Dual pulse labeling of S-phase population using click chemistry to measure changes in cell proliferation

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Introduction

The measurement of cellular proliferation is fundamental to the assessment of cell health, genotoxicity, and in the evaluation of drug efficacy. Changes in cell proliferation can give insights into mechanisms of cell growth, development, and cell cycle kinetics. A common method for detection of cell proliferation is using the incorporation of a thymidine analog during DNA synthesis. Incorporation of two 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) immunocytochemistry and 3H-thymidine radiography, or by combining BrdU with iododeoxyuridine (IdU) or chlorodeoxyuridine (CldU) using multiple BrdU antibodies[4] that cross-react with either IdU or CldU for detection.

With the introduction of the new thymidine analog, 5-ethynyl-2'-deoxyuridine (EdU) with detection via click chemistry[1,2,3], dual pulse labeling is simplified. Using sequential pulses of the thymidine analogs EdU and BrdU, a straightforward and reliable method of distinguishing BrdU- and EdU-labeled cells by flow cytometry is introduced. This dual pulse method uses a mouse monoclonal BrdU antibody for the detection of the incorporated BrdU, which shows no cross reactivity with EdU, clone MoBU-1. This is combined with click-chemistry detection of the incorporated EdU, which is bio-orthogonal and does not react with the incorporated BrdU. This method has broad applications and demonstrates the value of click chemistry combined with other antibody based methods.

Figure 1 – Click Chemistry Based Detection of EdU

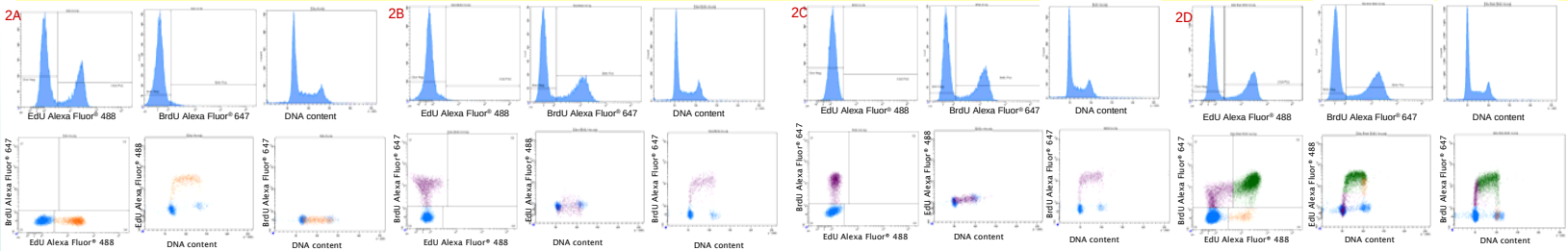
The click reaction[5,6] is a copper(I)-catalyzed variant of the Huisgen [3+2] cycloaddition between an azide and a terminal alkyne; a covalent bond results. In this application, the incorporated EdU contains a terminal alkyne which reacts with a fluorescently labeled azide dye, to covalently label the double stranded DNA.

Click chemistry is employed in this application because the reaction between an alkyne and an azide is bio-orthogonal; it occurs selectively, without the interference of any other functional groups present within complex biological systems. Alkyne and azide compounds are stable, inert functional groups which typically are not present in biological systems. The click reaction will detect the incorporated EdU, but does not cross-react with the incorporated BrdU.

References

1. Salic, A.; et al. Proc Natl Acad Sci U S A. **2008** Feb 19;105(7):2415-20.
2. Buck,S; et al. BioTechniques. June **2008**, 44:927-929.
3. Diermeier-Daucher, S; et al. Cytometry Part A, EPUB 20 Feb **2009**
4. Conboy,M; et al. PLoS Biology. May **2007** 5(5):1120-1126.
5. Rostovtsev, V.V.; et al. Angew. Chem. Int. Ed. **2001**, 41, 2596.
6. Tornøe, C.W.; et al. J. Org. Chem.**2001**, 67, 3057.
7. Current Protocols in Cytometry;vol 1; Robinson, J.P. ed; John Wiley & Sons Inc. **2007**.

Figure 2 – Labeling with single and dual pulses demonstrated with Jurkat T-cell leukemia cells



2A: Jurkat cells were treated with 20 µM EdU for one hour. The cells were then fixed in ETOH, and an acid denaturation method[7] was used before labeling with anti-BrdU-Alexa Fluor® 647 dye, Click-iT®-Alexa Fluor® 488 azide to detect EdU, and SYTOX® Blue nucleic acid dye (+RNase) for DNA content. No cross-reactivity of the azide dye and the incorporated BrdU is seen.

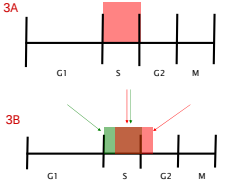
2B: Jurkat cells were treated with 10 µM BrdU for one hour. The cells were then fixed in ETOH, and the acid denaturation method was used before labeling with anti-BrdU-Alexa Fluor® 647 dye to detect the incorporated BrdU, Click-iT®-Alexa Fluor® 488 azide, and SYTOX® Blue nucleic acid dye (+RNase) for DNA content. No cross-reactivity of the BrdU antibody and the incorporated EdU is seen.

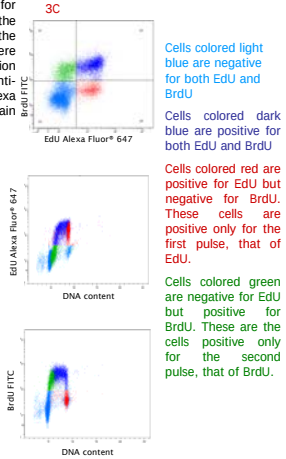
2C: Jurkat cells were treated with 20 µM EdU and 10 µM BrdU together for one hour. The cells were then fixed in ETOH, and an acid denaturation method was used before labeling with anti-BrdU-Alexa Fluor® 647 dye, Click-iT®-Alexa Fluor® 488 azide , and SYTOX® Blue nucleic acid dye (+RNase) for DNA content. Only detection of BrdU is seen, showing that BrdU is preferentially incorporated over EdU when both analogs are present.

2D: Jurkat cells were treated with 20 µM EdU for one hour and then 10 µM BrdU is added to the media for one hour, without removal of the EdU or washing of the cells. The cells were then fixed in ETOH, and an acid denaturation method was used before labeling with anti-BrdU-Alexa Fluor® 647 dye, Click-iT®-Alexa Fluor® 488 azide, and SYTOX® Blue nucleic acid dye (+RNase) for DNA content. Both analogs can be specifically detected.

Figure 3 – Dual pulse labeling

Jurkat cells were treated with 10 µM EdU for one hour and then 10 µM BrdU is added to the media for one hour, without removal of the EdU or washing of the cells. The cells were then fixed in ETOH, and an acid denaturation method was used before labeling with anti-BrdU-Alexa Fluor® 488 dye, Click-iT®-Alexa Fluor® 647 azide, and FxCycle™ Violet stain for DNA content.





3A: During the first pulse, EdU alone is incorporated into cells replicating DNA for one hour

3B: BrdU is added for the second pulse for one hour.

- Cells that have already completed DNA synthesis will not incorporate BrdU and will display EdU only.
- Cells newly entering S-phase will incorporate BrdU and display BrdU only.
- Cells in S-phase during both pulses will display BrdU and EdU

Cells colored light blue are negative for both EdU and BrdU

Cells colored dark blue 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 green are negative for EdU but positive for BrdU. These are the cells positive only for the second pulse, that of BrdU.

Figure 4 – TF-1 erythroleukemia cells with longer pulses

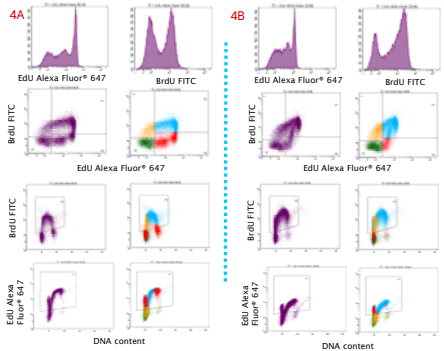
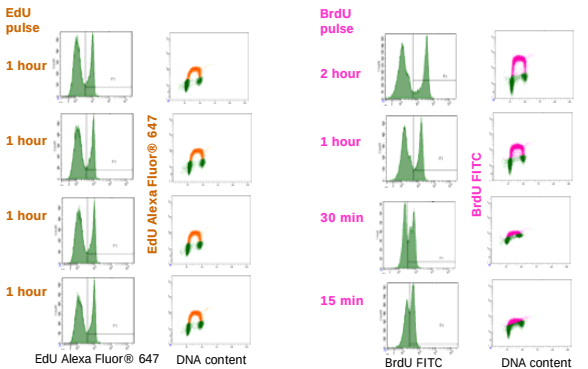


Figure 4A: TF-1 cells were treated with 10 µM EdU for 8 hours before 10 µM BrdU was added to the media for 2 hours.
Figure 4B: TF-1 cells were treated with 10 µM EdU for 2 hours before 10 µM BrdU was added to the media for 8 hours.

All the cells were labeled with anti-BrdU-Alexa Fluor® 488 dye, Click-iT® Alexa Fluor® 647 azide, and FxCycle™ Violet stain for DNA content
Plots show dual negative cells green, dual positive cells blue, EdU positive & BrdU negative cells red, BrdU positive and EdU negative cells yellow.

Figure 5 – THP-1 monocytic leukemia cells

Figure 5: THP-1 cells were treated with 10µM EdU for one hour, followed by 10µM BrdU for varying times of 15 minutes to 2 hours. All the cells were labeled with anti-BrdU-Alexa Fluor® 488 dye, Click-iT® Alexa Fluor® 647 azide, and FxCycle™ Violet stain for DNA content. Dual parameter plots show EdU positive cells orange, BrdU positive cells pink.



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