Quantitating responses of subpopulations in cellular assays using the IN Cell Analyzer 1000 Multi Target Analysis Module

Key words: *multiplex • classification • apoptosis • subpopulations • cell cycle • IN Cell Analyzer*

Cultured cell populations rarely behave in synchrony, yet many high-content analysis routines are designed to extract measurements that are averaged over the entire population. New analysis modules for the IN Cell Analysis platforms extract multiparametric measurements from each cell and employ advanced hierarchical classification tools to assign cells to user-defined subpopulations. The percentage of cells in each subpopulation is then reported together with the multiparametric data associated with cells in each class.

The Multi Target Analysis Module for the IN Cell Analyzer 1000 has been developed to analyze complex multicolor cell based assays with user-defined classification strategies. The module utilizes multidimensional graphical displays to visualize characteristics of cells within a population, whilst interactive filters permit the definition of subpopulations. The module allows the analysis of several object types including three organelles and two reference features and reports a wide range of intensity and morphology based output measures. Importantly, the module allows the user to build complex hierarchical classification systems by linking multiple subpopulation analyses.

This application note demonstrates the utility of the Multi Target Analysis Module in the visualization, definition, and analysis of subpopulations present in complex, multicolor, live and fixed cellular assays. The effects of compounds upon cellular integrity and viability have been assessed using combinations of the fluorescent indicators; calcein AM, propidium iodide (PI) and FITC-Annexin V. Whilst the effects of cell cycle inhibitors have been assessed using combinations of Hoechst™ 33342, G1S Cell Cycle Phase Marker (G1S CCPM) or G2M Cell Cycle Phase Marker (G2M CCPM) sensors, and the immunofluorescent detection of bromodeoxyuridine (BrdU) incorporation.

Materials

Products used

IN Cell Analyzer 1000*	28-4051-28
IN Cell Investigator, 1 seat license†	28-4089-71
IN Cell Investigator, 1 additional seat license†	28-4089-75
IN Cell Investigator, 5 seat license†	28-4089-72
IN Cell Translator Software	28-4047-40
G1S Cell Cycle Phase Marker Assay	25-9003-97
G2M Cell Cycle Phase Marker Assay	25-8010-50
Cell Proliferation Fluorescence Assay	25-9001-89

* IN Cell Analyzer 3000 may also be used for these experiments.

† A seat license is a cost-effective single-user or server license that gives access to all ready to use Image Analysis Modules provided for your IN Cell Analyzer instrument. License holders have access to all appropriate analysis software and more licenses can be purchased as the number of users grows.

Other materials required

Cell lines

SH-SY5Y, A549, U-2 OS (ECACC)

Culture medium

SH-SY5Y cells: 1:1 mixture of nutrient mixture F-12 Ham and minimum essential medium Eagle supplemented with 10% fetal bovine serum, 2-mM L-glutamine, 100-µg/ml penicillin-streptomycin and 1× non-essential amino acids (Sigma-Aldrich)

A549 cells: Nutrient mixture F-12 Ham supplemented with 10% fetal bovine serum, 2-mM L-glutamine, 100-µg/ml penicillin-streptomycin (Sigma-Aldrich)

U-2 OS cells: McCoy's 5A medium with supplements as for A549 cells (Sigma-Aldrich)

G1S CCPM and G2M CCPM cells: McCoy's 5A medium with supplements as for A549 cells and 500-µg/ml geneticin	(Sigma-Aldrich)
Test compounds	
Ionomycin, vinblastine, paclitaxel, nocodazole, roscovitine	(Sigma-Aldrich)
Reagents	
Calcein AM	(Sigma-Aldrich)
Hoechst 33342	(Molecular Probes)
Propidium iodide	(Sigma-Aldrich)
FITC-Annexin V	(Abcam)
96-well Viewplates™	(Perkin Elmer)
µClear™ 96-well microplates, black	(Greiner Bio-One GmbH)

Methods and analysis

Cells in log-phase growth were seeded into 96-well microplates (5000 cells per well) and incubated in culture medium for 24 h at 37°C, 5% CO_2. Culture medium was removed from the cells and replaced with culture medium (100 µl) containing test compounds (n = 8) and incubated at 37°C, 5% CO_2. Images were analyzed directly using the IN Cell Analyzer 1000 Multi Target Analysis Module. Hoechst 33342 emits fluorescence when bound to DNA and was used to identify and segment nuclei as objects. Where appropriate, additional cellular compartments were segmented to enable the classification of individual cells into subpopulations based on the relative fluorescence intensity and localization of appropriate reporters. The analysis module was configured to report the number and percentage of cells in each subpopulation together with the multiparametric data associated with cells in each class.

Cell viability assay

SH-SY5Y and A549 cells were exposed to ionomycin or paclitaxel for 24 h. Complete medium containing Hoechst 33342, calcein AM, and PI was added (to final concentrations of 5 µM, 0.1 µM, and 5 µM respectively) and cells were incubated for 10 min at 25°C. Images were acquired on the IN Cell Analyzer 1000 using a 10× objective and 360/40-nm excitation and 460/40-nm emission filters with 2000-ms exposure for Hoechst 33342; 475/20-nm excitation and 535/50-nm emission filters with 1000-ms exposure for calcein; 570/20-nm excitation and 620/60-nm emission filters with 100-ms exposure for PI.

Live and dead subpopulations were defined using a 2-D scatter plot, configured to identify and measure the green fluorescence of calcein and the red fluorescence of PI. Endogenous esterases active only in living cells hydrolyze calcein AM to the membrane-impermeant green fluorescent product calcein, which is retained within live cells. In contrast the nucleic acid intercalator, PI, is membrane impermeant and is a marker of dead cells.

Apoptosis assay

U-2 OS cells were exposed to ionomycin for 4 h or vinblastine or paclitaxel for 24 h. Complete medium containing Hoechst 33342, FITC-Annexin V, and PI was added (to final concentrations of 10 µM, 1:1000, and 5 µM respectively) and incubated for 10 min at 25°C. Images were acquired on the IN Cell Analyzer 1000 using a 10× objective and 360/40-nm excitation and 460/40-nm emission filters with 1000-ms exposure for Hoechst 33342; 475/20-nm excitation and 535/50-nm emission filters with 800-ms exposure for FITC-Annexin V; 570/20-nm excitation and 620/60-nm emission filters with 200-ms exposure for PI.

A hallmark of apoptotic and necrotic cell death is the translocation of phosphatidylserine (PS) to the outer leaflet of the plasma membrane (1). The exposure of PS can be detected using a fluorescently labeled annexin V conjugate, which when used in combination with PI enables the discrimination of apoptotic and dead cells. All cells were identified by Hoechst 33342 nuclear staining. Apoptotic cells expose PS early in the process and therefore exhibit additional FITC-Annexin V fluorescence, which can occur anywhere within the cell boundary. For dead cells, PS exposure occurs later once cells have lost membrane integrity and therefore these cells exhibit cellular fluorescence due to FITC-Annexin V and also nuclear fluorescence due to PI staining.

Note: *Cell viability and apoptosis are live-cell assays; removal of culture medium from wells or fixation of cells after treatment with test compounds should be avoided since dead, necrotic, and poorly attached cells will be detached by this process.*

G1S CCPM Assay

G1S CCPM cells were exposed to roscovitine for 24 h or nocodazole for 16 h. Cells were incubated with BrdU (1:500) for 1 h prior to fixation and staining with Hoechst 33342 (2 µM). BrdU was detected using anti-BrdU primary antibody and Cy™5 labeled secondary antibody (Cell Proliferation Fluorescence Assay). Images were acquired using 360/40-nm excitation and 460/40-nm emission filters with 750-ms exposure for Hoechst 33342; 475/20-nm excitation and 535/50-nm emission filters with 1000-ms exposure for GFP; 620/60-nm excitation and 700/75-nm emission filters with 400-ms exposure for Cy5.

Cells undergoing DNA replication (S-phase) incorporate BrdU and are therefore labeled using the Cell Proliferation Fluorescence Assay and exhibit an increased Cy5 nucleus: cell intensity ratio. The phenotype of G2-phase cells shows the G1S CCPM sensor to be mainly expressed in the cytoplasm, with very little expression in the nuclear region. The nucleus: cell intensity fluorescence ratio of the sensor in the G2-phase cells is therefore lower than for the remaining G1- and M-phase cells. In a fixed cell assay under standard, non-saturated imaging conditions, the nuclear integrated intensity of Hoechst

33342 fluorescence relates to the amount of DNA in the nucleus and is therefore useful for determining the nuclear DNA content of cells. Cells with an increased nuclear intensity were classified as in M-phase of the cell cycle and remaining cells were classified as G1-phase.

G2M CCPM Assay

G2M CCPM cells were exposed to a novel compound (A) for 24 h and 48 h. Cells were incubated with BrdU and Hoechst 33342 and BrdU staining was detected as described for the G1S CCPM Assay. Images were acquired using 360/40-nm excitation and 460/40-nm emission filters with 500-ms exposure for Hoechst 33342; 475/20-nm excitation and 535/50-nm emission filters with 2000-ms exposure for GFP; 620/60-nm excitation and 700/75-nm emission filters with 500-ms exposure for Cy5.

S-phase cells exhibited an increased Cy5 nucleus:cell intensity ratio as described for the G1S CCPM Assay. In G1- and G2-phase cells, the G2M CCPM sensor is found mostly in the cytoplasm and therefore the nuclear fluorescence intensity of the sensor is much lower than for the remaining M-phase cells. The fluorescence intensity of the G2M CCPM sensor in the cytoplasm of G2-phase cells is brighter than for G1-phase cells.

Results and discussion

Cell viability

Live and dead cell populations were vizualized for classification (Fig 1) using a 2-D scatter plot of the integrated (total) nuclear intensity of calcein (representing live cells) and integrated nuclear intensity of PI (representing dead cells). The populations were defined using two linear discriminants, which were moved manually to specify live, dead, and unclassified populations (unclassified objects arise due to inappropriate segmentation or represent false objects due to compound autofluorescence). The number and percentage of each population were then reported.

Treatment of A549 cells with the cytotoxic compound ionomycin, resulted in a dose-dependent decrease in both cell number and live cells (%) and a corresponding increase in dead cells due to cytotoxicity (Fig 2A). Similar results were obtained for ionomycin treatment of SH-SY5Y cells (Fig 2B). However, treatment of SH-SY5Y cells with low concentrations (between 2 and 500 nM) of the cell cycle inhibitor paclitaxel induced a reduction in cell number that was not associated with cytotoxicity (Fig 2B). At higher concentrations (> 30 µM), cytotoxicity caused a decrease in the number of live cells.

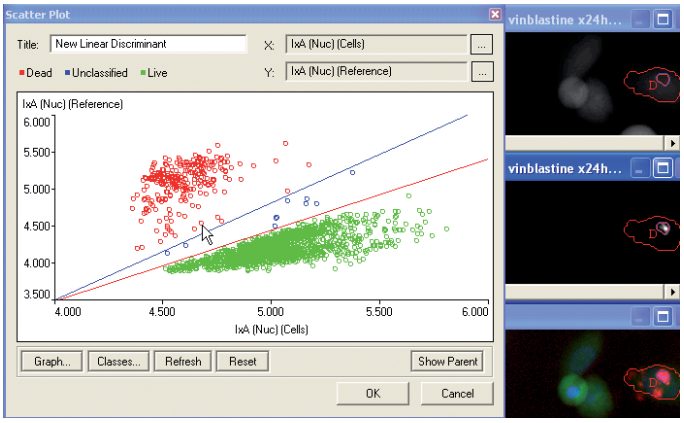

Fig 1. Visualization and classification of live and dead subpopulations in calcein-AM/PI cell viability assay. Live (green), dead (red) and unclassified (blue) subpopulations were visualized using a 2-D scatter plot of the integrated (total) nuclear intensity of calcein (green channel; x-axis; log10) and PI (red channel; y-axis; log10) for individual objects. The two user-defined linear discriminants are visible as blue and red lines. Scatter plots are interactive: clicking on a specific data point in the plot highlights the corresponding cell in the image display window (right) facilitating improvement/confirmation of the classification strategy.

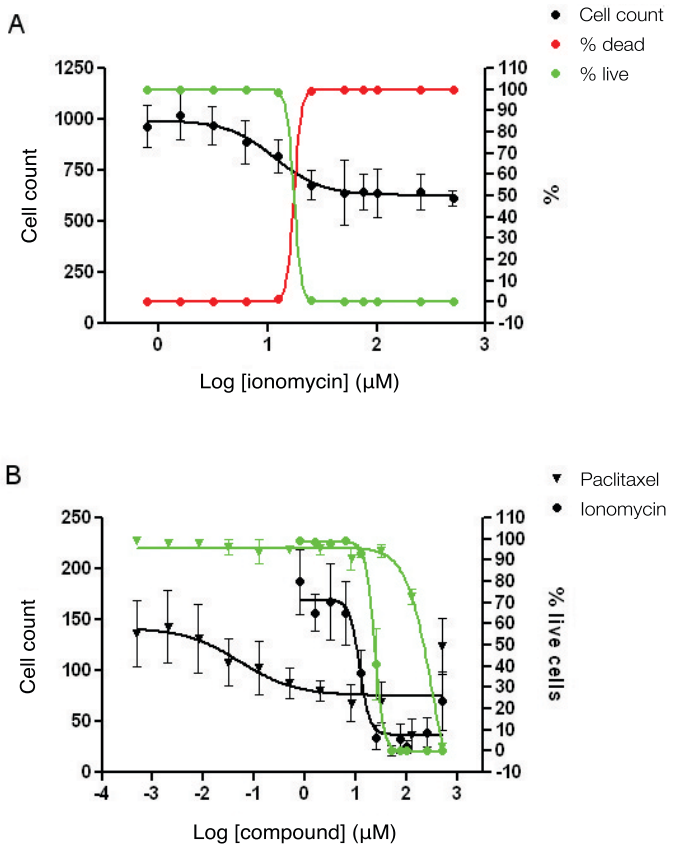

Fig 2. Dose response curves for cell viability assays. Subpopulations have been classified using the 2-D linear discriminant filter (Fig 1) in the Multi Target Analysis Module. (A) A549 cells were exposed to ionomycin for 24 h (EC_{50} values of 11.65 and 17.34 µM were obtained for cell number and live/dead subpopulations, respectively). (B) SH-SY5Y cells were exposed to ionomycin or paclitaxel for 24 h. Black and green curves denote cell number and % live cells, respectively (EC_{50} values of 11.9 and 23.2 µM were obtained for cell number and % live cells for ionomycin treatment, respectively; EC_{50} values of 0.053 and 330 µM were obtained for cell number and % live cells for paclitaxel treatment, respectively). Images were acquired on the IN Cell Analyzer 1000 and data analyzed by non-linear regression (mean ±SD, n = 8) using GraphPad™ Prism™ software.

Apoptosis and viability

In the Multi Target Analysis Module, classification of healthy, apoptotic and dead (secondary and primary necrotic) subpopulations present in annexin V/PI assays was achieved with a simple two-step hierarchical decision process (Fig 3). A 1-D frequency plot and user-defined threshold was used to visualize and discriminate the subpopulation of cells exhibiting PI fluorescence (dead cells). The remaining cells were visualized on a 2-D scatter plot of FITC-Annexin V fluorescence and Hoechst 33342 nuclear intensity and classified into healthy and apoptotic populations by applying a user-defined linear discriminant.

Ionomycin treatment of U-2 OS cells for 4-h caused cytotoxicity, resulting in a reduction in cell number and induction of apoptosis and necrosis, evident from the increased FITC-Annexin V and PI fluorescence (Fig 4A). In contrast, 24-h treatment of U-2 OS cells with vinblastine, caused cytotoxicity at higher concentrations (> 30 µM) shown by the decrease in both cell number and healthy cells but also induced a cytostatic effect at lower concentrations (between 1 and 25 nM) observed as a decrease in cell number only (Fig 4B). No apoptosis was observed after 24-h treatment with vinblastine.

In U-2 OS cells, exposure to the cell cycle inhibitor paclitaxel produced a biphasic, dose-dependent decrease in cell number and a decrease in viable cells at high concentrations due to cytotoxicity (Fig 5A). Analysis of selected multiparametric measures associated with the healthy subpopulation, demonstrated changes in both nuclear area and form factor (associated with nuclear symmetry; Fig 5B) that correlated with the cell number decrease effected by lower concentrations of drug. Stabilization of mitotic spindles resulting in G2/M arrest is characteristic of cells exposed to low concentrations of paclitaxel (2) and the cytostatic effect correlates with the observed changes in nuclear area and symmetry of the healthy population. No apoptosis was detected after 24-h treatment with paclitaxel.

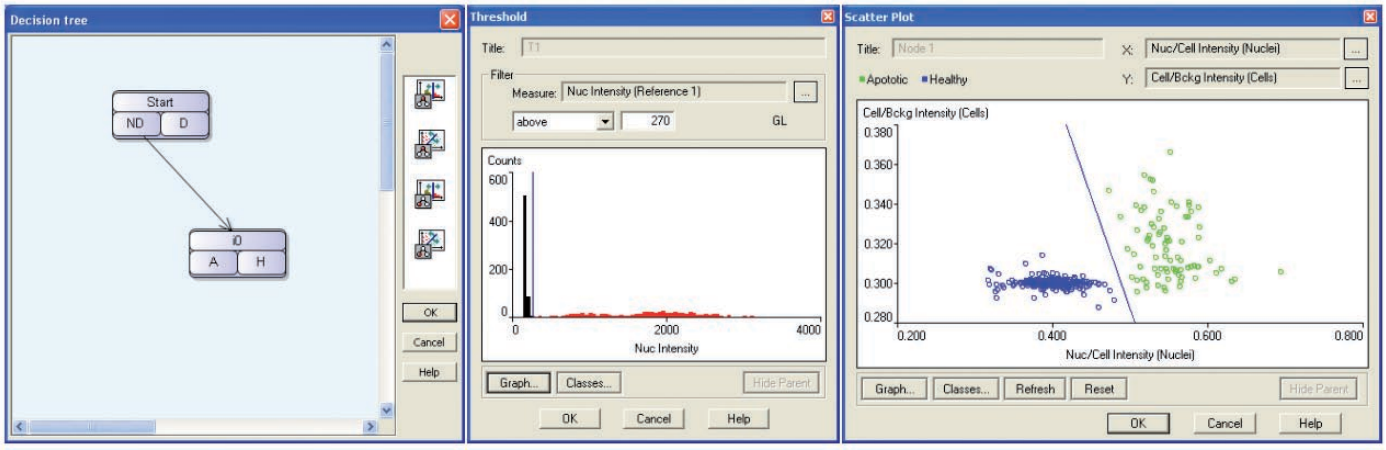

Fig 3. Visualization and classification of healthy, apoptotic, and dead subpopulations in FITC-Annexin V/PI apoptosis assay. Analysis employed a user-defined two-step hierarchical decision making protocol (left) to define three subpopulations. In Step 1, cells exhibiting nuclear PI fluorescence above background levels were visualized using a 1-D frequency plot (centre) and a user-defined threshold (blue line) was applied to discriminate the dead (red, dead population D) subpopulation of cells from cells that are not dead (population ND). In Step 2, population 'ND' was visualized using a 2-D scatter plot (right) of FITC-Annexin V fluorescence (y-axis) and Hoechst 33342 nuclear intensity (x-axis). Apoptotic cells (green, FITC-Annexin V stained population A) and healthy cells (blue-only, population H) were classified by applying a user-defined linear discriminant (blue line).

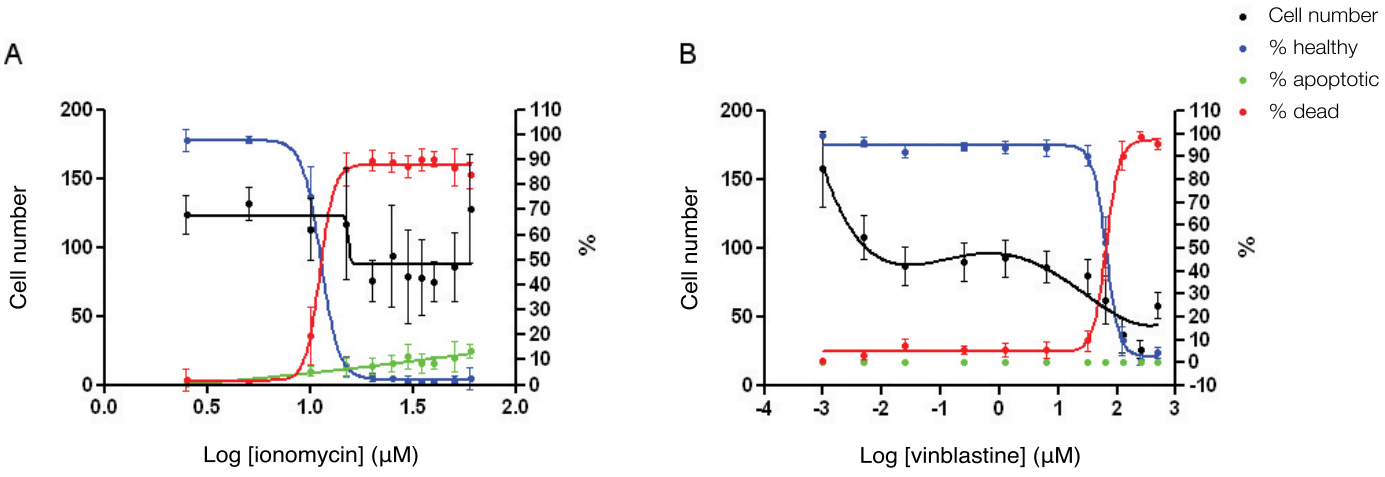

Fig 4. Dose response curves for apoptosis assays analyzed using a two-step hierarchical decision protocol in the Multi Target Analysis Module. U-2 OS cells were exposed to (A) ionomycin for 4 h (EC_{50} values of 11.25, 106, and 11.13 µM were obtained for healthy, apoptotic, and dead populations, respectively), and (B) vinblastine for 24 h (an EC_{50} value of 65.2 µM was obtained for healthy population). Images were acquired on the IN Cell Analyzer 1000. Data were analyzed by non-linear regression (mean ±SD, n = 8) using GraphPad Prism software.

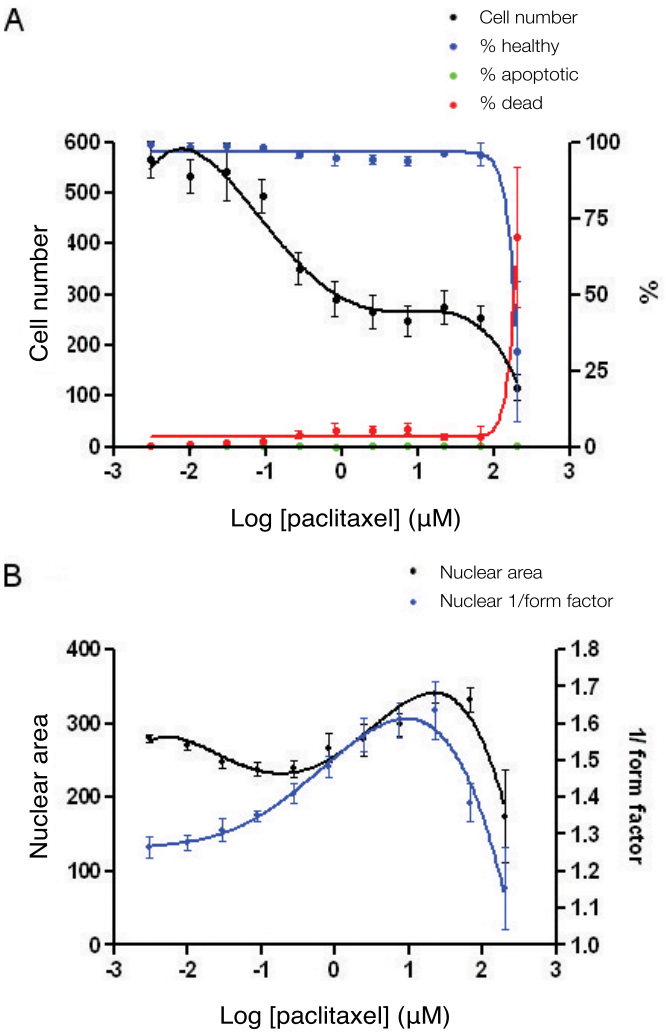

Fig 5. Dose response curves for apoptosis assay with U-2 OS cells exposed to paclitaxel for 24 h. Results shown are (A) whole population (EC_{50} values of 0.23, 295.6, and 291.6 µM were obtained for cell number, healthy, and dead populations respectively); and (B) reported measures for healthy subpopulation only. Images were acquired on the IN Cell Analyzer 1000. Data were analyzed by non-linear regression (mean ±SD, n = 8) using GraphPad Prism software.

Cell cycle

For the G1S CCPM Assay, the cell cycle subpopulations were classified using a hierarchical process consisting of three linked 1-D threshold filters (Fig 6). S-phase cells were first discriminated based on increased red fluorescence due to Cy5 labeled secondary antibody. Cells with a Cy5 nucleus: cell intensity ratio greater than the threshold are classified S-phase and reported. The remaining cells were classified further using a threshold filter to discriminate G2-phase cells. The nucleus: cell intensity fluorescence ratio of the sensor in the G2-phase cells is lower than for the remaining G1- and M-phase cells and a suitable threshold was selected for discrimination. Finally, G1- and M-phase cells were discriminated based on integrated (total) nuclear intensity of Hoechst 33342. Cells with an integrated nuclear intensity greater than the threshold value were classified as in M-phase of the cell cycle and remaining cells were classified as G1-phase.

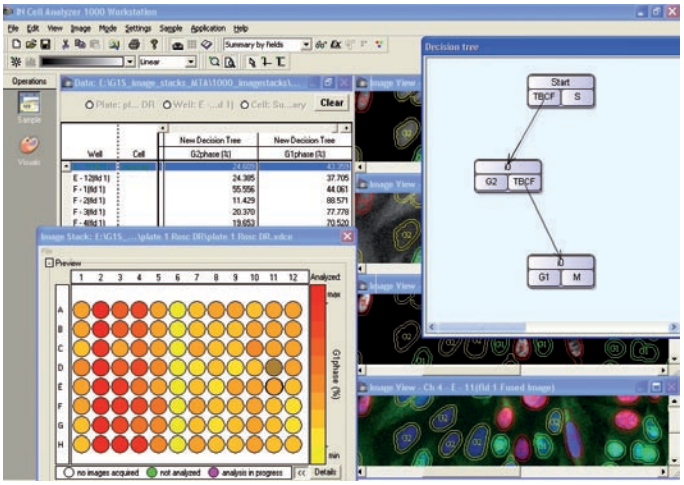

Fig 6. G1S cell cycle analysis using a three-step hierarchical process based on thresholded 1-D characteristic plots. In the first step of analysis strategy, cells were classified into S-phase (red, BrdU incorporation) through analysis of the red nuclear signal (anti-BrdU Cy5). G2-phase cells were highlighted in the second step due to the characteristically low green nucleus: cell intensity ratio of the G1S CCPM sensor in these cells. G1-phase and M-phase cells were discriminated in the final step on the basis of DNA content (2n or 4n) using the total blue nuclear intensity of the Hoechst 33342 signal. Individual cells are labeled in the image display window according to classification, whilst the heatmap can be used to display subpopulation analysis for the whole plate.

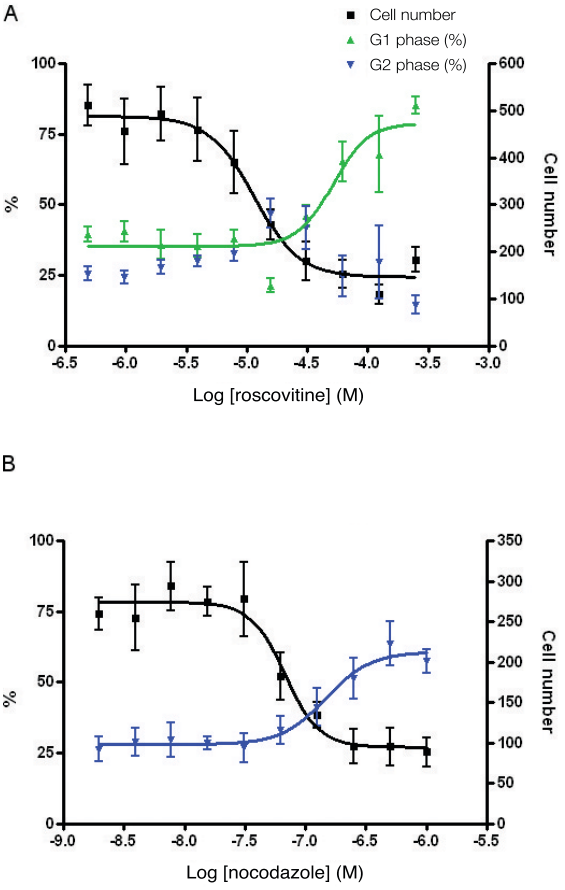

Fig 7. Dose response curves for G1S CCPM Assays. G1-, S-, G2-, and M-phase subpopulations were classified according to a three-step hierarchical decision process. U-2 OS cells stably expressing the G1S CCPM sensor were treated with increasing concentrations of (A) roscovitine for 24 h (EC_{50} values of 11.5, 51.2, and 2.25 µM were obtained for cell number, % G1, and % G2 respectively), and (B) nocodazole for 16 h (EC_{50} values of 67.9 and 150 nM were obtained for cell number and % G2 respectively). Images were acquired on the IN Cell Analyzer 1000. Data were analyzed by non-linear regression (mean ±SD, n = 8) using GraphPad Prism software.

Treatment of the G1S CCPM stable cell line with roscovitine, a cyclin-dependent kinase 1/2 inhibitor, resulted in cell cycle arrest in G1- and G2- phases (Fig 7A). At lower concentrations between 5 and 15 µM, a significant increase in the G2 population was observed. Treatment with high concentrations of roscovitine (> 30 µM) resulted in a dose-dependent increase in the G1 population and a reciprocal decrease in G2-phase cells (%). Exposure of the cells to increasing concentrations of nocodazole, a microtubule assembly inhibitor, induced cell cycle arrest in G2-phase (Fig 7B).

The cell cycle populations for the G2M CCPM Assay were classified based on a decision process involving two threshold filters and a 2-D scatter plot filter. The first threshold filter was set up to discriminate S-phase cells as for the G1S CCPM Assay. The second filter was a threshold filter to discriminate M-phase cells from G1- and G2-phase based on a threshold value for the nuclear fluorescence intensity of the G2M CCPM sensor. Finally, G1- and G2-phase cells were discriminated using a 2-D scatter plot filter, using a combination of the nucleus: cell intensity fluorescence ratio of the G2M CCPM sensor, and the cell intensity of Hoechst 33342 fluorescence.

Treatment of the G2M CCPM stable cell line with higher concentrations of a novel compound (compound A) for 24 h demonstrated a decrease in cell number, an increase in G1-phase cells (%) and a reciprocal decrease in S-phase cells, with no observed effect on G2-phase cells (Fig 8A). The effect is consistent with cell cycle arrest in G1-phase and is more evident after treatment for 48 h (Fig 8B). In contrast, closer examination of the nuclear characteristics of the cells after 48 h, demonstrated an increase in nuclear area with increasing concentration of compound A (Fig 9); indicative of a G2 phenotype and G2-phase arrest. However, an additional observation is the corresponding increase in nuclear asymmetry (evident from the observed increase in nuclear 1/form factor) suggesting an abnormal mitosis.

Table 1. EC_{50} concentrations of compound A for cell number, cell cycle populations, and nuclear characteristics measurements for 24 and 48-h treatment of the G2M CCPM cell line.

	EC_{50} (µM)	
	24 h	**48 h**
Cell number	0.049	0.064
% G1-phase	>10	0.014
% S-phase	>10	0.019
Nuclear area	ND*	0.108
Nuclear form factor	ND*	0.105

* Not determined.

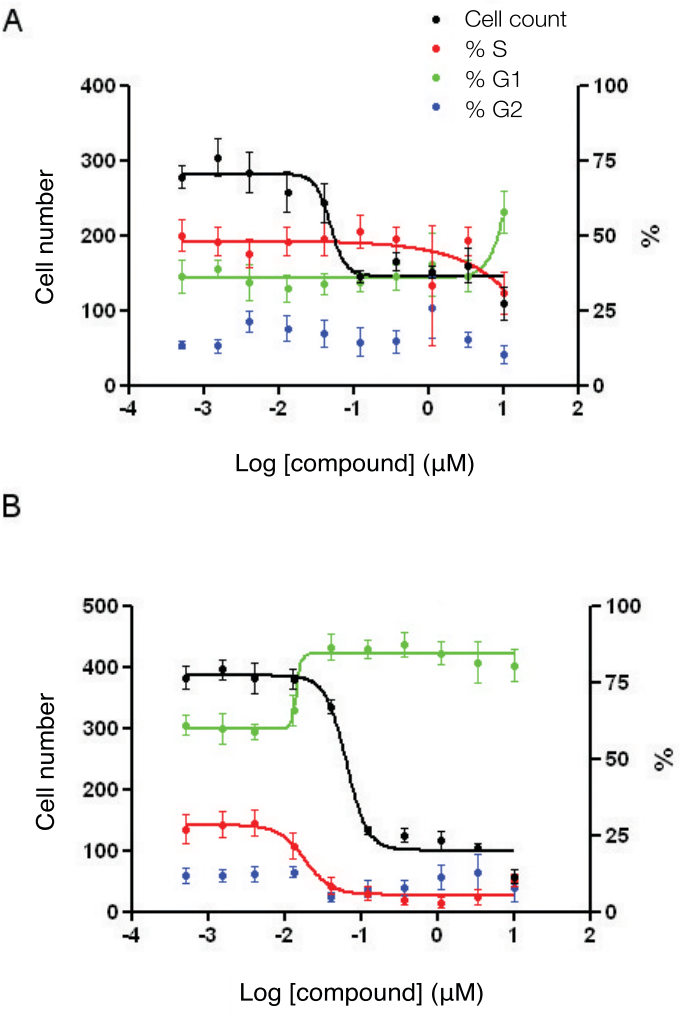

Fig 8. Dose response curves for G2M CCPM Assays analyzed using a three-step hierarchical decision process. U-2 OS cells stably expressing the G2M CCPM sensor were treated with increasing concentrations of compound A for (A) 24 h or (B) 48 h. Images were acquired on the IN Cell Analyzer 1000. Data were analyzed by non-linear regression (mean ±SD, n = 2; four fields of view) using GraphPad Prism software and EC_{50} values calculated (Table 1).

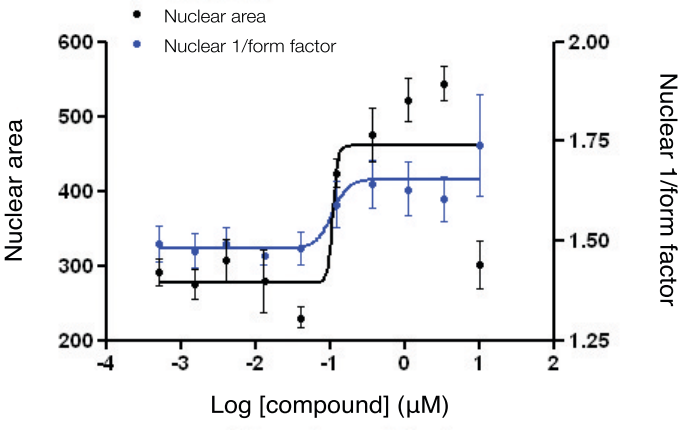

Fig 9. Dose response curves for nuclear characteristics measurements in G2M CCPM Assay. U-2 OS cells stably expressing the G2M CCPM sensor were treated with increasing concentrations of compound A for 48 h. Images were acquired on the IN Cell Analyzer 1000. Data were analyzed by non-linear regression (mean ±SD, n = 2; four fields of view) using GraphPad Prism software and EC_{50} values calculated (Table 1).

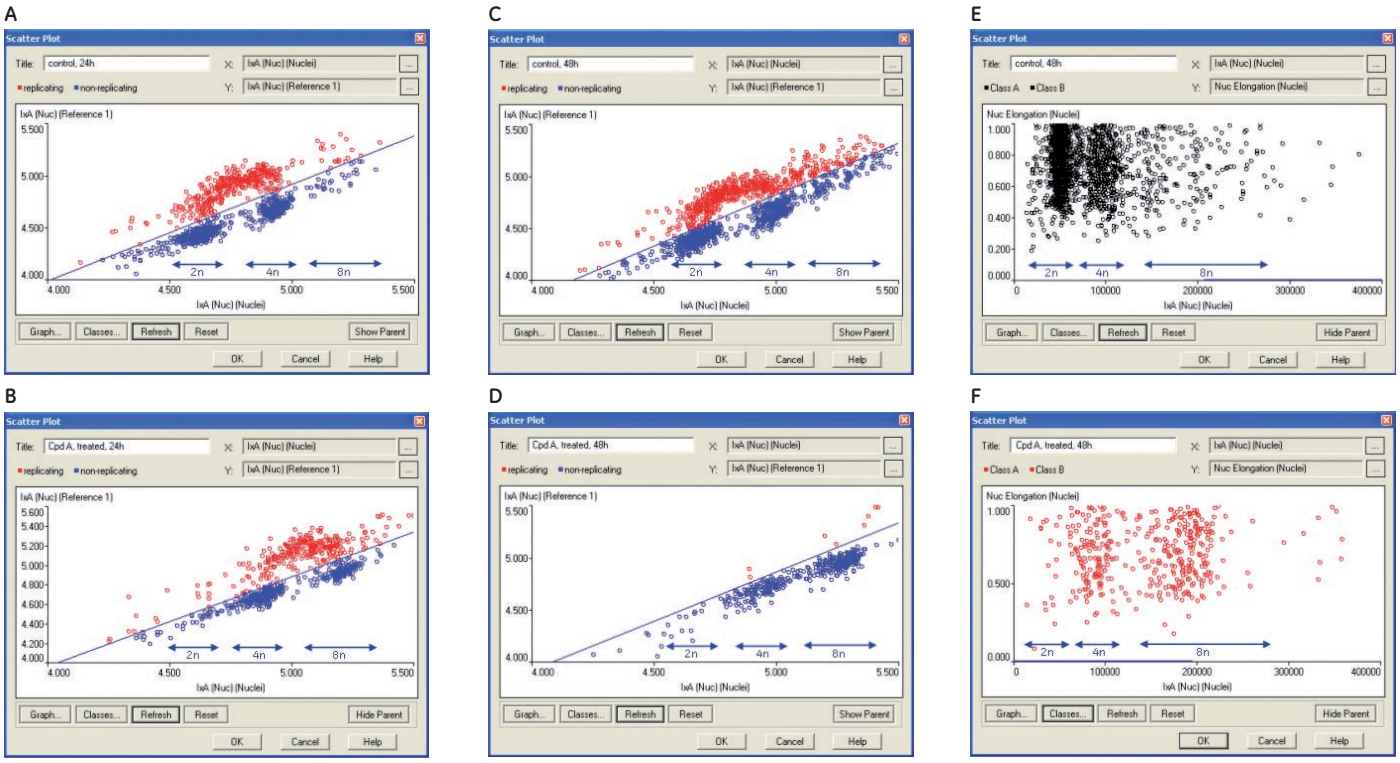

Fig 10. Analysis of DNA replication (BrdU incorporation), DNA content (integrated Hoechst 33342 intensity) and nuclear asymmetry (elongation) in G2M CCPM Assay using the 2-D scatter plot feature. U-2 OS cells stably expressing the G2M CCPM sensor were treated with culture medium (control, top panel; A, C, and E) or compound A (treated, bottom panel; B, D, and F) for 24 and 48 h. Interactive 2-D scatter plots were generated for A and B, total BrdU intensity (y-axis) and total Hoechst 33342 intensity (x-axis), 24 h; (C and D), total BrdU intensity (y-axis), and total Hoechst 33342 intensity (x-axis), 48 h (red data points represent replicating cells, blue data points represent non-replicating cells); (E and F), nuclear elongation (y-axis) and total Hoechst 33342 intensity (x-axis), 48 h (black and red data points represent control and treated cells respectively). Each plot shows individual cell data from control or treated (3.3-µM compound A) wells (n = 1; four fields of view).

Further analysis and visualization of the level of BrdU incorporation and total Hoechst 33342 intensity (representing DNA replication and genomic complement respectively) for cells treated with compound A, revealed a decrease in the number of replicating cells (further evidence of G1-phase arrest) and an increase in the number of cells demonstrating greater DNA complement (4n and 8n; Figs 10A–F). After 48 h, the number of replicating cells has significantly decreased (Fig 10D) and the majority of cells exhibit 4n and 8n DNA complement (Fig 10F). These results suggest that compound A has caused endoreduplication, leading to polyploidy and cell cycle arrest in G1-phase.

Conclusions

The advanced hierarchical classification tools within the Multi Target Analysis Module can be used to analyze a range of complex multicolor cell based assays and identify cells in multiple subpopulations. The example assays utilized a combination of fluorescent markers to discriminate cell viability, apoptosis, and cell cycle populations in both live- and fixed-cell format.

Ionomycin treatment of A549 cells produced a dose-dependent decrease in cell number and a corresponding decrease in cell viability (EC_{50} = 11.65 and 17.34 µM respectively). Ionomycin treatment of SH-SY5Y cells produced similar results (EC_{50} = 11.9 and 23.2 µM for cell number and viability, respectively). Treatment of SH-SY5Y cells with the cell cycle inhibitor paclitaxel, caused a decrease in cell number at lower concentrations (corresponding to a cytostatic effect) and a decrease in both cell number and viability at higher concentrations due to cytotoxicity.

In the detection of apoptosis, ionomycin treatment of U-2 OS cells caused rapid cytotoxicity and induced apoptosis and necrosis (EC_{50} = 11.25, 106, and 11.13 µM for healthy, apoptotic, and dead populations respectively). Exposure of U-2 OS cells to both vinblastine and paclitaxel produced a biphasic dose-dependent decrease in cell number and an increase in necrotic cells at high concentrations, due to cytotoxicity. Further investigation of the cytostatic effect of paclitaxel at lower concentrations, showed a correlation with an increase in both nuclear area and form factor, characteristic of G2/M arrest.

In the analysis of cell cycle populations, treatment of the G1S CCPM stable cell line with roscovitine, a cyclin-dependent kinase 1/2 inhibitor, resulted in cell cycle arrest in G1-phase at high concentrations (EC_{50} = 51.2 µM) and G2-phase at lower concentrations (EC_{50} = 2.25 µM). Exposure of the cells to increasing concentrations of nocodazole, a microtubule assembly inhibitor, produced an increase in the G2 population (EC_{50} = 150 nM). Analysis of the exposure of the G2M CCPM cell line to a novel compound, using the 2-D scatter plot as both classification filter and visualization tool, revealed that the compound caused endoreduplication, resulting in cells with increased DNA complement and cell cycle arrest in G1-phase.

Note: *Most countries have legislation governing the handling, use, storage, disposal, and transportation of mammalian cell lines. Readers must be aware of and observe the Local Regulations or Codes of Practice, which relate to such matters prior to experimentation.*

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Asia Pacific	Tel: +85 65 62751830	Fax: +85 65 62751829
Australasia	Tel: +61 2 8820 8299	Fax: +61 2 8820 8200
Austria	Tel: 01 /57606 1613	Fax: 01 /57606 1614
Belgium	Tel: 0800 73 890	Fax: 02 416 8206
Canada	Tel: 1 800 463 5800	Fax: 1 800 567 1008
Central & East Europe	Tel: +43 1 972 720	Fax: +43 1 972 722 750
Denmark	Tel: +45 70 25 24 50	Fax: +45 45 16 2424
Eire	Tel: 1 800 709992	Fax: +44 1494 542010
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Middle East & Africa	Tel: +30 210 96 00 687	Fax: +30 210 96 00 693
Netherlands	Tel: 0800-82 82 82 1	Fax: 0800-82 82 82 4
Norway	Tel: +47 815 65 777	Fax: +47 815 65 666
Portugal	Tel: 21 417 7035	Fax: 21 417 3184
Russia, CIS & NIS	Tel: +7 495 956 5177	Fax: +7 495 956 5176
Spain	Tel: 902 11 72 65	Fax: 935 94 49 65
Sweden	Tel: 018 612 1900	Fax: 018 612 1910
Switzerland	Tel: 0848 8028 10	Fax: 0848 8028 11
UK	Tel: 0800 515 313	Fax: 0800 616 927
USA	Tel: +1 800 526 3593	Fax: +1 877 295 8102

www.gelifesciences.com

GE Healthcare Bio-Sciences Corp
800 Centennial Avenue
P.O. Box 1327
Piscataway, NJ 08855-1327
USA

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GE Healthcare Bio-Sciences AB Björkgatan 30, 751 84 Uppsala, Sweden

GE Healthcare UK Ltd Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, UK

GE Healthcare Europe GmbH Munzinger Strasse 5, D-79111 Freiburg, Germany

GE Healthcare Bio-Sciences KK Sanken Bldg., 3-25-1, Hyakunincho, Shinjuku-ku, Tokyo 169-0073, Japan

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