Video Article

Heat-Induced Antigen Retrieval: An Effective Method to Detect and Identify Progenitor Cell Types during Adult Hippocampal Neurogenesis

Syed M.Q. Hussaini*[1], Heechul Jun*[1], Chang Hoon Cho[1], Hyo Jin Kim[1], Woon Ryoung Kim[2], Mi-Hyeon Jang[1,3]

[1]Department of Neurologic Surgery, Mayo Clinic College of Medicine

[2]Department of Anatomy, Korea University College of Medicine

[3]Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine

*These authors contributed equally

Correspondence to: Woon Ryoung Kim at wrkim@korea.ac.kr, Mi-Hyeon Jang at Jang.MiHyeon@mayo.edu

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Abstract

Traditional methods of immunohistochemistry (IHC) following tissue fixation allow visualization of various cell types. These typically proceed with the application of antibodies to bind antigens and identify cells with characteristics that are a function of the inherent biology and development. Adult hippocampal neurogenesis is a sequential process wherein a quiescent neural stem cell can become activated and proceed through stages of proliferation, differentiation, maturation and functional integration. Each phase is distinct with a characteristic morphology and upregulation of genes. Identification of these phases is important to understand the regulatory mechanisms at play and any alterations in this process that underlie the pathophysiology of debilitating disorders. Our heat-induced antigen retrieval approach improves the intensity of the signal that is detected and allows correct identification of the progenitor cell type. As discussed in this paper, it especially allows us to circumvent current problems in detection of certain progenitor cell types.

Video Link

The video component of this article can be found at https://www.jove.com/video/50769/

Introduction

Neurogenesis, the generation of new neurons from neural stem cells, is now known to constantly occur into adulthood in two specialized regions in the brain. These include the subventricular zone (SVZ) of the olfactory bulb and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. Over the past decade, the field of adult hippocampal neurogenesis has seen significant work. The region has attracted much interest since newborn neurons in the SGZ contribute to enhanced neural plasticity that can sustain specific brain functions[1-5]. Adult hippocampal neurogenesis has been implicated to play important roles in mood regulation, regeneration, and learning and memory. Thus, there has been a concerted effort to understand the development and regulation of neurons in this rich neurogenic niche.

An unavoidable and important aspect of studying adult hippocampal neurogenesis is the identification of separate stages that an activated neural stem cell passes through on its way to becoming a fully functional neuron. In this process, the quiescent neural stem cell is activated and proceeds through a series of early active and late active progenitor cell types (**Figure 1**). These distinct populations of neural progenitors can be identified by morphology and their expression of molecular markers such as MCM2, nestin, Tbr2 and Doublecortin (DCX) and NeuN that guide the conversion of RGLs into their respective progenitor cell types. Nestin is an intermediate filament protein that is expressed across the radial glia-like cells and some early progenitor cell types. Another marker is Tbr2 that is specifically expressed in the amplifying progenitor cells. The Tbr2 transgene expression is initially switched off in the Type-1 cells (radial-glia like), turned on across the Type-2a, Type-2ab and Type-2b progenitors and switched off among the more differentiated Type-3 and immature neurons (**Figure 1**). DCX is a neuronal migration marker which is expressed in the Type-2ab, Type-2b and Type-3 neural progenitors. Using this combination of three markers, we can label distinct subtypes of neural progenitors. These include the Type-1 (MCM2+nestin+Tbr2-), Type-2a (MCM2+nestin+Tbr2+), Type-2ab (partial MCM2+nestin-Tbr2+ and partial MCM2+Tbr2+DCX-), Type-2b (MCM2+Tbr2+DCX+) and Type-3 (MCM2+Tbr2-DCX+). Post-mitotic cells such as the immature and functionally integrated neurons can be identified by utilizing DCX and the mature neuronal marker, NeuN.

Traditional methods of IHC utilize antibodies to identify antigens for specific cell types based on their gene expression. Subsequent visualization through high resolution imaging techniques such as confocal microscopy can be utilized for their identification. However, currently there are problems that exist with these methods that don't allow efficient identification of the radial glia-like cells and early progenitor cell types. It is difficult to identify these particular cell types because the applied antibody is not able to penetrate and efficiently bind the nestin protein. Nestin is the intermediate filament protein that comprises the radial processes produced by the radial glia-like cell. The inefficient binding of an antibody to the antigen can be a result of many factors including fixation time, temperature and technique utilized[6-7]. To help solve such issues, we have

developed an antigen-retrieval or "boiling" method. Besides nestin, this method of antigen-retrieval also improves staining for other markers such as Ki67, BrdU, glial fibrillary acidic protein (GFAP), Tbr2 and MCM2. Our method combines together chemical and physical approaches. It involves chemical fixation of the tissue in paraformaldehyde and subsequent high temperature boiling of thin coronal hippocampal sections in a buffer of a denaturant and chaotropic treatment. This enhances accessibility of the antigen to the antibody, improves antibody specificity and allows improved identification of progenitor cell types. Our approach is simple to use requiring mostly already available tools and reagents in the laboratory. We have utilized it extensively to study development of neural stem cells and their regulation via intrinsic genetic or extrinsic environmental factors[8].

Protocol

1. Pre-immunohistochemistry: Perfusion and Histology

1. Perfuse mice as per a general transcardial protocol adapted for mice and extract the brains into 4% paraformaldehyde (PFA)[9].
2. Leave the brains in PFA overnight at 4 °C (*e.g.* a 10 ml conical tube filled with PFA). Keep in PFA for at least 12-24 hr, no more than 36 hr for full fixation. After overnight fixation, empty out PFA from conical tube but leave brains inside. Pour 30% sucrose into tube and wash the brains for a brief 5 sec to remove any residual PFA. Finally, fill tube containing brains with 30% sucrose again. Keep brains in sucrose at 4 °C until brains sink to the bottom of tube.
3. Cut 40 µm brain sections on a microtome. Consult a mouse brain atlas to start collecting sections from the beginning of the dentate gyrus till its end. Preserve sections in anti-freeze solution (a simple recipe is 300 g sucrose in 500 ml 0.1 M PBS and 300 ml ethylene glycol) until ready for immunohistochemistry.
4. Mount the cut tissue on microscope slides specifically designed to adhere strongly to tissue. These slides provide a strong hold to tissue during the boiling stage. After mounting the tissue, please let the slide dry for 10 min or until it is fully dry. Drying can be expedited if the slide is rested against a vertical surface with the slide's bottom edge on a paper towel. If more than one slide is being mounted, the other slide(s) may be left dry during that time (1-2 hr is okay) until ready to proceed to the next step.
5. Once slide is dry, wash with PBS three times for 5 min each and dry again (PBS buffer = 0.137 M NaCl, 0.0027 M KCl, 0.0119 M Na_2PO_4). This ensures any previous chemical (such as glycerol from anti-freeze storage solution) is sufficiently removed and cannot impair the tissue's adherence to the slide.

2. Preparation of Reagents and Minor Equipment

1. Prepare Solution A (0.1 M or 19.21 g/l citric acid) and Solution B (0.1 M or 24.9 g/l tris-sodium citrate) in which tissue sections will be boiled.
2. In a graduated cylinder, combine 9 ml of Solution A and 41 ml of Solution B. Add 450 ml of ddH_2O to this mixture.
3. Pour this mixture into an empty container (*e.g.* an empty pipette tips box) that is microwave safe.

3. Heat-induced Antigen Retrieval

1. Boil solution made in step 2 for 5 min at standard setting in a standard microwave. The solution will start boiling at or above 100 °C.
2. Once boiling is complete, carefully remove container from microwave and place dried slides into it, ensuring the slide-face with hippocampal sections is facing down and fully exposed to the liquid. If possible, place slides at an angle against the walls of the box and stack other slides around them. Ensure their "fit" inside the box is tight so the slides have no room to move on top of each other.
3. Boil this mixture with slides for 7 min at standard settings in the microwave. The solution will boil during this time at or above 100 °C.
4. During this boiling stage, fill two ice buckets halfway with ice.
5. Once boiling in step 3.3 is complete, remove the container from the microwave and place it inside one of the ice buckets. Pour the rest of the ice from the other bucket all around the container and completely cover it. Let it sit for 1 hr.

4. Primary and Secondary Antibody Staining

1. At the end of the 1 hr waiting period, remove the slides from the container and wash with TBS-T buffer 3x for 5 min each. Any apparatus may be used to perform these basic washes. (Buffer TBS = 50 mM Tris HCl, 150 mM NaCl, TBS-T = 0.05% Triton X-100 TBS buffer, TBS-TT = 4% donkey serum TBS-T buffer).
2. After washing, let the slides dry in a dark place (*e.g.* inside a bench drawer, place a paper towel inside the drawer and tilt the slide against it) for 5-10 min or until slide is fully dry. During this period, prepare a chamber for overnight primary antibody staining. A simple staining chamber may include a container with large surface area filled with ddH_2O and a stage inside the container, on top of which the slide may be rested above the ddH_2O.
3. Once the slide has dried, draw an outline around the tissue sections using a water-repellant pen. During step 4.5, this outline acts as a barrier preventing the antibody mixture from flowing over.
4. Prepare the primary antibody mixture in TBS-TT. At least 500 µl is required to cover one entire slide for instance, if antibody stock is at 1:500 concentration, add 1 µl of particular antibody to 500 µl of TBS-TT). Utilize primary antibodies as mentioned in the reagents table.
5. Place the dried slide on top of the stage in the staining chamber. Add 500 µl of the primary antibodies slowly and cover entire surface. Cover entire apparatus and wrap in aluminum foil to block external light. Let it sit overnight at room temperature.
6. The next day, wash the slide three times for 5 min each with TBS-T buffer. Let it dry in a dark area as described in step 4.2.
7. Prepare secondary antibody mixture similar to step 4.4. Refer to reagents table for secondary antibodies.
8. Place the dried slide in the staining chamber as described in steps 4.2 and 4.5. Add 500 µl of the secondary antibodies slowly and cover entire surface. Wrap the staining chamber in aluminum foil to protect it from external light. Let it sit covered and protected from external light for at least 2 hr at room temperature.

9. After completion of secondary staining, wash the slide three times for 5 min each with TBS-T buffer. Let the slide dry in a dark area. After drying, put mounting solution and cover the slide with a cover slip carefully preventing any introduction of bubbles. Let the slide sit in a dark area for at least 2 hr before subsequent analysis.
10. Perform time-lapse or confocal analysis on the stained tissue.

Representative Results

Adult hippocampal neurogenesis is a sequential process where the representative progenitor cell type at each stage may be identified with a combination of markers. Careful chemical fixation and antigen-retrieval improves the effectiveness and accessibility of markers that are otherwise difficult to detect. One of these is nestin which is expressed among the radial glia-like neural stem cells and some early active progenitor cell types. Nestin can be a key protein in identifying early progenitor cell types as its expression is lost when the stem cell or progenitor subsequently differentiates into a mature neuron.

A schematic diagram of the sequential process of adult hippocampal neurogenesis occurring in the SGZ is shown in **Figure 1A**. It is divided into the mitotic and post-mitotic stages. Genetic markers allowing for identification are shown across the spectrum of their expression. The radial glia-like neural stem cell may be identified through their expression of GFAP and nestin, an intermediate filament protein comprising the radial processes. Quiescent and actively dividing radial glia-like cells may be identified through their expression of MCM2, a proliferation marker expressed among the mitotic progenitor cell types. The intermediate progenitor cell types or transient amplifying progenitor cells express a variety of markers that can be used to identify them. These include Tbr2 that is transiently expressed across the early active progenitors, DCX which is a neuronal maturation marker and NeuN which is a mature neuronal marker. Using this combination of three markers, we labeled distinct types of transient amplifying cells; Type-1 ($MCM2^+nestin^+Tbr2^-$), Type-2a ($MCM2^+nestin^+Tbr2^+$), Type-2ab (partial $MCM2^+$nestin-$Tbr2^+$ and partial $MCM2^+Tbr2^+DCX^-$), Type-2b ($MCM2^+Tbr2^+DCX^+$) and Type-3 ($MCM2^+Tbr2^-DCX^+$). Once committed to the neurogenic lineage, the transient amplifying progenitors become post-mitotic and lose their proliferative ability. The migration of this post-mitotic immature neuron is characterized with the expression of DCX and NeuN. Once functionally integrated into the system, DCX expression is lost while NeuN expression is maintained.

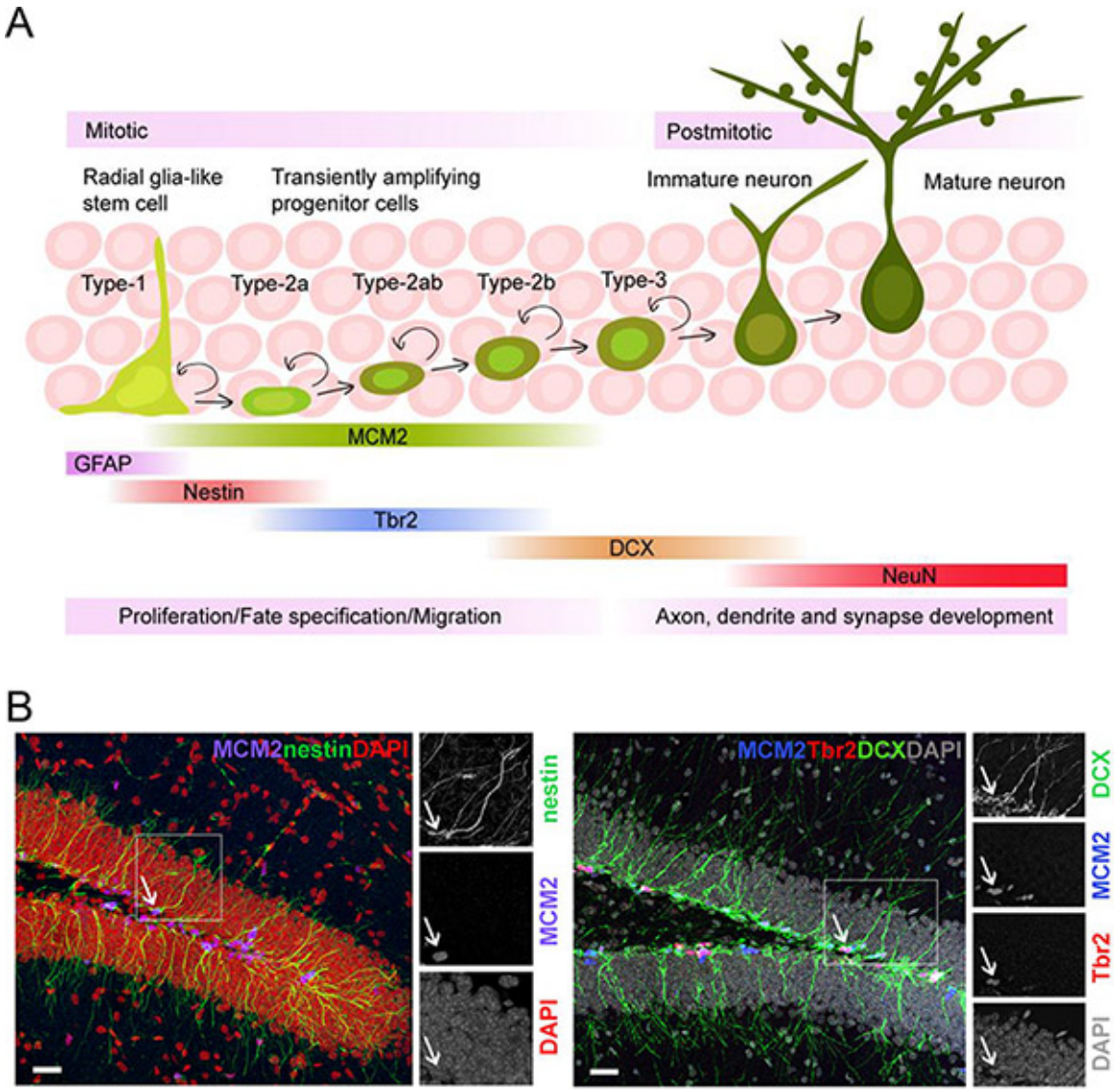

Figure 1. Development of neural stem cell during adult hippocampal neurogenesis. **(A)** Adult hippocampal neurogenesis is depicted across the subgranular zone of the dentate gyrus. An activated radial glia-like stem cell (Type-1) passes through distinct phases as it differentiates, matures and functionally integrates with an elaborate morphology into the neuronal circuit. Each of the phases and the respective cell types can be identified based on their expression of a combination of markers. MCM2 is a proliferation marker that is expressed across all the mitotic progenitor cell types while nestin is expressed only among the radial glia-like stem cell and Type-2a progenitors. As the Type-2a progenitors differentiate, nestin expression is first turned off and Tbr2 expression increases. In the post-mitotic progenitor, MCM2 expression is lost while DCX and NeuN expression dictate migration and maturation into a functional neuron. **(B)** Shown on the left is sample confocal image of MCM2 (purple), nestin (green) and DAPI (red) staining. The boxed magnification shows a radial glia-like progenitor expressing nestin, MCM2 and DAPI. Shown on the right is a sample confocal image of MCM2 (blue), Tbr2 (red), DCX (green) and DAPI (grey) staining. This is characteristic of a Type-2b progenitor cell type which is shown in the boxed magnification expressing MCM2, Tbr2, DCX and DAPI. Scale bars: 20 μm. Click here to view larger figure.

Discussion

The most critical steps for successful antigen-retrieval and staining of progenitor cell types are: 1) utilizing well perfused and fixed tissue of the optimal coronal thickness; 2) allowing sufficient time for boiling and subsequent cooling during antigen retrieval; 3) manual dexterity in mounting fixed coronal sections and preventing their damage when doing so.

It is critical in IHC to use perfused tissue that has undergone sufficient fixation but not over-fixation. A primary difficulty can be the inaccessibility of the antigen to the antibody. Our method was designed to improve the accessibility. Correctly perfusing the animal during surgery and subsequent fixation is essential as a first step. In our protocol, we allow the 4% PFA to perfuse through the animal for at least 5 min before extracting the brain. Subsequently, we believe it is important to leave the extracted brain in 4% PFA for at least 12 hr overnight to a maximum of 24-36 hr. This is an optimal duration in PFA and a longer period may over-fix the tissue introducing more protein cross links. After PFA fixation, the brain may be left in sucrose for an indefinite period of time without effecting tissue viability. When ready for histological analysis, the thickness of the tissue plays an important role. Hippocampal brains must be completely frozen before being cut to maintain tissue consistency in thickness

and integrity in structure. We have utilized sections from 40-60 µm for IHC. This thickness was primarily chosen because 1) it is of sufficient thickness for antigen-antibody accessibility and binding, and 2) utilizing confocal microscopy, we have been able to take pictures at separate focal points within this thickness and stitch the images together for three-dimensional resolution of the neurogenic niche. Coronal thickness more than 60 µm can impede antigen accessibility and effectiveness of the antigen-retrieval process as described.

In general, fixing of the tissue introduces protein cross links which are subsequently broken down by antigen-retrieval during the boiling step. The degree of this cross linking can be a result of fixation time, temperature and concentration[6]. Thus, careful thought must be given to the duration of fixation as described above. Our antigen-retrieval method utilizes both a chemical and physical approach. While our chemical approach is the careful fixing of the tissue in PFA, our physical approach is the subsequent boiling to break the protein cross links. Thermal denaturation using a microwave promotes an unmasking effect as it disrupts the secondary and tertiary structure of the proteins[6]. Other mechanisms have been proposed including molecular rapid oscillation[6,10,11]. Thus, boiling in the citric acid and tri-sodium citrate buffer is a key step in antigen-retrieval. Protein links are broken down and accessibility of the antigen to a particular antibody greatly improved. While 5 min of pre-heating have been provided in steps 3.1, it is vital to make sure the solution is boiling at the end of step 3.1 (at or above 100 °C). When submerging the tissue slides into this solution to boil, the slides should be facing down into the container and fully exposed to the solution. This will ensure the effect of boiling (and later antibody staining) is even across all sections. Finally, a certain amount of manual dexterity is helpful in mounting the hippocampal sections to the slides. While the sections adhere very strongly to the slides utilized in this protocol, it is helpful to make sure 1) the sections are mounted flat onto the slides and 2) after mounting, the hippocampal sections are dried, then washed with PBS and dried again before being boiled in step 3.3. The purpose of washing with PBS is to remove any chemicals (such as glycerol from previous storage of histological material in antifreeze solution) that may cause the sections to "slip" and come off the slides when being boiled. Moreover, for BrdU or Ki67 staining to label dividing cells, compared to an HCl-treated antigen-retrieval method, the boiling antigen-retrieval method presented here is more efficient in providing a consistent number of labeled cells. Further, in the case of paraffin sectioned tissue, any remaining paraffin substrate present in the tissue can be removed through this boiling method. Thus, our boiling method may also help in the immunostaining of paraffin-sectioned tissue.

The protocol described here provides an easy-to-use method of antigen-retrieval that improves the effectiveness with which progenitor cell types are identified in the neurogenic niche of the SGZ. It circumvents current problems in identification of radial processes of radial glia-like neural stem cells, and improves or maintains the efficiency of identification of other progenitor cell types. Both the integrity of the tissue and the specificity of the antibodies is maintained allowing for high resolution imaging. Adult hippocampal neurogenesis is a key process that maintains and enhances the plastic nature of the brain. Thus, an understanding of the development and regulation of a neural stem cell into a functionally integrated neuron can provide us clues to how neurogenesis enhances brain specific functions, and also how one may therapeutically manipulate it to treat debilitating brain disorders. Our method of antigen-retrieval is a simple yet powerful tool that can assist in this process.

Disclosures

The authors declare that they have no competing financial interests.

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