

Improved Click Chemistry Demonstrating EdU Cell Proliferation with GFP-expressing Cells and R-PE-based Immunophenotyping

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ABSTRACT

The current Click-IT® EdU kit for direct S-phase determination cannot be used for the simultaneous detection of GFP and R-PE fluorescence due to the presence of copper and reactive oxygen species mediated damage to fluorescent proteins. We present modifications to the click reaction resulting in "copper safe" catalysis of the click reaction. Flow cytometry measurements of cell proliferation using Click-IT® EdU are demonstrated to be compatible with immunophenotyping panels using R-PE conjugates and GFP-expressing cell lines. The basis of the click chemistry improvement is the use of a copper (I) ligand combined with a modified dye azide detection reagent. We optimized components in the improved click reaction and found those that best preserved R-PE fluorescence while obtaining a bright EdU signal. Jurkat cells were pulsed with EdU, labeled with R-PE conjugated antibodies and then fixed and permeabilized. EdU incorporation was then detected using click chemistry, demonstrating improved detection while maintaining R-PE signal. Additionally, cells expressing GFP were pulsed with EdU and used to demonstrate GFP and click chemistry compatibility. The modified click reaction is an improvement over the original copper based click reactions and will further enhance the utility of EdU based cell proliferation applications in multi-color flow cytometry.

INTRODUCTION

EdU (ethynyl-deoxyuridine) incorporation into nascent DNA detected with copper-catalyzed click chemistry (CuAAC) as a method for measuring cell proliferation was first described in 2008^{1,2}. This method reduces assay time and improves the work flow compared to the traditional method using antibody-based BrdU detection. However, copper and reactive oxygen species (ROS) mediated damage to fluorescent proteins prevents the simultaneous detection of EdU, GFP or R-phycoerythrin (R-PE) fluorescence. We present chemical modifications to the click reaction resulting in "copper safe" catalysis of the click reaction, whereby GFP and R-PE fluorescence are preserved while EdU based cell proliferation is detected. The basis of the improvement is the use of a copper (I) ligand combined with a modified dye azide detection reagent. Together, the copper ion is sequestered with the ligand and prevented from damaging protein fluorescence but still remains available to catalyze the click reaction. The use of the described modified click reaction is an improvement over earlier described click reactions and will further enhance the utility of EdU based cell proliferation assays by facilitating multiplexing using common fluorescent proteins.

MATERIALS AND METHODS

To study the effects of our modified reactants on GFP fluorescence, we used an A375 cell line constitutively expressing GFP-ERK2 under control of a CMV promoter. ERK2 A375 cells were pulsed with 10 μ M EdU for 2 hours prior to fix/permeabilization and click labeling with modified Alexa Fluor® 647 azide. For R-PE immunophenotyping experiments Jurkat cells were pulsed with 10 μ M EdU for 2 hours prior to surface staining with hCD3-RPE followed by fix/permeabilization and click labeling with modified Alexa Fluor® 647 azide. We identified the click reaction conditions that best preserved GFP and R-PE fluorescence while obtaining a bright EdU signal. Quintessential cell cycle bi-variate plots were generated using Jurkat cells pulsed with 10 μ M EdU for 2 hours prior to fix/permeabilization then click labeled with modified Alexa Fluor® 488 azide and DNA content cell cycle stained with FxCycle™ Violet. All flow cytometry data was collected and analyzed on the Attune® Acoustic Focusing Cytometer.

Figure 1 – Click chemistry-based detection of metabolically incorporated DNA analog 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 prepared 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.

Figure 2 – Use of the Click-IT® EdU imaging kit with proliferating A375 cells

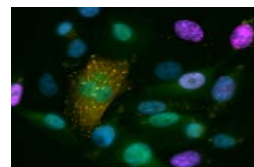


Figure 2 – ERK2 A375 GFP expressing cells pulsed with 10 μ M EdU for two hours are labeled with modified Alexa Fluor® 647 azide (pink), CellLight® Tailin-RFP® "BacMam 2.0" (orange), and Hoechst 33342 (blue). Proliferating cells have pink nuclei. Non-proliferating cells have blue nuclei.

Figure 3 – Use of modified click components preserves GFP fluorescence while maintaining a robust EdU signal

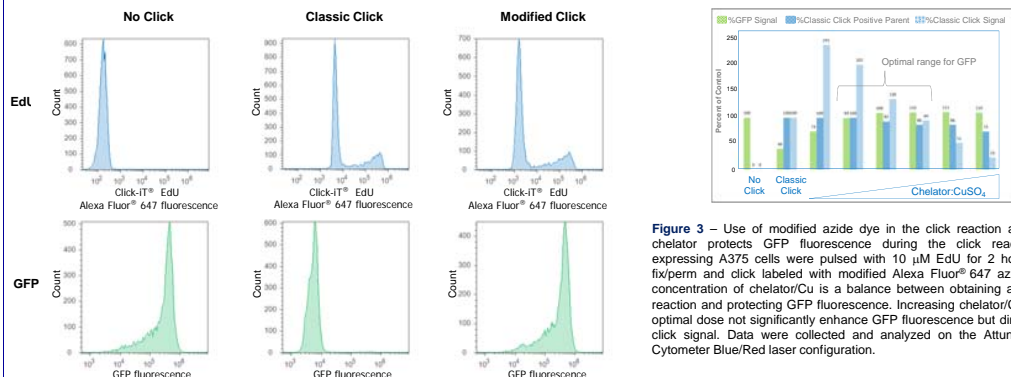


Figure 3 – Use of modified azide dye in the click reaction along with a chelator protects GFP fluorescence during the click reaction. GFP-expressing A375 cells were pulsed with 10 μ M EdU for 2 hours prior to fix/permeabilization and click labeled with modified Alexa Fluor® 647 azide. Optimal concentration of chelator/Cu is a balance between obtaining a bright click reaction and protecting GFP fluorescence. Increasing chelator/Cu ratio past optimal dose not significantly enhance GFP fluorescence but diminishes the click signal. Data were collected and analyzed on the Attune® Acoustic Cytometer Blue/Red laser configuration.

Figure 4 – Use of modified click components preserves R-PE fluorescence while maintaining a robust EdU signal

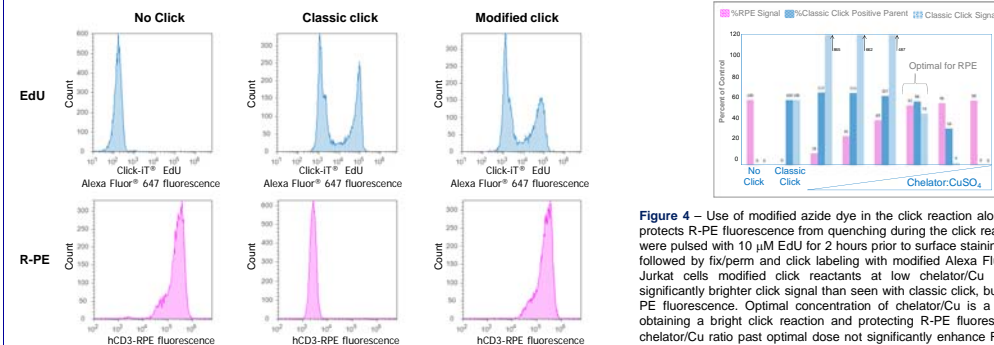


Figure 4 – Use of modified azide dye in the click reaction along with a chelator protects R-PE fluorescence from quenching during the click reaction. Jurkat cells were pulsed with 10 μ M EdU for 2 hours prior to surface staining with hCD3-RPE followed by fix/permeabilization and click labeling with modified Alexa Fluor® 647 azide. In Jurkat cells modified click reactants at low chelator/Cu ratios produce a significantly brighter click signal than seen with classic click, but fail to protect R-PE fluorescence. Optimal concentration of chelator/Cu is a balance between obtaining a bright click reaction and protecting R-PE fluorescence. Increasing chelator/Cu ratio past optimal dose not significantly enhance R-PE fluorescence but diminishes the click signal. Data was collected and analyzed on the Attune® Acoustic Cytometer Blue/Red laser configuration.

Figure 5 – Comparison of classic Click-IT® EdU and modified Click-IT® EdU in the determination of S-phase percent

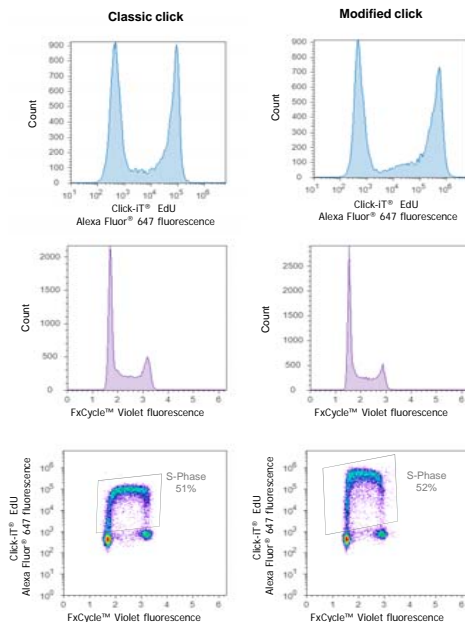


Figure 5 – S-phase % as measured by Click-IT® EdU and FxCycle™ Violet in Jurkat T lymphocyte cells. Measurements taken on the Attune® Acoustic Focusing Cytometer. Top Plots – click reaction; Middle Plots – DNA content cell cycle; Bottom Plots – Bivariate plots of click reaction and DNA content cell cycle showing S-phase population within the rectangular region. Data was collected and analyzed on the Attune® Acoustic Cytometer with Blue/Violet laser configuration.

CONCLUSIONS

The use of an optimized ratio of chelator to copper combined with a modified dye azide creates an ideal click reaction that sequesters Cu ions and protects GFP and R-PE fluorescence. These breakthrough conditions enable scientists to use the Click-IT® EdU cell proliferation technology with GFP-expressing cell lines and in conjunction with immunophenotyping panels containing R-PE.

REFERENCES

1. A. Salic, T. J. Mitchison, *Proc Natl Acad Sci U S A* 105, 2415 (Feb 19, 2008)
2. S. B. Buck et al., *Biotechniques* 44, 927 (Jun, 2008).