

High Content Screening Assay for Monitoring Cell Proliferative Activity by Multiplexed Measurements of DNA Content, BrdU and Ki67

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Introduction

Cell proliferation and the characterization of agents that either promote or inhibit cell proliferation are particularly critical areas of cell biology and drug-discovery research. Although cell proliferation can be measured by the incorporation of [3H]-thymidine into cellular DNA, this assay is slow, labor intensive and its use is limited by potentially hazardous radioactive materials. As an alternative to [3H]-thymidine, 5-bromo-2'-deoxyuridine (BrdU), a thymidine analog, can be used to detect the DNA replication in actively proliferating cells. Cells which have incorporated BrdU into DNA can be quickly detected by using monoclonal antibodies directed against BrdU and fluorophore-conjugated secondary antibody. BrdU staining facilitates the identification of cells that have progressed through the S-phase of the cell cycle during the BrdU-labeling period. The antibody against BrdU can detect DNA-incorporated BrdU only when the double stranded DNA is denatured or disintegrated. Conventional treatments with acid, base or heat to detect the BrdU which is incorporated during the DNA replication could also be disadvantageous for certain applications. These harsh procedures could result in destruction of cellular morphology and cell integrity, and may

destroy the epitopes of antibodies against cell surface and other protein markers. Therefore, these proliferation kits have been specifically designed to enable BrdU detection in nuclear DNA without such extreme treatments. This enables multiplexed detection of BrdU with antibodies towards other cellular targets. In addition to the DNA-incorporated BrdU, an S phase indicator, several other protein markers are known to be associated with cell proliferation, including the antigen Ki67. Ki67 is expressed during G₁, S, G₂, and M phases of the cycle but absent in G₀ phase, such as in quiescent and terminally differentiated cells. Ki67 antigen-positive cells give a more specific and accurate indication of proliferating cells compared to that of PCNA-positive cells since PCNA is detectable in almost all quiescent cells. Ki67 has also been found to correlate with poor survival in ovarian cancer in both univariate and multivariate analyses. The quantification of Ki67 provides independent prognostic information in breast cancer in addition to the tumor size and S-phase fraction. High content analysis (HCA) involves a fluorescence cell-based assay where the cells are automatically imaged and analyzed using quantitative fluorescence microscopy. HCA can be used to quantify the cell proliferation activity by measuring their DNA

content, the state of cell cycle-associated proteins, and morphological changes in individual cells and in cell populations. Cell Proliferation Cellomics HCS Reagent Kits are the optimized and validated reagent kits for robust, turnkey and multiplexed HCS assays that simultaneously monitor three multiplexed biomarkers in the same cells: DAPI (DNA content), bromodeoxyuridine (DNA replication) and Ki67 (cell proliferation). All the images were acquired and analyzed quantitatively using ArrayScan® HCS Reader with Cellomics Bioapplication. Thermo Fisher Scientific, Inc. provides an integrated set of products that work together to deliver a "total solution" platform for HCS. Integral components of this set are the Cellomics HCS Reagent Kits. These kits provide easy-to-use methods and reagents for preparing high quality samples for automated cell-based imaging assays.

Conclusions

- Cell proliferative activity can be measured effectively by BrdU and Ki67 detection
- Contact inhibition induces the G₀ phase characterized by the inhibition of BrdU incorporation and Ki67 expression.
- Different cell cycle inhibitors can be examined (or screened) by multiplexing DNA content, BrdU and Ki67 detection.
- DNA content analysis is an effective method to monitor the G₂/M blockers.
- The Cell Proliferation Cellomics HCS Reagent Kits can be used in HCS assays for the quantitative imaging and analysis of total proliferating cells, G₀/G₁ phase, S phase and G₂/M phase cells with robust performance (Z' values ? 0.4).

BrdU and Ki67 as Cell Proliferation Markers and Cell Cycle

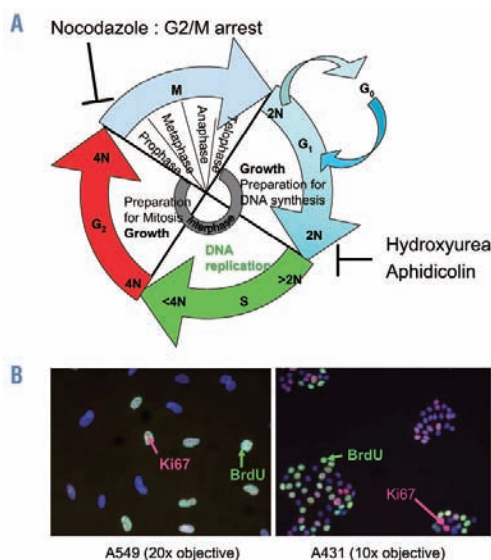


Figure 1: Cells were treated with Bromodeoxyuridine (BrdU, 40 μ M) for 30 min in 96 well microplate and then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and labeled with a anti-BrdU/anti-Ki67 primary antibodies. Primary antibodies were detected with DyLight™ 488- and DyLight™ 549-conjugated secondary antibodies, respectively. Images were acquired on ArrayScan® HCS reader.

1A shows schematic of the cell cycle and some of the cell cycle inhibitors that were used in this study. 1B shows BrdU (green) and Ki67 (red) detection in A549 (20X Objective lens) and A431 cells (10X Objective lens), respectively. Cells with only blue nuclei (DAPI stained) are in G₀ phase (no Ki67). Cells with green nuclei indicate the cells have undergone DNA replication and BrdU incorporation. Cells with red nuclei are the proliferative cells (Ki67 positive) that have not gone through the S phase (no BrdU).

細胞周期

- ArrayScanVTI
- HCS蛍光試薬キット

The Effect of Cell Cycle Inhibitors on BrdU and Ki67 Detection

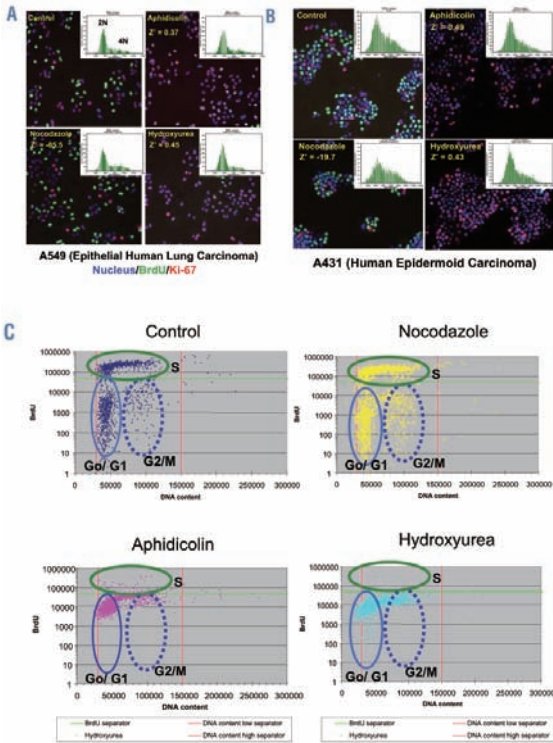


Figure 2: A549 and A431 cells were seeded at 5,000 cells per well on 96 well microplates the day before treating them with different drugs. Cells were incubated with the drugs for 2 hrs at 37°C, 5% CO₂ in a humidified incubator, incubated with 40 μM BrdU for 30 min and stained with BrdU and Ki67 antibodies. Cell nuclei were labeled with DAPI. The DNA content, BrdU and Ki67 intensities were quantitatively analyzed on Cellomics' ArrayScan[®] HCS Reader. (Aphidicolin, 1 μg/ml; Nocodazole, 0.5 μg/ml; Hydroxyurea, 100 mM) 2A: Fluorescence images acquired on the ArrayScan[®] HCS Reader show the inhibition of BrdU incorporation (for 30 min) in A549 cells with Aphidicolin or Hydroxyurea treatment. The untreated control cells have a population in S phase (BrdU, green), while the Aphidicolin- or Hydroxyurea-treated cells show no BrdU incorporation. DNA content of the cell population was analyzed at the same time on the ArrayScan[®] HCS Reader (inset). The level of Ki67 and DNA content were not significantly changed with 2 hrs treatment of these three drugs. 2B: Detection of DNA content, the levels of BrdU and Ki67 in A431 cells after drug treatment. 2C: Cell population analysis of BrdU intensity vs. DNA content of A549 cells after drug treatment. The cell detail data were acquired from the ArrayScan[®] HCS Reader and plotted in log scale only for BrdU intensity. Three different cell cycle phases i. e. G0/G1, S and G2/M phases were separated by cell population analysis with BrdU incorporation and DNA content.

DNA Content Analysis After Nocodazole Treatment

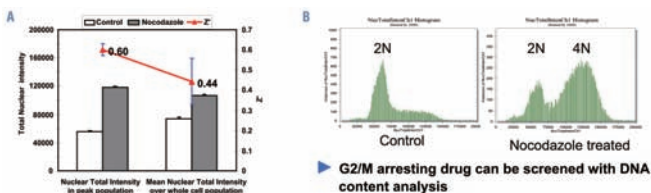


Figure 3: A549 cells were treated with 0.5 μg/ml Nocodazole for 18 hrs, fixed, permeabilized and stained with DAPI. The samples were automatically imaged and quantitatively analyzed on Cellomics' ArrayScan[®] HCS Reader. DNA content analysis itself allows screening G2/M blockers in cell cycle. 3A: Using cell cycle bioapplication integrated in the ArrayScan[®] HCS Reader, different output features of DNA content were analyzed; DNA intensity value of the majority of the cells and average value of the DNA content in the acquired cell population. Nocodazole treatment changed the DNA content in the population from 2N to 4N. Z' values are > 0.4 in both of DNA content output features. 3B: The histogram of cell population represented with DNA content.

DNA Content Analysis After Serum Starvation and Serum Stimulation

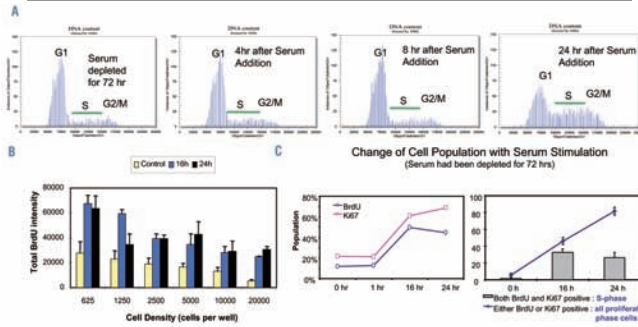


Figure 4: After A549 cells were incubated without serum for 72 hrs, 10% serum was added to the culture medium. A549 cells were incubated with 40 μM BrdU for 30 min, fixed, permeabilized and stained with DAPI, BrdU and Ki67 antibodies. The samples were automatically imaged and quantitatively analyzed on Cellomics' ArrayScan[®] HCS Reader. 4A: DNA content analyses of A549 cell after serum depletion and serum addition. Serum depletion induces cell cycle arrest and causes accumulation of cell population in G0/G1 phase. After 24 hrs of serum stimulation, cells in S phase and M phase were significantly increased. 4B: DNA synthesis (BrdU incorporation) in A549 cells depends on the cell density, which indicates cell cycling time is being reduced in high density cell culture by contact inhibition. 4C: Serum stimulation increases BrdU and Ki67 positive cells in serum-starved A549 cells. Population analysis with BrdU and Ki67 markers shows total proliferating cells as well as S-phase cells.

Contact Inhibition Suppress Cell Proliferation in A549 Cells

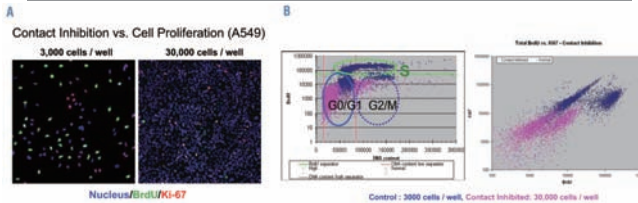


Figure 5: A549 cells were cultured in normal density (3,000 cells/well) and in high density (30,000 cells/well) and incubated with 40 μM BrdU for 30 min. High density cell culture shows the inhibition of both BrdU incorporation and Ki67 expression through contact inhibition. 5A: The image of A549 cells showing nucleus (blue), BrdU (green) and Ki67 (red). 5B: The features plotted are the incorporated BrdU intensity vs. nuclear DNA content and Ki67 vs. BrdU. Note the change in DNA content, BrdU intensity and Ki67 intensity by contact inhibition. Substantial inhibition of Ki67 expression in the contact inhibited sample suggests the majority of cells are in G0 phase.

Dose Response Curves for BrdU Incorporation Inhibition by Anti-Proliferative Drugs.

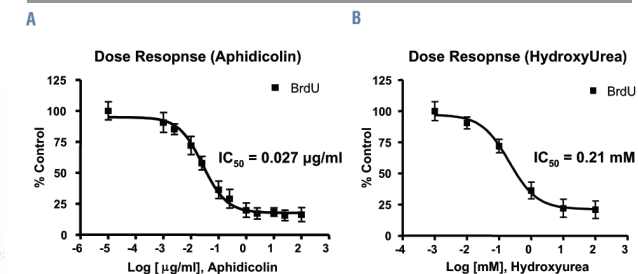


Figure 6: The IC₅₀ for the inhibition of BrdU incorporation was determined (from 3 plates, 16 points dose response curve per plate). The plot shows the dose dependence of Aphidicolin (6A) and Hydroxyurea (6B) for the inhibition of BrdU incorporation from each of the three plates.

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