CHARACTERIZATION OF DNA CONTENT, CYCLIN B1 AND PHOSPHORYLATED HISTONE H3 WITH DIRECT S-PHASE USING EDU INCORPORATION IN MULTIPARAMETER TESTING OF CELL LINES WITH CELL CYCLE BLOCKING AGENTS

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Introduction

Detailed characterization of cell cycle is critical in basic and applied immunologic and oncologic studies. By combining measurements of DNA content and DNA synthesis, the cell cycle can be resolved into G0+G1, S, and G2+M phases. By including other key indicators of cell cycle progression, multiparametric analysis of cell cycle by flow cytometry or imaging cytometry can provide further insights into the mechanistic aspects of pharmaceutical agents. We utilized a recently introduced method for determining direct S-phase synthesis using the incorporation of the nucleoside analog EdU (5-ethynyl-2'-deoxyuridine) coupled with click chemistry. Combining DNA content and direct S-phase measurement with cyclin B1 (clone GNS-1 antibody, normally expressed low in G_0G_1, increasing in S and maximally expressed in late-S, G_2, and M phases of the cell cycle) and with phospho-histone H3 (clone HTA28 antibody, phosphorylated at serine 28, associated with early stage mitosis), it is possible to identify different subpopulations and assess the effects of the pharmaceutical agents. In five-parameter flow cytometric analysis, an amine-reactive dye is utilized to eliminate dead cells from the analysis, which may non-specifically bind the antibodies used. Separate testing, using the monomeric cyanine YO-PRO®-1 iodide dye in combination with SYTOX® Red dead cell dye, is used to identify early-apoptotic and late-apoptotic/necrotic events. In addition to flow cytometry, we show that automated imaging cytometry can be employed in a similar manner to obtain comparable results.

Figure 1 – Click chemistry based detection of EdU

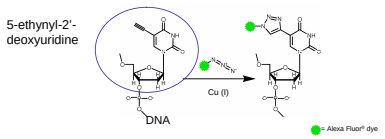

Figure 1 – Click chemistry is the copper(I) catalyzed reaction between a terminal alkyne and an azide. A solution containing the dye labeled azide plus copper(II) and a reductant is added to cells. Because the azide and the alkyne are abiotic, they are inert until catalyzed by copper. The highly selective bio-orthogonal covalent reaction occurs rapidly at room temperature. In this application, EdU is incorporated into actively growing cells. The EdU contains a terminal alkyne which reacts with a dye labled azide dye to fluorescently label the double stranded DNA.

Figure 2 – Flow cytometric comparison of cell cycle phase distribution effects using cell cycle blocking agents on four lymphocytic cell lines

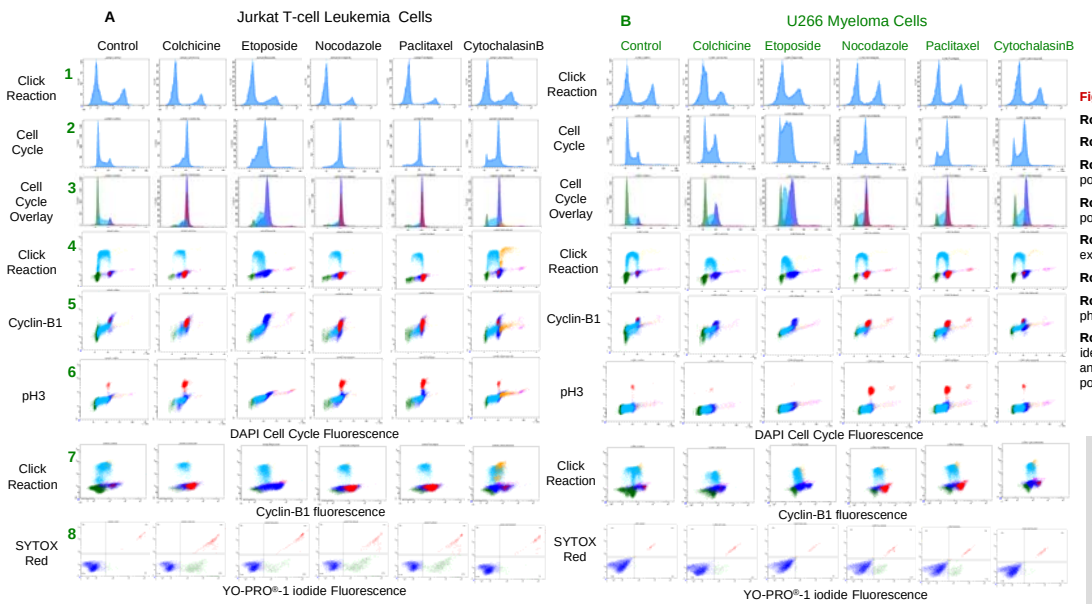

Figure 2

Row 1: positive click reaction shows S-phase cells

Row 2: DNA content cell cycle histogram

Row 3: Overlay DNA content histogram showing individual populations in the cell cycle

Row 4: Click reaction vs DNA content, showing three cell populations of G0+G1, S, & G2+M phases

Row 5: Cyclin B1 vs DNA content shows how cyclin B1 expression varies during the cell cycle

Row 6: pH3 vs DNA content identifies mitotic cells

Row 7: Click reaction vs cyclin B1 shows interaction of S-phase cells with cyclin B1 expression

Row 8: YO-PRO®-1 iodide vs SYTOX® Red dye identifies identifies late-apoptotic/necrotic cells (red), live cells (blue) and early apoptotic cells (green), with percentage of total population listed in each quadrant.

Color coding for overlay and plots rows 3-7:

click-positive S-phase (DNA diploid)

G0G1 (DNA diploid)

G2M (DNA diploid) or G0G1 (DNA tetraploid)

pH3 positive

click-positive (DNA tetraploid)

G2M (DNA tetraploid)

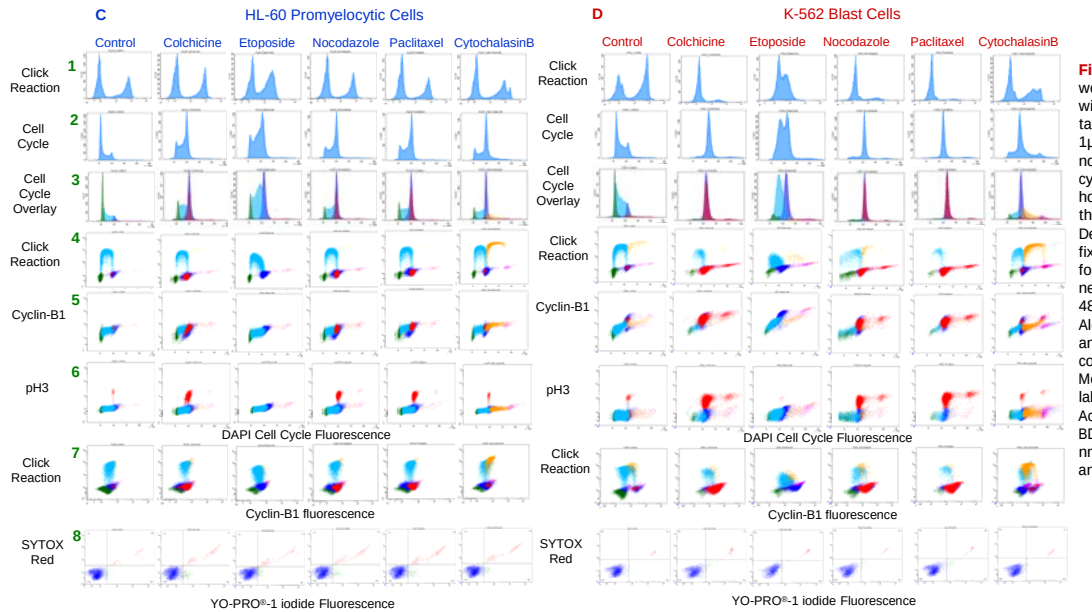

Figure 2 Four different lymphocytic cells lines were either untreated or treated for 15 hours with each of five cycle blocking agents which target the G2M transition of the cell cycle: 1µM colchicine, 1µM etoposide, 100nM nocodazole, 1µM paxlitaxel, or 5µM cytochalasin B. This was followed by one hour incubation with 10µM EdU. Cells were then labeled with LIVE/DEAD® Fixable Near-IR Dead Cell Stain (data not shown) before fixation with 4% paraformaldehyde in PBS, followed by saponin-permeabilization; labeling next with the click reaction using Alexa Fluor® 488 azide, followed by antibody labeling using Alexa Fluor® 647 Rat anti-Histone H3 (pS28) and purified mouse anti-Cyclin B1 (GSN-1) complexed with Zenon® R-Phycoerythrin Mouse IgG1 Labeling reagent. DNA content labeling was then performed using DAPI. Acquisition and analysis was performed on a BD LSRII flow cytometer using 355 nm, 488 nm, and 633 nm excitations in 5-color analysis.

A Jurkat T-cell Leukemia Cells

B U266 Myeloma Cells

C HL-60 Promyelocytic Cells

D K-562 Blast Cells

Figure 3 – Automated Imaging: DNA content with EdU proliferation and nuclear cyclin B1 in nocodazole treated Hela cells

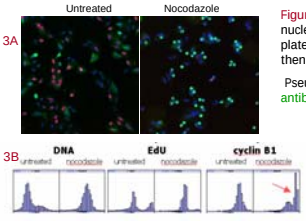

Figure 3A – Characterization of DNA content, cell proliferation with EdU labeling, and nuclear cyclin B1 intensity using automated imaging cytometry. A549 cells in 96 well plates were treated for 24 hours with the G2/M cell cycle blocker nocodazole (170 nM) then labeled with 10 µM EdU for 30 minutes before processing for detection.

Pseudo-colored in the images: Blue – Hoechst 33342; Green – anti-cyclin B1 antibody; Red – EdU positive

Figure 3B – Automated image acquisition and analysis performed with an Arrayscan VTI (Thermo Fisher / Cellomics, Pittsburgh, PA) were followed by data examination and graphing using Panmo (The Chi-Square Works, Inc. Seabeck, WA). The distributions of DNA content (Hoechst 33342 stain), EdU nuclear intensity, and cyclin B1 nuclear intensity are shown as histogram trellis plots. A shift in DNA content profile and decrease in cell proliferation (EdU incorporation) is revealed in nocodazole treated cells.

Results and Conclusions

Controls for each cell type show typical pattern of cell progression. Treatment with nocodazole and paclitaxel block cell division at the M phase in all four cell types. Colchicine blocks cell division at the M phase in Jurkat, HL60, and K562 cells while in U266 cells colchicine blocks the cell cycle at the G2 phase before mitosis. Etoposide blocks the cycle at the G2 phase before mitosis in all four cell types. Cytochalasin B allows nuclear division to proceed while inhibiting cell division in all four cell types, with a DNA tetraploid population demonstrated. The inhibitory effects on cell proliferation of each treatment leads to apoptosis/necrosis, as seen with the increase of apoptotic/necrotic cells in treated cells compared with control cells.

Changes of cell proliferation can be easily distinguished using the Click™-iT EdU Proliferation assay for S-phase measurement in multiparametric analysis using flow and imaging cytometry.