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Proliferation Assays (BrdU and EdU) on Skeletal Tissue Sections

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Abstract

Assessing cell proliferation in situ is an important phenotyping component of skeletal tissues from development to adult stages and disease. Various methods exist including immunostaining for proteins and protein modifications associated with specific steps of the cell cycle, but the gold standard is to quantify the percentage of DNA-synthesizing cells. The thymidine analog 5-bromo-2'-deoxyuridine (BrdU) has been widely used in the last decades for this purpose, with the inconvenience that its detection is lengthy and requires harsh treatment of tissue sections to give access of anti-BrdU antibody to nucleosides in genomic DNA. In 2008, Salic and Mitchison developed a new method and proved it to be quicker, simpler, and highly sensitive in non-skeletal tissues. This method relies on incorporation of 5-ethynyl-2'-deoxyuridine (EdU) into de novo DNA. This other thymidine analog is readily detected by click chemistry, i.e., covalent cross-linking of its ethynyl group with a fluorescent azide, a molecule small enough to diffuse freely through native tissues and DNA. Here, we describe and compare the BrdU and EdU approaches in skeletal tissues and conclude that in these tissues too EdU provides an easy and very sensitive alternative to BrdU.

Keywords

Bone; Cartilage; BrdU; EdU; In situ; Proliferation; Skeleton

1 Introduction

Achieving proper rates of cell proliferation in space and time is central to developing and maintaining a healthy, fully functional skeleton [1]. This is sadly illustrated by the fact that numerous types of skeletal dysplasias and acquired skeletal diseases are due, at least in part, to defective or excessive proliferation of skeletal progenitor or differentiated cells. For instance, premature growth arrest of growth plate chondrocytes is a main contributor to the most common form of dwarfism in humans, which is called achondroplasia and is due to activating mutations in the fibroblast growth factor receptor 3 (FGFR3) [2]. At the other side of the spectrum, osteosarcomas and chondrosarcomas, like many other types of tumors, involve increased, aberrant proliferation of osteogenic and chondrogenic cells, respectively [3]. Skeletal cell proliferation depends on many factors, such as cell types and differentiation stages, individual age, gender, genetic background, health, diet, and mechanical stimulus. Many molecular mediators have already been identified, including hormones, paracrine and autocrine growth factors, signaling components, and transcriptional factors [4, 5], but many

more undoubtedly remain to be uncovered to fully understand skeletal diseases and propose novel therapeutic approaches. Critical means to achieve this goal include highly sensitive and reliable cell proliferation assays.

As for other organs, several methods exist to assess cell proliferation in the skeleton [6]. Most rely on commercially available antibodies and kits to detect proteins and protein modifications involved in or associated with cell mitosis. Common assay targets are phosphorylated Thr3 in histone H3, which marks cells in pro-phase and metaphase; proliferating cell nuclear antigen (PCNA), which is abundant in cells during the S, G2, and M phases of mitosis; and Ki-67 nuclear protein, which is present in all active phases of the cell cycle but absent from resting cells (G₀). The assays of these targets are simple and sensitive but not always fully instructive as PCNA and Ki-67, for instance, are present in virtually every non-hypertrophic chondrocyte in the fetal growth plate. Moreover, they only reveal the cells that are proliferating at the time of tissue collection, but not the cells that were proliferating during a defined time period. These assays are therefore often used as secondary rather than primary means to assess cell proliferation.

The primary, gold standard approach to assess cell proliferation is to quantify the percentage of cells synthesizing DNA during a desired time period. The original protocol was developed in the late 1950s and used ³H-thymidine [7]. While greeted as a pioneer and still utilized for discrete applications, the method bears the inconvenience that it involves radioactivity, is lengthy and poorly sensitive, and yields low-resolution data. It has therefore been overtaken since the early 1980s by more sensitive, nonradioactive methods and in particular by methods using the thymidine analog 5-bromo-2'-deoxyuridine, referred to as BrdU [8]. This is true for a wide range of studies, including analyses of skeletal tissues from early development to adult-onset diseases [9–14]. BrdU is incorporated into de novo-synthesized DNA as a substitute for thymidine and thereby permanently labels proliferating and daughter cells until it is diluted out through multiple rounds of cell division. BrdU is detected by immunostaining of tissue sections with specific anti-BrdU antibodies. The latter can be revealed through direct coupling with an enzyme, biotin, or a fluorescent tag or through binding to secondary antibodies coupled in similar ways. A major disadvantage of BrdU methods is that harsh treatments of tissue sections are required to allow antibody access to BrdU inserted into genomic DNA. These treatments involve digesting or denaturing tissues and DNA with trypsin, HCl, heat, and/or DNase. In addition to being technically cumbersome and difficult, they can result in tissue damage and are rarely compatible with other staining assays.

An easier and highly sensitive method was developed by Salic and Mitchison in 2008 [15]. It utilizes 5-ethynyl-2'-deoxyuridine (EdU) to label newly synthesized DNA and applies a “click” chemistry reaction to detect the new thymidine analog in native tissues. The reaction is a copper-catalyzed [3 + 3] cycloaddition, whereby a stable triazole ring is formed by covalently coupling the alkyne group present in EdU to an Alexa Fluor®-conjugated azide group. Because of its small size and chemical nature, the fluorescent azide effectively diffuses through live and fixed tissues and readily accesses EdU nucleosides in genomic DNA. No harsh treatments of tissues are thus required, making the assay fast and reliable. In addition, the assay is very sensitive, and, because it preserves the physical integrity of

specimens, it can be multiplexed with BrdU in dual-pulse assays and with many other histological assays. It has been shown to work well in various types of tissues in adult mice and embryos [15–17], but not in skeletal tissues yet.

We use here commercially available reagents and kits to assess cell proliferation in mouse skeletal tissues using BrdU and EdU assays. We provide technical and scientific advice for these assays and, as shown in other tissues, demonstrate that EdU is a very easy and sensitive alternative to BrdU in skeletal tissues too.

2 Materials

BrdU, EdU, and several other reagents described below are classified as toxins, potential mutagens, and/or teratogens. It is therefore imperative that appropriate precautions be taken in compliance with all pertaining local regulations when working with these chemicals (*see* Note 1).

2.1 Labeling Reagents and Detection Kits

The BrdU and EdU reagents and kits utilized in the assays described in this chapter are listed below. Other, similar materials are commercially available and can be used as desired or needed (*see* Notes 2 and ³).

1. *BrdU labeling reagent* (Invitrogen). This reagent is an aqueous solution of BrdU and 5-fluoro-2'-deoxyuridine (10:1). It is ready to be injected in animals.
2. *BrdU Staining Kit* (Invitrogen). This kit provides a biotinylated mouse monoclonal anti-BrdU antibody, a streptavidin–peroxidase conjugate, and other specific complementary reagents.
3. *Click-iT® EdU Alexa Fluor® 488 Imaging Kit* (Invitrogen). The kit contains all components needed to label DNA-synthesizing cells and to detect EdU incorporated into DNA.
4. BrdU is best purchased as a ready-to-use solution, as this option reduces personnel exposure to this biohazard (Invitrogen). EdU is sold only as a powder. Prepare a 10 mM solution by dissolving the 5 mg vial content (component A of kit) in 2 ml DMSO (component C of kit) or sterile PBS. Store both solutions at 4 °C in a waterproof and lightproof secondary container.

¹BrdU, EdU, and Hoechst 33342 are classified as toxins and potential mutagens and teratogens. Special precautions must therefore be taken in compliance with all pertaining local regulations when working with these chemicals. They include working in a chemical fume hood or laminar flow hood; wearing double gloves, safety goggles, and a lab coat; storing and transporting the BrdU and EdU solutions in secured and properly labeled secondary containers; disposing of contaminated disposables (syringes, needles, mouse parts, mouse cage bedding, and paper towels) in appropriate sharp or biohazard containers to be incinerated; disposing of fixative and rinse solutions in properly labeled chemical waste bottles; and cleaning dissecting tools and contaminated areas with large volumes of water. Other reagents that require special precautions include PFA, H₂O₂, and DMSO. Thus, handle and dispose of them according to their biohazard nature and all pertaining regulations.

²Several types of anti-BrdU antibodies and detection methods are commercially available. They include primary antibodies conjugated with fluorochromes. If you wish to perform dual labeling with BrdU and EdU, keep in mind that some anti-BrdU antibodies cross-react with EdU (Accurate), whereas others do not (Sigma) [16].

³Other fluorochromes conjugated to azide for the detection of EdU include Alexa Fluor® 488, 555, and 647 (Invitrogen).

2.2 Other Materials

Other materials needed for the BrdU and EdU assays described in this chapter, but not provided in the kits, are as follows:

For both assays:

- 5 Secondary container tightly closed and protected from light to store and transport BrdU and EdU solutions (e.g., opaque dark-color Tupperware®).
- 6 1× Phosphate-buffered saline (1× PBS).
- 7 Paraformaldehyde aqueous solution (16 % stock).
- 8 Histo-Clear (nontoxic xylene substitute).
- 9 Ethanol at 100, 95, and 70 % in distilled water.
- 10 Distilled water.
- 11 Coplin jars.
- 12 Superfrost Plus slides.
- 13 Glass cover slips.
- 14 Optional: Immunostaining pen or pap pen.

For the BrdU assay only:

- 15 H₂O₂ (30 % stock).
- 16 Methanol.
- 17 Humid chamber: Line a large Petri dish with wet paper towels. Arrange sections on toothpicks, like railroad tracks, on the towels.

For the EdU assay only:

- 18 3 % (w/v) bovine serum albumin (BSA) in PBS.
- 19 Vectashield® Mounting Media (Vector Laboratories).

3 Methods

Figure 1 provides a schematic of the major steps involved in the generation of tissue sections, BrdU and EdU assays, and image acquisition and quantification of data. In particular, it emphasizes the complexity and duration of the BrdU assay in comparison to the EdU assay. All steps are described in detail below. Unless otherwise stated, they are carried out at room temperature.

3.1 In Vivo Labeling with BrdU and EdU

1. For mouse administration, aspirate 100 µl of BrdU or EdU solution per 10 g of mouse body weight using a sterile 30G1/2 needle attached to a 1-ml syringe and inject the solution intraperitoneally. Return the mice to their cage, and place a treatment card to indicate the presence of a biohazard. Generate biological

replicates by using as many animals per experimental group as needed for statistical analysis.

2. The labeling time for skeletal tissues is typically 30–60 min for embryos at the gestation days 10.5 (E10.5) to E13.5, 1–2 h for E14.5 to E18.5 fetuses, and 2–4 h for postnatal mice. When time is over, euthanize the mice by CO₂ asphyxiation, followed by cervical dislocation or decapitation.

3.2 Preparation of Tissue Sections

1. Dissect mouse embryos or body parts of interest, and briefly rinse them in ice-cold 1× PBS.
2. Fix samples in 4 % paraformaldehyde (PFA) in PBS at 4 °C. Embryos up to E15.5 can be fixed as a whole. From E16.5, fetuses should be skinned and cut in pieces (head, trunk, and limbs) to facilitate penetration of fixative and subsequent solutions. Fixation time is 1–2 h for E11.5 to E14.5 mouse embryos, 24–48 h for E15.5 to E18.5 fetuses, and 48 h for postnatal tissues.
3. Demineralize postnatal samples at 4 °C in 1 % PFA and 0.5 MEDTA, pH 8. Change this solution every day. Demineralization takes 1 day for a newborn mouse, 1 week for a 7-day-old mouse, and 3 weeks for an adult mouse. It is complete when samples are soft enough to be bent without fracture.
4. Rinse samples in 1× PBS for 3× 15–60 min, and further process them for paraffin or frozen embedding, following standard protocols. Both types of sections are suitable for the BrdU and EdU assays. Choose either type depending on other assays that you may want to perform on adjacent sections.
5. Sections should be 7- to 20-μm thick. Superfrost Plus slides, which bind tissue sections more efficiently than regular glass slides, are recommended. Generate technical replicates by dedicating at least three nonadjacent sections per sample to the BrdU/EdU assay.
6. Just before proceeding for BrdU/EdU detection, air-dry frozen sections on the bench for 1 h, and then remove the embedding medium by washing the slides in 1× PBS for 3× 2 min. If you use paraffin sections, deparaffinize them by dipping the slides in Histo-Clear for 2× 5 min. Then, rehydrate them in 100, 95, and 70 % ethanol for 2× 1 min and wash in 1× PBS for 3× 2 min.
7. Optional: Use an immunostaining pen to circle sections with a hydrophobic barrier (*see* Note 4), but do not let sections dry out.

3.3 BrdU Detection

The BrdU detection method described below is based on formation of complexes between BrdU, biotin-conjugated anti-BrdU antibodies, and peroxidase-conjugated streptavidin. In

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⁴Surrounding tissue sections with a hydrophobic barrier allows smaller volumes of solution to be used during incubations than when entire slides are covered with solution or to perform distinct assays with different sections on one slide. It is still recommended that such steps as 1× PBS washes be performed by dipping slides in Coplin jars. Note that the barrier must be added after deparaffinization of sections because it is insoluble in alcohol but completely removed by Histo-Clear.

our experience, this method is more sensitive than fluorescence-based methods (e.g., using FITC-labeled anti-BrdU antibodies, or complexes between biotin-conjugated anti-BrdU antibodies and Alexa Fluor®-conjugated streptavidin).

Perform this assay essentially as described in the BrdU staining kit (Invitrogen):

1. Quench the endogenous peroxidase activity of tissues by submerging slides in a 10 % H₂O₂ solution in methanol for 10 min. Rinse in 1× PBS for 3× 2 min.
2. Cover sections with trypsin solution (one drop of Reagent 1A mixed with three drops of Reagent 1B from BrdU staining kit) and incubate in a humid chamber at 37 °C. In our experience, this step is not necessary for embryos younger than E14.5, and 10–15 min of incubation is needed for fetal cartilage and bone tissue (E14.5 to E18.5). Longer incubations and higher concentrations of trypsin may be needed for postnatal samples. Rinse in distilled water for 3× 2 min.
3. Cover sections with DNA-denaturing solution (Reagent 2 from BrdU staining kit) for 20–30 min. Rinse in 1× PBS for 3× 2 min. Blot off the solution without touching the sections.
4. Apply the blocking solution (Reagent 3 from BrdU staining kit) for 10–30 min. Blot off the solution. Do not rinse.
5. Apply the biotinylated mouse anti-BrdU antibody solution (Reagent 4 from BrdU staining kit) and incubate for 60 min in a humid chamber (*see* Note 5). Rinse in 1× PBS for 2× 3 min.
6. Apply the streptavidin–peroxidase solution (Reagent 5 from BrdU staining kit) for 10–30 min. Rinse in 1× PBS for 2× 3 min.
7. Apply the peroxidase detection mixture (one drop of reagents 6A, 6B, and 6C from BrdU staining kit in 1 ml of distilled water, prepared just before use) and incubate for 5 min or more, i.e., until spotty brown staining is visible with the naked eye. Rinse abundantly with distilled water.
8. Counterstain with hematoxylin solution (Reagent 7 from BrdU staining kit) for 1 min and then immediately wash with water. Place the slides into 1× PBS for approximately 30 s, until the sections turn light blue. Do not overstain, as dark blue signals are difficult to distinguish from peroxidase-generated dark brown signals. Rinse in distilled water.
9. Dehydrate the slides in a graded series of alcohol (70, 95, and 100 % ethanol) for 2× 1 min, and clear with Histo-Clear for 2× 1 min.
10. Coverslip with Histomount mounting media (Reagent 8 from BrdU staining kit).

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⁵A 1-h incubation with the antibody is the minimum time required to obtain a strong signal. Extend to 2 h, if possible, or incubate overnight at 4 °C in a humid chamber.

3.4 EdU Detection

Perform all steps of the assay in the dark (*see* Note 6), and never let the slides dry out, as this creates fluorescence background. Proceed essentially as instructed in the Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Invitrogen):

1. Prepare the Click-iT® reaction cocktail, and apply it to each section for 30 min.
2. Wash slides with 3 % BSA in 1× PBS for 2 min and then with 1× PBS for 2 min (*see* Note 7).
3. Stain cell nuclei by incubating sections in Hoechst 33342 solution (Component G of the Click-iT® EdU Alexa Fluor® 488 Imaging Kit, diluted 1/2,000 in 1× PBS) for 2 min and then wash in 1× PBS for 2× 2 min.
4. Mount slides with Vectashield® media.

3.5 Image Acquisition and Quantification

1. Photograph tissue sections assayed for BrdU incorporation under regular bright-field microscopy conditions. BrdU-positive cell nuclei will appear light to dark brown, and BrdU-negative cell nuclei will appear light blue (Fig. 2, top left). Photograph sections assayed for EdU incorporation under epifluorescence microscopy conditions: use an excitation wavelength of 350 nm and an emission wavelength of 461 nm to detect Hoechst 33342-stained nuclei and an excitation wavelength of 495 nm and an emission wavelength of 519 nm to detect Alexa Fluor® 388-EdU-positive cells. EdU-positive cell nuclei will appear green and other cell nuclei blue (Fig. 2, top right). If needed, increase the color intensity and contrast of pictures using Adobe Photoshop or other appropriate software.
2. Judiciously select tissue areas of interest for quantification of cell proliferation. This step is critical, as rates of cell proliferation can vary greatly between tissue types (e.g., bone versus cartilage) and tissue regions. As appropriate, divide tissues into several areas (Fig. 2, top left and right).
3. Count the numbers of BrdU/EdU-positive and -negative cell nuclei in selected areas. Calculate the total number of cells by adding the two numbers and deduce the percentages of positive cells. As desired and appropriate, calculate averages and standard deviation of replicates, plot data on graphs, and use statistical tests to analyze experimental groups (Fig. 2, bottom left and right).

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⁶Total darkness is not necessary, but avoid direct exposure to light. Turn off room lights, and stay away from windows. Perform incubations in a lightproof drawer or incubator.

⁷If desired, perform complementary immunostaining or histochemistry assays on the slides at this time. Keep the samples protected from light during all incubations.

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Fig. 1.

Schematic and comparison of the major steps involved in the BrdU and EdU assays. The two assays involve similar procedures to (1) label proliferating cells in vivo, (2) prepare tissue sections, and (4) analyze data, but differ significantly from each other (3A and 3B) in the number and nature of steps required to detect labeled cells and in the time (*arrows*) required to perform these steps

Fig. 2.

Quantification of cell proliferation in mouse fetus cartilage growth plates using BrdU and EdU assays. Pregnant mice were injected at the gestation day 17.5 with either BrdU (*left*) or EdU (*right*). Fetuses were collected 2 h later, and longitudinal sections of tibias were generated in paraffin. Three nonadjacent sections (technical replicates) for each of the three littermates (biological replicates) were stained for BrdU or EdU. Images were acquired of the proximal half of tibias, and the percentages of BrdU- or EdU-positive cells were counted in 18 successive areas spanning the growth plate from the subarticular to the prehypertrophic zone (representative images are shown in the *top panels*). Results were plotted on graphs as averages with standard deviation of data obtained for each area (*bottom panels*). As previously described [12], the rate of chondrocyte proliferation increased in the epiphysis from the subarticular to the columnar zone; it reached a plateau in the proximal half of the columnar zone and rapidly declined in the distal half; chondrocytes were terminally growth arrested as they reached the prehypertrophic stage. Note that similar profiles and rates of cell proliferation were obtained for the BrdU and EdU assays, demonstrating equal efficiencies of these assays. Considering that the EdU assay is much faster and easier, we recommend this assay over the BrdU assay