# Dual pulse labeling using a new thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) to detect alterations in S-phase progression by fluorescence microscopy and flow cytometry

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## Abstract

The measurement of cellular proliferation is fundamental to the assessment of cell health, genotoxicity, and the evaluation of drug efficacy. Changes in DNA replication during S-phase can give insights into mechanisms of cell growth, cell cycle kinetics, and cytotoxicity. A common method for detection of cell proliferation is the incorporation of a thymidine analog during DNA synthesis. Incorporation of two different analogs at different time points can further define cell cycle kinetics. Traditionally the dual pulse method has been done by combining 5-bromo-2'-deoxyuridine (BrdU) with iododeoxyuridine (IdU) or chlorodeoxyuridine (CldU), using multiple BrdU antibodies that cross-react with either IdU or CldU for detection.[1] The objective of this study was to examine an alternative dual pulse labeling method using the new thymidine analog, 5-ethynyl-2'-deoxyuridine (EdU), with subsequent detection by click chemistry.[2-3] Using quantitative flow cytometry, multiple BrdU antibodies were screened for their specificity for BrdU. While many BrdU antibodies cross react with EdU, no cross reactivity with incorporated EdU was observed when using the BrdU antibody clone, MoBU-1. Flow cytometry and fluorescence microscopy confirmed labeling of BrdU did not interfere with the detection of EdU, and that incorporated BrdU was not detected by click chemistry. Using this method for dual pulse labeling, we examined the effect of several drugs with known biological effects such as DNA damage, cell cycle arrest, and apoptosis. Cells were pulsed with EdU, followed by drug treatment, and then pulsed with BrdU. Altered S-phase progression was observed after drug treatment, as evident from changes in the percentage of cells labeled with BrdU alone, EdU alone, or both EdU and BrdU. Evaluation of S-phase labeling patterns by fluorescence microscopy further defined cell cycle status of individual cells. In conclusion, this method eliminated concerns of antibody specificity associated when pulse labeling with two different halogenated deoxyuridine derivatives. Furthermore, dual pulse labeling with EdU and BrdU provided an accurate and simplified method of examining alterations in DNA replication during cell cycle progression.

## Figure 1: EdU and BrdU structures

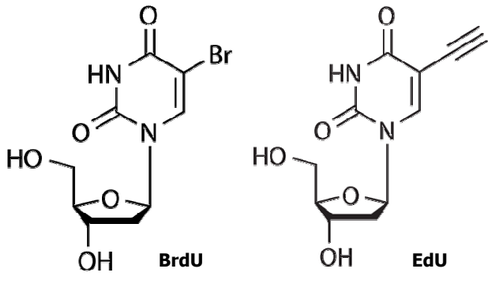
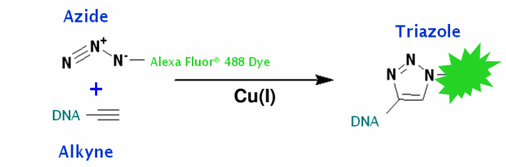
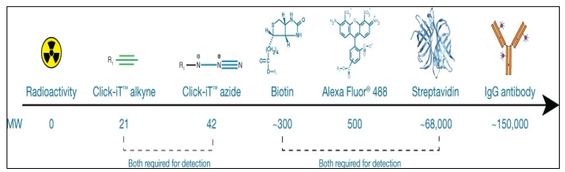



**Figure 1** The structures of BrdU and EdU are very similar: BrdU contains a bromine in the terminal position while EdU contains an alkyne group in the terminal position of the compound.

## Figure 2: Click chemistry based detection of EdU

The click reaction[4,5] is a copper(I)-catalyzed variant of the Huisgen [3+2] cycloaddition between an azide and a terminal alkyne, resulting in a covalent bond in the triazole product. In this application, the incorporated EdU contains a terminal alkyne which specifically reacts with a fluorescently labeled azide dye (Alexa Fluor® 488 azide shown as an example below), to covalently label nascent DNA after the EdU pulse. The click reaction will detect the incorporated EdU, but does not cross-react with the incorporated BrdU. Additionally, the small size of the azide detection molecule allows for access to EdU without denaturation.




## Figure 3: Flow cytometric identification of an anti-BrdU antibody clone that does not cross react with EdU

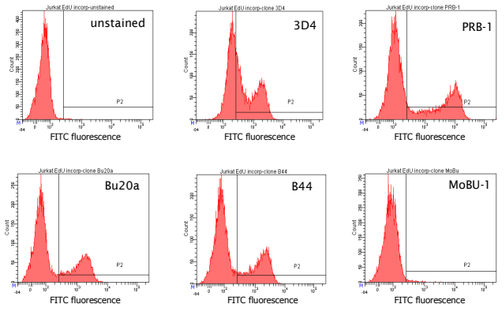



**Figure 3** The dual pulse technique requires a BrdU antibody that does no react with EdU. Jurkat T cells were treated with 10 µM EdU for one hour, followed by ethanol fixation, and an acid denaturation method[6] was used before labeling with FITC conjugates of five different and commonly used anti-BrdU antibody clones: 3D4, PRB-1, Bu20a, B44, & MoBU-1. Of these five antibodies tested, only the MoBU-1 clone did not react with EdU.

## Figure 4: Multiplex analysis of cell proliferation with EdU and BrdU and DNA content using flow cytometry

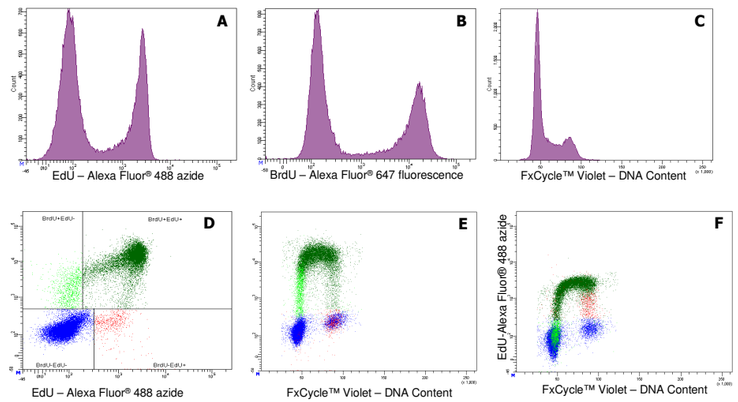



**Figure 4** TF-1 myeloid progenitor cells were treated with 20 µM EdU for one hour before adding 10 µM BrdU to the media for one hour; without removal of the EdU or washing of the cells. The cells were then fixed in ethanol, and an acid denaturation method[6] was used before labeling with anti-BrdU antibody (clone MoBU-1)-Alexa Fluor® 647 conjugate, EdU Click-iT®-Alexa Fluor® 488 azide, and FxCycle™ Violet stain for DNA content. Data was acquired on a Becton Dickinson™ LSRII flow cytometer using 405 nm, 488 nm, and 633 nm lasers and analyzed with DIVA™ 6.1 software. Both analogs can be specifically detected. Panels A-C show the individual parameter histograms of (A) EdU detection (B) BrdU detection and (C) DNA content. Panels D-F show dual parameter plots of (D) EdU vs. BrdU (E) DNA content vs. BrdU and (F) DNA content vs. EdU. In panels E and F the cells are colored as shown in the EdU vs. BrdU plot quadrant regions in plot D: Cells colored blue are negative for both EdU and BrdU. Cells colored dark green are positive for both EdU and BrdU. Cells colored red are positive for EdU but negative for BrdU. These cells are positive only for the first pulse, that of EdU. Cells colored light green are negative for EdU but positive for BrdU. These are the cells positive only for the second pulse, that of BrdU.

## Figure 5: Fluorescence microscopy demonstrates the anti-BrdU antibody (clone MoBU-1) does not cross react with EdU and the click reaction does not detect incorporated BrdU

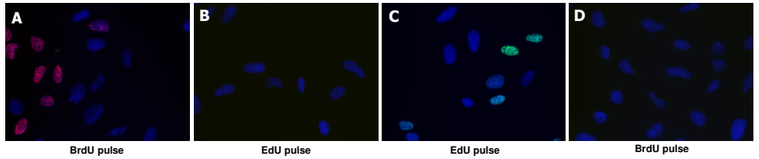



**Figure 5** U-2 OS cells pulsed with (A and D) 10 µM BrdU or (B and C) 10 µM EdU for 60 minutes. Panels A-B show cells labeled with anti-BrdU antibody (clone MoBU-1)-biotin conjugate followed by secondary detection with streptavidin Alexa Fluor® 594 conjugate (red). Panels C-D show cells subjected to click reaction with Alexa Fluor® 488 azide (green). Nuclei counterstained with Hoechst 33342 (blue). Images were acquired on a Nikon Eclipse E800 wide-field microscope using standard DAPI/FITC/Texas Red filters. Anti-BrdU antibody (clone MoBU-1) detects cells which have incorporated BrdU, but not cells that have incorporated EdU. Click-iT® azide detects cells which have incorporated EdU, but not cells that have incorporated BrdU.

## Figure 6: Dual pulse labeling with EdU and BrdU using fluorescence microscopy and high content imaging

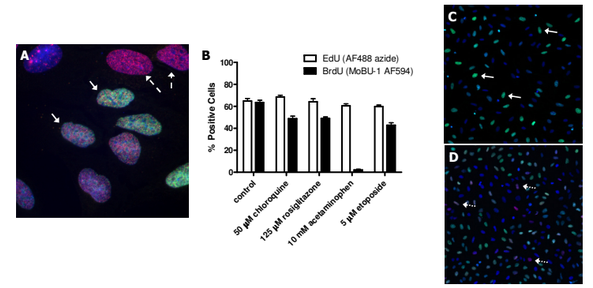



**Figure 6** Use of the dual pulse method with EdU and BrdU to analyze the effect of various drugs on DNA replication using high content imaging and analysis. U-2 OS cells were pulsed with 10 µM EdU for 60 minutes, washed twice with warm complete media, left untreated or drug-treated for 23 hours, and then pulsed with 10 µM BrdU for 60 minutes. Cells were fixed and denatured with HCl before performing click reaction with Alexa Fluor® 488 azide to detect EdU (green). Subsequent detection of BrdU was conducted using anti-BrdU antibody (clone MoBU-1)-Alexa Fluor® 594 direct conjugate (red). Nuclei were counterstained with HCS NuclearMask™ Blue stain (blue). (A) Visualization of a variety of staining patterns in untreated cells indicate differences in the extent of nascent DNA synthesis within subpopulations of cells during the EdU and BrdU pulses (solid arrows show cells which have incorporated EdU and BrdU, dashed arrows show cells which have incorporated BrdU only). (B) Quantitative analysis of drug effects on DNA replication as indicated by the decrease in percent of BrdU-positive versus EdU-positive cells. Images show (C) cells treated with 10 mM acetaminophen lack BrdU positive cells (mostly EdU positive – solid arrows), while (D) untreated cells are BrdU and EdU positive (dashed arrows). Imaging and analysis was performed on a Cellomics Arrayscan® VTI platform (Thermo Scientific).

## Conclusions

The benefits of dual pulse labeling with EdU and BrdU include:

- Eliminates concerns regarding antibody specificity associated with dual labeling with two different halogenated deoxyuridine derivatives
- The specific anti-BrdU antibody (clone MoBU-1) allows for detection of BrdU without cross reactivity with EdU
- EdU detection using click chemistry is bio-orthogonal and does not cross react with BrdU
- Sequential pulses of EdU followed by BrdU can be done with removing or washing out EdU
- This method has broad applications in flow cytometry, fluorescence microscopy, and high content imaging

## References

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