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Detection of markers of cell proliferation by immunofluorescent staining and microscopy imaging in paraffin-embedded tissue sections

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Abstract

This unit provides a step-by-step protocol to detect markers of cell proliferation (BrdU or Ki67) by immunofluorescent staining and confocal microscopy imaging in paraffin-embedded tissue sections.

Keywords

BrdU; Ki67; cell proliferation; immunofluorescence; immunohistochemistry

Introduction

Immunohistochemical staining of proliferating cells in tissue sections enables quantification of proliferative fraction of cells. It also provides information about localization of such cells in context of the whole tissue morphology. The purpose of this unit is to provide a step-by-step protocol for immunodetection of markers of proliferation by fluorescent confocal microscopy imaging in formalin-fixed paraffin-embedded tissue sections. Two complementary protocols are included. Basic Protocol 1 describes the immunodetection of thymidine analog bromodeoxyuridine (BrdU) incorporated into newly synthesized cellular DNA after administration of BrdU to living animals prior to tissue collection. Basic Protocol 2 describes the immunodetection of endogenous nuclear protein Ki67, as a marker of cell proliferation. Both protocols are based on general immunohistochemical staining methodologies and rely on indirect immunofluorescent detection of BrdU and Ki67 antigens with specific monoclonal primary antibodies, which are subsequently detected by fluorescently conjugated secondary antibodies. While the immunofluorescent staining protocols described in this unit have been specifically optimized for formaldehyde-fixed paraffin-embedded mouse heart tissue sections, they can be easily applied to other tissue sections with appropriate modifications.

Basic Protocol 1

Immunofluorescent Detection of Bromodeoxyuridine (BrdU) in Paraffin-Embedded Tissue Sections—BrdU is a thymidine analog that is readily incorporated

into DNA during S-phase of cell cycle and is a widely used and well-established reagent to label and quantify proliferating cells both *in vitro* and *in vivo*. Immunofluorescent detection of BrdU requires that the experimental animals are administered BrdU prior to sacrifice and tissue dissection. BrdU incorporation can be detected within 2-24 hours of administration, depending on the tissue of interest.

In this protocol, we first describe the steps to de-paraffinize and rehydrate 5 μ m tissue sections mounted onto glass slides (Steps 1-9), and then to retrieve the antigens that are masked through the process of formaldehyde fixation by heat-induced antigen retrieval (Steps 10-12) and nuclease digestion (Steps 13-15). Next, we present the steps required to detect BrdU antigen by specific primary monoclonal antibodies coupled to fluorescent dye-conjugated secondary antibodies for visualization of labeled nuclei by fluorescent confocal microscopy (Steps 16-23).

To prepare the tissue sections labeled with BrdU, adult mice were injected intraperitoneally for 3 consecutive days with BrdU solution and the heart tissue was dissected, as previously described (Teekakirikul et al., 2010). The dissected tissues were carefully rinsed in PBS, and fixed in freshly prepared 4% paraformaldehyde/PBS (w/v) at 4 C overnight (16-24 hours) with gentle agitation on a rocker and dehydrated through graded ethanol solutions and xylene for standard paraffin-embedding. 5 micron (μ m) tissue sections were cut on a microtome and mounted on positively charged glass slides. Please see Support Protocol, which describes in detail *in vivo* administration of BrdU as well as steps involved in preparation of paraffin-embedded tissue sections on glass slides.

Materials and Equipment: 5- μ m thick paraffin-embedded tissue sections mounted on positively charged glass slides (Superfrost plus microscope slides, Thermo Scientific J1800AMNZ)

Slide staining racks and vessels with lids (xylene- and heat-resistant) (Tissue-Tek Slide Staining Set, Sakura Finetek)

Solvent insoluble pen (EMS, 62053-B) or pencil

Incubator or hybridization oven, 60 C

Xylene (Histological grade) (SIGMA, 534056-4L)

Ethanol (Histological grade, SIGMA, 493546-1L)

Tris-buffered saline (TBS)

Tween-20 (SIGMA, P9416)

10 mM sodium citrate buffer, pH 6.0

EcoRI and 10X EcoRI buffer (NEB, R0101S)

Exonuclease III (Exo III) (NEB, M0206S)

Normal goat serum (Vector Labs, S-1000)

Antibody diluent (Dako, S0809)

Anti-BrdU antibodies: Clone Bu20a (Dako, M0744) or BU1/75 (ICR1) (Abcam, ab6326)

Mouse IgG1 (Dako, X0931)

Rat IgG (Vector Labs, I-4000)

Anti-Ki67 antibodies: Clone SolA15 (eBioscience, 14-5698-82)

Alexa Fluor-488 conjugated goat anti-mouse secondary antibodies (Life Technologies, A11001)

Alexa Fluor-488 conjugated goat anti-rat secondary antibodies (Life Technologies, A11006)

Humidified-chamber:

- Commercially available (StainTray, M920, Simport) or;
- A large tissue culture dish (Thermo Scientific Nunc, 240835) can be used to prepare the humidified chamber as described in Step 14 and depicted in Figure 2

Low-lint wipes (VWR, 115-0202)

Hydrophobic pen (Dako, S2002)

Mounting reagent containing DAPI (Prolong Gold Antifade Mountant with DAPI, Life Technologies, P36935)

Glass coverslips (VWR, Cat# 631-9430)

Programmable pressure-cooker (Biocare Medical Decloaking Chamber)

- Alternatives: vegetable steamer, microwave oven or water bath

Confocal microscope

De-paraffinization and re-hydration of paraffin-embedded tissue sections: Before starting the de-paraffinization and re-hydration steps, total number of slides/samples that need to be processed should be planned (up to 25 slides can be processed in the recommended staining vessels). We recommend 1 slide per condition to be tested and we generally use slides with 2 tissue sections mounted, which serve as replicates (Figure 1). At the very least, the experiment should be designed to include 5 different slides (Table 1) including the following:

- Negative control 1 slide contains tissue sections from the animal, which was not injected with BrdU: incubate with primary anti-BrdU antibodies and secondary antibodies.

- Negative control 2 slide contains tissue sections from the animal injected with BrdU: incubate with species-matched IgG instead of the anti-BrdU antibodies and secondary antibodies
- Negative control 3 slide contains tissue sections from the animal injected with BrdU: incubated with secondary antibodies only.
- Positive control slide contains tissue sections of an organ with high proliferative index from the animal injected with BrdU: incubate with incubate with primary anti-BrdU antibodies and secondary antibodies.
- Experimental sample slide contains tissue sections from the animal injected with BrdU: incubate with anti-BrdU antibodies and secondary antibodies.

The slides should be labeled with a solvent insoluble pen (EMS, 62053-B) or pencil to include sample ID and slide number (Figure 1).

1. Place slides to be stained on a staining rack (solvent-resistant), immerse in xylene (in a fume hood) and incubate for 3 min at room temperature (RT). Repeat with 2 more washes (3 min each) in xylene.

Xylene is a clearing agent, which removes paraffin from tissue sections. Removal of paraffin is a critical step for allowing antibody access to antigens. Warming the slides to 60 C for 10-30 min prior to xylene immersion may help in removing the paraffin wax. Xylene is a toxic and highly flammable solvent and care must be taken and appropriate personal protective equipment (PPE) must be used when handling it. Always use xylene in a chemical fume hood.

2. Incubate slides in 100% ethanol for 2 min at RT. Repeat with 2 more washes (2 min each) in 100% ethanol.

100% ethanol incubation steps should be carried out in a fume hood as in Step 1 to avoid fumes due to contamination of ethanol with carry-over xylene from step 1. Graded ethanol washes (Steps 2-5) ensure removal of xylene and rehydrate the tissues.

3. Incubate slides in 95% ethanol for 2 min at RT.
4. Incubate slides in 80% ethanol for 2 min at RT.
5. Incubate slides in 70% ethanol for 2 min at RT.
6. Incubate slides in distilled water for 2 times 5 min each at RT.
7. Incubate slides in phosphate buffered saline (PBS) for 5 min at RT.
8. Incubate slides in PBS-Tween-20 (0.1%, v/v) for 2 times 5 min each at RT.
9. Rinse slides in distilled water for 2 times at RT.

After re-hydration (Steps 2-9), do not allow the sections to dry at any point by keeping the slides in solution.

Heat-induced antigen retrieval: 10. Remove the slides with the staining rack from water (Step 9) and quickly immerse into the antigen retrieval buffer (10 mM citrate buffer, pH 6.0) in a heat-resistant polypropylene container (Tissue-Tek Slide Staining Set, Sakura Finetek). Cover the container with the lid (provided with the staining set) and place in the pressure cooker (containing 500 ml water). Apply heat according to manufacturer's instructions. We find that 125 C for 30 sec (typically reaching pressure level between 20-24 psi), and 90 C for 10 sec works well in a programmable pressure cooker (Biocare Medical Decloaking Chamber). Glass staining dishes should not be used during heat-induced antigen retrieval step as they may crack.

Heat-induced antigen retrieval (HIAR) is an essential step to unmask the formaldehyde-fixed epitopes for recognition by antibodies in subsequent steps. If a pressure-cooker is not available, HIAR can be performed using a water-bath, microwave oven, or vegetable steamer for 20 minutes; however the antigen retrieval buffer should be pre-heated to 95 C (during rehydration steps) prior to immersion of slides and the temperature of the retrieval solution should be monitored closely to ensure that it is maintained at 95-100 C. If a microwave is used, the power level of the microwave oven should be optimized to prevent vigorous boiling of antigen retrieval solution during heating to prevent damage to tissue morphology. Also as power levels of microwave ovens vary, this should be optimized and standardized for consistent results. One disadvantage of microwave method is that it can cause non-uniform heating resulting in inconsistent staining and therefore, may not be preferable.

11. Remove the container with the slides in the antigen retrieval buffer (appears cloudy when hot) from the heat source and allow to cool for about 20 minutes at RT (solution becomes clear again).

Caution should be observed when removing the container as the antigen retrieval solution is very hot!

12. Wash slides 3 times 5 min with TBS at RT with gentle agitation on a rocker.

Nuclease treatment: 13. Dilute Exo III (100,000 units/ml) and EcoRI (20,000 units/ml) to 10 units/ml final concentration in 1× EcoRI reaction buffer (supplied by the manufacturer).

Digestion with nucleases (Step 15) helps expose the BrdU-labeled DNA epitope for anti-BrdU antibody recognition by generating single-stranded DNA (Dinjens et al., 1992) (Montuenga et al., 1992).

14. Carefully remove one slide at a time from the staining rack in the TBS wash-buffer, and gently wipe and dry excess buffer around the sections using low-lint wipes, being cautious not to dry-out the tissue. Draw a circular border around the tissue with hydrophobic pen. Place the slide horizontally on the raised rails of the humidified chamber and add ~50-100 ul nuclease solution (prepared in Step 13) to cover the tissue section. Repeat step 14 for each slide and incubate at 37 C for 30 minutes in a humidified chamber.

Hydrophobic border around the tissue sections limits the volume of solution needed to cover the section, reducing costs. During use, if the felt-tip of hydrophobic pen dries, moisten it by

gently pressing it on a piece of paper. Do not press the tip when drawing a border around the tissue as this may result in release of excess liquid adversely affecting the immunostaining quality as the liquid fluoresces when viewed under a fluorescent microscope. Generally, to cover sections of small tissues from mice, 50-100 μ l of solution is enough for a diameter of circular border of up to 1 cm and 150-200 μ l of solution is enough for a diameter of circular border of up to 1.5 cm; however, the volume of buffer should be adjusted for larger tissue sections.. Incubation in a humidified chamber is critical to prevent drying-out of tissue sections during incubations. Commercially available humidified chamber can be used. Alternatively, position and fix 2 plastic pipets in parallel on the bottom of a large tissue culture dish to form a raised level on which the slides can be placed horizontally (Figure 2) next to each other. Add layers of water-soaked filter papers on the bottom of the dish to create a humidified chamber. The plastic pipets keep the slides at a raised level preventing the contact with the wet filter paper at the bottom of the chamber.

15. Gently remove the nuclease solution, immerse the slides into the staining rack placed in the vessel with TBS-T and wash 3 times 3 min with gentle agitation at RT.

16. Remove one slide at a time from the staining rack in TBS-T and gently wipe and dry excess buffer around the tissue being careful not to dry the tissue (re-apply the hydrophobic border if needed). Place the slide horizontally on the raised rails of the humidified chamber. Add 50-100 μ l blocking buffer (10% normal goat serum/TBS) per section and repeat step 16 for each slide. Incubate in the humidified chamber for 1 hour at RT or overnight at 4 C.

Blocking buffer should be prepared with serum from species in which the secondary antibody was raised.

17. In the meantime, prepare anti-BrdU antibody dilutions. Dilute mouse monoclonal anti-BrdU clone Bu20a (Dako) 1:100 or rat monoclonal anti-BrdU clone BU1/75 (ICR1) (Abcam) 1:250 in antibody diluent (Dako).

Species-matched IgGs (mouse IgG for clone Bu20a and rat IgG for BU1/75) at the same final concentration as the respective antibodies should be prepared as a control for non-specific binding.

18. Gently remove the blocking buffer by tipping the slide to the side and use low-lint wipes to gently absorb any excess buffer (without touching the tissue section). Place the slide horizontally back on the raised rails of the humidified chamber and add 50-100 μ l of anti-BrdU antibodies or control IgGs per section on the appropriate slide (Table 1) and incubate slides at RT for 1 hour or at 4 C overnight in a humidified chamber.

19. Place slides in the staining rack in the vessel containing TBS-T and wash slides with TBS-T 3 times 5 min each at RT with gentle agitation.

20. Carefully remove one slide at a time from the staining rack in the vessel containing TBS-T wash buffer and gently wipe/dry excess buffer around the tissue section being careful not to dry the tissue. Place the slide horizontally on the raised rails of the humidified chamber and add 50-100 μ l of Alexa-Fluor 488 conjugated anti-mouse (for detection of anti-BrdU

Clone Bu20a) or Alexa-Fluor 488 anti-rat (for detection of anti-BrdU Clone BU1/75 (ICR1)) secondary antibodies diluted 1:500 in TBS. Incubate for 1 h at RT in the dark (covering the humidified chamber with aluminum foil).

Fluorescent conjugated secondary antibodies should be protected from light and steps 20-22 should be performed in dark to prevent photobleaching of the fluorescent antibodies. Prior to use, centrifuge the fluorescent-conjugated secondary antibodies for 1 min at 14000 rpm in a tabletop centrifuge and use only the supernatant to prepare required dilution to prevent non-specific staining of sections due to possible protein aggregates formed during storage. Even though Alexa-Fluor conjugated secondary antibodies are used in this protocol due to its intense fluorescence and photostability, there are wide range of fluorescent conjugated secondary antibodies commercially available and can be used with this protocol with proper optimization. The following link is a very useful tool, which displays a fluorescence spectra viewer with information on excitation and emission wavelengths of various fluorophores: <http://www.lifetechnologies.com/uk/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html>

21. Place slides in the staining rack in the vessel containing TBS-T and wash slides with TBS-T 3 times 5 min each at RT with gentle agitation protected from light exposure.

22. Carefully remove one slide at a time from the staining rack in the vessel containing TBS-T wash buffer and gently wipe and dry excess buffer around the tissue section. Add 10-20 μ l of mounting reagent with DAPI on tissue section and carefully drop a glass coverslip allowing the mounting reagent to spread slowly. If bubbles are present, gently press on the coverslip to remove the bubbles being careful not to damage the tissue. Cure slides for 24 h at RT in the dark. Store slides at 4 C in the dark until viewing on the fluorescent microscope, protected from light.

For longer lasting fluorescent signal, it is recommended to use a mounting reagent with antifade properties. We also recommend staining of nuclei with a fluorescent DNA-binding dye such as DAPI (4', 6-diamidino-2-phenylindole) to enable quantification of total nuclei in a region of interest. In our hands, ProLong Gold antifade (Life Technologies) works well, and even several months after staining/mounting, the slides can be viewed and imaged with no significant loss of fluorescence.

23. Capture images with a fluorescent confocal microscope using appropriate excitation/emission filters and quantify the total number of BrdU positive (or Ki67 positive-Basic Protocol 2) nuclei as well as total number of nuclei (DAPI stained) to calculate the fraction of cells proliferating in a section of interest. Figure 4 and Figure 5 show tissue sections stained with anti-BrdU antibodies and anti-Ki67 antibodies, respectively.

As tissue sections can be heterogenous depending on the field of view, it is recommended that images should be taken from 5-10 areas per section and nuclear BrdU staining or Ki67 staining (Basic Protocol 2) should be overlaid with DAPI staining to confirm the specificity of the immunolabeling (should also be located in the nucleus). The total nuclei quantification can be carried out using an image processing program such as ImageJ (freely available <http://imagej.nih.gov/ij>).

Basic Protocol 2

Immunofluorescent Detection of Ki67 Antigen in Paraffin-Embedded Tissue

Sections—Ki67 protein is a large nuclear protein expressed during all phases of cell cycle except G₀ and is a well-accepted marker of cellular proliferation. This protocol for immunofluorescent detection is provided as an additional method of detecting proliferation and allows assessment of proliferation in tissue sections, which were not necessarily injected with BrdU. Furthermore, if identification of cells undergoing proliferation during a specific time period (e.g. specific developmental or disease stage) is not required; or if identification of cells that have undergone DNA synthesis is not needed, then Ki67 staining can be a faster and cheaper alternative to BrdU immunostaining (Please refer to Figure 3(A) for a flowchart to help decide which method may be more appropriate when designing experiments). The Protocol steps described in Basic Protocol 1 can be applied for Ki67 immunostaining with the following modifications:

Steps 13-15: Omit

Steps 17-18: Dilute rat anti-Ki67 (Clone SolA15) antibodies (eBiosciences, Cat no. 14-5698-82) 1:100 dilution and dilute species-matched IgG (rat IgG) at the same final concentration as the antibody to serve as a negative control.

Step 20: Use Alexa-Fluor 488 anti-rat secondary antibodies diluted 1:500 in TBS.

Support Protocol

BrdU Injection and Tissue Collection/Processing

Materials and Equipment: Sterile PBS

Sterile BrdU solution (filter-sterilized through a 0.22 μ m filter, see recipe)

Sterile needles (e.g. 30 G for mice)

Sterile 1 ml syringes

Anesthetics (e.g. isofluorane)

Dissection tools (forceps, scissors)

Bijou sample container

4% paraformaldehyde (see recipe)

BrdU labeling of tissues in vivo and tissue collection/processing: 1. Thaw BrdU (6 mg/ml) solution at 37 C prior to injection.

It is important to warm up the BrdU solution to prevent discomfort to the animals during injection. BrdU is toxic and should be handled carefully in the fume hood and any contaminated material/equipment (e.g. gloves, needles, wipes, etc) should be disposed according to local regulations.

2. In the fume hood, restrain the mouse and carefully inject intraperitoneally in the lower left or right quadrant of the abdomen (to avoid any internal organs such as liver or bladder) with 100 mg/kg BrdU (e.g. ~420 μ l BrdU solution should be administered to a 25 g mouse) or PBS as a control.

We have injected mice with BrdU for 3 consecutive days and were able to detect proliferating cells (non-myocytes) in the heart (Teekakirikul et al., 2010). If BrdU injection over a longer time period is needed, alternative methods of BrdU administration (e.g. BrdU in drinking water or subcutaneously implanted osmotic mini-pumps) should be used to prevent injection site injury, discomfort and stress induced by repeated handling.

24 hours after the last injection, anesthetize and sacrifice the mouse. Carefully, dissect the desired tissues (e.g. heart) avoiding damage to tissues collected. In addition, dissect a tissue with a high proliferative index (e.g. small intestine) to use as a positive control during staining. For helpful information about anatomy of mouse please see: <http://www.informatics.jax.org/cookbook/index.shtml>

4. Carefully rinse dissected tissues in PBS, and immersion-fix in 4% paraformaldehyde/PBS (w/v) in bijou containers at 4 C overnight (16-24 hours) with gentle agitation on a rocker. Once fixed, the tissues can be processed for standard paraffin-embedding and sectioning.

Before the fixed tissues can be embedded in paraffin wax, they need to be infiltrated with a series of dehydrating (ethanol) and clearing (xylene) baths to replace water as paraffin wax is hydrophobic and therefore, immiscible with water. The dehydration and clearing steps ensure that paraffin wax can completely infiltrate the tissues. The tissues can then be placed in molds containing melted paraffin wax to obtain “tissue blocks” once the paraffin solidifies. Properly fixed and processed paraffin-embedded tissue blocks are stable and can be kept at room temperature for long-term storage (years). To prepare tissue sections for histological staining or immunohistochemistry, the paraffin blocks can be sectioned using a microtome to generate 5 μ m tissue sections, which stick together and form “ribbons” as they are cut. The tissue ribbons can then be floated on warm water bath (to flatten) and mounted on positively charged microscope slides. We prefer to mount tissue ribbons containing 2 or 3 sections per slide, which allows replicate or triplicate staining per sample on the same slide. For more detailed information on sample processing for paraffin-embedding and subsequent sectioning, the following links may be helpful: <http://www.leicabiosystems.com/pathologyleaders/an-introduction-to-specimen-preparation/> <http://www.leicabiosystems.com/pathologyleaders/an-introduction-to-specimen-processing/>

Reagents and Solutions

BrdU Solution for injection: Dissolve BrdU (Sigma B5002) in sterile PBS at a final concentration of 6 mg/ml and if it does not dissolve easily, warm it to 37 C for 5-10 minutes with occasional vortexing. Filter-sterilize through a 0.22 μ m filter. BrdU solution can be stored at 4 C for 1 week or aliquoted and stored at -20 C for long-term storage. If white precipitates are visible after thawing, warm the solution at 37 C. The powder and the solution should be handled carefully in the fume hood and with proper personal protective equipment (PPE) as BrdU is toxic and a suspected mutagen.

4% Paraformaldehyde/PBS (w/v) (should be prepared in a fume hood): Warm up 150 ml of PBS in a glass beaker to ~60 C while stirring with a magnetic stir bar. Add 8 g of paraformaldehyde and continue stirring. Add 1N NaOH dropwise until all the paraformaldehyde dissolves completely. Adjust the volume to 200 ml with PBS and check to make sure that the pH is maintained at about 7.0. Once it cools to room temperature, filter-sterilize through a 0.22 μ m filter. It can immediately be used to fix the tissue sections or alternatively aliquoted and stored at -20 C for up to 6 months. Paraformaldehyde is toxic and should be handled in the fume hood with care.

10X Tris-buffered Saline (TBS) (1.37 M NaCl, 200 mM Tris, pH 7.6): Add 24 g Tris base (MW:121.1 g/mol), 88 g NaCl (MW: 58.4 g/mol) in ~900 ml distilled water and pH to 7.6 with 12N HCl. Adjust volume to 1 L. Store at room temperature (stable at least 1 year). *Prior to use dilute 1:10 in distilled water.*

10X Sodium Citrate Buffer: Dissolve 29.4 g sodium citrate trisodium salt dehydrate and 5 ml Tween-20 in 900 ml distilled water, adjust pH to 6.0 and volume to 1 L. 10 $\times$ buffer can be stored at 4 C for up to 1 year. *Prior to use dilute 1:10 in distilled water and check that pH is 6, otherwise adjust with 2N HCl or 2N NaOH.*

Commentary

Background Information

Historically, proliferation of cells *in vitro* and *in vivo* was assessed by incubating the cells or injecting experimental animals, respectively, with tritiated thymidine (^3H -TdR) and measuring or detecting the incorporation of ^3H -TdR into newly synthesized DNA by autoradiography (Hughes et al., 1958). However, over the last 2 decades, the radioisotope labeling of newly synthesized DNA to detect proliferation has been widely replaced by the development of monoclonal antibodies to detect bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU), a thymidine analog (Gratzner, 1982). Similar to ^3H -TdR, BrdU is incorporated into newly synthesized DNA during S-phase of the cell cycle and has several advantages. First, it enables faster detection time (long exposure time is generally required with autoradiography to detect incorporated ^3H -TdR). It also permits multiple immunofluorescence detection of other antigens within the same tissue section, providing additional information about the identity or cell fate of proliferating cells. Importantly, it eliminates the need for radioactive isotope handling. Even though BrdU is toxic, at the concentrations used in rodents *in vivo* (50-200 mg/kg) it is generally well tolerated. One of the disadvantages of BrdU immunostaining is that in order to expose the epitopes for antibody recognition, the labeled DNA has to be denatured by acid pre-treatment, which may damage tissue morphology and/or destroy other antigens to be detected in multiple-immunolabeling experiments. To avoid such problems, acid denaturation step can be replaced with heat-induced antigen retrieval step (Basic Protocol 1: Step 10), which, combined with nuclease-digestion (Exonuclease III and EcoRI endonuclease, Basic Protocol 1: Step 15) (Dinjens et al., 1992; Montuenga et al., 1992) can efficiently expose the BrdU antigen for immunodetection.

Recently, a click chemistry method utilizing another thymidine analog, 5-ethynyl-2'-deoxyuridine (EdU), has been developed to detect DNA synthesis, and hence cell proliferation, as an alternative to BrdU (Salic and Mitchison, 2008). The main advantage of EdU compared with BrdU is that it does not require any acid denaturation or HIAR step, since its detection is based on a chemical reaction (Salic and Mitchison, 2008). EdU can be used as a replacement for BrdU labeling and detected by commercially available reagents. EdU in combination with BrdU can also be useful in dual-pulse labeling experiments to detect cellular proliferation at defined time periods (Gomez-Nicola et al., 2011). Administration of BrdU and EdU at two different time points (Figure 3(B)) can provide information about cell cycle kinetics of different populations of cells.

While detection of incorporation of BrdU (or other thymidine analogues such as EdU) are well accepted methods of detecting cellular proliferation, BrdU (and EdU) can also get incorporated into DNA during DNA damage repair (Kao et al., 2001) (Limsirichaikul et al., 2009) and thus may not be a preferable methodology in certain experimental conditions when one needs to differentiate between BrdU incorporation into DNA as a result of DNA damage-repair vs. DNA synthesis during proliferation. Thus, additional immunohistochemistry methods to detect markers of proliferation should be used to complement the experiments with BrdU labeling.

Assessment of proliferation by detecting the expression of Ki67 antigen is an alternative or complementary method to quantify cellular proliferation. Ki67 protein is a large nuclear protein expressed during all phases of cell cycle (G₁, S, G₂, M), except quiescent G₀ phase. Ki67 is a well-established marker of cellular proliferation (Scholzen and Gerdes, 2000) and its expression has been shown to correlate well with BrdU incorporation (Goodson et al., 1998) (Kee et al., 2002) (Muskhelishvili et al., 2003). While determination of proliferation by BrdU requires injection of experimental animals with BrdU prior to sacrifice, Ki67 immunostaining protocol relies on detection of endogenously expressed protein, eliminating any additional handling of animals for BrdU injections. Furthermore, it can be used on archived tissue slides (obtained from animals not injected with BrdU) enabling quantification of proliferation on valuable experimental samples, reducing the need for further experimental animals. One drawback of Ki67-based detection of proliferation is that Ki67 positive cells reflect only a snapshot of amount of proliferation at the time of tissue dissection/fixation rather than during a defined period of interest. Despite this drawback, Ki67 is a reliable marker of cells in active cell cycle and is a suitable method complementing BrdU-based (or other thymidine analog) approaches.

Additional alternative methods to detect proliferation include immunostaining for other endogenous proteins expressed during cell cycle, such as phosphorylated histone H3 (p-HH3) and proliferating cell nuclear antigen (PCNA). Phosphorylation of histone H3 (p-Ser10, p-Ser28) has been shown to correlate well with mitotic index (Hendzel et al., 1997) (Van Hooser et al., 1998) (Goto et al., 1999) and can be used to quantify proliferative fraction. On the other hand, despite being used widely, correlation of PCNA expression with proliferative index has been questioned due to its long half-life and its involvement in DNA repair (Scott et al., 1991) (Sarli et al., 1995) (Muskhelishvili et al., 2003). Therefore, its

suitability for assessment of proliferation in tissue sections will need to be determined depending on the tissue type and experimental conditions.

Critical Parameters and Troubleshooting

Fixation—One of the most important parameters for the success of the immunohistochemical/immunofluorescent detection in paraffin-embedded tissue sections is the nature and the duration of the fixation, which aims to preserve the tissue morphology and antigens. For small tissues (e.g., dissected from mice) overnight fixation (16-24 hours) at 4 °C in freshly prepared 4% paraformaldehyde works well. While longer fixation times can adversely affect the quality of the immunodetection due to the inability to retrieve the antigens, shorter fixation times may not sufficiently preserve the antigens compromising detection (Table 2). Larger tissues may need to be cut into smaller pieces (~ 5 mm thick) to allow sufficient penetration of the fixative, and the fixation time may need to be extended. Volume of the fixative is also critical and at least 20× volume/tissue weight of fixative should be used to ensure proper fixation. (However, we prefer 50× volume/tissue weight for best results (e.g., for a 100 mg tissue, 2-5 ml of fixative should be sufficient).

Slides—It is highly recommended to mount tissue sections cut on a microtome on positively charged slides to avoid loss of tissue sections during heat-induced antigen retrieval step or multiple wash steps, since the charge of normal glass and cell surfaces are negative.

Deparaffinization and Rehydration—Xylene and ethanol solutions can be re-used at least 5 times to save reagents and reduce toxic waste. However, they may need to be replaced sooner depending on the total number of slides processed or if there are problematic unstained areas on tissue sections, which may be due to insufficient paraffin removal.

Antigen Retrieval—During fixation of tissue in 4% paraformaldehyde, most antigens are masked due to formation of covalent cross-links (methylene bridges) between proteins. In order to expose these masked antigens for antibody recognition, enzymatic or heat-induced antigen retrieval step (reviewed in (Shi et al., 2011)) is generally required. Heat-mediated antigen retrieval works well for most antigens and is dependent on buffer, pH, temperature, as well as the length of time of heat application, and often requires optimization for different specific antigens. Steps 10-11 of Basic Protocol 1 and 2 are based on citrate buffer (pH 6.0)-induced antigen retrieval and work well for both BrdU (Basic Protocol 1) and Ki67 antibodies (Basic Protocol 2). However, depending on the heat source used to retrieve the antigen, the duration of heat application may need to be modified to reach the optimum conditions in which a strong signal-to-noise ratio of nuclear BrdU or Ki67 staining is detected

Immunostaining—Prior to incubation of tissue sections with specific antibodies, appropriate blocking reagent (serum from species in which the secondary antibodies were raised) should be added to eliminate or reduce high background (i.e., non-specific staining). Wash steps and durations can also be increased to reduce background. In order to prevent any non-specific staining, it is critical that the tissue sections are not allowed to dry at any

point after rehydration steps and antibody incubation steps should always be performed in a humidified chamber. In addition, primary and secondary antibody concentrations may need to be further diluted to reduce background staining. Use of mouse-monoclonal antibodies on mouse sections may also result in non-specific staining due to recognition of endogenous mouse IgG by secondary anti-mouse antibodies. While the specific nuclear BrdU staining can be distinguished from the non-specific staining by co-localisation of BrdU signal with nuclear DAPI staining (Teekakirikul et al., 2010), if non-specific staining is significant, rat monoclonal anti-BrdU antibodies (Clone Bu1/75 ICR, Abcam) are recommended. Alternatively, endogenous mouse IgG can be blocked with unconjugated anti-mouse IgG Fab fragment antibodies (Ivell et al., 2014) (Brown et al., 2004) or commercially available mouse-on-mouse (mouse primary antibodies on mouse tissue) immunodetection kits (e.g. Vector Laboratories BMK-2202) can be used to reduce non-specific staining. Finally, Tween-20 in TBS-T wash buffer may be replaced with a stronger detergent, TritonX-100, to eliminate non-specific background staining.

All the antibodies described in this unit work well with paraffin-embedded tissue sections; however if alternate antibodies are used, the manufacturer's recommendations should be followed to determine whether a selected antibody works on paraffin-sections. During antibody immunostaining, it is critical to use appropriate positive and negative control slides processed in parallel to experimental slides (Table 1 lists the recommended positive and negative controls for BrdU staining). As a positive control for both BrdU and Ki67 antibodies, a tissue with high proliferative index can be used (e.g. small intestine). As negative controls for both BrdU and Ki67 antibodies, additional serially-cut tissue sections should be incubated with species-matched immunoglobulins instead of the primary antibodies. A control tissue isolated from animals injected with PBS (instead of BrdU) should be included as an additional negative control for BrdU antibodies. Finally, the fluorescent secondary antibody incubations and subsequent washes should be performed in the dark (Steps 20-22) to avoid prolonged exposure to light, which may photo-bleach the fluorescent-dye conjugated to the secondary antibody reducing signal intensity.

If weak or no specific nuclear signal is detected, this can be due several factors (Table 2). The tissues may have been over-fixed or under-fixed leading to inability to retrieve the antigen or loss of antigen, respectively. The antibody incubation steps may need to be extended; even though the dilutions provided in this unit work well for mouse heart tissue, for other tissues or species, appropriate dilutions may need to be determined. Furthermore, duration of the antigen retrieval step-duration may need to be optimized depending on the source of heat.

Anticipated Results

Ki67 and BrdU immunofluorescence staining with their respective antibodies should result in a specific nuclear staining and negative controls should not have any nuclear immunofluorescence signal. Co-localisation of Ki67 and/or BrdU with nuclei staining (DAPI) will further confirm the specificity of the staining (Figure 4 and Figure 5). If every nucleus stains positive for Ki67 or BrdU, this may indicate a non-specific staining and

protocol should be followed as outlined along with appropriate positive and negative controls.

Time Considerations

The complete immunostaining protocol (Basic Protocols 1 and 2) can be completed in one day (primary antibody incubation for 1 hour at RT) or 2 days (primary antibody incubation overnight at 4 C). Once the slides are deparaffinised and rehydrated, it is recommended that the protocol is completed as described to avoid compromising tissue integrity and staining quality due to extended wash or incubation steps. The approximate time line for the protocol is as follows:

Deparaffinization and rehydration: ~ 45 min

Heat-induced antigen retrieval/washes: ~1 hr

Nuclease digestion: 35-50 min

Serum blocking: ~ 1hr

Primary antibody incubation/washes: ~1-2 hr at RT or overnight at 4 C.

Secondary antibody incubation/washes/mounting: ~1-2 hr at RT.

An experienced investigator can easily process 25 slides in one day. If needed, it is possible to process >25 slides at the same time; however, we recommend processing in smaller batches to avoid extending the time required to complete some steps (14,16, 18, 20, 22), which require individual handling and to prevent introducing variables.

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Figure 1. Depiction of consecutive tissue sections on positively charged microscope slides
Each slide must be labeled with Sample ID and slide number as well as any additional information that may be needed using a solvent insoluble pen or regular pencil prior to de-paraffinization/rehydration steps.

Figure 2. Humidified-chamber

Position and fix 2 plastic pipets in parallel on the bottom of a large tissue culture dish to form a raised level on which the slides can be placed horizontally next to each other. Add layers of water-soaked filter papers on the bottom of the dish to create a humidified chamber. The plastic pipets keep the slides at a raised level preventing the contact with the wet filter paper at the bottom of the chamber.

Figure 3.

(A) Flowchart to decide which immunodetection method to use for assessing markers of cellular proliferation in tissue sections. (B) Schematic depiction of experimental design to pulse-chase with BrdU and EdU.

Figure 4. Detection of BrdU-labeled nuclei in tissue sections

Confocal immunofluorescence images show BrdU labeled nuclei (red) in heart sections from mice injected with BrdU and total nuclei stained with DAPI (blue). Overlaid image shows overlap (arrowheads) of BrdU stained nuclei with DAPI. Scale bar = 78 μm .

Figure 5. Detection of Ki67 positive (+) nuclei in tissue sections

Confocal immunofluorescence images show Ki67+ nuclei (red) in heart sections from mice and total nuclei stained with DAPI (blue). Overlaid image shows overlap (arrowheads) of Ki67+ nuclei with DAPI. Scale bar = 50 μ m.

Table 1
Recommended experimental design, which includes positive and negative controls for BrdU immunostaining

The table includes details of recommended three negative controls and a positive control to be processed in parallel to the experimental sample to ensure that the results can be appropriately interpreted.

Slide	Sample/Tissue	Primary Antibodies	Secondary Antibodies
Negative Control 1	Animal injected with PBS/tissue sections of interest	anti-BrdU (Clone Bu20a)	Alexa-fluor-488 anti-mouse antibodies
Negative Control 2	Animal injected with BrdU/ tissue sections of interest	mouse IgG control	Alexa-fluor-488 anti-mouse antibodies
Negative Control 3	Animal injected with BrdU/ tissue sections of interest	Incubation buffer only	Alexa-fluor-488 anti-mouse antibodies
Experimental Sample	Animal injected with BrdU/ tissue sections of interest	anti-BrdU (Clone Bu20a)	Alexa-fluor-488 anti-mouse antibodies
Positive Control	Animal injected with BrdU/ tissue sections with high-proliferative index, e.g. small intestine	anti-BrdU (Clone Bu20a)	Alexa-fluor-488 anti-mouse antibodies

Table 2

List of common problems that maybe encountered in the protocol, possible causes and suggested remedies.

Problem	Cause	Remedy
Uneven staining	Over- or under-fixation of tissues	Shorten or lengthen the fixation time
	Insufficient paraffin removal	Increase the incubation time in xylene and replace xylene and ethanol with fresh solutions
High background or Non-specific staining	Insufficient blocking	Increase the incubation time in blocking buffer
	Inappropriate blocking buffer	Use serum from species in which the secondary antibodies were raised
	Insufficient washing	Increase the duration and the number of washes. Incubate sections with TBS-0.1% TritonX-100 instead of TBS-0.1% Tween-20
	High concentration of primary or secondary antibodies	Use a higher dilution of antibodies
Weak or no specific staining	Slides allowed to dry- out	Always keep sections covered with solution and in a humidified chamber
	Over- or under-fixation of tissues	Shorten or lengthen the fixation time
	Incorrect primary antibody dilution	Optimise primary antibody concentration
	Too short antibody incubation time	Incubate with primary antibody for longer time
	Insufficient antigen retrieval	Optimise the antigen retrieval step by using varying lengths of time for heat-induced antigen retrieval