Application Note

Cancer Biology

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Monitoring Cell Cycle Progression in Cancer Cells

Using nuclear staining to assess cellular DNA content

Author

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Abstract

Cell cycle progression is a tightly regulated process that involves the duplication of nuclear DNA content before cell division. The control mechanisms that regulate this process are often disrupted in tumor cells and serve as viable targets for therapeutic compounds in the treatment of cancer. Cell cycle progression has historically been monitored using flow cytometry. This application note describes the use of a microplate reader to rapidly image and analyze nuclear-stained tissue culture cells for nuclear content.

Introduction

The progression through the cell cycle and cell division of an organism is a tightly regulated process associated with proliferation and differentiation. Generally, most cells are quiescent and do not undergo division unless signaled to enter the active segments of the cell cycle. In a number of disease states (e.g., cancer, psoriasis, hyperplasia), this regulation is diminished or disrupted. In these instances, it is important to identify the genetic basis and develop therapies to preferentially target those cells with abnormalities. One screening method for potential therapeutic drugs, or the effect of specific genes on cell cycle regulation, is to measure changes in cell cycle kinetics and DNA content using a nuclear stain.

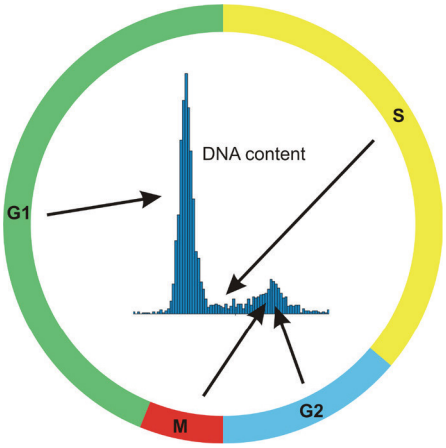

Figure 1. Relationship between cycle and DNA content histogram. As cells progress through the cell cycle, their DNA content doubles before mitosis. Cells treated with the nuclear stain Hoechst 33342 exhibit fluorescence proportional to their DNA content.

Cell cycle analysis by DNA content measurement is a method that until recently used flow cytometry to distinguish cells in different phases of the cell cycle. Before analysis, the cells are treated with a fluorescent dye that stains DNA quantitatively. Propidium iodide is commonly used with flow cytometry due to its ability to be excited with a 488 nm laser common in many systems. The drawback for this dye is that it also binds RNA, necessitating the treatment of cells with RNase before analysis. Nuclear stains such as 4,6-diamino-2-phenylinoloe (DAPI) or Hoechst 33342 require UV excitation, but are specific to DNA. Regardless of the dye used, the fluorescence intensity of the stained cells correlates with the amount of DNA they contain.

As the DNA content doubles during the S phase, the intensity of fluorescence increases in proportion. Thus, cells in G0 and G1 phase (before S) have half the fluorescent signal as those in G2 or M phase (Figure 1). This study used these elements in conjunction with image-based analysis rather than flow cytometry to assess cell cycle phase status and identify compounds that effectively stall cell cycle progression.

Materials and methods

Cell culture

PC-3 cells were cultured in Hams F12K media supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO_2. Cultures were routinely trypsinized (0.05% trypsin-EDTA) at 80% confluency. For experiments, cells were plated into Corning 3904 black-sided clear-bottom 96-well microplates.

Thymidine double block

PC-3 cells were seeded into Corning 3904 plates at a density of 4,000 cells per well in a volume of 100 µL and allowed to attach overnight at 37 °C, 5% CO_2 in a humidified environment. The following day, thymidine was added to all the wells for a final concentration of 2 mM. Cells were treated for 16 hours then released by washing once with fresh media, followed by 100 µL of media without thymidine. Cells were allowed to grow for 9 hours, after which thymidine was added to a final concentration of 2 mM for a second time. Cells were thymidine-treated for 16 hours and released as described previously. Plates were immediately loaded into an Agilent BioTek BioSpa 8 automated incubator for cell cycle progression.

Cell cycle progression

PC-3 cells synchronized using double thymidine block were released from blockade with fresh complete media. Cells were then fixed at various times following release. Using a BioSpa 8 to control timing and maintain the necessary environmental control, individual strips of two plates were fixed with 4% PFA for 10 minutes at 1-hour intervals using an Agilent BioTek MultiFlo FX multimode dispenser attached to the system. Fixed cells were maintained with 200 µL of PBS until the completion of the experiment, after which wells were stained with 10 µM Hoechst 33342 for 30 minutes followed by washing three times with PBS. Cells were maintained in a hydrated state using PBS.

Drug treatments

Twenty-seven cytoactive-agents were obtained from R&D Systems/Tocris and were reconstituted to 10 mM with DMSO and stored at –80 °C. Working stocks of the compounds were adjusted to a concentration of 2 mM with DMSO and stored at –20 °C. On the day of drug induction, the compounds were thawed and further diluted to 20 µM in media. Negative controls (media only), and positive controls (nocodazole, vinblastine, and mevinolin) were also assayed in the same plates. PC-3 cells were seeded into Corning 3904 plates at a density of 4,000 cells per well in a volume of 100 µL and allowed to attach overnight at 37 °C, 5% CO_2 in a humidified environment. The following day, 100 µL of drug treatment was added to wells (n = 3) resulting in a final drug concentration of 10 µM. Compound exposure lasted for 24 hours after which cells were washed three times with PBS, then fixed for 10 minutes at room temperature with 4% paraformaldehyde in PBS. Fixed cells were washed three times with PBS and stained for 30 minutes with 10 µM Hoechst 33342 at room temperature. Excess stain was removed by washing three times with PBS. Fixed and stained cells were kept hydrated during imaging with 200 µL per well of PBS.

Compound titration

Two compounds identified in the screening assay as possible hits in regards to their ability to affect PC-3 cell cycle progression were further analyzed with a dose titration. For these experiments, PC-3 cells were seeded at 4,000 cells per well in 100 µL and allowed to attach overnight. The following day, 100 µL of compound serial titrations in media were added as 2x solutions in replicates of eight. Cells were exposed for 24 hours after which cells were fixed and stained as described previously.

Imaging

Montage (6 × 6) images of each well were obtained with an Agilent BioTek Cytation 5 cell imaging multimode reader configured with a DAPI LED cube using a 10x objective. The DAPI cube was configured with a 377/50 excitation filter and a 447/60 emission filter in conjunction with a 409 nm cutoff dichroic mirror. The imaging parameters, set automatically using Agilent BioTek Gen5 microplate reader and imager software, used a LED intensity setting of 5, an integration time of 72 ms, and a gain of 0.

Analysis

Table 1. Image processing and image analysis parameters for nuclear content determination.

Parameter	Value
Stitching	
Channel	DAPI
Size	6,731 × 4,843 (62.18 Mb)
Crop Edges	Yes
Reduce Image	40%
Preprocessing	
Background	Dark
Flattening	Auto
Rolling Ball Diameter	746 µm
	462 pixels
Image Smoothing	0
Background Flattening Size	Auto
Object Counting	
Channel	Tsf[Stitched[DAPI 377,447]
Threshold	
Value	5,000
Background	Dark
Split Touching Object	Yes
Fill Holes in Masks	Yes
Advanced Options	
Smoothing	0
Background	5% lowest pixels
Object Selection	
Minimum	5 µm
Maximum	50 µm
Include Edge Object	No
Entire Image	Yes

Images were automatically stitched into a single file using Gen5 software. After stitching, montage images were preprocessed to subtract background fluorescence before analysis (Table 1). Primary mask analysis identified objects using a threshold of 5,000 and a lower and upper size limitation of 5 µm and 50 µm, respectively. Histograms relating total (integral) object fluorescence (X-axis) to % count (Y-axis) were generated using Gen5 software with the bin number set to 500. The histogram plots were subsequently used to identify G1 and G2 cells and set upper and lower signal thresholds from their respective count peaks. The intervening region between the G1 and G2 was used to identify S-phase cells.

Results and dicussion

Cell cultures exposed to a double thymidine block are enriched for cells in G1 phase of the cell cycle. The presence of high levels of thymidine results in the disruption of the deoxynucleotide metabolism pathway halting cell cycle progression at the G1/S border.[1] As demonstrated in Figure 2, cells released from thymidine block quickly enter S-phase and begin replicating DNA content. This is observed by an increase in cellular fluorescence over time. Upon completion of DNA replication, the cells are in G2 phase with fluorescent staining double that of cells in G1. After mitosis, their nuclear staining returns to initial levels (Figure 2).

Histograms relating total fluorescence to object count from Figure 2 were used to define the G1 and G2 subpopulations. The initial (time 0) histogram exhibited G1 and G2 peaks that allowed minimum and maximum fluorescent gating values to be applied to identify these subpopulations. The region between the maximum G1 subpopulation value and the minimum G2 subpopulation value was used to define S-phase cells.

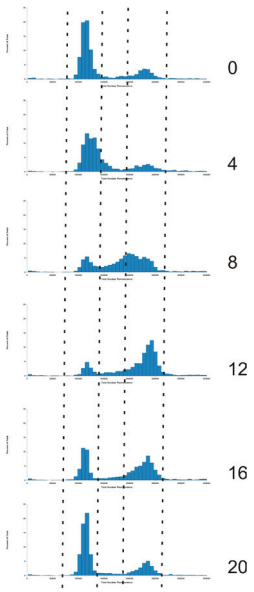

Figure 2. Cell cycle progression of PC-3 cells released from thymidine block. Histograms of cell population count versus fluorescence intensity taken at various times after thymidine block release. Dashed lines represent gates defining G1, S, and G2/M subpopulations.

Using subpopulation analysis, the temporal relationship between DNA content and cycle progression becomes apparent. As shown in Figure 3, cells released from thymidine blockade are nearly synchronous with respect to their DNA content. Initially, approximately 70% of the cells have a 2N chromosome number. Within 6 hours, this percentage has dropped to 33%, while the percentage of cells in S-phase has increased from near 0 to approximately 33%. The number of cells exhibiting a 4N chromosome number has also increased slightly by this time. By 12 hours, most cells have duplicated their DNA content and are in G2 phase of the cell cycle. During cellular mitosis, nuclear DNA is divided equally between the two daughter cells, returning the cells to G1 phase. This is observed by the rapid decline in the G2 percentage after 15 hours concurrent with an increase in the percentage of cells with a G1 DNA content such that all subpopulations return to their original states.

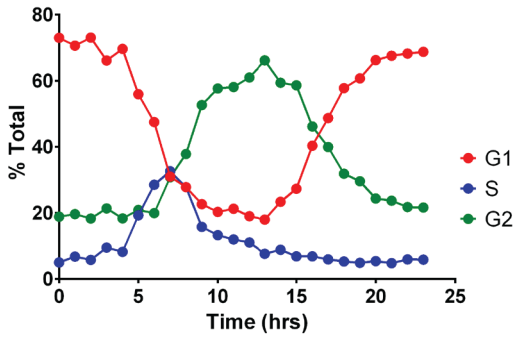

Figure 3. Cell cycle progression of cynchronized PC-3 cells. PC-3 cells synchronized with double thymidine block were fixed and stained at timed intervals after release. The percentages of cells in G1, S, and G2, determined by nuclear staining analysis, were plotted versus time. Data points represent the mean of eight determinations.

Cell cycle phase determination made from image-based DNA content can be used to screen compounds for their ability to inhibit cell cycle progression. Threshold gates for G1, S, and G2 phases based on nuclear fluorescence can be determined for histogram analysis (Figure 4) using known cell cycle inhibitors as controls to enrich cell populations in these specific cell cycle phases. Mevinolin has been demonstrated to stall cells in G1[2], while Nocodazole blocks mitosis and enriches cell populations in G2.[3]

Using these data to define subpopulations, compounds with unknown efficacy can be tested for cell cycle blockade. As shown in Figure 5, PC-3 cells were exposed to 27 different compounds for 24 hours and the percentage of cells in G1, S, and G2 phases of the cell cycle calculated. Control wells contained compounds known to stall cells in either G1 or G2 phases of the cell cycle. Cutoff thresholds from these wells were used to define G1, S, and G2 subpopulations. Deviation of greater than two standard deviations from untreated cells was used to identify compounds that stalled cell growth in G1, S, or G2 phases of the cell cycle. Compounds effecting PC-3 progression were flagged using color-coding to quickly identify the cell cycle phase. Of the compounds tested, one compound, Mevinolin, was shown to enrich cells in G1, while seven compounds caused an increase in the percentage of G2 cells. Note that Mevinolin was also used as a G1 control compound. When it was treated as an unknown compound, it was independently identified as blocking cells in G1, which confirms the hit criteria used. Some of the G2 positive hits also increased the percentage of cells deemed to be in S-phase.

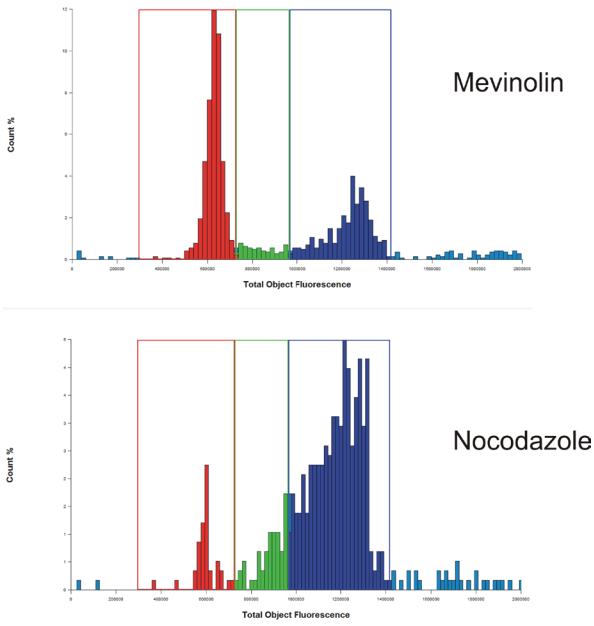

Figure 4. Histogram analysis of nuclear staining from Mevinolin and Nocodazole treated PC-3 cells. Mevinolin and Nocodazole treatments were used as controls to define G1 (red) and G2 (blue) subpopulations. S-phase (green) was defined as the region between G1 and G2.

	1	2	3	4	5	6	7	8	9	10	11	12
A				Cisplatin	Cisplatin	Cisplatin	Metranidazole	Metranidazole	Metranidazole	Mevinolin	Mevinolin	Mevinolin
	45.977	46.104	48.322	42.767	43.708	48.720	49.815	46.849	52.868	56.448	57.480	58.378
	14.778	14.228	13.053	16.610	16.982	12.651	14.299	14.622	13.217	6.448	7.821	7.162
	39.245	39.668	38.625	40.623	39.310	38.629	35.886	38.528	33.915	37.104	34.699	34.459
B	G1 POS Control	G1 POS Control	G1 POS Control	Colchicine	Colchicine	Colchicine	Cycloheximide	Cycloheximide	Cycloheximide	Nocodazole	Nocodazole	Nocodazole
	55.712	58.880	59.977	11.485	17.415	12.297	43.763	44.031	43.449	8.910	10.763	14.385
	7.285	6.181	8.028	10.891	12.567	9.946	11.316	10.436	10.115	16.090	14.033	10.919
	37.003	36.939	31.995	77.624	70.018	77.758	44.922	45.533	46.436	75.000	75.204	74.697
C	G2 POS Control A	G2 POS Control A	G2 POS Control A	Hydroyurea	Hydroyurea	Hydroyurea	Chloramphenicol	Chloramphenicol	Chloramphenicol	Vinblastin	Vinblastin	Vinblastin
	12.025	8.578	11.349	49.471	51.043	49.761	49.312	46.225	45.760	12.705	12.725	15.898
	18.143	12.190	12.829	14.165	15.249	13.244	14.272	12.206	14.590	12.842	11.480	9.062
	69.831	79.233	75.822	36.364	33.708	36.995	36.415	41.569	39.649	74.454	75.795	75.040
D	G2 POS Control B	G2 POS Control B	G2 POS Control B	CCCP	CCCP	CCCP	Verapamil	Verapamil	Verapamil	Azothioprine	Azothioprine	Azothioprine
	11.911	12.945	14.921	48.000	47.617	45.284	49.307	47.233	48.347	49.716	48.290	45.329
	11.911	8.252	9.206	13.863	16.553	16.529	14.010	17.520	15.117	13.400	14.941	15.634
	76.179	78.803	75.873	38.137	35.830	38.187	36.683	35.248	36.536	36.883	36.768	39.036
E	Hydrocortisone	Hydrocortisone	Hydrocortisone	Picropodophyllin	Picropodophyllin	Picropodophyllin	Albendazole	Albendazole	Albendazole	Fenbendazole	Fenbendazole	Fenbendazole
	49.587	53.405	46.150	12.834	11.870	12.382	8.466	11.514	10.125	12.930	11.514	13.365
	12.658	11.957	16.589	11.230	12.520	11.442	9.265	12.934	13.176	6.190	9.832	7.075
	37.755	34.638	37.261	75.936	75.610	76.176	82.268	76.552	76.699	80.880	78.655	79.560
F	Chlorpromazine	Chlorpromazine	Chlorpromazine	Monastrol	Monastrol	Monastrol	5'-Azocytidine	5'-Azocytidine	5'-Azocytidine	Mechlorethamine	Mechlorethamine	Mechlorethamine
	51.907	49.861	50.212	47.222	48.621	40.612	34.749	36.939	38.560	12.078	10.855	10.314
	7.415	10.794	11.653	14.423	13.208	24.347	7.312	10.952	7.712	15.645	14.722	15.770
	40.678	39.345	38.136	38.355	38.171	35.040	57.939	52.109	53.728	72.278	74.423	73.916
G	Carbachol	Carbachol	Carbachol	Mimosine	Mimosine	Mimosine	5-Fluor-5-deoxyurid...	5-Fluor-5-deoxyurid...	5-Fluor-5-deoxyurid...	Tamoxifen	Tamoxifen	Tamoxifen
	45.226	46.173	44.288	46.967	46.729	47.224	47.228	46.876	44.616	57.448	53.808	48.489
	12.791	14.031	16.314	15.188	14.239	13.754	14.013	14.796	14.181	11.508	13.264	13.602
	41.983	39.796	39.398	37.845	39.032	39.022	38.758	38.328	41.203	31.044	32.929	37.909
H	Chloroquine	Chloroquine	Chloroquine	Sodium Butyrate	Sodium Butyrate	Sodium Butyrate	Methotrexate	Methotrexate	Methotrexate			
	53.736	50.764	50.224	47.245	50.429	50.335	53.436	48.391	52.274	49.593	47.837	50.737
	12.716	13.849	13.901	14.541	14.286	14.874	13.345	14.700	10.466	11.739	14.208	11.851
	33.549	35.387	35.874	38.214	35.285	34.792	33.219	36.910	37.260	38.668	37.955	37.412

Figure 5. Agilent BioTek Gen5 screenshot of a cell cycle phase screen of compounds in a 96-well plate. PC-3 cells were exposed to 30 different compounds, and controls in triplicate. Histograms from control compounds were used to define minimum and maximum fluorescence gates for subpopulation analysis. Each well lists percentages of cells in G1, S, and G2. Deviations of percentages of cells in different cell cycle phases of greater than two standard deviations from untreated control wells were used as the cutoff to identify potential compounds. G1 enriching compounds were identified with blue, while G2 was outlined with red, and S-phase with green.

One of the identified positive hits was examined in detail. PC-3 cells were exposed to half-log titrations of mechlorethamine for 24 hours, which resulted in a dose-dependent increase in the percentage of G2 cells after a 24-hour exposure (Figure 6).

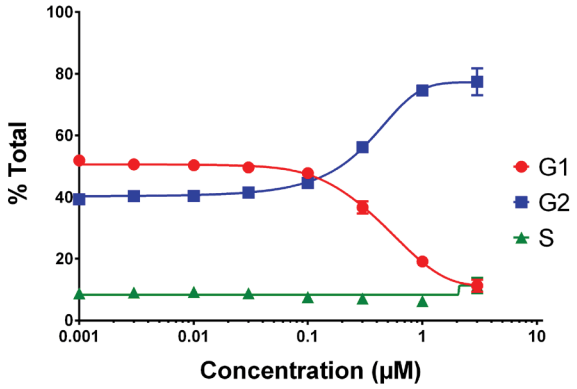

Figure 6. Effect of mechlorethamine concentration on cell cycle progression. PC-3 cells were treated with various concentrations of mechlorethamine for 24 hours. After compound exposure, cells were fixed and stained for nuclear content. Image-based analysis was used to determine the percentage of cells in G1, S, and G2 phases of the cell cycle. Data points represent the mean of eight determinations.

Conclusion

These data indicate that using a nuclear stain to quantify nuclear DNA content along with image-based analysis can be used to assess cell cycle progression in adherent cells. Cell cycle analysis has traditionally been performed using flow cytometry. While convenient for nonadherent cell lines such as lymphocytes, its use with adherent lines requires trypsinization or scaping of the cells to suspend them. This leads to a bottleneck in terms of throughput. The use of image-based analysis of adherent cells fixed and stained in place in microplates allows the high throughput necessary for compound screening.

Several of the compounds tested significant enrichment of cells in one or more phases of the cell cycle as compared to untreated control cells. Mevinolin, nocodazole, and vinblastine were used as assay controls to define subpopulation thresholds. Under these criteria, these same compounds were also identified correctly as potential hits in the assay screen. Because exposure to a single high concentration of a compound can be cytotoxic without necessarily stalling cells in any particular cell cycle phase, drug titrations on potential screen hits is a necessary confirmatory experiment.

Mechlorethamine was further investigated due to screening results, which suggest that it is a good candidate for a cell cycle inhibitor. This compound is a nitrogen mustard-alkylating agent that works by binding and crosslinking DNA strands and preventing cell duplication. As such, this drug will halt cell cycle progression at the S/G2 border. With high doses, cell cycle progression will be halted during S-phase as highly cross-linked DNA cannot be repaired or replicated.

The Agilent BioTek Cytation 5 cell imaging multimode reader and Agilent BioTek Gen5 microplate reader and imager software are an ideal combination of value and performance. The system is capable of making multiple images to form a montage, which allows a larger cell sampling for each sample. The multiple files are stitched into a single image file before analysis. Preprocessing of the image subtracts background, eliminating any well-to-well differences before object identification. Gen5 histogram analysis of object information allows the researcher to visually apply threshold gates to define G1 and G2 populations. A quick check of multiple wells can then be used to confirm goodness of the fit. The percentages of each subpopulation is then calculated and reported. Long-term live cell experiments can be carried out using an Agilent BioTek BioSpa 8 automated incubator to maintain environmental control of temperature, CO_2 and humidity levels.

References

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