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Analysis of Cell Cycle Phases and Progression in Cultured Mammalian Cells

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Abstract

Fluorescence Activated Cell Sorting (FACS) analysis has become a standard tool to analyze cell cycle distributions in populations of cells. These methods require relatively large numbers of cells, and do not provide optimal resolution of the transitions between cell cycle phases. In this report we describe in detail complementary methods that utilize the incorporation of nucleotide analogs combined with microscopic examination. While often more time consuming, these protocols typically require far fewer cells, and allow accurate kinetic assessment of cell cycle progression. We also describe the use of a technique for the synchronization of adherent cells in mitosis by simple mechanical agitation (mitotic shake-off) that eliminates physiological perturbation associated with drug treatments.

1. Introduction

Among the crucial events required for the development of malignancies are the loss of responsiveness to negative regulators of cell cycle progression and/or the acquirement of independence from mitogenic signals (Hanahan and Weinberg, 2000). Not surprisingly, expression profiles of genes involved in governing cell cycle progression can be used as molecular markers to predict responsiveness to therapeutic intervention and patient survival in various human neoplasias (reviewed in Singhal et al., 2005, Yasui et al., 2005, Quinn et al., 2005). Over the last 30 years, beginning with the revolutionary discoveries of the genes involved in cell cycle control by Hunt, Nurse and Hartwell (reviewed in Nurse, 2000), intensive research efforts have led to significant progress in identifying the molecular machinery involved in cell cycle progression. Today this information is widely used for the development of highly specific therapeutic interventions in cancer treatment.

While FACS is a useful technique that has become a standard tool to analyze the DNA content of cells, it provides only a snapshot of the cell cycle distribution at any given point in time. FACS also requires relatively large numbers of cells to achieve adequate statistical significance, suffers from a variety of sample preparation artifacts, and does not distinguish accurately between closely spaced events, for example, late G1 phase from early S phase. In contrast, incorporation of nucleotide analogs, such as Bromo deoxyuridine (BrdU), even for periods as short as a few minutes, can very clearly and reproducibly mark cells in S phase when combined with sensitive immunological detection methods and microscopic observation. In conjunction with physiological methods for cell synchronization that avoid the use of drugs,

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these approaches allow the accurate determination of dynamic cell cycle phase progression in living cells.

The methods presented in this article have been developed using the Rat-1 cell line (Prouty et al., 1993). This is an established, immortalized fibroblast cell line derived from a mid-gestation rat embryo. It shows good contact inhibition and does not display any significant transformed phenotypes such as anchorage independent growth or tumor formation in immuno-compromised mice. In most aspects it is very similar to the several murine fibroblast cell lines established by the 3T3 protocol: NIH-3T3, Balb/c-3T3, Swiss-3T3, etc. The techniques described here can also be readily adapted to primary fibroblast cultures, such as mouse embryo fibroblasts (MEF) or normal human diploid fibroblasts (HDF) from a variety of sources. Other cell types may require significantly different culture conditions, and transformed cells typically cannot be adequately synchronized in the G0 cell cycle phase by serum deprivation and/or contact inhibition; however, the methods for labeling and sample processing in exponential phase should be readily adaptable.

2. BrdU labeling

BrdU and uridine (Sigma, St. Louis, MO, cat. no. B5005, U3003) are made up as 1000 x and 100 x stock solutions, respectively, in distilled water (dH₂O), filter sterilized, and stored protected from light at 4 °C. To avoid unequal distribution and locally high concentrations, both solutions should be pre-added to the medium, rather than added to plated cells. Uridine is added to prevent incorporation of BrdU into RNA. The final concentration in medium for BrdU is 1 µg/ml and for uridine is 1 mg/ml. While BrdU can have cytotoxic effects, at the concentrations used in our studies we did not detect any deleterious effects. It is however important to stress that once BrdU has been added all subsequent handling of the cells should be done under safe light conditions (orange or red illumination), as even brief exposure to standard overhead fluorescent lights can elicit toxicity. Incorporation of BrdU is terminated by addition of L-ascorbic acid (Sigma, cat. no. A4544) directly to the culture medium to a final concentration of 0.067 M (Moscovitis et al., 1980). This has the effect of rapidly killing the cells without perturbing their morphology or causing detachment. The particular advantage of this method is that L-ascorbic acid can be rapidly pipetted into a single well of a multi-well plate, which can then be returned to the incubator for continued culture of cells in adjacent wells. This is very useful in time course experiments that may take 24 h or longer to complete. Addition of ascorbic acid will change the color of the medium to bright yellow. The stock solution of L-ascorbic acid is made as 0.4 M in dH₂O, filter sterilized, and stored in the dark at 4 °C. It can be used until the color of the stock solution changes from pale opaque to yellow. After termination of the experiment cells can be kept under the medium/ascorbic acid mixture for up to 48 h at 4°C without detrimental effects on subsequent staining.

3. Experiments to analyze cell cycle progression

3.1. Culture conditions

Both the quality of the serum used to supplement the medium as well as the culture conditions can dramatically affect the proliferation rates of cells. It is highly recommended to carefully test different batches of serum for their effects on the parameters under study. We test our Rat-1 fibroblasts using two criteria: 1) maximum rate of proliferation under exponential growth conditions; 2) minimum apoptosis during a 48 h serum-deprivation period (0.25% serum) under 100% confluent conditions. Other assays may be applicable in other systems. We have observed variations as large as 25–30% in exponential growth rates between individual serum batches. Once the desired batch of serum has been identified sufficient amounts should be purchased for all planned experiments.

In terms of culture conditions that may affect cell cycle progression, frequent replenishment of the medium and keeping the cells in a well dispersed and subconfluent state are the most important. In our hands, for cells with population doubling times of < 24 h, replacing the medium every 2 days and not allowing the cells to exceed 50% confluency results in homogeneously growing cultures and highly reproducible doubling times. For slower growing cells less frequent media changes may be used, but confluency should not be allowed to exceed 50%. We define confluency as the % of the total culture vessel surface that is occupied by cells. This can be estimated from phase contrast micrographs taken at low magnification. Although maximum desirable cell density may vary between cell types, the main objective is to avoid cell-cell contact, which can lead to contact inhibition and hence a departure from exponential growth kinetics. Transformed cell lines typically do not show contact inhibition, so this criterion may perhaps be relaxed; however, such cells tend to metabolize rapidly so that close attention should be paid to medium changes.

We have also found that subculture of cells at ratios between 1 : 4 and 1 : 6 is optimal, because it tends to minimize growth in patches where only the outer cells are free from significant contact inhibition. While this may be more labor intensive because it necessitates a frequent subculture regimen, it also promotes maximum exponential phase cycling of the cultures. Finally, to ensure asynchronous and homogeneous cycling, any experiment should start with a minimum of two passages under the above conditions before any measurements are made.

3.2. Synchronization of cultures

For kinetic analyses of cell cycle progression it is highly desirable to synchronize the cells. One frequently used method is to arrest the cells in the G₀ phase, which can be achieved by growing the culture to confluency followed by serum deprivation (0.1% to 0.25% serum) for 48 h. At the start of the serum deprivation period it is important to thoroughly rinse the culture dishes several times with PBS to remove residual serum. The degree of growth arrest is best determined by FACS of ethanol fixed and propidium iodide stained cells (0.05 µg/ml final concentration; Shichiri et al., 1993). Good growth arrest should result in 95% or greater cells with a G₁/G₀ DNA content.

Some cell types, for example MEF or Balb/c-3T3 can be adequately synchronized by contact inhibition alone. In these cases the cultures are simply allowed to reach 100% confluency and then incubated without medium change for a further 48–72 h. Release into the cell cycle is best achieved by subculture; while re-feeding with medium containing fresh serum without subculture should induce some cell cycle entry, this may not recruit all cells and also may not produce adequate synchrony of progression.

Cells that have been synchronized by the combination of contact inhibition and serum deprivation can typically be recruited into the cell cycle very efficiently (>95% entry in Rat-1 cells) by simple re-feeding with medium containing fresh serum. While both methods are believed to induce minimal physiological perturbations, contact inhibition alone would be considered to be the gentler of the two. Transformed cell lines typically cannot be synchronized by these methods. Serum deprivation of subconfluent cultures is usually avoided because it commonly results in apoptosis.

The second commonly used synchronization point is mitosis (M). A method that produces good yields of cells is incubation with the microtubule inhibitor nocodazole (Sigma, cat. no. M1404) at 40–200 ng/ml final concentration (depending on cell type), diluted from a stock solution of 2 mg/ml in DMSO. Ideally, incubation time should be for a period of at least one cell cycle (to allow all cells to accumulate in M), but this may produce toxic effects, and is almost certain to perturb subsequent cell cycle progression as the cells recover from the drug treatment. Shorter periods of incubation will result in lower yields of mitotic cells, which then

need to be separated from the non-synchronized cells. Fortunately, since cells in mitosis become rounded and lose most of their attachment to the substratum, they can be dislodged using relatively gentle mechanical agitation.

The optimal procedure to minimize physiological perturbations, commonly called "mitotic shake-off", is to eliminate the drug treatment altogether. This technique is rapid, very gentle, and produces highly synchronized cultures; however, the yields can be very low. Since mitosis lasts approximately 30 min, in a culture with a 24 h doubling time only ~5% of the cells will be in M phase, and not all can be harvested by the shake-off. Thus, a considerable number of dishes as well as some teamwork may be needed to reduce the processing time as much as possible, since otherwise the synchronization of the culture is compromised. 30 to 40 10-cm plates of exponentially growing cells will yield enough mitotic cells for 12 wells of a 24 well microtiter plate. To harvest the mitotic cells it is easiest to gently tap 3 stacked plates at a time against a hard surface for about one minute while turning the dishes in 90° steps. In order to avoid contamination it is important to avoid spillage of the medium onto the lid, and hence this technique requires some practice. The medium from all plates is pooled and cells are harvested by centrifugation. At least two people should do the tapping of the plates while a third person handles the pooling and centrifugation. The dishes can be recycled by adding back fresh medium, returning to the incubator, and harvesting additional mitotic cells at a later time (e.g., the next day).

3.3 Pulse labeling

A straightforward experiment to determine the fraction of cells in S phase is to pulse label an asynchronous culture with BrdU for 15 to 60 min and immediately harvest for analysis. Using this highly sensitive method, it is possible to capture cells at the very beginning and end of S phase, and thus to obtain more accurate representation of S phase content than is possible by FACS.

The pulse labeling experiment can be augmented by using synchronized cells. For example, after mitotic shake-off cells are seeded in 24 well microtiter plates, BrdU/uridine containing medium is added to the wells as successive time points (every 2–4 h), and after 30 min incubation with BrdU each well is quenched with ascorbic acid (Fig. 1A). Using this protocol one should see a relatively rapid rise in BrdU-positive cells as the culture enters S phase. As cells exit S phase the labeling should drop off, resulting in a clearly defined peak of BrdU incorporation. If the synchrony of the culture is maintained, a second increase in BrdU labeling will occur when the cells enter the subsequent S phase. In this type of experiment cultures with shorter cell cycles typically maintain better synchrony than those with longer cell cycles (Fig. 1A; compare c-myc $+/+$ cells with c-myc $-/-$ cells). Under ideal conditions it should be possible to determine the length of one entire cell cycle (midpoint of the first rise to the midpoint of the second rise), the length of G1 phase (beginning of the experiment to the midpoint of the first rise), and the length of S phase (mid point of the first rise to the midpoint of the first decline). G2 can then be estimated indirectly by subtracting the length of G1 and S from the entire cell cycle (under most conditions M phase can be estimated to be 30–60 min).

There are two reasons why the S phase peak may not reach 100%. First, not all the cells in the culture may be cycling; the fraction of active cells can best be estimated by continuous labeling (below). Second, if the culture synchrony is not good, the fastest cells may exit S phase before the slowest cells enter it. Thus, even if all cells are cycling, there may not be any one time when all the cells are in S phase.

3.4 Continuous labeling

In a continuous labeling protocol BrdU is added at the beginning to all the wells, and individual wells are quenched with ascorbic acid at successive time points. This provides a very accurate determination of S phase entry either from M phase or G0 phase that is not compromised by S phase exit. In a well synchronized, active culture one should see a rapid, continuous rise to 100% labeling (Fig. 1B). The half point (50% BrdU positive cells) is usually taken to represent the G0 to S or M to S transition interval. In most normal cell types the interval from G0 to S is significantly longer (up to 2-fold) than the interval from M to S.

In an asynchronous culture one should see all the S phase cells labeled at the first time point, followed by a straight rise to 100% labeling (the interval from the start of the experiment to the point of 100% labeling represents the length of G2+M+G1 phases). In either case failure to reach 100% labeling indicates that not all the cells are cycling. Significant departures from a linear rise in labeling indicate the presence of subpopulations with different cell cycle kinetics.

If cells are seeded at the start of the experiment (for example, after mitotic shake-off) the volume can be reduced by up to 50%; the dishes should then be kept absolutely still in the incubator to promote attachment. We have routinely taken the first time point at 2 h at which point the cells, although not completely spread out, are sufficiently attached to allow processing. With many fibroblast cell lines it should be possible to push back the first time point to 1 h. If attachment is a problem (or if earlier time points are desired), gentle centrifugation (500 x g, room temperature, 5 min) in a swinging bucket rotor equipped to accommodate microtiter plates is a good option. Another option is to coat the wells with adhesion-promoting substrates (e.g., collagen, fibronectin, matrigel, etc.).

3.6 Determination of the restriction point

The restriction point separates G1 into 2 sub-phases referred to as G1pm (G1 post mitosis) and G1ps (G1 pre synthesis) (Zetterberg, et al. 1995). G1pm is necessary for several signaling processes that are required for cell cycle commitment, whereas G1ps is characterized by rapid macromolecular synthesis in preparation for S phase. The exact molecular landmarks defining the restriction point are still debated, but there is a general agreement that the key events are the phosphorylation and successive inactivation of the retinoblastoma protein. Thus once a cell has passed the restriction point it becomes largely independent of mitogenic signaling, and is committed to complete one cell cycle even if exogenous stimuli are withdrawn (Fig. 2A). Cells synchronized either in M or G0 can be used in these experiments. Typically, the G1pm interval in exponentially cycling cells (M synchronization) is very short (Fig. 2B,C), whereas during the cell cycle recruitment of resting cells (G0 synchronization) it is much longer. In contrast, the duration of G1ps is similar under both conditions (Schorl and Sedivy, 2003).

Based on the property of mitogen-independence, we modified earlier assays to allow the facile determination of the restriction point (Fig. 2A–C). Synchronized cells are seeded in 24 well clusters in the presence of BrdU/uridine-containing complete medium and allowed to attach. At successive time points one well is washed 3 times with PBS, serum-free medium containing BrdU/uridine is added back, and incubation is continued for a time previously determined by continuous labeling to give complete S phase entry (high labeling index). In this experimental setup, the key (and only) variable is the time at which serum is withdrawn, since all cells are incubated for the same total time and BrdU is present throughout. The halfway point in the rise of BrdU-positive cells thus demarks the time at which S phase entry becomes non-responsive to serum withdrawal.

3.6 Labeled mitoses

This assay allows a direct kinetic determination of G2 length in exponentially cycling asynchronous cultures. The assay is based on the ability to distinguish mitotic cells microscopically by their characteristic dumbbell shape (mitotic figures). Asynchronously growing cells are pulse labeled with BrdU for 30 min, washed several times with PBS, incubation is continued in normal medium in the absence of BrdU, and successive time points are taken (Fig. 3A). We use 6 cm dishes and score the entire dish for each time point. The brief pulse labels all S phase cells, and the initial appearance of BrdU-labeled mitotic figures thus denotes the time needed for cells labeled in late S phase to traverse into mitosis. Typically, all mitotic figures become labeled rapidly, and the G2 interval is taken as the half point of this rise. The number of BrdU-positive cells should drop sharply again as the cells labeled in early S phase complete the traverse, and the steepness of the down curve is a good indicator of synchrony. In such cases, the width of the peak at the half way point is another independent means of calculating the length of S phase (Fig. 3A).

Several possible complications need to be taken into account. First, because of their rounded shape, it may not be easy to distinguish BrdU-positive and BrdU-negative cells under phase illumination, and observations should thus be verified by examination under bright field illumination. Second, since mitosis is the shortest phase of the cell cycle, large numbers of total cells need to be scanned to find a statistically significant number of mitotic cells. Finally, mitotic cells are relatively loosely attached to the plates and can thus be easily washed off during the antibody staining and processing steps.

3.7 Double labeling

One parameter that is not easily measured in unperturbed cultures is the time for one complete cell cycle. Since even gentle synchronization such as mitotic shake-off can affect cell cycle progression, the ideal experiment would simply employ exponential, asynchronously growing cultures. Such an approach is made possible by the existence of antibodies specific for two different nucleotide analogs, Chloro deoxyuridine (CldU) and Iodo deoxyuridine (IdU). Cells are marked in S phase using the first analog, and entry of these cells into the next S phase is then monitored with the second analog. In practice, exponentially growing cells are pulse labeled with CldU for 30 min, washed twice with PBS, and cultured with excess thymidine for 1 h to dilute the intracellular pool of CldU. The thymidine-containing medium is then removed, and incubation is continued with normal medium. At subsequent time points a second 30 min pulse using IdU is delivered, the samples are quenched, fixed, and stained for immunofluorescent observation using the CldU- and IdU-specific antibodies. A representative experiment is shown in Fig. 3B.

4 Processing and analysis

4.1 Fixation

While ascorbic acid is a superb quenching reagent, and cells can be kept in its presence in culture medium for up to 48 h, we prefer to proceed to the fixation steps as soon as the experimental regimen allows. Prior to fixation, the plates should be washed quickly but very thoroughly 2–3 times with PBS. During all steps it is important to avoid pipetting directly on top of the cells, as this can dislodge them. Likewise, it is important to prevent the cells from drying out at any point. We therefore do not stain more than 8 to 10 plates at one time, and remove solutions by simply decanting the medium into a large beaker, as opposed to using an aspirator, which takes much longer. After the PBS washes to remove the medium and ascorbic acid, cells are fixed with 100 % ice-cold methanol for 10 min at 4 °C, followed by 3 washes with PBS. At this point the plates can be stored under PBS sealed with parafilm (to avoid desiccation) at 4 °C for several weeks.

4.2. Denaturation

In order to provide accessibility to the antibodies, the DNA needs to be rendered single-stranded to expose the incorporated nucleotide analogs. The classical method is to denature the DNA with acid (1.5 M HCl final concentration, 1 h at room temperature, gentle rocking). Immediately afterwards the plates are washed 3 times (3–4 min each wash) with 0.1 M borate buffer (boric acid, BioRad, Hercules, CA, cat. no. 161-0751, pH adjusted to 8.5 with NaOH), followed by 3 similar washes with PBS. At this point it is possible to store the plates under PBS at 4°C as described before.

Another way to expose the incorporated nucleotide analogs is by partial degradation with benzonase, a genetically engineered nuclease which degrades both single and double stranded DNA (VWR International, West Chester, PA, cat. no. 80108-808). In this protocol the exposure of single-stranded regions and binding of the antibody are accomplished simultaneously. Cells are incubated in a humidified chamber at 37°C for 2 h with anti-BrdU monoclonal antibody (Becton Dickinson, San Jose, CA, cat. no. 555627), diluted 1 : 200 in PBS containing 1 mM Mg²⁺ and 125 U/ml benzonase. This is followed by the desired secondary antibody (Herbig et al., 2004).

4.3 Staining

Non specific staining is prevented by first blocking with 0.1% PBS-BSA (PBSA) solution (0.1% w/v BSA Fraction V, Fisher Scientific, cat. no. BP1600-100) dissolved in PBS in a humidified chamber at 37°C for 1 h without shaking. Primary anti-BrdU monoclonal antibody (Becton Dickinson, cat. no. 555627, 0.5 mg/ ml stock concentration) is used at 1 : 200 dilution in PBSA for 1 h, also at 37 °C in the humidified chamber. The diluted antibody can be re-used 4- to 5-times without loss of signal intensity and should be stored at –20 °C. All subsequent steps are carried out at room temperature and utilize the Vectastain ABC Elite Mouse IgG Kit from Vector Laboratories (Burlingame, CA, cat. no. PK-6102), combined with the Vector Laboratories NovaRED Substrate Kit for peroxidase (cat. no. SK 4800).

Plates are first rinsed briefly several times with PBS, and then agitated gently on a rotating platform for 10 min under PBS before adding the biotinylated secondary antibody. We have adopted the protocol suggested by the manufacturer in order to avoid over-staining. The secondary antibody solution is comprised of 10 ml PBS, 115 µl normal horse serum, and 23.5 µl biotinylated anti-mouse secondary antibody. 750–1000 µl of this solution are added per well of a 24 well culture dish, and incubated for 30 min.

During this time Vectastain solution AB is prepared (10 ml PBS, 4 drops solution A, 4 drops solution B, mixed thoroughly after adding each solution). Solution AB is pre-incubated for 30 min at room temperature before addition to the cells. The time required for a PBS rinse followed by a 10 min PBS wash (above) after the secondary antibody should be taken into account while preparing solution AB. 750–1000 µl of solution AB is used per well, incubated for 30 min, and again followed by a PBS rinse and a 10 min PBS wash (above). Finally, a brief rinse with dH₂O is used to remove salts, which is critical for even staining. The cells can be kept in dH₂O while the NovaRED staining solution is prepared by mixing 10 ml dH₂O with 3 drops each of reagents 1 through 3 (immediate mixing after adding each component is important). Finally, 2 drops of hydrogen peroxide solution are added, mixed in, and the staining solution is added to the cells. The staining intensity is checked immediately and frequently in a microscope at 20 x magnification. After good BrdU incorporation nuclear staining is typically easily detectable after 30 to 40 sec (but can be strong enough as early as 10 sec), at which time the reaction can be stopped by performing several dH₂O washes. Prolonged exposure of the cells to the staining solution is unnecessary and will result in non-specific staining. Stained

plates can be stored at 4°C under dH₂O (sealed with parafilm) for several weeks without loss of signal intensity.

4.4 Two color immunofluorescent detection of CldU and IdU incorporation

This procedure is adapted from the one published by Aten et al. (1992). CldU and IdU can be purchased from Sigma (cat. no. C6891 and I7125, respectively). While histochemical detection of BrdU incorporation can be performed directly on cells grown in plastic culture plates, for immunofluorescent detection cells should be seeded on glass coverslips which can then be cultured in appropriately sized culture vessels. Exponentially growing cells are incubated initially for 30 min with 2.6 µg/ml CldU (10 µM final, stock solution is 1000 x or 10 mM prepared in the same way as for BrdU), followed by 2 washes with PBS and incubation for 1 h in medium containing 200 mM thymidine. Cells are again washed twice with PBS and incubation is continued using normal medium. At subsequent times the medium is changed for a 30 min labeling period to medium containing 3.5 µg/ml IdU (10 µM final, stock solution is 1000 x or 10 mM prepared in the same way as for BrdU). If needed, the labeling is quenched with ascorbic acid as indicated above.

To process the samples the cells are first washed three times in 0.05 % Tween-PBS (PBS-T). All antibody incubations are carried out in a humidified chamber at room temperature. Rat monoclonal antibody clone BU1/75 (ICR1; Harlan, Indianapolis, IN, cat. no. MAS 250) diluted 1 : 520 in PBSA-T (PBS-T containing 0.1% BSA) was used to detect CldU. For detection of IdU we used mouse monoclonal antibody clone B44 (BD Biosciences, San Jose, CA, cat. no. 347-580) diluted to a final concentration of 2.5 µg/ml in PBSA-T. The secondary antibodies were a Cy3-conjugated donkey anti-rat antibody (Jackson ImmunoResearch, West Grove, PA, cat. no. 712-165-153), and Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR, cat. no. A-11029), both used at a final concentration of 3.0 µg/ml.

The antibodies were used sequentially, with the anti-CldU antibody being used first, followed by the donkey anti-rat antibody, then the anti-IdU antibody, and finally the goat anti-mouse antibody. A high salt wash (28 mM Tris, pH 8.0, 500 mM NaCl, 0.5% Tween 20) for 10 min followed by a 10 min wash in PBSA-T was used after each antibody incubation to reduce non-specific signals. Finally, cells were counterstained with 0.1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) for 15 min to visualize the nuclei, followed by a final PBS wash. Microscopic analysis was performed at 200 x magnification. Randomly selected fields were photographed with a Spot-II digital camera (Diagnostics Products, Los Angeles, CA) and the images were scored. Exposure to the excitation beam as well as to overhead fluorescent lighting should be minimized to avoid photo bleaching.

5.1 Analysis of proliferative capacity using CFSE staining

This procedure is very useful to assess the longer term proliferative capacity of cultures, specifically, whether all cells are cycling uniformly and continuously, and to address the possible existence of slower or faster growing sub-populations (Lyons, 1999, Lyons et al., 2001). Under ideal conditions it allows the monitoring of cultures for multiple cell cycles, to a degree that cannot be achieved using nucleotide incorporation methods. 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Molecular Probes, cat. no. C-1157) diffuses into cells where it attaches to amine groups of cytoplasmic proteins and is metabolized by cellular esterases to a fluorescent dye. CFSE has very similar spectral properties to fluorescein and hence can be detected in the FL-1 channel of a flow cytometer.

CFSE is prepared as a 5 mM stock solution in DMSO, and stored at -20 °C protected from light (do not freeze or thaw more than 3 times). 1–3 x10⁷ cells are trypsinized, collected by low speed centrifugation, resuspended in 1 ml complete medium containing 10 µM CFSE, and

incubated for 10 min at 37°C. 14 ml of ice-cold complete medium is added and the incubation is continued on ice for another 5 min. Cells are again collected by centrifugation and washed once in CFSE-free medium. A small aliquot is removed and immediately analyzed by FACS as intact, live cells (without fixation).

The remaining cells are returned to culture in 10 cm dishes, making sure to seed them at a low enough density to allow multiple cell cycles. One dish is analyzed by FACS each subsequent day (the time intervals should be matched roughly to the doubling time). This method relies on the fact that after an initial rapid turnover period (manifested as a large decrease in fluorescence during the first 12–24 h), subsequent turnover is minimal and the dye-modified proteins are distributed equally to the daughter cells. Thus, most dye dilution results from cell growth and division, such that daughter cells are roughly half as bright as mother cells. These successive generations can be visualized as discrete peaks by FACS, shifted to the left in the FL-1 channel compared with the peak of the initial cell population. The reduction in fluorescence intensity can be followed over several cell cycles, and shoulders or small peaks remaining to the right of the main peak indicate the existence of sub-populations of non-dividing cells.

Conclusion

In this review we have provided detailed protocols for several methods that allow kinetic analyses of cell cycle progression in living cells. These protocols can be easily modified to the experimental needs of the investigator. Undoubtedly the near future will see great progress in the understanding of the cell cycle and cell cycle progression, and the knowledge gained from studying the cell cycle will result in improved therapies for numerous diseases. Indeed, small inhibitor molecules targeting the dysregulated activities of key cell cycle regulators are currently undergoing clinical trials, and it is hoped that they will significantly improve cancer therapy.

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Figure 1. Pulse labeling (A) and continuous labeling (B) of c-myc^{+/+} and c-myc^{-/-} cells synchronized by mitotic shake off (reproduced from Schorl and Sedivy (2003) Mol. Biol. Cell 14, 823–835).

Figure 2. (A) Schematic representation of the method to determine the restriction point. Phases of the cell cycle: M, mitosis; G0, quiescence (resting cells); G1pm, G1 phase post mitosis; G1ps, G1 phase pre DNA synthesis; S, S phase; G2, G2 phase. R designates the restriction point. Filled nuclei depict cells that have incorporated BrdU. For further details see text. (B) and (C) Restriction point determination in cycling cells using mitotic shake off for synchronization (reproduced from Schorl and Sedivy (2003) Mol. Biol. Cell 14, 823–835). (B) c-myc $^{+/+}$ cells. (C) c-myc $^{-/-}$ cells.

Figure 3. (A) Labeled mitoses experiment performed with exponentially cycling *c-myc* ^{+/+} cells. (B) Double labeling with CldU and IdU to determine entry into the second S phase in exponentially cycling *c-myc* ^{+/+} and *c-myc* ^{-/-} cells. The data (labeling indices) are presented as the percentage of CldU/IdU double-labeled cells in the total pool of CldU-positive cells. For further details see text (reproduced from Schorl and Sedivy (2003) *Mol. Biol. Cell* 14, 823–835).