DETECTION OF S-PHASE CELL CYCLE PROGRESSION USING 5-ETHYNYL-2'-DEOXYURIDINE INCORPORATION WITH CLICK CHEMISTRY

Jolene A. Bradford[1], Scott T. Clarke[1], Suzanne B. Buck[1], Dani Hill[1], Yih-Tai Chen[1], Kyle Gee[1], Brian Agnew[1], Adrian Salic[2]

[1]Molecular Probes® - Invitrogen Detection Technologies • 29851 Willow Creek Rd • Eugene, Oregon 97402 • USA

[2]Harvard Medical School, Boston MA • USA

Introduction

Labeling, detection, and quantification of cells in the synthesis phase of cell cycle progression are not only important in characterizing basic biology, but also in defining the cellular responses to drug treatments. S-Phase has classically been assayed with incorporation of either radioactive thymidine or 5-bromo-2'-deoxyuridine (BrdU). We introduce the novel use of click chemistry for the labeling and detection of DNA synthesis. This labeling strategy uses the incorporation of the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU)[1], followed by chemical coupling of the analog with an azide conjugated fluorophore for detection. This protocol uses standard aldehyde fixation and detergent permeabilization, avoiding the need for denaturation steps required for BrdU labeling. Results using click chemistry detection of EdU are shown using flow cytometry, fluorescence microscopy and automated fluorescence microscopy (HCS) platforms. Comparison data of EdU and BrdU detection are shown. Results demonstrate that click chemistry-based detection of incorporated EdU provides a rapid and accurate assessment of S-phase using standard fixation and permeabilization protocols. Results obtained with EdU detection, without having to denature the DNA, are equivalent to results obtained with BrdU detection. The novel method using click chemistry-based detection of EdU can be used to measure S-phase cell cycle progression in multiple platforms.

Figure 1 – Click Chemistry Based Detection of Metabolically Incorporated DNA Analog

1A: The click reaction[2,3] is a copper(I)-catalyzed variant of the Huisgen [3+2] cycloaddition between an azide and a terminal alkyne; a covalent bond results.

1B: 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 bioorthogonal; 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.

Figure 2 – Thymidine Analogues

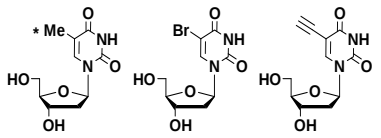


Figure 3 – EdU vs. BrdU Detection of Cell Proliferation

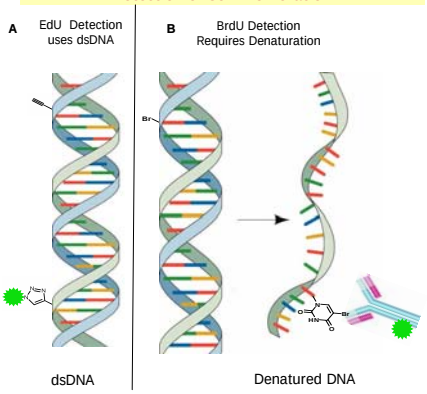


Figure 3. Panel A: The Click-iT™ EdU method obviates the need for DNA denaturation by acid, heat or nucleases; the incorporated EdU is detected directly on the dsDNA via click reaction of the alkyne with the Alexa Fluor® 488 azide. Panel B: BrdU detection with an anti-BrdU antibody requires denatured DNA.

Figure 5 – Determination of S-phase % with Click-iT™ EdU

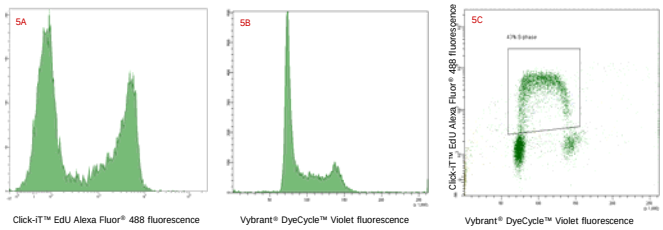


Figure 5 – S-phase percentage as measured by Click-iT™ EdU and Vybrant® DyeCycle™ Violet stain in Jurkat human leukemia T cells. Measurements taken on BD LSRII using 405 nm and 488 nm excitation lasers.

5A – click reaction

5B – cell cycle stain

5C – Bivariant plot of click reaction and cell cycle showing S-phase population within the boxed region.

Figure 6 – Comparison of EdU detection to BrdU detection using BrdU alcohol fixation and acid denaturing method[4]

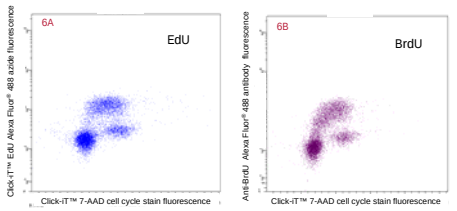


Figure 6 – Acid denaturation comparison of EdU to BrdU labeling.

6A - Jurkat cells treated with 10 µM EdU were processed using the acid denaturation protocol: 7-AAD with Click-iT™ EdU Alexa Fluor® 488 azide.

6B - Dual parameter plot showing Jurkat cells treated with 10 µM BrdU and processed using the acid denaturation protocol: 7-AAD with anti-BrdU-Alexa Fluor® 488 dye.

Conclusion: Although DNA denaturation is not required for EdU detection, results equivalent to BrdU detection are demonstrated.

Figure 7 – Comparison of EdU to BrdU detection using EdU method - paraformaldehyde fixative & saponin permeabilization

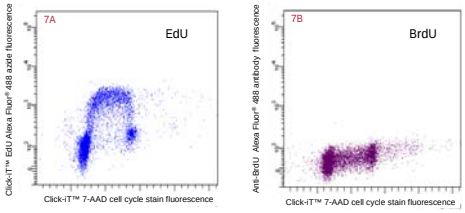
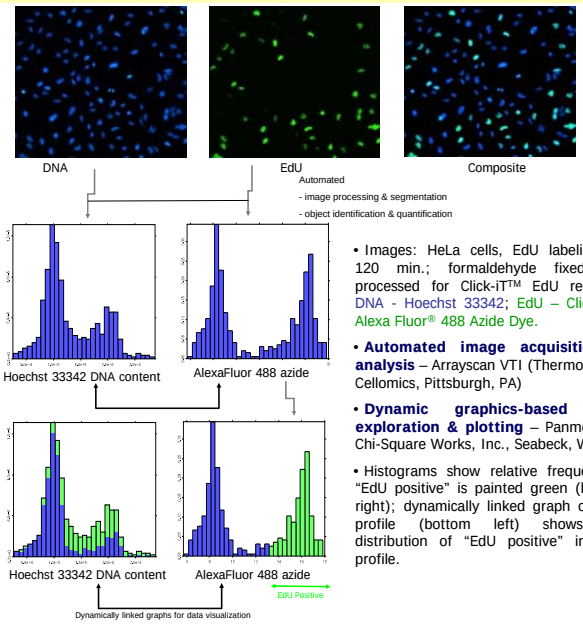


Figure 7 – Comparison of EdU to BrdU labeling of aldehyde/saponin treated cells without DNA denaturation.

7A Jurkat cells treated with 10 µM EdU were processed using the Click-iT™ EdU protocol: 7-AAD vs. Click-iT™ EdU Alexa Fluor® 488 azide. S-phase fraction is clearly seen.

7B Dual parameter plot showing Jurkat cells treated with 10 µM BrdU and processed using the Click-iT EdU aldehyde-based protocol: 7-AAD vs. anti-BrdU-Alexa Fluor® 488 antibody. No S-phase fraction is observed using antibody based detection when cellular DNA is not denatured.

Figure 4 - DNA profile and Click-iT™ S-phase detection using automated imaging



• Images: HeLa cells, EdU labeling for 120 min.; formaldehyde fixed and processed for Click-iT™ EdU reaction; DNA - Hoechst 33342; EdU – Click-iT™ Alexa Fluor® 488 Azide Dye.

• **Automated image acquisition & analysis** – Arrayscan VTI (Thermo Fisher Cellomics, Pittsburgh, PA)

• **Dynamic graphics-based data exploration & plotting** – Panmo (The Chi-Square Works, Inc., Seabeck, WA)

• Histograms show relative frequencies; "EdU positive" is painted green (bottom right); dynamically linked graph of DNA profile (bottom left) shows the distribution of "EdU positive" in DNA profile.

Figure 8 – Imaging with EdU Incorporated Cells Labeled with Click-iT™ Proliferation kit

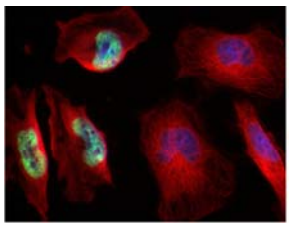


Figure 8 - HeLa cells were grown in 10 µM EdU for 2 hours and labeled with Click-iT™ EdU Alexa Fluor® 488 (green), a-tubulin/GAM Alexa Fluor® 555 conjugate (red), and a nuclear stain (DAPI, blue).

Proliferating cells have green nuclei.

Non-proliferating cells have blue nuclei.

Results and Conclusions

- A novel method of measuring cell proliferation by nucleoside incorporation using click chemistry detection is presented.
- The thymidine analog EdU incorporates into DNA, thus providing a terminal alkyne which reacts with an azide labeled with a fluorescent compound to form a covalent bond, thus labeling synthesizing DNA.
- Click chemistry detection of incorporated EdU uses standard fixation and permeabilization protocols.
- No denaturation of DNA is required for EdU detection using click chemistry.
- A simplified protocol enables
  - accurate, consistent performance
  - shorter time to answer, basic assay is complete in 2 hours
  - comprehensive data, compatible with multiplexing
- The Click-iT™ EdU cell proliferation assay can be used with flow cytometry, imaging, and automated imaging platforms.

References

1. Salic, A.; et al. Proc Natl Acad Sci U S A. **2008** Feb 19;105(7):2415-20. Epub 2008 Feb 12.

2. Rostovtsev, V.V.; et al. *Angew. Chem. Int. Ed.* **2001**, *41*, 2596.

3. Tornøe, C.W.; et al. *J. Org. Chem.* **2001**, *67*, 3057.

4. Current Protocols in Cytometry; vol 1; Robinson, J.P. ed; John Wiley & Sons Inc. **2007.**

Molecular Probes®
invitrogen detection technologies

invitrogen™
www.invitrogen.com