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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.

Keywords: Cell cycle analysis; DNA analogues; BrdU; Synchronization; Mitotic shake-off

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 [2]. 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 [13,14,10]). 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 [8]), 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, 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 [9]. 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

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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 × and 100 × stock solutions, respectively, in distilled water (dH_20), 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 [7]. 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 dH_2O, 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%–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; [12]). Good growth arrest should result in 95% or greater cells with a G1/G0 DNA content.