Multiplexed Mitosis and Apoptosis Analysis

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Introduction

Cell proliferation and apoptosis, which connote life and death of the cell, are two key areas in cell biology and drug discovery research.[1] Understanding the signaling pathways in cell proliferation and apoptosis is important for new therapeutic discovery because the imbalance between these two events is predominant in the progression of many human diseases, including cancer. We have developed a high content screening (HCS) assay that identifies cell proliferation, p53 induction and Caspase-dependent cell death, enabling systematic investigation of apoptosis and mitosis events in the cell.

This assay is conducted using multiplexed Mitosis-Apoptosis HCS Reagents, which measure cell proliferation (cell number, DNA replication), cell cycle (DNA content, BrdU incorporation), and apoptosis (Caspase 3 activation and p53 induction) simultaneously using a fixed, end-point assay based on immunofluorescence detection in cells grown on standard high-density microplates.[2] The DNA binding dye DAPI is used to determine the nuclear size and nuclear morphology as well as cell cycle phases by DNA content. As a cell proliferation marker, 5-bromo-2´-deoxyuridine (BrdU), a thymidine analog, can be used to label the actively proliferating cells. BrdU staining with specific monoclonal antibody facilitates the identification of cells that have progressed through the S-phase of the cell cycle during the BrdU-labeling period. Caspase 3 is a key executioner of both intrinsic and extrinsic pathways of apoptosis. The activation of Caspase 3 can be detected by indirect immunofluorescence using primary antibody against the cleaved portions of Caspase 3. The tumor suppressor protein p53 is a key critical protein in regulating the cell cycle and apoptosis through its regulation of downstream signaling events. By using the Thermo Scientific™ ArrayScan™ XTI High Content Analysis (HCA) Reader and Thermo Scientific™ Mitosis-Apoptosis HCS Reagents, the complex network of key players controlling proliferation and apoptosis can be reduced to several sentinel markers for analysis.

Methods and Materials

BrdU Primary Antibody, Active Caspase 3 Primary Antibody, p53 Primary Antibody, Thermo Scientific™ DyLight™ 488 Conjugated Goat Anti-Mouse IgG, Thermo Scientific™ DyLight™ 549 Conjugated Goat Anti-Rabbit IgG, Thermo Scientific™ DyLight™ 649 Conjugated Donkey Anti-Goat IgG BrdU, Magnesium Chloride (1M), DAPI Dye, wash buffer (10X Dulbecco's PBS), Permeabilization Buffer (10X Dulbecco's PBS with 1% Triton™ X-100), blocking buffer (10X), thin plate seals, Actinomycin D (Fisher Scientific Product No BP606-5) or other Caspase 3 and p53 inducers, paraformaldehyde (16%) (Thermo Scientific Product No. 28908 or 28906), collagen-I coated clear-bottom 96-well microplates (e.g., BD BioCoat™ Plate, Product No. 354407), cells, and medium.

Cells and Cell Culture

This protocol is optimized for live A549 lung carcinoma cells (American Type Culture Collection Product No. CCL-185) growing on standard high-density microwell plates. MDCK, IMR-90 and NIH 3T3 cell lines have been tested successfully for Caspase 3 and p53. Using cell lines other than A549 might require protocol optimization.

For routine culture of A549 cells, use EMEM Complete Media (Thermo Scientific™ HyClone™ Product No. SH30024) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100µG/mL streptomycin, 1X non-essential amino acids and 1mM sodium pyruvate. Split cells when they reach 70-80% confluence (2-3 times per week) at a ratio of 1:3-1:8. Use cells at a passage number ≤ 20. Harvest cells by trypsinization, dilute into EMEM Complete Medium, and determine cell density. Dilute cells to 10^5 cells/mL in EMEM Complete Medium and add 100µL of the cell suspension per well of a 96-well microplate (recommended plating density = 10,000 cells/well).

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Procedure (with a 96-well plate)

1. Plate 10,000 cells of A549 in 100µL EMEM complete media per well and incubate 16-24 hours at 37°C in 5% CO_2. Assign control wells in the microplate.

2. Prepare 1.6mM actinomycin D stock solution in sterile DMSO. Dilute Actinomycin D to 4.8µM in culture medium (e.g., 30µL of 1.6mM actinomycin D in 10mL EMEM complete media) or other test compound to appropriate concentration. Add 50µL of 4.8µM actinomycin D to each treatment well and add 50µL of culture medium to the control wells. Incubate plate for 24 hours at 37°C in 5% CO_2.

3. Dilute BrdU (100mM) to 160µM in warm (37°C) culture medium (e.g., 12µL of BrdU in 7.5mL of medium per plate). Add 50µL of BrdU in culture medium to each control and treatment well and incubate for 30 minutes at 37°C in 5% CO_2.

4. Add 60µL/well of warm 16% formaldehyde and incubate in a fume hood at room temperature (RT) for 30 minutes.

5. Aspirate formaldehyde and wash plate twice with 100µL/well of 1X Wash Buffer.

6. Aspirate buffer, add 100µL/well of 1X Permeabilization Buffer and incubate for 15 minutes at RT.

7. Aspirate Permeabilization Buffer, add 100µL/well of 1X Blocking Buffer and incubate for 15 minutes at RT.

8. Aspirate Blocking Buffer and add 50µL/well of Primary Antibody Solution. Incubate plate for one hour at 37°C.

9. Aspirate Primary Antibody Solution and wash plate twice with 100µL/well of 1X Blocking Buffer.

10. To avoid cross-reactivity between the secondary antibodies, sequentially add antibodies. Aspirate Blocking Buffer and add 50µL/well of Staining Solution 1 containing DyLight 649 Donkey Anti-Goat secondary antibody. Incubate for 30 minutes protected from light at RT.

11. Wash plate twice with 100µL/well of 1X Blocking Buffer.

12. Aspirate buffer and add 50µL/well of Staining Solution 2. Incubate plate for 30 minutes at RT protected from light.

13. Wash plate twice with 100µL/well of 1X Wash Buffer. Aspirate buffer and add 100µL/well of 1X Wash Buffer.

14. Seal plate and evaluate on the ArrayScan XTI HCA Reader. Store plates at 4°C.

Imaging and Analysis

Images were acquired on an ArrayScan XTI HCA Reader using 10X magnification. For analysis, the Thermo Scientific™ Compartmental Analysis bioapplication was used. Readout measurements included cell number, cell size, nuclear area and intensity. Values for output features were imported into GraphPad™ software and dose response curves were generated.

Results

The basis for this assay is to quantify cell cycle and apoptosis features under different conditions and drug treatments and determine if the cell cycle arrest and apoptosis progress occur as interrelated events in response to various cytotoxic insults. A549 cells were treated with Actinomycin D (1.6mM) or Camptothecin (50mM) for 24 hours. BrdU incorporation, active Caspase 3 and p53 were detected using reagents for measuring mitosis and apoptosis. Negative and positive controls for BrdU, active Caspase 3 and p53 indicate a measurable model for BrdU incorporation, Caspase 3 activation, and p53 modulation by cytotoxic chemicals. (Figure 1)

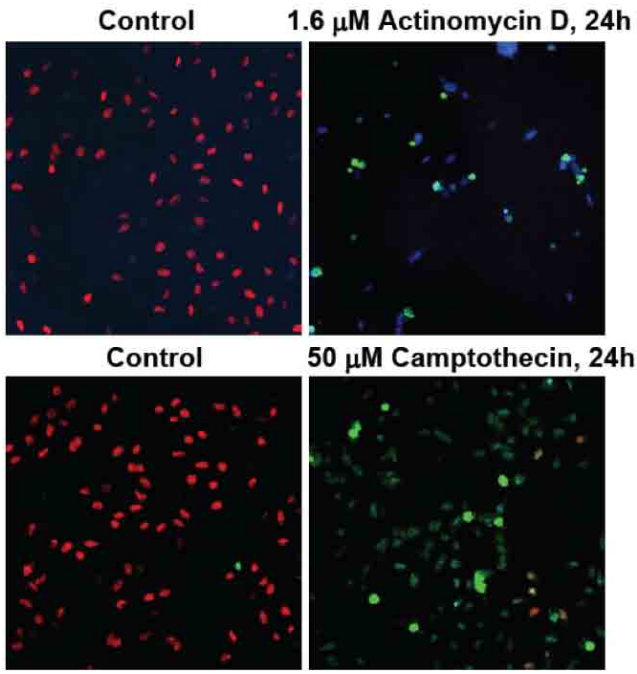

Figure 1: Images of A549 cells at 10x magnification showing untreated and treated (Actinomycin D or Camptothecin) controls for BrdU, Active Caspase 3 and p53.

As above, cells were treated with Actinomycin D, however, a time course was used for dosing. After 24 hour incubation, the multiwell plates were scanned, and A549 cells showed differing responses for BrdU, Caspase 3 and p53, showing a time dependent response to Actinomycin D. (Figure 2)

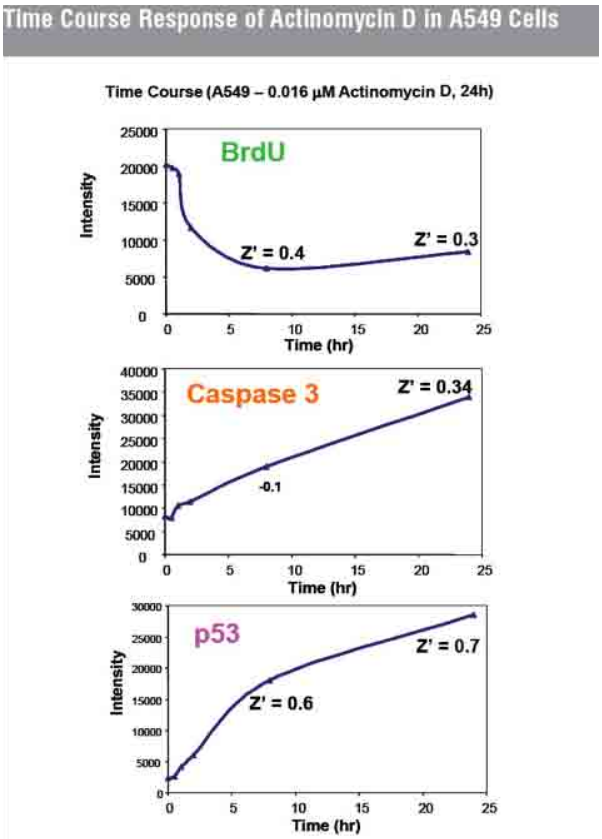

Figure 2: Time dependent drug effects on cell proliferation and induction of apoptosis markers were measured with Actinomycin D treatment. Cell proliferation is inhibited rapidly after Actinomycin D treatment. Apoptosis markers, such as active Caspase 3 and p53, show gradual response with Actinomycin D treatment. (t1/2, BrdU=1.7 h, t1/2, Caspase 3=20.1 h, t1/2, p53=7.3 h)

Conclusions

This application note presents an image-based assay that allows the simultaneous monitoring of cell proliferation and apoptosis in cells to provide insight on specific mechanisms involving cell cycle arrest and cell death progression. Measuring Caspase 3 activation, p53 modulation and BrdU incorporation may be performed simultaneously in an automated manner using the ArrayScan XTI HCA Reader.

References

1. Roberts, R.A., *et al.* (2007) Perturbation of the mitosis/apoptosis balance: A fundamental mechanism of toxicology. *Fundam. Appl. Toxicol.* **38:**107-15.

2. Taylor, D.L., *et al.* (2007) High content screening: A powerful approach to systems cell biology and drug discovery. *Method Mol Biol* **356.** Humana Press, Totowa, N.J.

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