Using Near-infrared Fluorescence and High-resolution Scanning to Measure Protein Expression in the Rodent Brain

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Abstract

Neuroscience is the study of how cells in the brain mediate various functions. Measuring protein expression in neurons and glia is critical for the study of neuroscience as cellular function is determined by the composition and activity of cellular proteins. In this article, we describe how immunocytochemistry can be combined with near-infrared high-resolution scanning to provide a semi-quantitative measure of protein expression in distinct brain regions. This technique can be used for single or double protein expression in the same brain region. Measuring proteins in this fashion can be used to obtain a relative change in protein expression with an experimental manipulation, molecular signature of learning and memory, activity in molecular pathways, and neural activity in multiple brain regions. Using the correct proteins and statistical analysis, functional connectivity among brain regions can be determined as well. Given the ease of implementing immunocytochemistry in a laboratory, using immunocytochemistry with near-infrared high-resolution scanning can expand the ability of the neuroscientist to examine neurobiological processes at a systems level.

Keywords

Neuroscience; Issue 147; immunohistochemistry; near-infrared; high resolution; mPFC; Amygdala; Hippocampus

Introduction

The study of neuroscience concerns an investigation of how cells in the brain mediate specific functions¹. These can be cellular in nature such as how glia cells confer immunity in the central nervous system or can involve experiments that aim to explain how the activity of neurons in the dorsal hippocampus leads to spatial navigation. In a broad sense, cellular function is determined by the proteins that are expressed in a cell and the activity of these

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Video Link

The video component of this article can be found at <https://www.jove.com/video/59685/>

proteins². As a result, measuring the expression and/or activity of proteins in brain cells are critical for the study of neuroscience.

A number of techniques are available to measure protein expression in the brain. These include in vivo methods such as positron emission topography for receptor densities³ and micro-dialysis for small peptides⁴. More commonly, ex vivo methods are used to examine protein function and expression. These include mass spectrometry techniques⁵, western blot and enzyme-linked immunosorbent assay (ELISA)⁶, and immunocytochemistry⁷. Immunocytochemistry is widely used in the field of neuroscience. This technique involves the use of a primary antibody to detect a protein (or antigen) of interest (e.g., c-Fos) and a conjugated secondary antibody to detect the protein-primary antibody complex (Figure 1). To enable detection of the protein-primary antibody-secondary antibody complex, secondary antibodies have oxidizing agents such as horseradish peroxidase (HRP) conjugated to them. This allows for the formation of precipitates in cells that can be detected using light microscopy⁷. Secondary antibodies can also have chemicals fluoresce conjugated to them (i.e., fluorophores). When stimulated these chemicals emit light, which can be used to detect protein-primary antibody-secondary antibody complexes⁷. Lastly, sometimes primary antibodies have reducing agents and fluorescence chemicals attached to them directly negating the need for secondary antibodies⁷ (Figure 1).

Interestingly, many immunocytochemistry methods allow for visualization of proteins in brain cells, but not the ability to quantify the amount of protein in a specific cell or brain region. Using light microscopy to detect precipitates from reduction reactions allows for visualization of neurons and glia, but this method cannot be used to quantify protein expression in cells or in a specific brain region. In theory, fluorescence microscopy can be used for this, because the light emitted from the fluorescent secondary antibody is a measure of the protein-primary antibody-secondary antibody complex. However, autofluorescence in brain tissue can make it difficult to use fluorescence microscopy to quantify protein expression in brain tissue⁸. As a result, light emitted from fluorescent images of brain tissue is rarely used to quantify protein expression in the brain.

Many of these issues can be addressed using near-infrared immunocytochemistry in conjunction with high-resolution scanning^{9,10}. In this article, we describe how immunocytochemistry coupled with fluorophores in the near-infrared emission spectra can be combined with high resolution scanning (e.g., 10–21 μm) to obtain sharp images that allow for semi quantification of protein in distinct brain regions.

Protocol

The following protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Delaware. Male Sprague Dawley rats approximately 55–75 days old were used for this protocol.

1. Brain Extraction and Tissue Preparation

1. Anesthetize rat with isoflurane in anesthesia induction chamber until rat no longer exhibits a response to foot pinch.

2. Sacrifice rats via rapid decapitation using a guillotine.
3. Cut skin on skull posterior to anterior and clear from top of skull.
4. Use rongeurs to carefully remove the back part of skull, and then using small dissecting scissors cut down midline of skull, pushing upward against the top of skull at all times to avoid damaging the brain tissue.
5. Use rongeurs to peel away right and left half of skull to expose the brain.
6. Use a small spatula to scoop under brain and sever nerves, carefully elevate brain and freeze brains in isopentane chilled on dry ice (at least -20°C). After this, store brains in a -80°C freezer until slicing.
7. Slice brains in regions of interest at $30\text{--}50\text{ }\mu\text{m}$ in a cryostat maintained between -9°C and -12°C . Directly mount slices onto glass slides and store in a -80°C freezer until time of immunocytochemistry assay.

NOTE: In this protocol brain regions of interest were the hippocampus and the amygdala.

2. Single Immunohistochemical Reaction

NOTE: For double immunohistochemical reaction, the protocol is the same as the single immunohistochemical reaction except this reaction has two primary antibodies of different hosts (e.g., rabbit and mouse) and two secondary antibodies for the corresponding primaries should be from a single host (e.g., goat antirabbit and goat antimouse). The secondary antibodies also have to be from two different spectra that are available in high-resolution scanners. For example, one secondary antibody with an emission spectrum peak at 680 nm and one secondary antibody 800CW (emission spectrum peak at 780 nm).

1. Remove glass slides from the freezer and allow to equilibrate to room temperature for 30 min.
2. Under a fume hood, fix brain tissue in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 1–2 h at room temperature.
3. Rinse slides in 0.1 M tris buffered saline (TBS) three times for 10 min each. Permeabilize cell membranes by incubating slides in a mild detergent (e.g., 0.01% detergent) for 30–60 min.
4. Rinse slides in TBS three times for 15 min each.
5. Dilute primary antibody for protein of interest in PBS in the correct concentration. For example, to detect the immediate early gene c-Fos, prepare a rabbit primary anti c-Fos antibody in a concentration of 1:500.
6. Pipette primary antibody solution directly onto the brain tissue (approximately $200\text{ }\mu\text{L}$ per 3 inch x 1 inch slide).
7. Use coverslips to incubate the brain tissue on glass slides in primary antibody dilution for either 1–2 h at room temperature or overnight ($\sim 17\text{ h}$) at 4°C .

8. Remove coverslips and wash slides in TBS that has a small amount of detergent added to it (e.g., 0.01% detergent, “TBS-T”) four times for 15 min each.
9. Use coverslips to incubate brain sections in a secondary antibody at room temperature for 2 h.

NOTE: The secondary antibody has to be to the correct dilution and in a diluent containing TBS, detergent, and 1.5% of host serum. For example, a goat secondary antibody in a dilution of 1:2,000 would contain 1.5% goat serum and 0.05% detergent.

10. Rinse slides in TBS-T four times for 20 min each, and then in TBS four times for 20 min each.
11. Dry slides at room temperature in the dark overnight. When slides are dry, they are ready for imaging.

3. Imaging

1. Place slides onto the near-infrared scanning interface with the tissue facing down. Either image one glass slide or multiple slides at a time using a selection tool.
2. Image slides using the highest quality setting with an offset of 0 nm and a resolution of 21 μm . The scanning will typically take 13–19 h depending on the scanning equipment used.
3. Import images into the image analysis software (e.g., ImageStudio) to view and mark for semi-quantitative protein analysis.

4. Protein Expression Analysis

1. Open the image analysis software and select the **Work Area** into which the image was scanned.
2. Open the scanned image in the image analysis software to view the scan, and adjust which wavelengths are viewed, as well as the contrast, brightness, and magnification shown without altering the raw image or the total quantified emission.
3. Identify the key regions for quantification and select the **Analysis** tab along the top of the page, then select **Draw Rectangle** (or **Draw Ellipse/ Draw Freehand**) to draw a rectangle over the area that will be quantified.
4. To view the size of the rectangle, select **Shapes** along the bottom left of the screen, then select **Columns** along bottom right. Add **Height** and **Width** columns to identify the shape size.

NOTE: It is important to control for shape size when comparing quantification. It is recommended to use identical shapes placed within the desired quantification location, in order to get an accurate sampling of the emissions for that region.

5. Then name the shape and repeat. Once all regions are sampled the data available from the **columns** tab can be aggregated and analyzed.

Representative Results

Prior to using high-resolution scanning for immunohistochemistry, one should verify that the protocol works. This can be accomplished using a validation assay where brain sections from the same animal are incubated with primary and secondary antibodies, secondary antibody alone, or neither primary nor secondary antibody. Results for such a validation assay are shown in Figure 2. In this reaction we were detecting the immediate early gene c-Jun in the dorsal hippocampus and amygdala. C-Jun expression was only observed when primary and secondary antibodies were applied to the brain tissue.

Figure 3 shows dual protein detection in amygdala nuclei. In this experiment we assayed the GluR1 subunit of the α -amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (AMPA) receptor and the NR2A subunit of the N-methyl-D-aspartate receptor (NMDA) receptor in the same brain tissue. This allowed us to examine the ratio of AMPA/NMDA receptors in sub-nuclei of the amygdala. This ratio is a neurobiological signature of learning and memory^{11,12}. As can be seen in Figure 3, signal for GluR2 (780 nm light pseudo colored in green) and NR2A (680 nm light pseudo colored in red) can only be observed in the brain tissue that was exposed to primary and secondary antibodies.

Figure 4 shows how mean and normalized measures of protein expression can be obtained from high-resolution scans in the ventral hippocampus. Using the image analysis software, a rectangle is placed in the region of interest (molecular layer of CA1) and mean intensity of light from the shape can be used as a measure of fluorophore expression (Figure 4A). In turn, this is a measure of protein expression (i.e., the antigen-primary antibody-secondary antibody complex). The shape can also be placed across a region that expresses high signal and low signal to obtain a normalized curve (Figure 4B). In this example, a rectangle was placed across the molecular layer of CA1, but covered the dendritic fields as well, which did not express significant amounts of c-Jun in this assay. The area under the curve can then be used as a measure of protein expression. If the setup in an experiment involves treatment groups (e.g., stress exposure) and a control, relative changes in protein expression in the brain can be obtained.

Discussion

The results presented in this article show that near-infrared immunocytochemistry in combination with high-resolution scanning can be used to obtain semi-quantitative measures of protein expression in brain tissue. It can also be used to label two proteins simultaneously in the same brain region. We have previously used near-infrared immunohistochemistry to measure immediate early gene expression in multiple brain regions^{9,10}. Immediate early genes can be used as a measure of neural activity. We also subjected these semi-quantitative measures of protein expression to statistical analyses that allowed us to group brain regions with correlated levels of immediate early genes (IEGs). We used this as a measure of functional connectivity to examine how stress affects functional connectivity among nodes

within the fear circuit during emotional learning and memory^{9,10}. We showed how AMPA/NMDA ratios (a signature of learning and memory) can be measured using near-infrared immunocytochemistry with high resolution scanning¹³. A similar technique can be used to measure pan and phospho-proteins to determine molecular signaling. This is accomplished using western blot¹⁴. However, this requires dissecting brain regions out of thick brain slices and is not amenable to small brain regions. This issue can be circumvented using near-infrared immunocytochemistry with high-resolution scanning. Finally, all immunocytochemistry images are digitized, which allows for unlimited storage and convenient re-analysis of previous assays.

As with all methods there are drawbacks. There is no magnification in high-resolution scanners and the treatment of tissue does not readily allow for probing using fluorescence microscopic techniques. Even taking alternate slices from the brain may not work, since confocal microscopy may work best in perfused brain tissue, but near-infrared imaging with high-resolution scanning is typically done on flash frozen brains. Protein expression in specific neurons (e.g., interneuron vs. pyramidal neuron) or different cell types in the brain (e.g., neurons vs. glia) cannot be determined using near-infrared immunocytochemistry with high-resolution scanning. Performing validation assays are critical as this is still a relatively new method for examining protein expression in brain tissue.

When used appropriately, near-infrared immunocytochemistry with high-resolution scanning offers advantages. Autofluorescence in brain tissue is reduced in the near-infrared range, semi-quantitative measures of protein expression can be obtained, expression of two proteins in the same brain region can be obtained, and images of the assay can be stored indefinitely. When paired with the correct protein and/or statistical method this technique can be used to examine protein expression, neural activity, molecular signaling, and functional connectivity within the brain.

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Antigen-Primary-Secondary
Reaction Antigen-Primary
Reaction

Figure 1: Illustration of the immunohistochemical reaction using primary (1st) and secondary (2nd) antibodies or just primary antibody.

Filled black circles represent a label, which could be an oxidizing agent such as horseradish peroxidase or a fluorophore such as borondipyrromethene (BODIPY). Green squares represent antigen (on protein of interest) being detected in the immunohistochemical reaction.

Figure 2: Images of a validation assay for detection of c-Jun.

In this assay, tissue from the same animal was treated with a rabbit primary antibody that recognizes c-Jun and goat antirabbit secondary antibody with attached fluorophore with emission at 780 nm (pseudo colored in green, left panel). Adjacent tissue was treated with either secondary antibody alone (middle panel) or no antibody (right panel). Scale bar = 1 mm.

Figure 3: Validation assay for double labeling immunohistochemical reaction in the amygdala. Triplicate brain sections from the same rat was either exposed to rabbit antibody that recognizes GluR1 and mouse antibody that recognizes NR2A (left panels), secondary antibody (middle panels), or no antibody (right panels). GluR1 was visualized with goat anti rabbit 800CW secondary antibody (780 nm, pseudo colored in green) and NR2A was visualized using a goat antimouse 680RD secondary antibody (680 nm, pseudo colored in red). Scale bar = 1 mm. ot = optic tract; ic = internal capsule; ec = external capsule.

A) Measuring mean protein expression in the vHipp

B) Measuring normalized protein expression in the vHipp

Figure 4: Obtaining semi quantitative measures of protein expression in brain tissue. (A and B) screenshots of scored images in the image analysis software that is used to analyze images from the scanner. Tissue was from the ventral hippocampus and treated to visualize c-Jun.