Cell analysis

In vivo use of Click-iT EdU cell proliferation assays

Introduction

Invitrogen™ Click-iT™ labeling technology provides an exceptional alternative to traditional assays for cell proliferation and DNA synthesis, which are fundamental to understanding cell health, genotoxicity, and drug efficacy. Click-iT assays help reduce the need for harsh cell treatment or DNA denaturation, which is required for traditional antibody-based detection. Here we provide a brief background on click chemistry for animal models, protocols using Click-iT kits for *in vivo* EdU (5-ethynyl-2´-deoxyuridine) detection, the benefits of Click-iT assays and Click-iT Plus EdU Cell Proliferation Kits, and scientific references.

Click-iT EdU assays used to measure *in vivo* cell proliferation

In vivo cell proliferation assays are used to study tumor proliferation, cellular and neural development, inflammation, hearing and vision loss, and cellular regeneration [1-22]. However, due to the ubiquitous distribution of amines and thiols within cells and their presence in cell culture, *in situ* labeling of proteins and newly synthesized nucleic acids is often difficult. Click-iT labeling technology overcomes this obstacle by employing bioorthogonal reactive chemistry with reaction partners, including EdU, that are not endogenously present in biological molecules, cells, tissues, or model organisms [23].

EdU can be delivered in drinking water, by injection (intraperitoneal, intramuscular, subcutaneous), or direct incubation in certain organisms (e.g., *Drosophila* and zebrafish larvae), whereby EdU becomes incorporated into actively dividing cells (Figure 1). In initial experiments, we recommend testing a range of EdU concentrations to determine the optimal dosage required. EdU concentrations can be based on BrdU-based assays [24]. It should be noted that lower quantities of EdU can achieve brightness equivalent to that of BrdU labeling [2]. The optimal concentration may also vary depending on the duration of the pulse, with lower concentrations recommended for longer incubations.

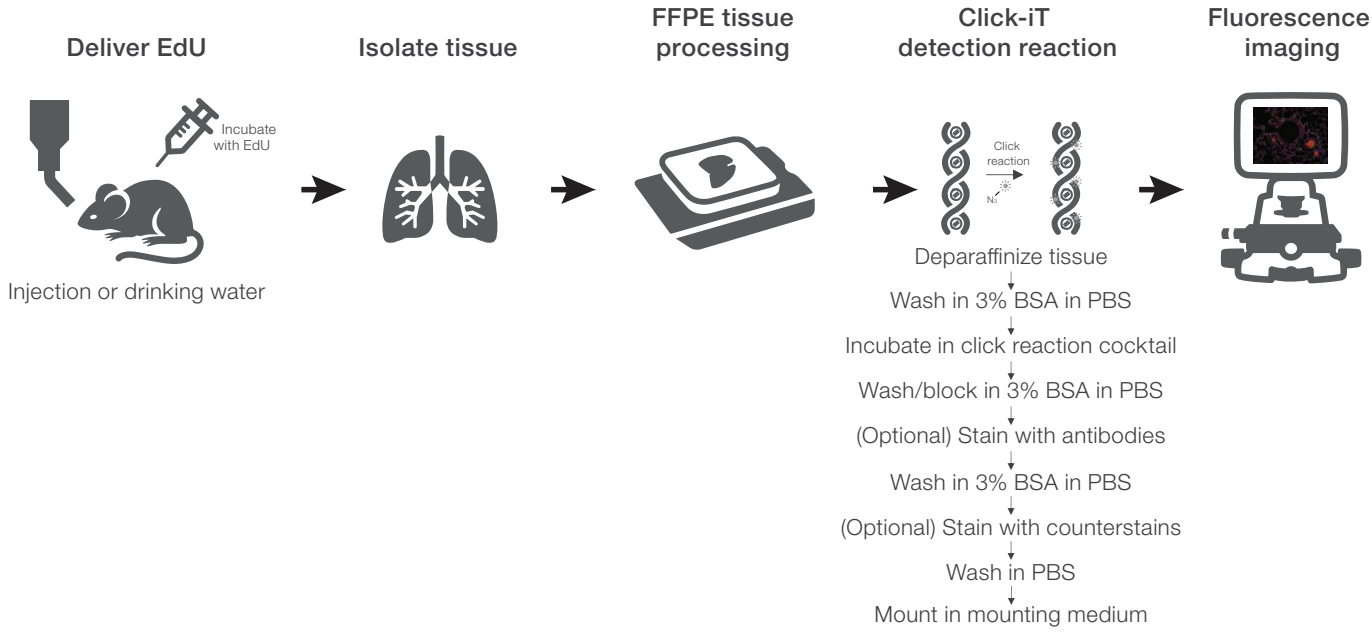

Figure 1. Workflow of Click-iT EdU cell proliferation assays to detect EdU incorporation in an animal model.

EdU detection using Click-iT assays can be performed on cryopreserved tissue, formalin-fixed, paraffin-embedded (FFPE) tissue (Figure 2), or whole-mount samples for fluorescence microscopy, as well as on cells harvested for flow cytometry.

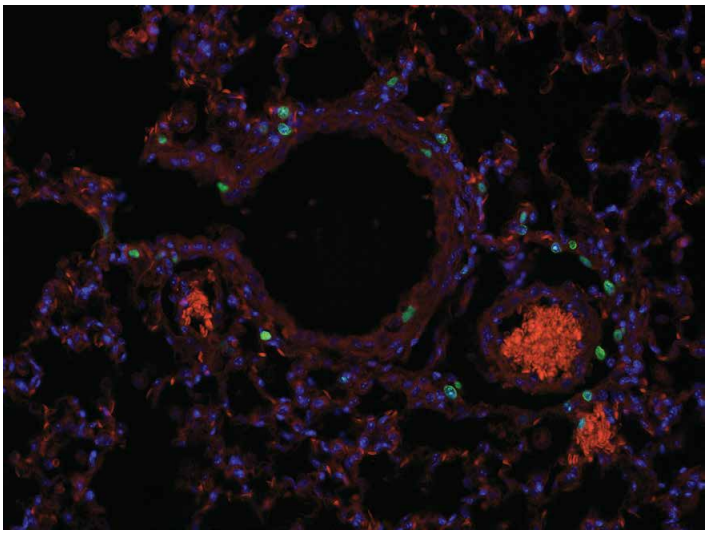

Figure 2. Cell proliferation in mouse lung tissue, detected using a Click-iT EdU assay. Mouse lung tissue sections were formalin-fixed and paraffin-embedded following EdU injection. FFPE tissue sections were labeled with the Invitrogen™ Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 488 dye, to detect proliferating cells (green). Nuclei were stained with DAPI (blue). Red is background from autofluorescence.

Click-iT vs. Click-iT Plus EdU Cell Proliferation Kits

The Click-iT EdU Cell Proliferation Kits utilize copper-mediated azide-alkyne click cycloaddition for EdU detection. Given that copper can damage proteins and nucleic acids, **Click-iT Plus EdU Cell Proliferation Kits** were developed and include Invitrogen™ Alexa Fluor™ picolyl azides for EdU detection in the presence of copper-sensitive compounds. Picolyl azides react efficiently with chelated copper, so free copper in the click reaction is minimized, protecting against undesired interactions with proteins (e.g., green fluorescent protein (GFP), R-phycoerythrin (R-PE)), nucleic acids (e.g., RNA, oligos), and small molecules. Picolyl azides offer increased sensitivity and faster reaction times than standard azides, and allow multiplexing while retaining the benefits of the standard azide-alkyne click reaction (Table 1). Click-iT Plus assays can determine cell proliferation while preserving cell morphology, DNA integrity, antigen-binding sites, and fluorescent signals for better DNA staining to examine cell cycles.

Reagents

- **Invitrogen™ Image-iT™ Fixative Solution**, 4% formaldehyde in PBS (methanol-free) (Cat. No. R37814)
- **Thermo Scientific™ Xylenes, 99%, for biochemistry and histology** (Cat. No. 447240010)
- **Gibco™ Phosphate Buffered Saline** (e.g., Gibco™ PBS (10X), pH 7.4, Cat. No. 70011044)
- **Thermo Scientific™ Absolute Ethanol**, 200 proof, Molecular Biology Grade (Cat. No. T038181000CS)
- **Invitrogen™ EdU (5-ethynyl-2′-deoxyuridine)** (Cat. No. E10187)
- **Click-iT EdU Cell Proliferation Kit for Imaging** (Cat. No. C10338, C10340, C10337, C10339)
- **Invitrogen™ Image-iT™ Fixation/Permeabilization Kit** (Cat. No. R37602)
- 3% bovine serum albumin (BSA) in PBS, pH 7.4
- For dual-pulse labeling: **Thermo Scientific™ 5-Bromo-2′-deoxyuridine (BrdU)**, 99% (Cat. No. H27260.06)
- For dual-pulse labeling: Invitrogen™ BrdU Monoclonal Antibody (**MoBU-1**) (e.g., Cat. No. **B35128**)

Table 1. Comparison of Click-iT EdU and Click-iT Plus EdU Cell Proliferation Kits.

	Click-iT EdU kits	Click-iT Plus EdU kits
Detection label functional group	Azide	Picolyl azide
Signal intensity	Bright	As bright or brighter
Reaction rate	Fast	As fast or faster
Compatibility:		
• Organic dyes such as Invitrogen™ Alexa Fluor™ dyes, fluorescein isothiocyanate (FITC)	✓	✓
• Fluorescent proteins such as GFP, mCherry	Not recommended	✓
• Phalloidin	Not recommended	Not recommended
• Invitrogen™ Qdot™ probes	Post-label staining	Post-label staining
• R-PE and R-PE tandems	Post-label staining	✓
• PerCP, allophycocyanin (APC), and APC-based tandems (e.g., Alexa Fluor 680–APC conjugate)	✓	✓
• Brilliant Violet™ dyes compatibility	✓	✓

EdU labeling in FFPE tissue

Table 2 shows publications that describe the use of click chemistry for EdU labeling to measure *in vivo* proliferation of cells in different model organisms, using various delivery methods.

Table 2. Examples of EdU concentrations and incorporation in animal model systems.

Species	Cell or tissue type	Cell or tissue preparation	EdU amount	Incubation time	Administration method	Detection*	Reference (link)
Mouse	Colon	Frozen, fixed tissue	1 mg/mouse	4–144 hours	Intraperitoneal	Alexa Fluor 488 dye	*J Immunol* 188(5):2427–2436 (2012).
	Small intestine and brain	FFPE tissue (brain and small intestine); fresh, fixed tissue (small intestine)	100–200 µg, in PBS	24–96 hours	Intraperitoneal	Alexa Fluor 568 dye and TMR	*Proc Natl Acad Sci USA* 105(7):2415–2420 (2008).
	Retina (dissociated cells)	Fixed cells	25 µg per g of body weight	4 hours	Intraperitoneal	Alexa Fluor 647 dye	*Mol Biol Cell* 23(22):4362–4372 (2012).
	Embryonic kidney	Fixed tissue	25 µg per g of body weight	1 hour	Intraperitoneal (pregnant dams)	Click-iT EdU	*Development* 138(6):1161–1172 (2011).
	Embryonic brain	Cryosection tissue	5 µg per g of body weight	24–72 hours	Intraperitoneal (pregnant dams)	Alexa Fluor 594 dye	*Proc Natl Acad Sci USA* 110(8):3113–3118 (2013).
	Organ of Corti (cochlear neurosensory epithelium)	Whole-mount tissue	50 µg per g of body weight	4 hours	Subcutaneous injection	Alexa Fluor 488 dye	*J Neurosci* 31(24):8883–8893 (2011).
	Optic nerve	Cryosection, fixed tissue	0.2 mg/mL	2–8 weeks (water exchanged every 72 hours)	Drinking water	Alexa Fluor 594 dye	*J Neurosci* 32(36):12528–12542 (2012).
Rat	Lung	Cryosection, fixed tissue	50 µg per g of body weight	24 hours	Intraperitoneal	Alexa Fluor 488 dye	*Anal Cell Pathol (Amst)* 2015:326385 (2015).
	Olfactory epithelium	Cryosection, fixed tissue	50 µg per g of body weight	2 hours	Subcutaneous	Alexa Fluor 594 dye	*J Neurosci* 38(21):5022–5037 (2018).
	Bone (hematopoietic stem and progenitor cells from bone marrow)	Fresh and fixed cells	60 µg per g of body weight	24 hours	Intraperitoneal (pregnant dams)	Alexa Fluor 647 dye, Alexa Fluor 488 dye for flow cytometry	*Nat Commun* 13(1):1327 (2022).

Table 2. Examples of EdU concentrations and incorporation in animal model systems (continued).

Species	Cell or tissue type	Cell or tissue preparation	EdU amount	Incubation time	Administration method	Detection*	Reference (link)
Zebrafish	Larval intestine	FFPE tissue	100 µg/mL	16 hours	Soaking	Alexa Fluor 488 dye	*Proc Natl Acad Sci USA* 108 Suppl 1(Suppl 1):4570–4577 (2011).
	Larval jaw	Fixed tissue	400 µM	24 hours	Soaking	Click-iT EdU	*FASEB J* 35(11):e22002 (2021).
Drosophila	Larval brain, eye disc, and trachea	Fixed tissue	20–100 µM	10–60 minutes	Soaking	Alexa Fluor 594 dye	*Development* 138(23):5201–5212 (2011).
	Larval imaginal wing discs	Fixed tissue	10 µM	30 minutes	Soaking	Alexa Fluor 555 dye	*Genes Dev* 26(18):2027–2037 (2012).
Maize	Anther	Fixed tissue	20 µg/mL	6 hours	N6 culture medium	Alexa Fluor 488 dye	*Plant Physiol* 176(2):1610–1626 (2018).
Xenopus **(tadpole)**	Intestine	FFPE tissue	10 mg/mL	30–60 minutes	Intraperitoneal	Alexa Fluor 594 dye	*Cold Spring Harb Protoc* 2017(9):pdb.prot097717 (2017).
Chicken	Cochlea	Fixed whole-mount tissue	50 µg per g of body weight	4–8 hours	Subcutaneous injection	Alexa Fluor 488 dye, Alexa Fluor 594 dye	*Laryngoscope* 119(9):1770–1775 (2009).
Nematode (*C. elegans*)	Germ line cells	Fixed cells	20 µM	3–4 hours	Fed through EdU-labeled *E. coli* plates	Click-iT EdU assay	*Genetics* 183(1):233–247 (2009).

* All detection methods are by fluorescence microscopy unless otherwise indicated as flow cytometry.

Generalized protocol for EdU detection in FFPE tissue

1. After incorporation of EdU, isolate the target tissue, fix in formalin, embed in paraffin, section, and mount the tissue on slides using a standard FFPE protocol.
2. Deparaffinize the tissue using a standard deparaffinization rehydration protocol. Slides can be placed in a rack and washed in a coplin jar using the sequential steps listed below.

Deparaffinization and rehydration protocol

Solution	Incubation time
Xylene	5 minutes
Xylene	5 minutes
100% ethanol	5 minutes
100% ethanol	3 minutes
95% ethanol	3 minutes
85% ethanol	3 minutes
75% ethanol	3 minutes
50% ethanol	3 minutes
1X PBS	5 minutes

3. Wash tissue in 3% BSA in PBS.
4. Detect EdU by the click reaction according to the **Click-iT EdU** or **Click-iT Plus EdU** Cell Proliferation Kit protocol. Incubate tissue sections in the Click-iT reaction cocktail for 30 minutes at room temperature, protected from light.
5. Wash tissue in 3% BSA in PBS.
6. (Optional) Stain the tissue with primary and secondary antibodies for non-EdU protein detection. Wash 3x in 3% BSA in PBS.
7. Stain the tissue with Hoechst™ 33342 dye (from a Click-iT EdU Cell Proliferation Kit) or other appropriate counterstains. Wash the tissue 3x in PBS.
8. Prepare the stained tissue in a mounting medium (such as one of the **Invitrogen™ ProLong™ antifade mountants**) and image the slide.

Dual-pulse labeling with EdU and BrdU *in vivo*

Cell proliferation is traditionally assessed by incubating cells with a single "pulse" of a nucleoside analog that is incorporated into the DNA of actively dividing cells, followed by detection using radioactivity, antibodies, or click chemistry. Many applications benefit from the incorporation of two different analogs at different time points (dual-pulse labeling), which can further distinguish cell proliferation and define cell cycle kinetics. Dual-pulse labeling for cell proliferation is commonly done with BrdU and another analog such as 5-chloro-2′-deoxyuridine (CldU) or 5-iodo-2′-deoxyuridine (IdU), which also require antibodies that have high antigen specificity without cross-reactivity.

The BrdU labeling technique can be combined with click chemistry detection for a simplified method of **dual-pulse labeling using EdU** when utilizing highly specific anti-BrdU antibodies [25]. For dual-pulse applications, the use of EdU as one of the nucleoside analogs greatly simplifies the procedure, as there is no reactivity between the Click-iT azide detection reagent and the incorporated BrdU. Moreover, several anti-BrdU antibodies, including the **MoBU-1 clone**, do not cross-react with EdU. Of importance is that BrdU is preferentially incorporated over EdU, thus requiring EdU to be administered prior to BrdU in dual-pulse labeling, avoiding the need to remove excess EdU.

Table 3 shows publications that describe the use of Click-iT EdU kits in combination with BrdU to measure *in vivo* proliferation of cells in different model organisms, using various delivery methods.

Learn more about **single-pulse or dual-pulse cell proliferation Click-iT EdU assays**.

Generalized protocol for dual-pulse EdU and BrdU labeling in FFPE tissue

1. After incorporation of EdU followed by BrdU *in vivo*, isolate the target tissue, fix in formalin, embed in paraffin, section, and mount the tissue on slides using a standard FFPE protocol.
2. Use a standard deparaffinization rehydration protocol to deparaffinize the tissue (Table 3).
3. Perform antigen retrieval for BrdU detection. A common method is pH 6 citrate-based heat-induced epitope retrieval (HIER), but other methods can be used.
4. Perform DNA denaturation for BrdU detection. A common method is incubation in 1–2.5 M HCl at room temperature, but other methods such as treatment with nucleases can be used.
5. Neutralize the tissue in 0.1 M sodium borate buffer (pH 8.5) for 10 minutes at room temperature.
6. Wash the tissue 3x in 3% BSA in PBS.
7. Detect EdU by the click reaction according to the Click-iT EdU or Click-iT Plus EdU Cell Proliferation Kit. Incubate tissue sections in the Click-iT reaction cocktail for 30 minutes at room temperature, protected from light.
8. Wash the tissue in 3% BSA in PBS.
9. Incubate the tissue with an anti-BrdU primary antibody that does not cross-react with EdU (e.g., **clone MoBU-1**).
10. Wash the tissue 3x in 3% BSA in PBS.
11. Incubate with an appropriate secondary antibody.
12. Wash the tissue 3x in 3% BSA in PBS.
13. Stain the tissue with Hoechst 33342 dye (from the Click-iT EdU Cell Proliferation Kit) or other appropriate counterstains. Wash the tissue 3x in PBS.
14. Prepare the stained tissue in a mounting medium (such as one of the **ProLong antifade mountants**) and image the slide.

Table 3. Examples of dual BrdU and EdU concentrations and incorporation in animal model systems.

Species	Cell or tissue type	Cell or tissue preparation	EdU and BrdU amounts	Incubation time	Administration method	Detection	Reference (link)
Mouse	Brain (embryonic)	Frozen tissue	EdU: 20 µg/g body weight; BrdU: 200 µg/g body weight	1–2 days	Intraperitoneal (pregnant dams)	Alexa Fluor 488 dye	*J Neurosci* 31(17):6440–6448 (2011).
	Brain	Fixed tissue	EdU: 7.5 mg/mL, 0.1 mL/10 g mouse; BrdU: 7.5 mg/mL, 0.1 mL/10 g mouse	EdU at 0–20 hours then BrdU at 24–44 hours	Intraperitoneal	Alexa Fluor 488 dye	*Mol Biol Cell* 22(12):1960–1970 (2011).
	Skin tumors	Cryosections (fixed) tissue	6 x 50 µg (both)	EdU: 4 weeks BrdU: 2 hours	Intraperitoneal	Click-iT EdU assay	*Proc Natl Acad Sci USA* 109(52):21468–21473 (2012).
Zebra finch	Brain, liver, intestine	Cryosections (fixed) tissue	50 µg/g BrdU and 41 µg/g EdU	2–8 hours	Intramuscular	Alexa Fluor 488 dye	*Biology (Basel)* 9(11):356 (2020).

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