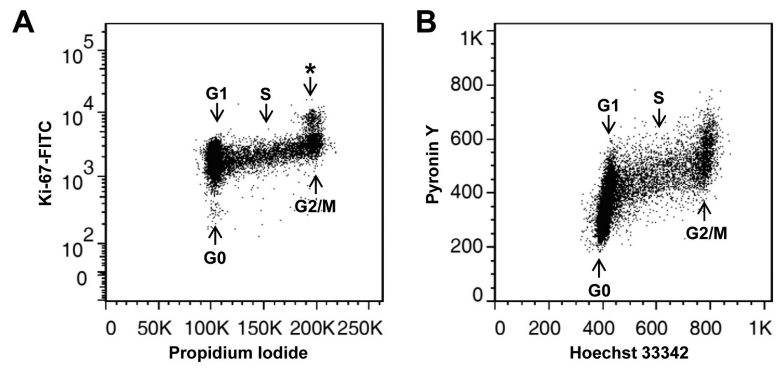


Figure 1. Analysis of cell cycle status by differential staining of Ki-67/DNA and DNA/RNA. HeLa cells were fixed and subsequently stained with Ki-67-FITC and PI (A) or Hoechst 33342 and Pyronin Y (B) according to the BASIC PROTOCOL 1 and 2.

Table 1

Lists of common fluorescent DNA dyes

Commercial Name	Ex _{max}	Em _{max}	Ex. Laser	Manufacturer
DAPI	345	455	UV	Various
Hoechst 33342*	350	461	UV	Various
Propidium Iodide (PI)	535	617	UV, 488, 532 or 561 nm	Various
7-AAD	546	647	488 or 532 nm	Various
DRAQ5*	647	681/697	633 nm	Various
DRAQ7	599/644	694	633	Various
FxCycle™ Violet	358	461	405 nm	Life Technologies
Vybrant® DyeCycle™ Violet*	369	437	UV or 405 nm	Life Technologies
Vybrant® DyeCycle™ Green*	506	534	488 nm	Life Technologies
Vybrant® DyeCycle™ Orange*	519	563	488 or 532 nm	Life Technologies
Vybrant® DyeCycle™ Ruby*	638	686	561 or 633 nm	Life Technologies
SYTOX® Blue	444	480	405 nm	Life Technologies
SYTOX® Green	504	523	488 nm	Life Technologies
SYTOX® Orange	547	570	488 or 532 nm	Life Technologies
SYTOX® AADvanced™	546	647	488 or 532 nm	Life Technologies
SYTOX® Red	640	658	633 nm	Life Technologies
FxCycle™ Far Red	640	658	633 nm	Life Technologies

* cell permeable dye which can stain DNA in both live or fixed cells.

Table 2

Lists of fluorophores for dye dilution proliferation assay

Commercial Name	Ex _{max}	Em _{max}	Ex. Laser	Manufacturer
CFSE	495	519	488 nm	Various
CellTrace™ Violet	405	450	405 nm	Life Technologies
BD Horizon™ Violet Cell Proliferation Dye (VPD450)	404	448	405 nm	BD Biosciences
Cell Proliferation Dye eFluor®450	405	450	405 nm	eBioscience
CytoTrack™ Blue	403	454	405 nm	BIO-RAD
Oregon Green 488 carboxylic acid diacetate, SE (carboxy-DFFDA SE)	496	524	488 nm	Life Technologies
SNARF-1 carboxylic acid, acetate, SE	514	586	488 nm	Life Technologies
CytoTrack™ Green	511	525	488 nm	BIO-RAD
CytoTrack™ Yellow	542	556	532 nm	BIO-RAD
CellTrace™ Far Red DDAO-SE	647	657	633 nm	Life Technologies
Cell Proliferation Dye eFluor®670	647	670	633 nm	eBioscience
CytoTrack™ Red	628	643	633 nm or 640 nm	BIO-RAD

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