Click chemistry based detection of DNA synthesis by imaging: a new, versatile, and user-friendly method compared with BrdU methods

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Abstract

Accurate characterization of the effect of pharmaceuticals or other biologically active reagents on DNA synthesis and cell cycle profession 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 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 BrdU method with a new method of pulse-labeling cells with 5-ethynyl-2′-deoxyuridine (EdU) and the subsequent fluorescent 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 that while both methods showed excellent sensitivity with a 5-minute pulse labeling of cells with nucleosides, the BrdU method, due to its DNA denaturation requirements, gave poor performance in co-labeling with an anti-cyclin B1 antibody. On the other hand, the EdU method not only gave excellent cyclin B1 staining, its staining procedure is much simpler and with a 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 a protein target of cell cycle importance (cyclin B1), 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 should also open the possibility of multiplexing with other functional probes to further enrich the information content of image based assays of cellular activities.

Figure 1

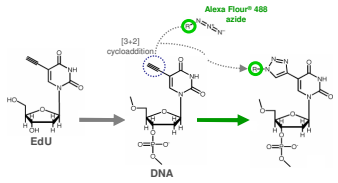

Figure 1 – Click chemistry[1,2] is the copper(I) catalyzed reaction between a terminal alkyne and an azide. EdU, a thymidine analog, is incorporated into the DNA and detected with Click-iT™ EdU Alexa Fluor® azide reagent

Figure 2 – Comparison of EdU and BrdU labeling protocols for imaging

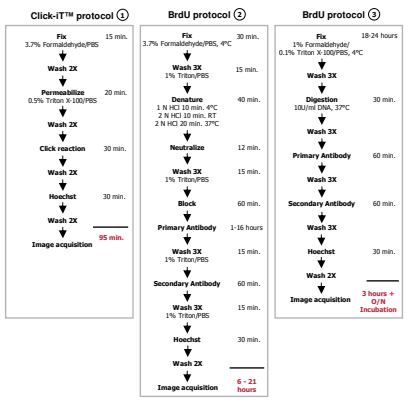

Figure 3 – Multiplexing proliferation using EdU or BrdU with DNA cell cycle and cyclin B1 in nocodazole treated HeLa cells

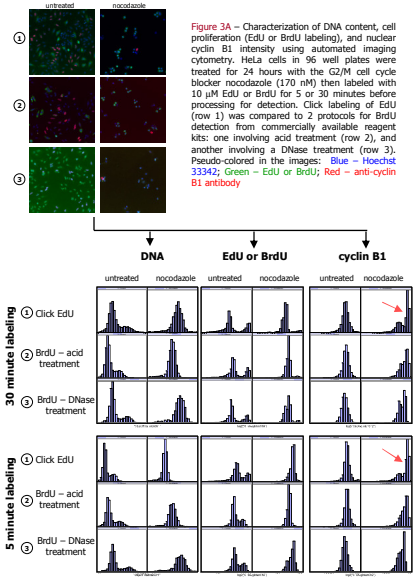

Figure 3A – Characterization of DNA content, cell proliferation (EdU or BrdU labeling), and nuclear cyclin B1 intensity using automated imaging cytometry. HeLa cells in 96 well plates were treated for 24 hours with the G2/M cell cycle blocker nocodazole (170 nM) then labeled with 10 µM EdU or BrdU for 5 or 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 – DNA profile and Click-iT™ S-phase detection by imaging – data exploration with dynamic graphics

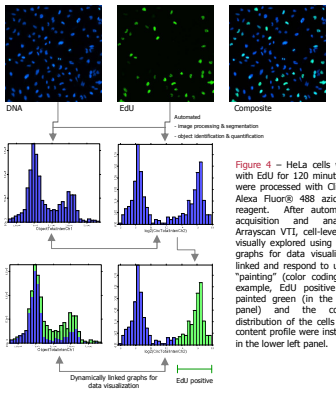

Figure 4 – HeLa cells were pulsed with EdU for 120 minutes and then were processed with Click-iT™ EdU Alexa Fluor® 488 azide detection reagent. After automated image acquisition and analysis with Arrayscan VTI, cell-level data were visually explored using Panmo. All graphs for data visualization were linked and respond to user-directed "painting" (color coding). In this example, EdU positive cells were painted green (in the lower right panel) and the corresponding distribution of the cells in the DNA content profile were instantly shown in the lower left panel.

Figure 5 – DNA profile and Click-iT™ S-phase detection: comparison of flow cytometry and imaging cytometry

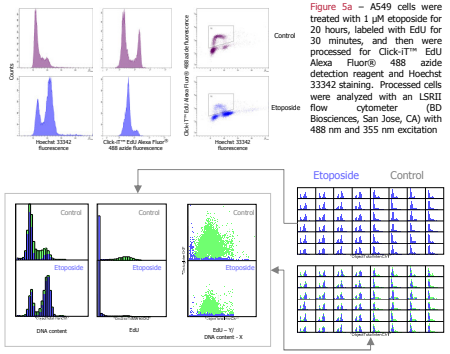

Figure 5a – A549 cells were treated with 1 µM etoposide for 20 hours, labeled with EdU for 30 minutes, and then were processed for Click-iT™ EdU Alexa Fluor® 488 azide detection reagent and Hoechst 33342 staining. Processed cells were analyzed with an LSRII flow cytometer (BD Biosciences, San Jose, CA) with 488 nm and 355 nm excitation

Figure 5b – A549 cells were treated with 1 µM etoposide for 20 hours, labeled with EdU for 30 minutes, and then processed with Click-iT™ EdU Alexa Fluor® 488 azide detection reagent and Hoechst 33342 staining. Automated image acquisition and analysis was performed as illustrated in the Figure 4. Histogram trellis plots (above right) show performance across wells in a microplate. Graphs in the left show profiles of parameters using drug treatment as the conditioning attribute for cell-level data pooled from multiple wells. Note: histograms show relative frequencies.

Figure 6 – DNA profile and Click-iT™ S-phase detection – Drug effects

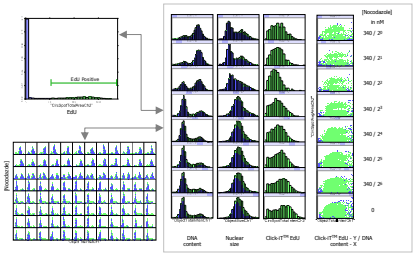

Figure 6 – A549 cells were treated with nocodazole (concentration step gradient as illustrated) for 20 hours, then labeled with EdU for 30 minutes and were processed with Click-iT™ EdU Alexa Fluor® 488 azide detection reagent. Image acquisition and analysis were performed as illustrated in the previous figures. In this plate cells in each row were treated with one drug concentration (steps of ½ from top, with bottom row received no drug treatment). Upper left graph shows the EdU signal distribution with "EdU positive" painted in green. The lower left trellis graph illustrates the changes in DNA content profile in the drug concentration step gradient. The trellis plots in the right show various parameters with drug concentration as the conditioning attribute. Note: histograms show relative frequencies.

Figure 7 – Click-iT™ S-phase detection by imaging - multiplexing

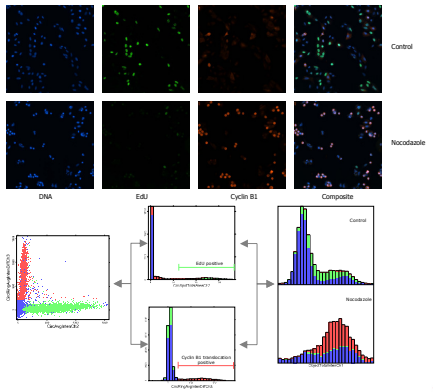

Figure 7a – A549 cells were treated with 170 nM nocodazole for 20 hours, labeled with EdU for 30 minutes, and then processed with Click-iT™ EdU Alexa Fluor 488® azide detection reagent; the cells were then stained with anti-cyclin B1 antibody (Invitrogen) and Hoechst 33342. DNA – Hoechst 33342; EdU – Click-iT™ Alexa Fluor® 488 Azide Dye; Cyclin B1 – Alexa Fluor® 555 conjugated secondary antibody.

Figure 7b – A sub-set of graphs from visual data exploration of the data set illustrated by the micrographs of Figure 7a. (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.

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 doesn't 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 and is readily applicable to adherent cells.
- Combining Click-iT™ EdU with other probes is easy and results in content-rich assays.

References

1. Rostovtsev, V.V.; Green, L.G.; Fokin, V.V.; Sharpless, K.B. *Angew. Chem. Int. Ed.* **2002,** *41*, 2596.
2. TornØe, C. W., Christensen, C., Meldal, M. **2002**. *J. Org. Chem. 76*:3057-3064.