Video Article

Batch Immunostaining for Large-Scale Protein Detection in the Whole Monkey Brain

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Abstract

Immunohistochemistry (IHC) is one of the most widely used laboratory techniques for the detection of target proteins *in situ*. Questions concerning the expression pattern of a target protein across the entire brain are relatively easy to answer when using IHC in small brains, such as those of rodents. However, answering the same questions in large and convoluted brains, such as those of primates presents a number of challenges. Here we present a systematic approach for immunodetection of target proteins in an adult monkey brain. This approach relies on the tissue embedding and sectioning methodology of NeuroScience Associates (NSA) as well as tools developed specifically for batch-staining of free-floating sections. It results in uniform staining of a set of sections which, at a particular interval, represents the entire brain. The resulting stained sections can be subjected to a wide variety of analytical procedures in order to measure protein levels, the population of neurons expressing a certain protein.

Video Link

The video component of this article can be found at https://www.jove.com/video/1286/

Protocol

Immunohistochemisty is one of the most widely used techniques for characterizing protein expression in the brain of various experimental animal models. It is relatively easy to conduct systematic immunohistochemical procedures on the brains of rodents and other common experimental models with similar brain size. However, there is no published work to our knowledge that provides a comprehensive account of how to carry out such immunodetection procedures across an entire monkey brain. What follows is a detailed description of how to prepare a whole monkey brain for large-scale immunohistochemical detection of various target proteins. This work has emerged as a result of collaboration between commercial and academic endeavours. As such, details pertaining to tissue embedding and sectioning remain a proprietary knowledge of NSA.

Part 1: Animal treatment and tissue preparation

The brain from an adult vervet monkey (*Cercopithecus aethiops*) is used for the present protocol. All procedures are carried out in compliance with the Canadian Council on Animal Care (CCAC) guidelines for the use and care of animals in biomedical research[1].

1. Animal is deeply sedated with ketamine hydrochloride (10 mg/kg, i.m.), euthanized with an overdose of sodium pentobarbital (25 mg/kg, i.v.) and perfused transcardially with 0.1 M PBS until completely exsanguinated.
2. This is followed by a 4% paraformaldehyde solution in PBS for 5 min (~1 liter).
3. The externalized brain is placed in graded PBS-buffered sucrose solutions (10, 20 and 30%, in sequence) containing 0.02% sodium azide and maintained at 4˚C until the brain sinks to the bottom of the container. The solution is replaced every few days until brain undergoes freeze-sectioning.
4. The brain is sent to NeuroScience Associates (NSA; Knoxville, TN) to be subjected to their proprietary MultiBrain™ embedding and freeze-sectioning technology.
5. Seven (7) alignment landmarks are placed in the embedding matrix so that sections can be oriented properly (i.e., left-right orientation) and re-aligned after histological processing is complete.
6. The frozen block is digitally photographed at the block face before each serial section is collected from the block. Digital images as well as sections from this brain can later be used for 3D reconstruction of anatomical and histological maps, respectively.
7. Serial free-floating coronal sections, at a thickness of 50 µm per section, are collected from the entire brain.

Part 2: Histological processing

Sections are chosen at a given spatial interval (e.g., 500-µm) and for our experimental purposes were processed with the following antibodies: fragile-X mental retardation protein (FMRP; Chemicon; Temecula, CA), SMI32 (Sternberger Monoclonals Inc.; Baltimore, MD), and NeuN

(Chemicon; Temecula, CA). FMRP is a cytoplasmic protein that abundantly found in neurons of normal and permutation carrier brains[2]. NeuN (Neuronal Nuclei) specifically recognizes the DNA-binding neuron-specific protein NeuN which is present in most neurons and is distributed in neuronal nuclei, perikarya and some proximal neuronal processes[3]. SMI-32 is a monoclonal antibody that recognizes the non-phosphorylated epitope on neurofilament proteins[4].

1. At an interval of 500 µm, approximately 140 sections are used per antibody.
2. All incubations, washes and staining steps are carried out with mild agitation using specialized staining dishes and baskets (HistoTools; Knoxville, TN).
3. Sections are first incubated in a solution containing 0.1 M PBS/0.3% Triton X-100 (TX)/5% normal hoarse serum (NHS) for 60 min in order to reduce nonspecific binding of antibody molecules and resulting background staining.
4. Sections marked for FMRP immunostaining undergo an additional step of antigen recovery by using Antigen Unmasking Solution (Vector Labs; Burlingame, CA) according to vendor's instructions.
5. Sections are then incubated overnight and on separate days in their respective antibody solutions containing 0.1 M PBS/0.3% TX/3%NHS (with a dilution factor of 1/5,000 for FMRP and SMI-32 and 1/2,000 for NeuN antibodies.
6. The next day, sections are washed for three 10-min periods in washing solution (0.1 M PBS/0.3% TX) followed by incubation for 2 h at room temperature in biotinylated anti-mouse secondary antibody raised in horse (Vector Labs; 1/1,000 dilution in 0.1 M PBS/0.3% TX/3% NHS).
7. After a further set of 3 10-min washes, the sections are placed in a solution of avidin-biotin conjugated horseradish peroxidase complex (Vector Labs) for 1 h at room temperature.
8. Finally, after another set of washes, the sections are subjected for 10 min to a nickel enhanced diaminobenzidine (Ni-DAB) reaction which produces a dark blue stain within immunoreactive neurons.
9. At the completion of the histological procedure, sections are mounted on gelatin-coated glass slides.
10. All sections are air-dried overnight and coverslipped according to standard procedures.

Part 3: Representative Results:

This method produces a complete expression profile of a target protein of interest across an entire monkey brain. Here we show representative coronal sections that provide a snapshot of FMRP, NeuN and SMI32 expression in the same monkey brain.

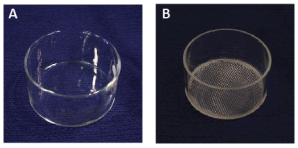

Figure 1: Staining dish (A) and basket (B) used for large-scale batch processing of free-floating sections for immunodetection.

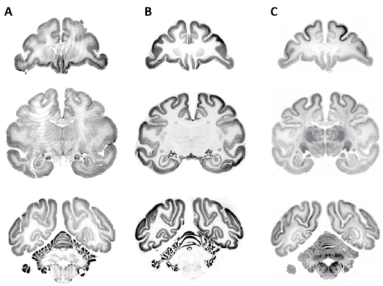

Figure 2: Representative coronal sections from a vervet monkey brain stained for FMRP (A), NeuN (B) and SMI32 (C) antibodies.

Discussion

There are two critical steps in this procedure that make large-scale detection of proteins in an entire monkey brain possible. One is the embedding and sectioning protocol, which remains the proprietary knowledge of NSA. The other is the use of staining dishes and baskets provided by HistoTools. The latter allows for easy and quick handling of many (~40) sections at a given time. It also provides the means for uniformly treating all sections across the brain and makes for a scientifically sound histological treatment. In addition, the use of embedded landmarks provides the added advantage of being able to digitally acquire the staining patterns from dried and coverslipped slides and to subject the digitized files to various forms of analyses. Although we provide examples of three antibodies, this procedure can be applied to a wide range of other target proteins as well as histological stains, particularly those that reveal the cytoarchitecture various cortical areas and hence used for mapping purposes.

Disclosures

The authors have nothing to disclose.

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