

EdU, a new thymidine analogue for labelling proliferating cells in the nervous system

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Abstract

(100 words)

Labelling and identifying proliferating cells is central to understanding neurogenesis and neural lineages *in vivo* and *in vitro*. We present here a novel thymidine analogue, ethynyl deoxyuridine (EdU) for labelling dividing cells, detected with a fluorescent azide which forms a covalent bond via the “click” chemistry reaction (the Huisgen 1,3-dipolar cycloaddition reaction of an organic azide to a terminal acetylene). Unlike the commonly used BrdU, EdU detection requires no heat or acid treatment. It is quick and easy and compatible with multiple probes for fluorescence immunochemistry, facilitating the characterisation of proliferating cells at high resolution.

Keywords: immunofluorescence, cell proliferation, neurogenesis, neural precursor, cell differentiation, thymidine analogue, cell labelling, Brain

Background

(222 wds)

Currently the method of choice to label dividing cells, *in vivo* and *in vitro*, is the incorporation of the thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU), into dividing cells during S-phase. BrdU is detected, after fixation, with a BrdU-specific antibody (Dean et al., 1984; Miller and Nowakowski, 1988; Nagashima et al., 1985; Takamiya et al., 1988; Tang et al., 2007). This method is very widely used, much more convenient than [³H]-thymidine, and compatible with high resolution microscopic methods, including immunochemistry. BrdU immunochemistry can be problematic because strong DNA denaturing conditions, such as strong acids and heating, are required to reveal the epitope which is masked within the DNA. This introduces significant variability within and between experiments. Loss of antigenicity due to BrdU processing can be overcome using antigen retrieval methods (Tang et al., 2007) requiring further acid and heat treatment that can degrade cellular structure.

An alternative thymidine analogue is 5-ethynyl-2'-deoxyuridine (EdU), in which the terminal methyl group is replaced with an alkyne group, which allows detection using a fluorescent azide that covalently binds to the alkyne group in a reaction known as "click chemistry" (Hsu et al., 2007; Sawa et al., 2006; Tornøe et al., 2002) (Fig. 1). This detection method is fast and specific without requiring DNA denaturation (Buck et al., 2008; Cappella et al., 2008; Salic and Mitchison, 2008). The aim of the present study was to investigate the efficacy of EdU for the analysis of proliferating cells *in vitro* and in the brain after intraperitoneal injection. We compared EdU with BrdU and investigated EdU compatibility with high resolution, multiple-fluorescence labelling, a major difficulty when using BrdU.

Materials and Methods

(1150 wds)

EdU chemistry

The 50 assay Click-iT™ EdU Cell Proliferation Assay Kit (Cat# C35002, Invitrogen) contains 12 reagents named component A to component L. Components A, B, E, G, H and I are required. The component description as provided by the supplier: Component A, EdU; Component B, Alexa Fluor® 488 azide; E, saponin-based permeabilization and wash reagent; G, reaction buffer; H, copper sulphate; I, buffer additive. Cells or tissue sections were incubated in component E for 30 minutes on a rocker, followed by incubation with the reaction cocktail for 30 minutes at room temperature, protected from light. 1500µl of reaction cocktail contained 7.5µl of component B, 30µl of component H, 1313µl of component G and 150µl of component I. The cells or sections were washed for 3X5 minutes in component E and cover slipped using Vectashield DAPI (4',6-diamidino-2-phenylindole-2HCl, Vector Labs) mounting media which labels cell nuclei. The reaction cocktail was used within 15 minutes of preparation.

EdU *in vitro*

Neurospheres were derived from human olfactory mucosa (Murrell et al, 2005). The cells were passaged using trypsin (Invitrogen), and seeded in medium (DMEM/F12 with 10% FBS and 1% penicillin/streptomycin) onto 8 well plastic chambers slides (Nunc) at a concentration of 10,000 cells/cm². The cells were grown overnight in medium (DMEM/F12 with 10% FBS and 1% penicillin/streptomycin) in a humidified incubator at 37°C with 5% CO₂. The cells were then grown for 24 hr in medium containing EdU at different concentrations (1µM-20µM). Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (pH 7.4; PBS). The EdU positive cells were labelled with the fluorescent azide probe (see above), followed by immunofluorescence labelling.

Samples were blocked and permeabilised for 30 minutes at room temperature with 2%BSA in saponin-based permeabilization reagent (wash buffer; Component E, Cat# C35002, Invitrogen).

Samples were then incubated in α Actin primary antibody (1:200, Cat# A2546, Sigma) which was diluted in 2%BSA in wash buffer for 30 minutes at room temperature before being washed with the wash buffer 3X for 5 minutes. Cells were then incubated with donkey anti-mouse Alexa fluor 594 secondary antibody (1:800, Cat# A21203, Invitrogen) for 30 minutes at room temperature, rinsed in wash buffer, and incubated with DAPI (1:1000, Cat# H1200, Vector Labs) diluted in 0.1M PBS for 20 minutes. For each EdU experiment, three random fields were imaged at 100X magnification and the numbers of EdU-positive cells were counted. EdU-positive cells were expressed as a percentage of the total number cells in each field, identified by DAPI nuclei staining. Each experiment was done in triplicate and the results presented as mean \pm SEM.

EdU *in vivo*

9 Pregnant C57B6 mice and their pups were used in the experiment. All procedures were carried out with the approval of the Griffith University Animal Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia. For acute labelling *post-partum*, Group 1 received a single intraperitoneal injection of both BrdU and EdU at post-natal day 1 (P1) and euthanized at post-natal day 2 (P2) to harvest tissues. For acute labelling *in utero*, Group 2 pregnant dams received an intraperitoneal injection of EdU at embryonic day 20 (E20) and the pups were killed at birth (P0). For long term labelling *post-partum*, Group 3 pregnant dams received an intraperitoneal injection of BrdU at embryonic day 16 (E16), the pups were given an intraperitoneal injection of EdU in post-natal day 7 (P7) and then killed at post-natal day 30 (P30).

EdU (Cat# C35002, Invitrogen) and BrdU (Sigma B 5002, St. Louis, MO) were given at a dose of 50 mg/kg body weight in a solution of 10 mg/ml PBS (pH 7.35) and 20 mg/ml in PBS, respectively. Control animals received an intraperitoneal injection of 0.1 M PBS, without EdU. Tissues from these animals were processed with those from EdU-injected animals. To establish an optimal dose of EdU, a group of 6 animals was injected with 12, 25, and 50mg/kg EdU (n = 2 per dose). Tissues were prepared as described in the next section (see below). From each animal, four sections were selected from the same regions of the olfactory epithelium and the subventricular

zone and processed for EdU detection. All concentrations of EdU labelled a similar population of cells within the neurogenic zones. Images were taken with the same fixed exposure to allow assessment of the EdU incorporation. The highest fluorescence intensity of individual nuclei was noted at a dose of 50 mg/kg dosage allowing the easiest detectability of EdU-positive cells. Lower doses labelled similar numbers of cells (not shown) with lower fluorescence intensity and consequently lower detectability. Because 50 mg/kg is the same concentration commonly used for BrdU uptake in the literature, it was selected for both EdU and BrdU in this study. A dose response curve was also generated by Cappella et al (2008).

Tissue fixation and preparation

Mice were killed by cervical dislocation and the heads fixed by immersion in 4% paraformaldehyde in PBS at room temperature for 2 hr (P0, P2) or 4 hr (P30). Following fixation, the P30 heads were decalcified in 20% disodium ethylenediaminetetraacetic acid (EDTA) in PBS. The heads were placed in an embedding matrix (O.C.T. compound, Miles Scientific, Naperville, IL) and snap frozen by immersion in iso-pentane that had been cooled with liquid nitrogen. Cryostat sections (18 µm) of the nasal cavity and brain were cut, mounted on to gelatinized slides and stored at -20°C before processing first for EdU chemistry and then for immunohistochemistry.

Tissue preparation for BrdU immunohistochemistry

Tissue sections were pre-treated to denature DNA, before immunolabeling for BrdU according to our protocol which was optimised for fluorescence *in situ* hybridisation using oligonucleotide probes (Robinson et al., 2005). Firstly, sections were treated with Pepsin (0.05mg/ml in 0.12M HCl) for 20 mins at 37°C, followed by a 10 minute wash in 5X sodium chloride-sodium citrate buffer (SSC). A 20X stock solution of SSC was prepared as follows: 173.3g sodium chloride and 88.2g tri-Sodium citrate (Cat# SA034, Chem-Supply, Pty, Ltd) were dissolved in 950 ml distilled H₂O and the pH was adjusted to 7.4; H₂O was added to bring the final volume to 1000 ml. For 5X

and 2X SSC, the 20X SSC was diluted in distilled H₂O. Sections were then placed on a heating block at 95°C for 5 mins incubated in a denaturing solution, 1 ml of which contained: 450µl of 100% formamide (Cat# F9037, Sigma), 250µl of 20X SSC, 250µl of 10% chondroitin sulphate (Cat#C9819, Sigma) 25µl of ssDNA (herring sperm, single stranded DNA, Cat#D7290, Sigma) and 25µl of distilled H₂O. After washing in 2X SSC for 10 minutes, the standard immuno-labelling protocols were applied. For detecting cells incorporating both BrdU and EdU, the protocol was performed in the following order: DNA denaturation, EdU chemistry, BrdU immunohistochemistry.

Immunohistochemistry

Sections were incubated in DMSO (neat) for 15 min before being washed with 0.1M PBS and 0.1% Triton-X-100 for 2 min. They were then incubated with 10% normal donkey serum (Sigma Chemical Corporation) in 0.1M PBS with 0.1% Triton X-100 for 1 hr at room temperature. The primary antibodies used for single and multiple labelling are listed in Table 1. These were diluted in 10% normal donkey serum /PBS/ Triton X-100 and the sections incubated overnight at room temperature. Sections were then washed with PBS/Triton-X-100 and incubated in the appropriate secondary antibody (Table 1) for 3 hrs at room temperature, washed in PBS/Triton X-100 and coverslipped with Vectashield DAPI mounting medium (neat).

Image capture and image preparation

Images were captured using an Axio Imager Z1 epi-fluorescence microscope with Apotome and an AxioCam Mrm camera (Carl Zeiss, Germany). Serial optical sections were captured using AxioVision software and projected to provide two-dimensional maximum brightness images. Figures were compiled in Adobe Photoshop 7.0 and Adobe Illustrator 10.0 (Adobe Systems Incorporated).

Quantification of EdU- and BrdU- labelled cells

Two mice were co-injected with BrdU and EdU at post-natal day 1 (P1) of age and killed at P2 by decapitation. Tissues were prepared as described above for EdU and BrdU detection. Sections were chosen from four neurogenic areas of the nervous system which have high numbers of dividing cells namely: olfactory epithelium; subventricular zone; rostral migratory stream (RMS); and hippocampus. Within each neurogenic area, two fields were selected. The 16 fields (8 per animal) were chosen to optimise and maximise signals in both fluorescent channels to make sure that all EdU and BrdU-labelled cells were being detected and that all fields had many cells represented in both channels. Serial optical sections of the selected fields were captured to provide three-dimensional maximum brightness images through the nuclei to capture all the EdU and BrdU labelling throughout the nucleus. The number of EdU- and BrdU-positive cells was counted, noting single- and double-labelled cells. Statistical significance was assessed using a paired t-test comparing the numbers of cells detected in each field through each fluorescence channel.

Results

(584 wds)

EdU labelled cells *in vitro*

Cells incorporated EdU *in vitro* and showed intense nuclear fluorescence after labelling with Alexa-Fluor 488 azide (Fig 2). The detection of EdU was compatible with the fluorescent nuclear label, DAPI, and immunochemistry (Fig. 2 A-D). The fluorescent azide probe for EdU was nuclear-specific, showing clear co-localisation with DAPI (Fig. 2D, arrowheads), with other nuclei clearly negative for EdU (Fig. 2D, arrow). EdU fluorescence was not located in the cytoplasm, identified by immunolabelling with anti - α actin (Fig. 2A, D). Maximal labelling of dividing cells was achieved at 5 μ M EdU concentration (Fig.2E). EdU did not effect the total cell number over the concentration range 0-20 μ M (one-way analysis of variance, $p=0.107$).

EdU labelled cells in neurogenic brain regions

After an intraperitoneal injection of EdU into the pregnant mother at E20, many EdU-positive cells are seen in the brain of the pup at birth (P0). Many labelled cells were present in neurogenic zones such as the subventricular zone and hippocampal granular layer (Fig 3). Cells in the RMS and olfactory bulb were labelled (Fig. 3A) as well as numerous other cells throughout multiple brain regions (Fig 3A). Moreover, EdU also labelled proliferative cells of the skull and choroid plexus of the third ventricle. The extensive co-localization of EdU with DAPI in cell nuclei of the RMS demonstrates the extensive labelling of proliferative cells in this area (Fig. 3B-D). At P2, after intraperitoneal injection of EdU at P1, numerous cells were labelled in the subventricular zone (Fig. 3E-G). 21 d after peritoneal injection of EdU at P7, labelled cells were detected throughout the brain. Cells in hippocampus showed clear localisation of EdU within nuclei (Fig. 3H-J). EdU labelled cells were not detected in sections of the control animals which were not injected with EdU (data not shown).

EdU and BrdU labelled the same cells

At P2, 1 d after a single injection of EdU and BrdU, EdU labelled cells were labelled green and BrdU were labelled red (Fig.4). Proliferative cells in neurogenic zones such as the RMS were co-labelled with the two markers, (Fig 4.A-D, arrowheads). EdU labelling was more discretely localised to the nucleus and readily distinguished as positive labelling when colocalised with DAPI labelling, in comparison to BrdU (Fig. 4A-D, arrows).

Comparison of the numbers of EdU- and BrdU-labelled nuclei in the same sections revealed a consistent difference in the 16 fields chosen from 4 neurogenic regions in 2 animals. EdU-labelled nuclei were more numerous in 15 of the 16 images. The numbers of EdU- and BrdU-labelled nuclei per field were 114.25 ± 10.06 and 101.5 ± 10.00 (Mean \pm SEM), respectively. These group differences were significantly different ($p < 0.0001$, paired t-test, two-tailed, $t=11$, $df=15$).

After injection of the pregnant mother with BrdU at E16, followed by EdU injection of the offspring at P7, double-labelled and single-labelled cells were observed at P30 (Fig. 4E-H).

Dividing cells labelled with EdU differentiated into neurons

Cells labelled after intraperitoneal injection of EdU in neonatal mice were identified in the brain and olfactory epithelium at 30 days of age. As expected, EdU-positive cells differentiated into multiple cellular phenotypes, revealed by multiple immunofluorescent labelling. For example, in the olfactory epithelium, cells double-labelled with EdU included immature neurons (expressing β -tubulin III) and horizontal basal cells (expressing cytokeratin 14; Fig. 5). Other EdU positive cells were also distributed within the Bowman's glands (data not shown) and within the sustentacular cell layer (Fig. 5). In the olfactory bulb, EdU-labelled cells were located in multiple layers including periglomerular cells (expressing tyrosine hydroxylase; Fig. 6A-D). Numerous EdU-positive cells were observed in the cerebellum among the granule cells, double-labelled with an antibody to the neuronal nuclear marker, NeuN (Fig. 6E-H).

Discussion

(399 wds)

Our findings show that EdU robustly labelled dividing cells *in vitro* and *in vivo*. Tissue processing for EdU was fast and easy, without the harsh DNA- denaturing condition required for BrdU processing. The EdU protocol maintained structural and antigenic integrity of neural tissues to accommodate high resolution, multiple fluorescence microscopy with antibodies to neuronal markers such as β -tubulin III, NeuN and tyrosine hydroxylase and non-neuronal markers such as GFAP and cytokeratin 14. The EdU protocol was also compatible with the DNA intercalating dye, DAPI. We show here that EdU chemistry can be combined with BrdU immunocytochemistry, provided care is taken with the BrdU tissue processing (Robinson et al., 2005; Tang et al., 2007). Sequential labelling using these two thymidine analogues allows identification of neural precursors undergoing multiple divisions and provides a technique for cell cycle analysis in neurogenic regions.

With the recognition that neurogenesis continues to occur in the adult brain, including human brain (Eriksson et al., 1998), the interest in labelling dividing cells in the nervous system has grown rapidly in the last 20 years. Historically, the first method involved the incorporation of [3 H]-thymidine into DNA during S-phase of the cell cycle (Swierkowska et al., 1973). This method is very useful for quantitative analyses of cell division but has shortcomings due to the labour-intensive and time-consuming processing required for detecting the radioactivity (Taupin, 2007). In addition, radioactive methods are not compatible with modern high-resolution microscopy because the emulsion used to detect the label lies outside the plane of focus of the labelled DNA.

Consequently, BrdU has taken over from [3 H]-thymidine for the histological analysis of dividing cells and their progeny in the nervous system. The perennial difficulty with BrdU is the care that must be exerted in processing the tissue to provide access of the anti-BrdU antibody to the BrdU epitope, incorporated into the DNA. In practice, tissue processing for BrdU is often capricious, which may result in variable levels of targeting of the BrdU antigen by BrdU antibodies, even within the same tissue section. The processing for BrdU can damage other antigens, reduce binding of DNA intercalating dyes and disrupt tissue structure, making it difficult to combine reliably with

other immunofluorescence techniques (Tang et al., 2007) . These can be overcome with careful attention to technique as reported here and previously (Robinson et al. 2005; Tang et al. 2007). Consistent with the recent finding of Cappella et al (2008), our data suggest that EdU offers an excellent alternative for the identification of proliferating cells. EdU does not require DNA denaturation and its terminal acetylene group forms a very specific covalent bond with the fluorescent azide probe because there is no other acetylene group in the cell. This azide probe is small and penetrates the cell and nucleus without harsh treatment leading to very little background when the specimen is washed appropriately.

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Table 1: Primary and secondary antibodies

Antibody	Host	Company	Catalogue#	Dilution
Olfactory marker protein OMP	Goat	Wako	544-10001	1:1500
Tyrosine hydroxylase (TH)	Mouse	Immunostar, Inc	22941	1:1000
Cytokeratin 14 (CK14)	Rabbit	Abcam	C8791	Neat
NeuN	Mouse	Chemicon	MAB377	1:200
Neuronal Class III β -Tubulin	Rabbit	Covance	PRB-435P	1:1000
BrdU	Mouse	Dako	M0744	1:100
Glial fibrillary acidic protein(GFAP)	Rabbit	Dako	Nr-Z.0334	1:500
Anti-Actin, anti-Smooth muscle	Mouse	Sigma	A2547	1:200
Alexa Fluor 594 mouse IgG	Donkey	Invitrogen	A-21203	1:800
Alexa Fluor 594 rabbit IgG	Donkey	Invitrogen	A-21207	1:800
Alexa Fluor 647 rabbit IgG	Donkey	Invitrogen	A-31573	1:800

Figure Legends

Figure 1 “Click” chemistry.

A: The Cu(I) catalysed 1,3-dipolar cycloaddition reaction of organic azides (R^1-N_3) with terminal acetylenes ($R^2-\equiv$). This “click” chemistry reaction affords exclusively the 1,4-disubstituted 1,2,3-triazole regioisomer. **B:** Click-iT™ assay by Invitrogen™.

Figure 2 EdU is detected *in vitro*.

A-D: After 24 hr exposure to EdU, dividing cells had incorporated EdU (A: green) and counterstained with DAPI (B: blue) and anti - α actin (C: red). EdU was confined to nuclei, demonstrated by co-localisation with DAPI (arrowheads) but, as expected, not all cells had divided (arrow). Scale bar: 50 μ m. **E:** An EdU concentration of 5 μ M was sufficient for maximal labelling of dividing cells.

Figure 3 EdU is detected in neurogenic zones of the brain.

A: Parasagittal sections through the brain at P0 after EdU incorporation at E20. Rostral is to the right, dorsal is to the top. Proliferative cells labelled with EdU were present in the cortex (CX), olfactory bulb (OB), skull (S), and the choroid plexus of the third ventricle (3V). **B-D:** High power view of the rostral migratory stream (boxed region in A) showing multiple nuclei double-labelled (arrowheads) with EdU (green) and DAPI (blue). **E-G:** High power view of the subventricular zone showing EdU labelled nuclei at P2 after EdU injection at P1. **H-J:** High power view of the dentate gyrus of the hippocampus showing EdU labelled nuclei (green) at P30 after EdU injection at P7. Scale bar: 410 μ m for A and 50 μ m (B-J).

Figure 4 EdU and BrdU label the same cells.

A-D: Section through rostral migratory stream showing cells at P2, double-labelled for EdU (green) and BrdU (red) after injection of both at P1. EdU labelled cells more robustly than BrdU (arrows).

E-H: Section through olfactory epithelium showing cells at P30 labelled with EdU (green) and BrdU (red) after EdU injection at P7 and BrdU injection at E16. Double-labelled cells are indicated by arrowheads, single-labelled cells are indicated by arrows. a: apical layer, m: middle layer. Scale bar: 20 μ m.

Figure 5 EdU-labelling birth-dates differentiated cells.

After injection of EdU at P7, EdU was detected in differentiated cells at P30 in the olfactory

epithelium. **A-D:** EdU-labelled cells (green) differentiated into neurons, double-labelled for β -

tubulin III (red). **E-H:** EdU-labelled cells (green) were identified as horizontal basal cells, double-

labelled with cytokeratin 14 (red). Double-labelled cells are indicated by arrowheads. Unidentified

EdU-labelled cells, probably supporting cells, are indicated by arrows. Scale bar: 20 μ m.

Figure 6 EdU-labelling is compatible with multiple fluorescence labelling.

After injection of EdU at P7, EdU-labelled cells were detected in differentiated cells at P30 in

olfactory bulb (**A-D**) and cerebellum (**E-H**). **A-D:** EdU-labelled cells (green) double-labelled for

tyrosine hydroxylase (red) in the glomerulus (Gl) of the olfactory bulb. Some EdU-labelled cells

lacked TH (double arrowhead). **E-H:** EdU-labelled cells in the cerebellum (green), double-labelled

for NeuN (purple). Double-labelled cells are indicated by arrowheads. One nucleus is not labelled

with NeuN and may be a glial cell (double arrow) identified by GFAP immunoreactivity (red). The

EdU labelled nucleus next to the GFAP positive cell may be the nucleus of the glial cell. Scale bar:

20 μ m