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Application Note

High Throughput Screening of Compounds Affecting Distinct Phases of the Cell Cycle Using Mix-and-Read Assays

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Introduction

The cell cycle is a series of events that a cell must complete in order to properly grow and divide. A high degree of molecular regulation highlights the importance of a properly functioning cell cycle for individual cell health as well as for the overall health of multicellular organisms. Perturbation to the cell cycle is linked to a broad set of diseases including cancer, inflammation, and neurodegenerative disorders. Additionally, potential therapeutics can exhibit toxicity by causing abnormal cell cycle modulation, making perturbations to the cell cycle an attractive high throughput screening endpoint.

Flow cytometry using a DNA staining dye is widely recognized as one of the most effective techniques for cell cycle analysis. While powerful, using flow cytometry in high throughput screens has been difficult because of low sample throughput and laborious staining methods. The most common cell cycle stain, propidium iodide, exemplifies many of the workflow disadvantages inherent to most currently available cell cycle stains: i) cells must be permeabilized for the dye to access the cell's nucleus, ii) a fixative must be added to immobilize the cell's contents within the now porous cell membrane and iii) many dyes, including propidium iodide, are incapable of discriminating between RNA and DNA, causing a problematic high background signal if the cells are not treated with RNase.

IntelliCyt® has developed a "no wash" Cell Cycle Screening Kit that addresses the workflow problems of traditional cell cycle assays (**Figure 1A**). IntelliCyt's live cell stain can be added to cells without the need to permeabilize, fix, or perform an RNase treatment, and only a single-addition staining step is required. This is followed by a one-hour incubation prior to reading plates on an IntelliCyt screening system.

It is well understood that compounds that perturb the cell cycle often have high potential toxicity to cells, and that cells that are dead or dying often confound the cell cycle profile of the bulk population. Because IntelliCyt's screening systems are based on a flow cytometry detection engine, the MultiCyt™ Cell Cycle Screening Kit improves the accuracy of cell cycle analysis by flagging cells as damaged. This provides a simple, mix-and-read solution that unites gold-standard cell cycle analysis with significantly enhanced sample throughput.

In this application note, we demonstrate how the MultiCyt Cell Cycle Screening Kit and the iQue™ Screener can be employed for high throughput assays that accurately determine cell cycle perturbations across of wide variety of known cell cycle inhibitors. These compounds were selected for their diverse mechanisms of action, including DNA polymerase inhibitors, topoisomerase inhibitors, and compounds that alter microtubule structure. Using both a hand-picked set of eight compounds and a larger, randomly selected 320-compound subset of the NCI Mechanistic Diversity Set library, we observed strong cell cycle blocks in G0/G1, S, and G2/M phases.

Assay Principles and Methodology

The MultiCyt Cell Cycle Screening Kit uses our Cell Cycle Stain, which is a fluorescent dye that intercalates into DNA (**Figure 1B**). The intensity of the fluorescent signal will be proportional to the DNA content of the cell. Diploid cells will have 2N DNA content at G0/G1 phase, 4N DNA content at G2/M phase, and an intermediate DNA content at S phase (**Figure 2A**).DNA content is reported with sufficient sensitivity to discriminate between these three phases (**Figure 2B**, blue histogram). A culture will have a certain percentage of cells undergoing each phase of the cell cycle. Under abnormal conditions, such as treatment with a cell cycle inhibitor, the cell cycle profile will be altered (**Figure 2B**, red histogram).

Using an analysis strategy based on positive and negative control wells, gates are employed that identify the cell population into

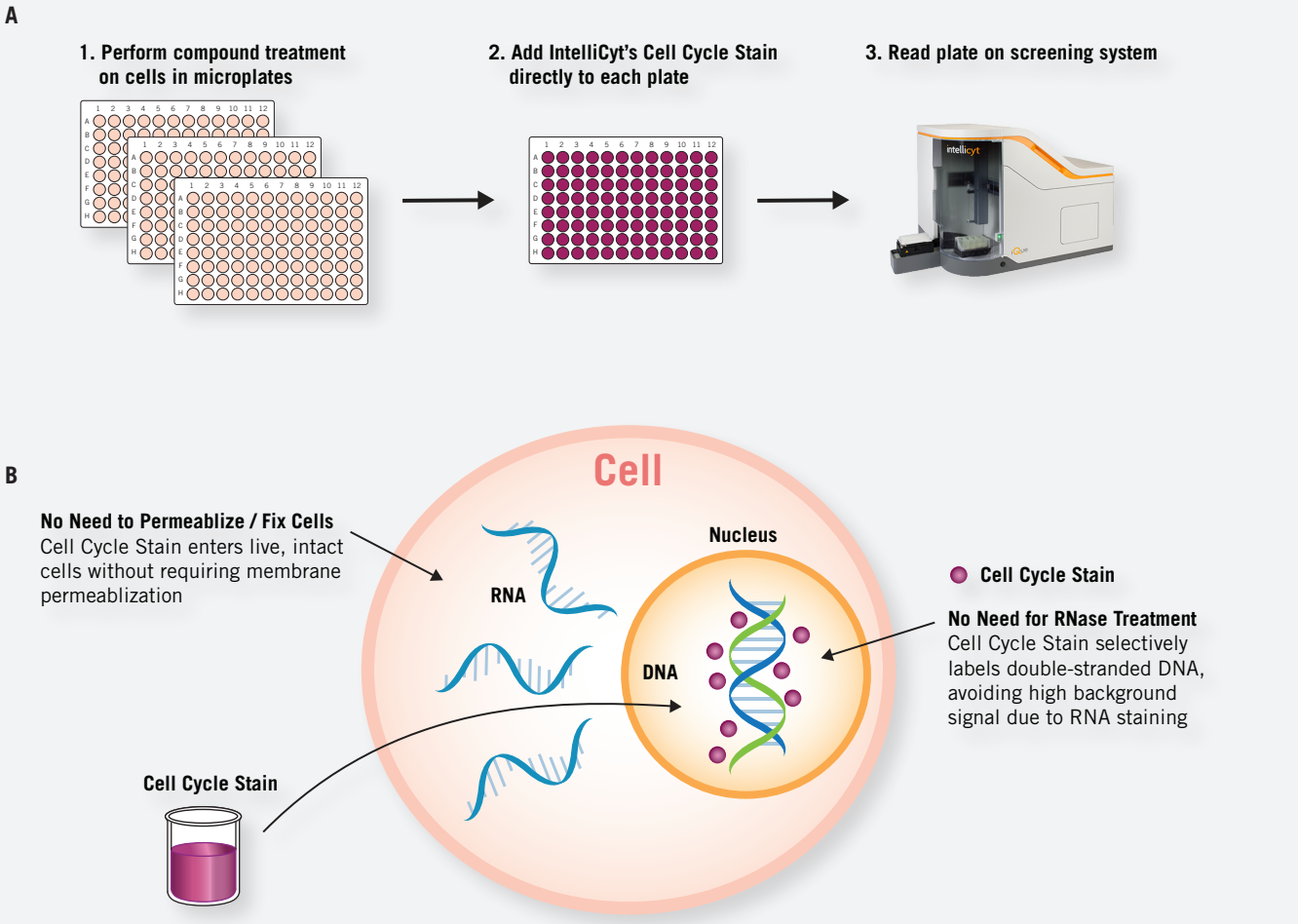

Figure 1. How the MultiCyt Cell Cycle Screening Kit works. Each kit contains a dye that stains DNA much like the traditional cell cycle stain propidium iodide. As an improvement over propidium iodide, IntelliCyt's Cell Cycle Stain does not require cell permeabilization, fixation, or RNase treatment. These dye properties allow the MultiCyt Cell Cycle Screening Kit to be used in a mix-and-read workflow by simply adding the dye directly to your assay volumes. Following a one-hour incubation, plates are ready to read on IntelliCyt screening systems.

each of the three cell cycle stages. To enable objective analysis of screening data, this control-based analysis strategy can be uniformly and automatically applied to every well of every plate in a high throughput screen, making it easy to identify compounds that perturb the cell cycle. (**Figure 2C**, data shown as heat maps indicating intensity of different cell cycle blocks).

PROFILING COMPOUNDS WITH KNOWN MECHANISMS OF ACTION

To determine if the MultiCyt Cell Cycle Screening Kit could detect cell cycle perturbation by compounds with different mechanisms of action, we selected eight known cell cycle inhibitors with unique mechanisms of action (**Table 1**). Jurkat cells were treated with compounds in a dose response, starting at a high concentration of 10 µM and proceeding in a 1:2 titration series across 12 doses. A constant DMSO concentration of 0.5% was maintained in all wells. Cells were incubated with compounds for 24 hours before staining with the MultiCyt Cell Cycle Screening Kit.

SCREENING COMPOUNDS FROM THE NCI MECHANISTIC DIVERSITY SET

We further tested the MultiCyt Cell Cycle Screening Kit by screening a subset of the NCI Mechanistic Diversity Set for cell cycle inhibitors. Four 96-well microplates from the Mechanistic Diversity Set (plate numbers: 4521, 4524, 4525, and 4526) were randomly selected and reformatted into a 384-well microplate. In

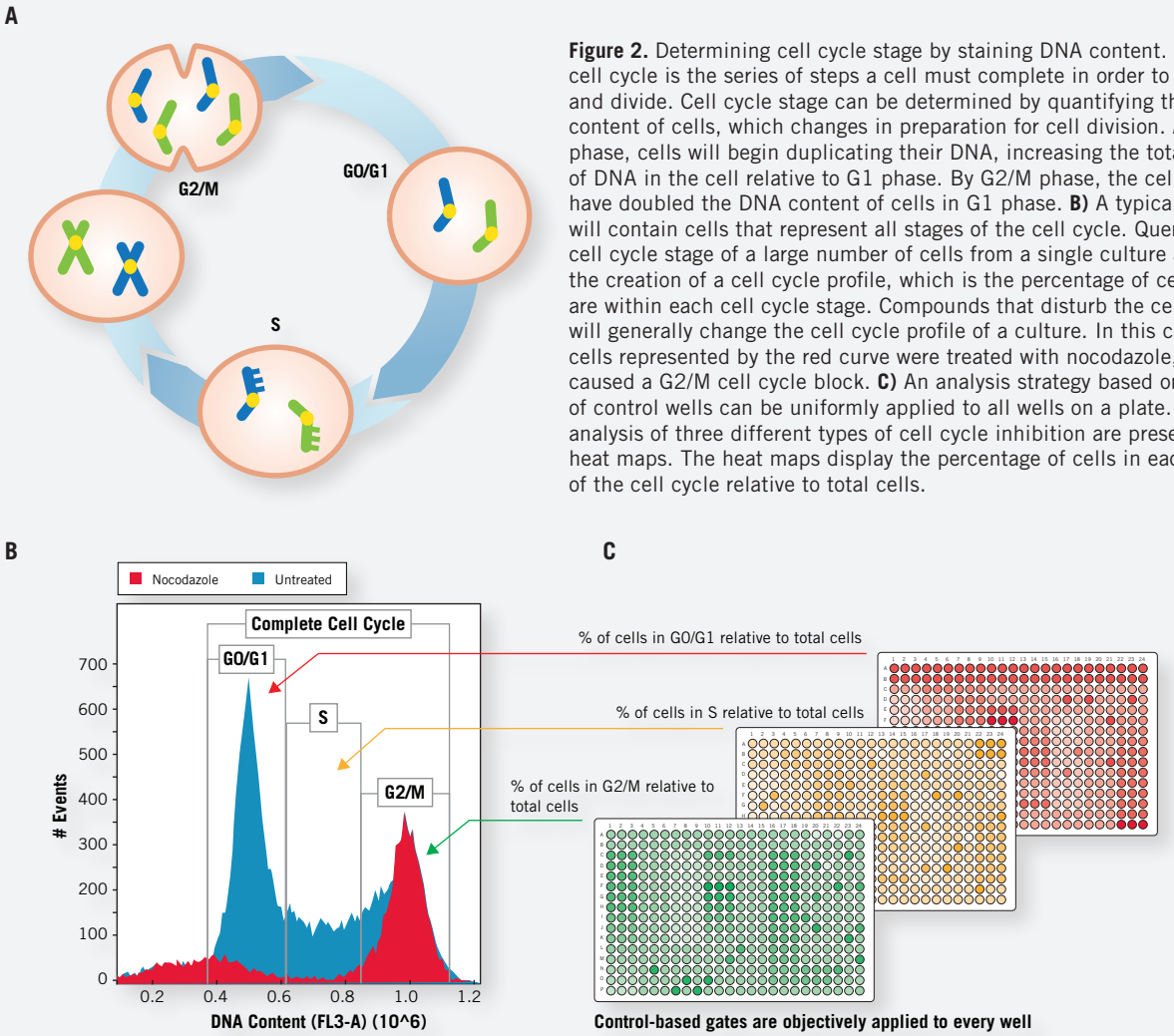

Figure 2. Determining cell cycle stage by staining DNA content. The cell cycle is the series of steps a cell must complete in order to grow and divide. Cell cycle stage can be determined by quantifying the DNA content of cells, which changes in preparation for cell division. **A)** At S phase, cells will begin duplicating their DNA, increasing the total amount of DNA in the cell relative to G1 phase. By G2/M phase, the cells will have doubled the DNA content of cells in G1 phase. **B)** A typical culture will contain cells that represent all stages of the cell cycle. Querying the cell cycle stage of a large number of cells from a single culture allows the creation of a cell cycle profile, which is the percentage of cells that are within each cell cycle stage. Compounds that disturb the cell cycle will generally change the cell cycle profile of a culture. In this case, cells represented by the red curve were treated with nocodazole, which caused a G2/M cell cycle block. **C)** An analysis strategy based on gating of control wells can be uniformly applied to all wells on a plate. Here, an analysis of three different types of cell cycle inhibition are presented as heat maps. The heat maps display the percentage of cells in each phase of the cell cycle relative to total cells.

total, 320 compounds were screened for cell cycle inhibition on Jurkat cells using a final compound concentration of 10 µM.

Results & Discussion

We considered compounds positive for a cell cycle block if they increased the percentage of cells in a given cell cycle phase by more than three standard deviations above the mean of negative control treated wells (wells containing 0.5% DMSO). **Figure 3** shows the cell cycle profiles for compounds chosen for their known mechanisms of action. Seven of the eight compounds tested caused a cell cycle block in at least one cell cycle phase (**Table 1**). Importantly, a block was detectable in all major phases of the cell cycle. Taken together, this set of compounds confirmed the ability of the MultiCyt Cell Cycle Screening Kit to observe blocks in G0/G1, S, and G2/M.

The Mechanistic Diversity Set compound library was created from a larger 1,990 member library (the original Diversity Set) by choosing compounds that caused growth inhibition on the NCI Human Tumor 60 cell line. Because all the compounds in the library are demonstrated to be biologically active and known to specifically have an effect on cell growth, we expected a high percentage of cell cycle inhibitors when compared with an unbiased library.

Compound	Mechanism of Action	G0/G1	S	G2/M
Methotrexate	Dihydrofolate reductase inhibitor	+	+	–
Fluorouracil	Pyrimidine analog	–	–	–
Aphidocolin	DNA polymerase inhibitor	+	+	–
Etoposide	Topoisomerase II inhibitor	–	–	+
Roscovitine	Cyclin dependent kinase inhibitor	+	+	–
Camptothecin	Topoisomerase I inhibitor	+	–	–
Nocodazole	Microtubule destabilizer	–	–	+
Paclitaxel	Microtubule stabilizer	–	+	–

Table 1: Compounds with known mechanism of action, tested for cell cycle inhibition. Compounds marked with "+" demonstrated a block at the indicated cell cycle phase. A block was defined as an increase in the percentage of cells in a particular phase that was 3 standard deviations greater than the mean of negative control treated cells. Compounds marked with "–" did not meet this criteria for a cell cycle block.

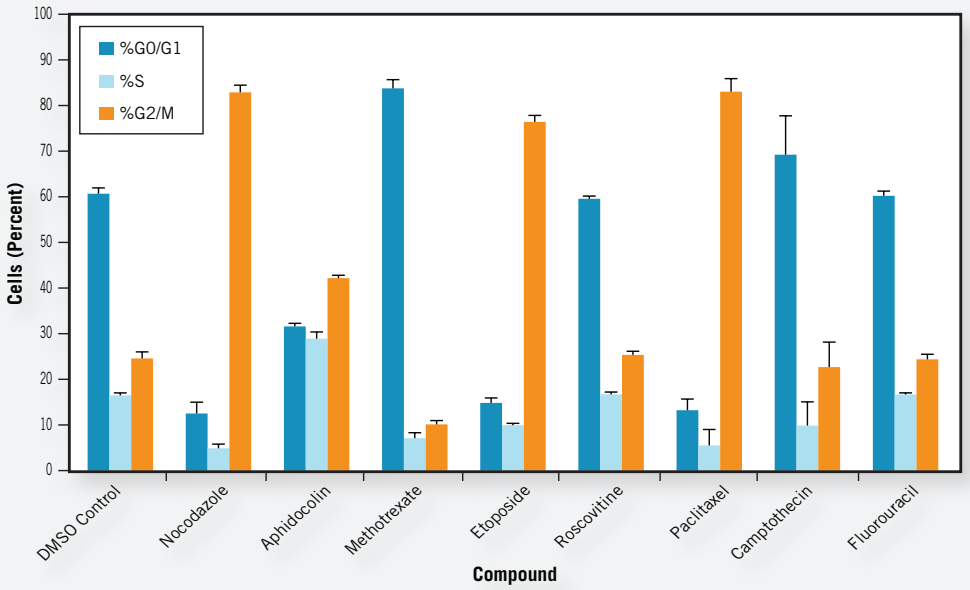

Figure 3. Cell cycle profiles for Jurkat cells treated with known cell cycle modulators. Jurkat cells were treated with compounds in a 12-point, 1:2 dose response starting with a top dose of 10 µM. The cell cycle profiles for cells treated at the 0.313 µM concentration of compound are shown. Error bars represent the standard deviation of three replicates.

Of the 320 compounds screened, 82 compounds (25.6%) scored positive for a cell cycle block, 56 compounds caused a block in G0/G1, 18 compounds caused a block in S, 8 compounds caused a block in G2/M, and 2 compounds caused a block in both S and G2/M. **Table 2** lists the inhibitors in the top 20th percentile of inhibitory activity. Reinforcing the utility of this assay in a screening scenario, several known cell cycle inhibitors returned as strong hits, including: M-AMSA, nagilactone C, peltatin B, and clanfenur.

A single compound, methotrexate, was present in both the initial eight compound profile as well as the subset chosen from the NCI Mechanistic Diversity Set. Interestingly, methotrexate did not score positive for cell cycle inhibition when tested in a single dose from the NCI library. It did, however, test positive with a clear dose response in the eight-compound profile. This divergent finding highlights the utility of quantitative high throughput screening, where increased throughput actually enables more in-depth compound characterizations rather than limiting screening campaigns to evaluating single concentrations of each compound. Compounds that may have been missed or shown as false negatives in single-point screening can be identified with a more robust data set enabled by profiling in dose response.

Summary

The mix-and-read workflow of the MultiCyt Cell Cycle Screening Kit makes it practical to screen compounds in a dose response series.

- Utilizing a set of compounds with a diverse set of inhibitory mechanisms, we were able to clearly observe cell cycle blocks in G0/G1, S, and G2/M and rapidly establish cell cycle “profiles” for each of these mechanisms.
- The utility of this reagent chemistry in a high throughput screening context was successfully demonstrated by screening of a 320-compound subset of the NCI Mechanistic Diversity library.
- By combining the unique capabilities of the iQue Screener with the MultiCyt Cell Cycle Screening Kit, we were able to accurately profile cell cycle perturbing compounds of several different mechanistic classes in approximately 45 minutes per 384-well microplate with a simple, turnkey mix-and-read assay protocol.

G0/G1	S	G2/M
Carbamic acid, [1-[[[3-chloro-2-oxo-1-(phenylmethyl)propyl]amino]carbonyl]-3-methylbutyl]-, phenylmethyl ester	4-Piperidinone, 1-(1-oxo-2-propenyl)-3,5-bis(phenyl- methylene)-	4-Piperidinone, 1-(1-oxo-2-propenyl)-3,5-bis(phenyl- methylene)-
Bisantrene hydrochloride	5-Fluorouridine	4-(2,4,5-Trimethoxystyryl)quinoline
	Sterigmatocystin	Peltatin B
	M-AMSA	Pyridine, 2-(p-chlorostyryl)-4-[[4-(diethylamino)-1-methylbutyl]amino]-, (E)-
	Acodazole HCl	Clanfenur (INN)
	Shikoccin	2H-1-Benzopyran, 6-methoxy-3-nitro-2-[2,2,2’,2’-tetramethyl (4,4’-bi-1,3-dioxolan)-5-yl]-
		9,10-Anthracenedione, 1,4-dihydroxy-2-[[2-[(2-hydroxyethyl)amino]ethyl]amino]-
		Pseudocoralyne
		3-Desmethylcolchicine

Table 2: Cell cycle modulators discovered as hits by screening a subset of the NCI Mechanistic Diversity Set compound library. Each compound listed scored in the top 20th percentile of hits. A compound was scored as a hit if it increased the percentage of cells within a cell cycle stage by greater than 3 standard deviations above the mean of DMSO control-treated cells.

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IC-004 Rev A