DETECTION OF DNA SYNTHESIS BY AUTOMATED MICROSCOPY AND IMAGE ANALYSIS: COMPARISON OF BRDU METHOD AND A NEW CLICK CHEMISTRY BASED EDU METHOD

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Introduction

Accurate characterization of the effect of pharmaceuticals or other biologically active reagents on DNA synthesis and cell cycle progression is of great importance not only in drug discovery but also in the study of basic cell biology. The traditional approach for DNA synthesis detection utilizes an antibody to detect the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into the newly synthesized DNA after pulse labeling. To facilitate the access by the antibody to the BrdU incorporated in the chromosomal DNA, the BrdU labeling and detection method involves harsh treatments such as nuclease digestion or acid treatment, leading to inevitable negative consequences when used in combination with other functional probes. With automated microscopy and image analysis, we compared the traditional BrdU method with a new method of pulse-labeling cells with 5-ethynyl-2'-deoxyuridine (EdU) and the subsequent fluorescence detection via a Cu(I) catalyzed click reaction in a mild reaction buffer (pH 7.4). We evaluated these two approaches for their sensitivity, time requirement, ease of use, and compatibility with other antibody labeling. We found both methods showed excellent sensitivity with a 5-minute pulse labeling of cells with nucleosides. However, the BrdU method, due to its DNA denaturation requirements, gave poor performance in co-labeling with an anti-cyclin B1 antibody while the EdU method gave excellent cyclin B1 staining. The EdU staining procedure is much simpler and utilizes shortened processing time: 2 hours for EdU vs. at least 4 hours plus an overnight incubation for BrdU. Besides the detection of newly synthesized DNA and co-staining of protein targets of cell cycle importance (cyclin B1 and phospho-Histone H3), we also demonstrate the profiling of DNA content in the same preparation of cells after drug treatments. This new click chemistry based EdU labeling method for detection of DNA synthesis not only simplifies and expedites the analysis of DNA synthesis at the cellular level but also opens the possibility of multiplexing with other functional probes to further enrich the information content of image based assays of cellular activities.

Figure 1 – Comparison of EdU and BrdU labeling protocols for imaging

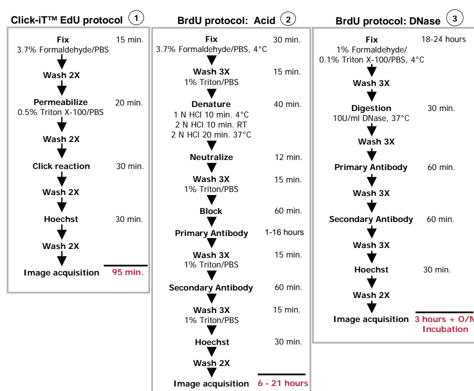


Figure 2 – Click-iT™ EdU S-phase detection by imaging - multiplexing

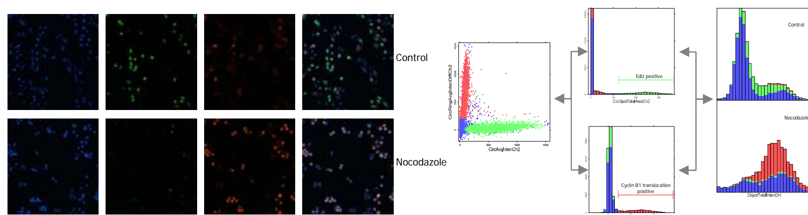


Figure 2a – A549 cells were treated with 170 nM nocodazole for 20 hours, labeled with EdU for 30 minutes, and then processed with Click-iT™ EdU Alexa Fluor® 488 azide detection reagent; the cells were then stained with anti-cyclin B1 antibody (Invitrogen) and Hoechst 33342. DNA – Hoechst 33342; EdU – Click-iT™ Alexa Fluor® 488 Azide Dye; Cyclin B1 – Alexa Fluor® 555 conjugated secondary antibody.

Figure 2b – A sub-set of graphs from visual data exploration of the data set illustrated by the micrographs of Figure 2a. (Note: histograms show relative frequencies.) Distinct distribution of EdU incorporation (green) and cyclin B1 nuclear translocation (red) in the data space. Note the effects of nocodazole on various parameters as revealed in the graphs: most prominently, shift of DNA profiles and increase in the number of cells with cyclin B1 nuclear translocation.

Figure 3 – Multiplexing proliferation using EdU or BrdU with DNA cell cycle and cyclin B1 in nocodazole treated HeLa cells

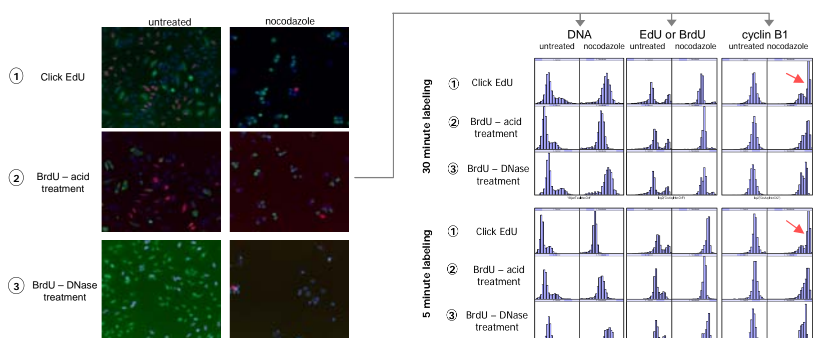
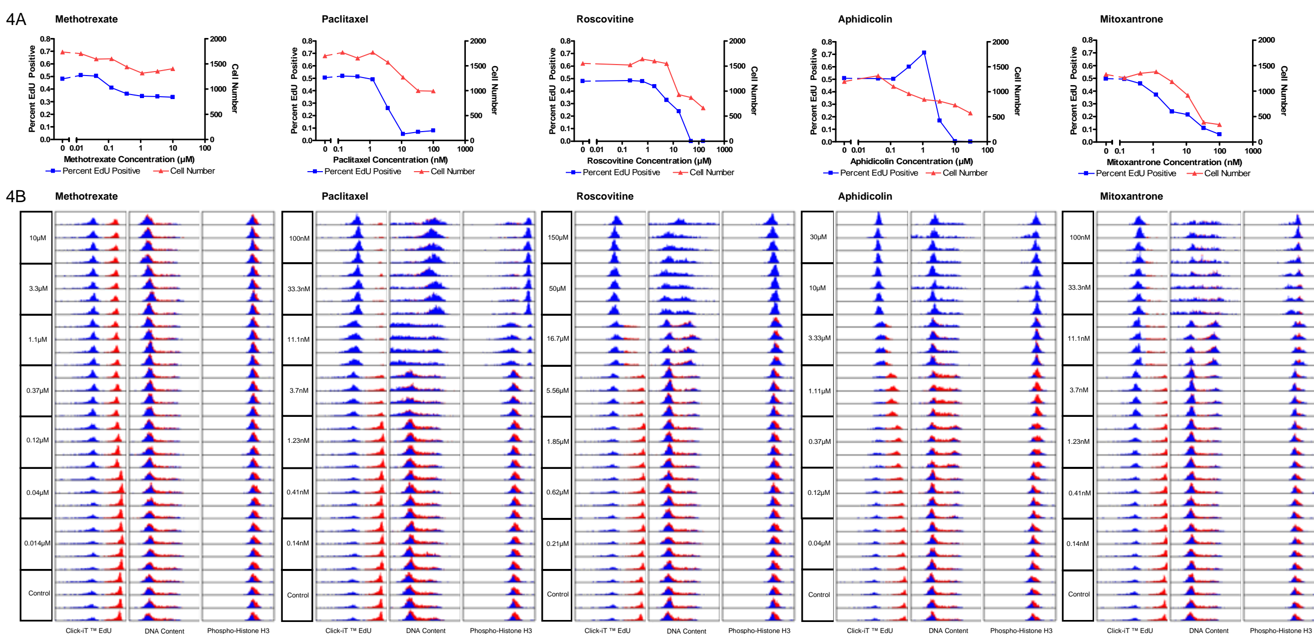


Figure 3A – Characterization of DNA content, cell proliferation (EdU or BrdU labeling), and nuclear cyclin B1 intensity using automated imaging cytometry. HeLa cells in 96 well plates were treated for 24 hours with the G2/M cell cycle blocker nocodazole (170 nM) then labeled with 10 µM EdU or BrdU for 30 minutes before processing for detection. Click labeling of EdU (row 1) was compared to 2 protocols for BrdU detection from commercially available reagent kits: one involving acid treatment (row 2), and another involving a DNase treatment (row 3). Pseudo-colored in the images: Blue – Hoechst 33342; Green – EdU or BrdU; Red – anti-cyclin B1 antibody

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 or BrdU 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 or BrdU incorporation) is revealed in nocodazole treated cells. Anti-cyclin B1 antibody only achieved good signal-background separation in cells processed for click EdU detection (red arrows) but not in cells processed for BrdU detection.

Figure 4 - Effects of drug treatment on DNA synthesis and cell cycle content analyzed with automated image analysis



A549 cells (ATCC CCL-185) were treated with drugs for 24 hours before fixation and processed for Click-iT™ EdU reaction (Alexa Fluor® 594 Azide Dye) The cells were then stained with anti-phospho-histone H3 antibody (Upstate 60-570) (Alexa Fluor® 488 Dye). Automated Image acquisition and analysis were performed using Arrayscan® VTI. Graphics-based exploration and graphing of cell-level data were performed using Panmo 4. Well-level data as calculated by Panmo 4 were then graphed with GraphPad Prism 4.

Figure 4A - Well-level data
EdU-positive cells were defined based on the fluorescence intensity of EdU (as exemplified in figure 4B and described below). The percentage of EdU positive cells (blue trace, left Y-axis) and cell counts from the images (red trace, right Y-axis) were graphed against drug concentrations with 4 replicates for each concentration. Data points show medians.

Figure 4B - Cell-level data
Each panel of the trellis plots represents data from one well of a 96-well plate. The distribution is presented as density. The typical number of cells sampled by imaging is around 1,500 for control group and low drug concentrations. The EdU total intensity profiles were plotted using a logarithmic scale (the left column of each drug group). The DNA content profiles (the total Hoechst 33342 intensity, the middle column of each drug group) were plotted with linear scale. The pH3 total intensity profiles (mitotic marker, the right column of each drug group) were plotted with logarithmic scale. EdU-positive sub-population was painted red in the EdU intensity histograms (the left columns of each drug group). The corresponding distribution of EdU positive cells in the DNA content profile (Hoechst 33342 intensity, middle columns) and pH3 stain profile (for mitotic cells, the right column) is also labeled red since, within the same drug group, all graphs were dynamically linked.

Results and Conclusions

- Click-iT™ EdU reaction for S-phase detection is performed in a mild reaction buffer, with a simple and speedy protocol which does not require any overnight incubations.
- In cultured cells, 5 minute pulses of BrdU or EdU produced good signals in adherent cells; however, denaturation requirements for the BrdU protocol drastically reduced its compatibility in multiplex labeling with other antibody probes.
- When combined with EdU or BrdU for detecting DNA synthesis, anti-cyclin B1 antibody achieved good signal-to-noise only with the EdU method.
- Click-iT™ EdU detection and analysis via automated imaging gave comparable results with flow cytometry data (data not shown) and is readily applicable to adherent cells.
- Combining Click-iT™ EdU with other probes is easy and results in content-rich assays.

References

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2. Tornøe, C. W., Christensen, C., Meldal, M. **2002**. *J. Org. Chem. 76*:3057-3064.

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