

# Copper safe Click-iT® Plus EdU proliferation assay: Improved compatibility with simultaneous phycobiliprotein and fluorescent protein detection

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## Abstract

Immunodetection of incorporated bromodeoxyuridine (BrdU) has until recently been the standard method for measuring cell proliferation by flow cytometry. This method has never been wholly satisfactory, however, due to the large size of antibodies and the necessity to chemically disrupt cells and their chromatin for detection.

In 2007, a small molecule technique for detecting 5-ethynyl-2'-deoxyuridine (EdU) incorporation to cells was developed and commercialized by Life Technologies (now a division of Thermo Fisher Scientific). The Click-iT® small molecule chemistry allows fluorescent tagging of incorporated EdU nucleotides without the DNA damage normally associated with nucleotide immunolabeling assays, and has become a dominant technique for proliferation analysis. However, a weakness of the technology was its incompatibility with two major biomolecule-based technologies often used simultaneously with proliferation detection: phycobiliprotein (PB) immunodetection and expressible fluorescent proteins (FPs). Copper (a necessary co-factor for the click reaction) inhibited both PB and FP fluorescence, and therefore prevents simultaneous detection of EdU, fluorescent proteins, and PB fluorescence. Recently, a chemical modification to the click reaction was made to make it "copper safe", rendering it compatible with PB labeling and FP expression. In this study, the "copper safe" Click-iT® Plus EdU incorporation assay was used to measure cell proliferation in mouse T splenocytes and cell lines both expressing a variety of fluorescent proteins, and with simultaneous immunolabeling with phycoerythrin and allophycocyanin. This modification maintained both PB and FP fluorescence in comparison with the standard procedure. The "copper safe" modification to the Click-iT® EdU assay therefore improved assay compatibility with these important groups of fluorescent molecules.

## Materials and Methods

The conventional Click-iT EdU labeling technology, while permitting the identification of incorporated EdU molecules into replicating DNA, has been found to be incompatible with phycoerythrin (PE) immunolabeling carried out **before** the Click-iT reaction, as well as *Aequorea* jellyfish derived expressible fluorescent proteins including fluorescent proteins like GFP and YFP. Sensitivity to the copper included in the assay required to catalyze the Click-iT reaction. A "copper-safe" formulation of this assay (the Click-iT Plus variant) was tested in comparison to the standard assay chemistry. In addition, copper was omitted from the reaction in the newer assay formulation to determine if and to what degree its presence affected phycobiliprotein or fluorescent protein fluorescence.

For extracellular labeling experiments, EL4 mouse lymphoma cells incubated with EdU at 20  $\mu$ M for 2 h and labeled for CD90 with the appropriate fluorochrome prior to Click-iT EdU labeling. SP2/0 cell expressing the relevant fluorescent protein were also incubated with EdU at 20  $\mu$ M for 2 h.

## Results

The "copper-free" Click-iT Plus assay formulation was found to preserve both PE and fluorescent protein fluorescence. Small losses of some fluorescent protein signal still occurred in the assay, but there were due to the permeabilization process, not the presence of copper ions.

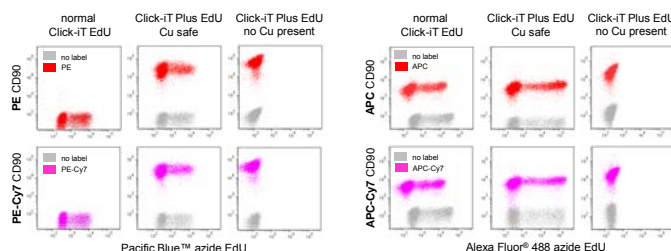
As determined previously, some phycobiliproteins (including APC) and some coral-derived fluorescent proteins (like DsRed) were not greatly affected by the presence of copper, and functioned well in both variations of the assay. Intracellular labeling with PE after the reaction (as opposed to extracellular labeling before the assay) was also preserved with both assays. In the case of quantum nanoparticles (Qdot® or eFluorNC probes), their exquisite sensitivity to copper did not allow their use with either assay variant. However, recently developed fluorochrome technology like the Brilliant Violet dyes were well-preserved in both variants.

The Click-iT Plus EdU assays also incorporate a modification in the azide linker chemistry to a picolyl moiety. This resulted in improved EdU labeling and a noticeable increase in assay sensitivity.

## Phycobiliproteins

**Below left.** **Phycoerythrin (PE)** and its tandems are copper sensitive and do not survive the conventional Click-iT EdU labeling chemistry (left column). However, the "copper-safe" Click-iT Plus EdU was compatible (middle column). Omission of the copper from the Plus reaction produced minimal change in fluorescence (right column).

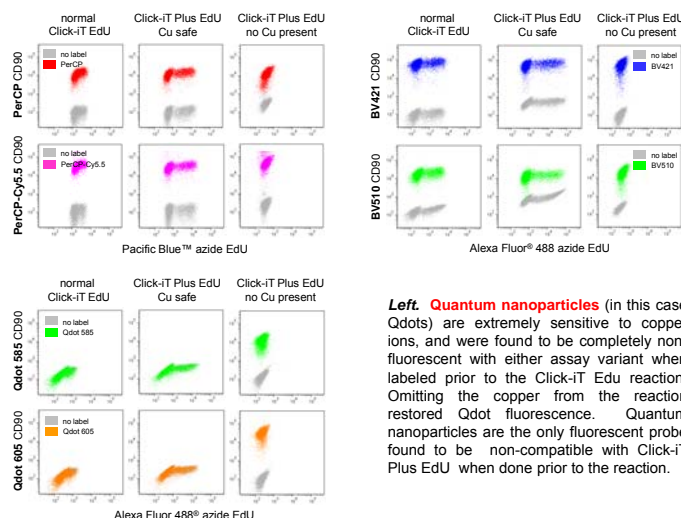
**Below right.** **Allophycocyanin (APC)** and APC tandem fluorescence was unaffected by either variant.



## Other fluorochromes

**Below left.** The dinoflagellate derived fluorochrome **peridinin chlorophyll PerCP** and its tandem conjugate **PerCP-Cy5.5** were both found to be copper-insensitive and could be used with both assay variants either before or after the Click-iT reaction.

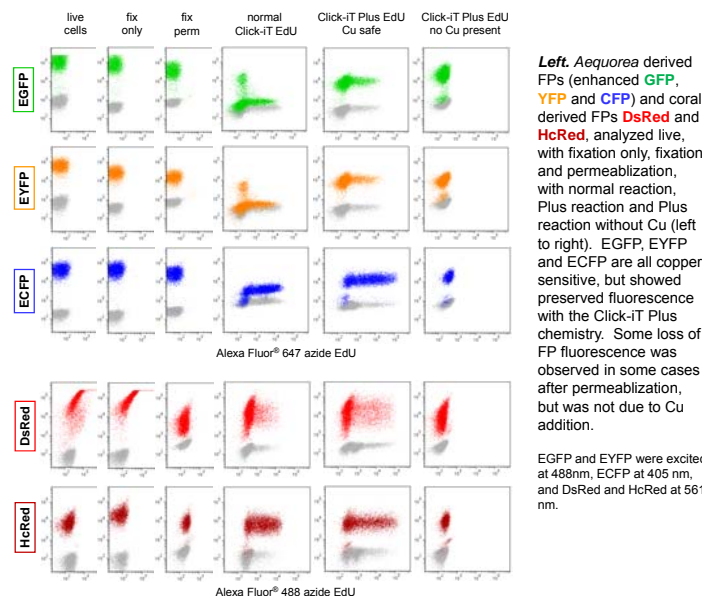
**Below right.** The **Brilliant Violet dyes BV421** and **BV510** were unaffected by either assay variant.



**Left.** **Quantum nanoparticles** (in this case Qdots) are extremely sensitive to copper ions, and were found to be completely non-fluorescent with either assay variant when labeled prior to the Click-iT EdU reaction. Omitting the copper from the reaction restored Qdot fluorescence. Quantum nanoparticles are the only fluorescent probe found to be non-compatible with Click-iT Plus EdU when done prior to the reaction.

## Fluorescent proteins

A variety of fluorescent proteins were also tested for Click-iT® Plus EdU compatibility, including *Aequorea* jellyfish derived FPs EGFP, EYFP and ECFP, and coral derived proteins DsRed and HcRed.



**Left.** *Aequorea* derived FPs (enhanced GFP, YFP and CFP) and coral derived FPs **DsRed** and **HcRed**, analyzed live, with fixation only, fixation and permeabilization, with normal reaction, Plus reaction and Plus reaction without Cu (left to right). EGFP, EYFP and ECFP are all copper sensitive, but showed preserved fluorescence with the Click-iT Plus chemistry. Some loss of FP fluorescence was observed in some cases after permeabilization, but was not due to Cu addition.

EGFP and EYFP were excited at 488nm, ECFP at 405 nm, and DsRed and HcRed at 561 nm.

The "copper-free" formulation of the Click-iT Plus EdU proliferation kit preserved prior PE labeling and *Aequorea* jellyfish derived FP fluorescence in comparison with earlier variants of this assay. This increased compatibility with other fluorescent markers will increase the utility of this technology for measuring cell proliferation in complex multicolor labeling systems.

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