Multiplexed High Content Screening Assays for Cell Cycle Analysis and Cytotoxicity Profiling

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Abstract

Cell proliferation and cell cycle analysis play critical roles in drug discovery. Modulation of cell division has implications for a broad variety of target processes e.g. oncogenesis, angiogenesis, apoptosis and inflammation. Cellular proliferation also represents a sensitive marker of cytotoxicity. High Content Screening (HCS) is a powerful tool for detailed investigation of cellular responses to pharmacological compounds. Millipore has developed a panel of platform-independent HCS assays for cell cycle analysis. Each is immunofluorescence-based, utilizing high quality reagents and validated protocols. Extensively characterized primary antibodies against key cell cycle markers phospho-histone H3, Ki-67, cyclin B1 and BrdU are employed in combination with FITC- or Cy3-conjugated secondary antibodies and a nuclear stain. The assays permit flexibility with regard to primary antibody species, secondary antibody fluorophore, and the choice of single or multiplexed immunolabeling, for fine discrimination of compound effects on cell cycle. We present cell cycle profiling data using human HeLa and A549 cells demonstrating the high signal quality achieved with these reagents, and provide dose responses for cell cycle modulating drugs, including vinblastine, etoposide, aphidicolin and paclitaxel. We use the assays to calculate mitotic and proliferation indices, and to discriminate between cell populations at various phases of the cell cycle. Our data also indicate AC50 values for compounds causing cytotoxicity. Additionally, we show that at working concentrations the reagents exhibit stability for >24 hours at room temperature, offering a great benefit for large-scale screening applications. These assays represent a simple, ready-to-use, validated strategy for characterization of agents which modulate the cell cycle, for cancer drug screening, aurora kinase profiling, and for in vitro toxicology studies - representing broad potential for drug screening and development.

Introduction

Major efforts in drug discovery research are focused on the identification of compounds that modulate cell division, whether for proliferation-targeted processes such as angiogenesis and neural repair, or arrest-targeted applications such as oncogenesis and inflammation. Also, proliferation assays have been demonstrated to be among the most sensitive tests for cytotoxicity. Drug-induced toxicity/apoptosis may be monitored by analysis of proliferation and cell cycle modulation. The development of High-Content Screening (HCS) technology represents a major step towards improving the drug discovery process, enabling accurate characterization of intracellular events. Successful HCS assays rely on high-quality detection reagents. Millipore's Proliferation/Cell Cycle HCS Assays have been specifically designed for High-Content Imaging and multiplexed analysis. The platform-independent assays are immunofluorescence-based, provide validated reagents/protocols, and utilize high quality primary antibodies against the well-established proliferation/ cell cycle markers BrdU, cyclin B1, Ki67 and phospho-histone H3. These assays provide flexibility with regard to primary antibody species and single or multiplexed immunolabeling, and offer a convenient, ready-to-use strategy for enhancing productivity in cell proliferation research, cytotoxicity testing, and drug screening.

Methods

1. Human HeLa or A549 cells were plated in growth media on clear-bottom 96-well plates suitable for HCS imaging, at a concentration of 15,000-21,000 cells/cm^2 (4,500-6,300 cells/well in 90 µL). Cells were cultured for a total of 48 hrs.
2. For the last 4 hrs of culture, serial dilutions of 10X cell-cycle arresting compounds (or DMSO negative control) were added to the culture media (10 µL/well) for a final 1X cell treatment concentration.
3. For the last 1 hr of culture, cells were pulsed with a final media concentration of 20 µM 5-bromo-2'-deoxyuridine (BrdU).
4. Following the culture period, cells were rinsed, fixed with HCS Fixation Solution, then rinsed twice with 200 µL of HCS Immunofluorescence Buffer.
5. Post-fixation, 50 µL of primary antibody solution was added to each well, followed by incubation for 1 hr at room temperature. Wells were rinsed three times with HCS Immunofluorescence Buffer.
6. Following rinsing, 50 µL of secondary antibody/nuclear stain solution was added to each well, followed by incubation for 1 hr at room temperature, protected from light.
7. Wells were rinsed twice each with HCS Immunofluorescence Buffer and Wash Buffer. Plates were sealed prior to HCS imaging/analysis.
8. Plates were imaged on the GE IN Cell Analyzer 1000 (3.3) at 10X objective magnification (10 fields/well) and analyzed using the GE IN Cell Analyzer 1000 Workstation (3.4) Multi Target Analysis algorithm.

Assays Currently Available

	Primary Antibody						Fluorophore-Conjugated Secondary Antibody		Nuclear Stain
Catalog Number	Mouse anti-BrdU	Mouse anti-Cyclin B1	Mouse anti-Ki67	Rabbit anti-Ki67	Mouse anti-Phospho-Histone H3	Rabbit anti-Phospho-Histone H3	FITC	Cy3	Hoechst
HCS201	✓							✓	✓
HCS202		✓						✓	✓
HCS203					✓			✓	✓
HCS204						✓		✓	✓
HCS205				✓				✓	✓
HCS206			✓					✓	✓
HCS209			✓			✓	✓	✓	✓
HCS210		✓		✓			✓	✓	✓
HCS211		✓				✓	✓	✓	✓
HCS212	✓					✓	✓	✓	✓
HCS213	✓			✓			✓	✓	✓

Figure 1. Proliferation/ cell cycle assays available from Millipore.

Reagent Stability

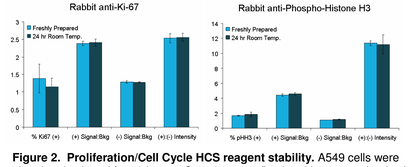

Figure 2. Proliferation/Cell Cycle HCS reagent stability. A549 cells were plated and cultured for 48 hours. Samples were fixed and immunostained using either fresh buffers and antibody/Hoechst solutions, or buffers and solutions that had been allowed to sit at room temperature (protected from light) for 24 hours prior to staining. No statistically significant difference in signal quality was observed between freshly prepared and 24 hour samples. 24 hr stability was observed for all antibodies used in Millipore's proliferation/cell cycle HCS kits. Data available on request.

Results

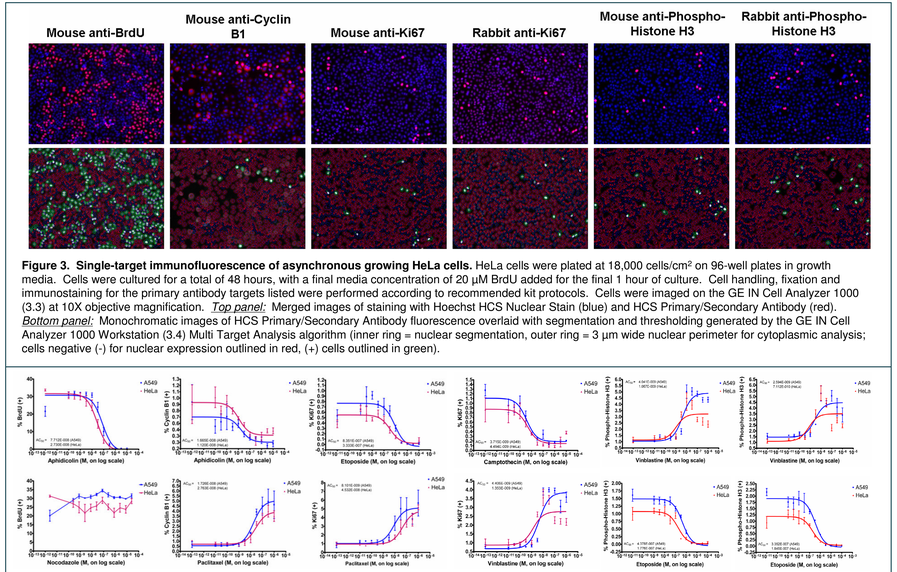

Figure 3. Single-target immunofluorescence of asynchronous growing HeLa cells. HeLa cells were plated at 18,000 cells/cm^2 on 96-well plates in growth media. Cells were cultured for a total of 48 hours, with a final media concentration of 20 µM BrdU added for the final 1 hour of culture. Cell handling, fixation and immunostaining for the primary antibody targets listed were performed according to recommended kit protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.3) at 10X objective magnification. *Top panel:* Merged images of staining with Hoechst HCS Nuclear Stain (blue) and HCS Primary/Secondary Antibody (red). *Bottom panel:* Monochromatic images of HCS Primary/Secondary Antibody fluorescence overlaid with segmentation and thresholding generated by the GE IN Cell Analyzer 1000 Workstation (3.4) Multi Target Analysis algorithm (inner ring = nuclear segmentation, outer ring = 3 µm wide nuclear perimeter for cytoplasmic analysis; cells negative (-) for nuclear expression outlined in red, (+) cells outlined in green).

Figure 4. Dose response of A549 and HeLa cells to cell-cycle arresting agents. HeLa cells were plated at 18,000 cells/cm^2 on 96-well plates in growth media. Cells were cultured for a total of 48 hours, with a final media concentration of 20 µM BrdU added for the final 1 hour of culture. Cell handling, fixation and immunostaining for the primary antibody targets listed were performed according to recommended kit protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.3) at 10X (10 fields/well) and analyzed (nuclear segmentation and (+)/(-) intensity thresholding) using the GE IN Cell Analyzer 1000 Workstation (3.4) Multi Target Analysis algorithm. Data presented are mean ± SEM, n = 4.

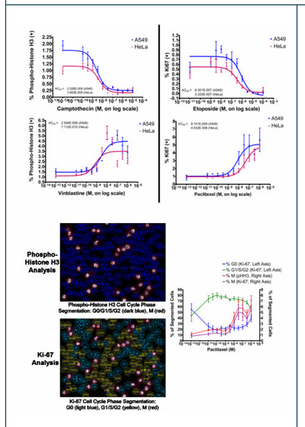

Figure 5. Multiparameter analysis of phospho-histone H3 and Ki-67. Upper Panels. Phospho-histone H3 and Ki-67 dose responses of A549 and HeLa cells to cell cycle-arresting agents. Lower Panels. Cell cycle phase analysis of paclitaxel-treated A549 cells. Lower left panels: Phospho-histone H3 or Ki-67 fluorescence overlaid with segmentation and thresholding. Cells were classified for nuclear expression (M-phase) or no expression (G0, G1, S or G2) of phospho-histone H3. Cells were also classified for homogeneous nuclear Ki-67 expression (M), punctate nuclear expression (G1, S or G2) and no Ki-67 expression (G0). Lower right panel: Percentages of cells in each of the phase segments are plotted as a function of paclitaxel concentration.

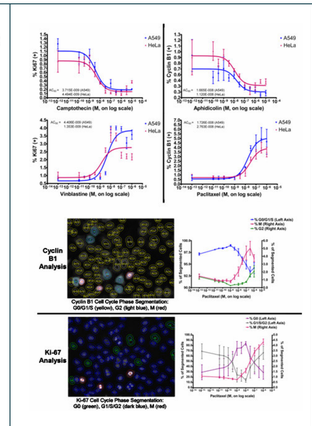

Figure 6. Multiparameter analysis of cyclin B1 and Ki-67. Upper Panels. Cyclin B1 and Ki-67 dose responses of A549 and HeLa cells to cell cycle-arresting agents. Lower Panels. Cell cycle phase analysis of paclitaxel-treated HeLa cells. Lower left panels: Cyclin B1 or Ki-67 fluorescence overlaid with segmentation and thresholding. Cells were classified for nuclear expression (M-phase), cytoplasmic expression (G2) and no expression (G0, G1 or S) of cyclin B1. Cells were also classified for homogeneous nuclear Ki-67 expression (M), punctate nuclear expression (G1, S or G2) and no Ki-67 expression (G0). Lower right panels: Percentages of cells in each of the phase segments are plotted as a function of paclitaxel concentration.

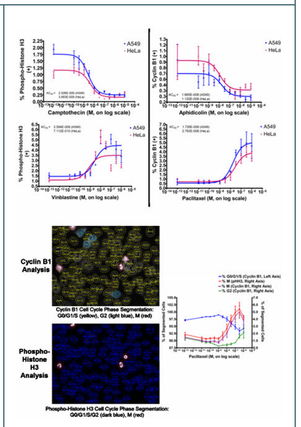

Figure 7. Multiparameter analysis of cyclin B1 and phospho-histone H3. Upper Panels. Cyclin B1 and phospho-histone H3 dose responses of A549 and HeLa cells to cell cycle-arresting agents. Lower Panels. Cell cycle phase analysis of paclitaxel-treated HeLa cells. Lower left panels: Cyclin B1 or phospho-histone H3 fluorescence overlaid with segmentation and thresholding. Cells were classified for nuclear expression (M-phase), cytoplasmic expression (G2) and no expression (G0, G1 or S) of cyclin B1. Cells were also classified for nuclear expression (M-phase) or no expression (G0, G1, S or G2) of phospho-histone H3. Lower right panels: Percentages of cells in each of the phase segments are plotted as a function of paclitaxel concentration.

Kit Components

1. Proliferation/Cell Cycle HCS Primary Antibody/Antibodies, 100X: One vial each containing 300 µL (ASSAY-DEPENDENT)
2. HCS Secondary Antibody/Antibodies (FITC- or Cy3-conjugated), 200X: One vial each containing 150 µL (ASSAY-DEPENDENT)
3. Hoechst HCS Nuclear Stain, 200X: 1 vial containing 150 µL
4. HCS Fixation Solution with Phenol Red, 2X: 1 bottle containing 100 mL
5. HCS Immunofluorescence Buffer, 1X: 1 bottle containing 1000 mL
6. HCS Wash Buffer, 1X: 1 bottle containing 500 mL
7. Cell Cycle Arrest Control Compounds (e.g., Paclitaxel, Etoposide, Vinblastine Sulfate, Camptothecin), 250X: 2-4 vials each containing 100 µL (ASSAY-DEPENDENT)
8. DMSO for Compound Serial Dilution: 1 bottle containing 10 mL
9. Compound Dilution Buffer: 1 bottle containing 25 mL
10. BrdU, 250X: 1 vial containing 100 µL (ASSAY-DEPENDENT)
11. Plate Sealers: 10 plate sealers

Summary

- Cell proliferation and cell cycle analysis are of broad potential and significance in the fields of cytotoxicity assays, drug screening and development.
- Millipore's Proliferation/Cell Cycle Assays for High-Content Screening represent a simple, ready-to-use, validated strategy for the investigation of multiple cell proliferation parameters.
- High quality immunodetection reagents against the proliferation markers BrdU, cyclin B1, Ki67 and phospho-histone H3 allow for highly sensitive and specific detection of cell cycle stage.
- Reagents are stable for at least 24 hours at room temperature, facilitating large scale screening experiments.
- Multiplexed immunolabeling allows for fine discrimination of compound effects on cell cycle, often within a single assay.
- HCS platform-independence and the of assays with regard to primary antibody species and single or multiplexed immunolabeling provide a high degree of flexibility.
- Assays can used to evaluate mitotic and proliferation indices, percentages of cells in specific phases, and the effect of both G1/S and G2/M arresting agents on cell populations, as demonstrated by data in human HeLa and A549 cells.
- Assays facilitate increased productivity in cell proliferation research, cytotoxicity studies, and drug discovery.

Related Products

HCS100 CellCiphr™ – Cytotoxicity assay, human HepG2 cells
HCS214 HCS Toolkit for use with mouse primary antibodies
HCS215 HCS Toolkit for use with rabbit primary antibodies
HCS220 Neurotoxicity and Neurite Outgrowth HCS assay

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