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Using Near-Infrared Fluorescence and High-Resolution Scanning to Measure Protein Expression in the Rodent Brain

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TITLE:

Using Near-Infrared Fluorescence and High-Resolution Scanning to Measure Protein Expression in the Rodent Brain

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SUMMARY:

Here, we present a protocol that uses near-infrared dyes in conjunction with immunohistochemistry and high-resolution scanning to assay proteins in brain regions.

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.

INTRODUCTION:

The study of neuroscience concerns an investigation of how cells in the brain mediate specific functions[1]. 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 proteins[2]. 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 densities[3] and micro-dialysis for small peptides[4]. More commonly, ex vivo methods are used to examine protein function and expression. These include mass spectrometry techniques[5], western blot and enzyme-linked immunosorbent assay (ELISA)[6], and immunocytochemistry[7]. 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 microscopy[7]. 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 complexes[7]. Lastly, sometimes primary antibodies have reducing agents and fluorescence chemicals attached to them directly negating the need for secondary antibodies[7] (**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 tissue[8]. 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

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

1.2. Sacrifice rats via rapid decapitation using a guillotine.

1.3. Cut skin on skull posterior to anterior and clear from top of skull.

1.4. 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.

1.5. Use rongeurs to peel away right and left half of skull to expose the brain.

1.6. 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.

1.7. Slice brains in regions of interest at 30–50 µm 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).

2.1. Remove glass slides from the freezer and allow to equilibrate to room temperature for 30 min.

2.2. 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.

2.3. 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.

2.4. Rinse slides in TBS three times for 15 min each.

2.5. 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.

2.6. Pipette primary antibody solution directly onto the brain tissue (approximately 200 µL per 3 inch x 1 inch slide).

2.7. Use coverslips to incubate the brain tissue on glass slides in primary antibody dilution for either 1−2 h at room temperature or overnight (~17 h) at 4 °C.

2.8. 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.

2.9. 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 1:2000 would contain 1.5% goat serum and 0.05% detergent.

2.10. Rinse slides in TBS-T four times for 20 min each, and then in TBS four times for 20 min each.

2.11. Dry slides at room temperature in the dark overnight. When slides are dry, they are ready for imaging.

3. Imaging

3.1. 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.

3.2. 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.

3.3. Import images into the image analysis software (e.g., ImageStudio) to view and mark for semi-quantitative protein analysis.

4. Protein expression analysis

4.1. Open the image analysis software and select the **Work Area** into which the image was scanned.

4.2. 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.

4.3. 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.

4.4. 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.

4.5. 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-5-methyl-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 (panel **A**). 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 (panel **B**). 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.

FIGURE LEGENDS:

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 boron-dipyrromethene (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 represents 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). GlurR1 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 represents 1 mm. ot, optic tract; ic, internal capsule; ec, external capsule.

Figure 4: Obtaining semi quantitative measures of protein expression in brain tissue. Panels **A** and **B** are 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.

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 scanning[13]. A similar technique can be used to measure pan and phospho-proteins to determine molecular signaling. This is accomplished using western blot[14]. 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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DISCLOSURES:

The authors have nothing to disclose.

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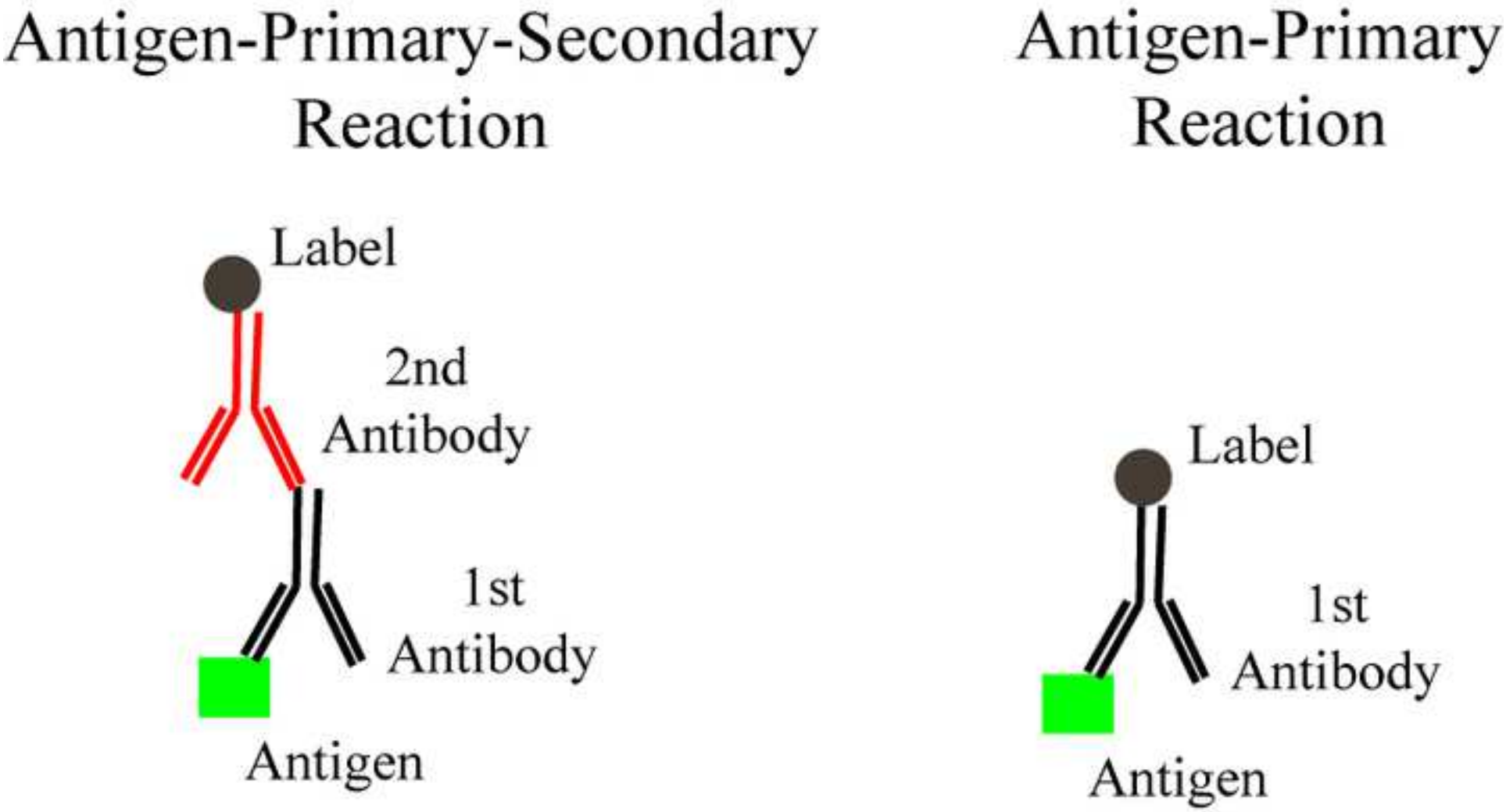


Figure 2

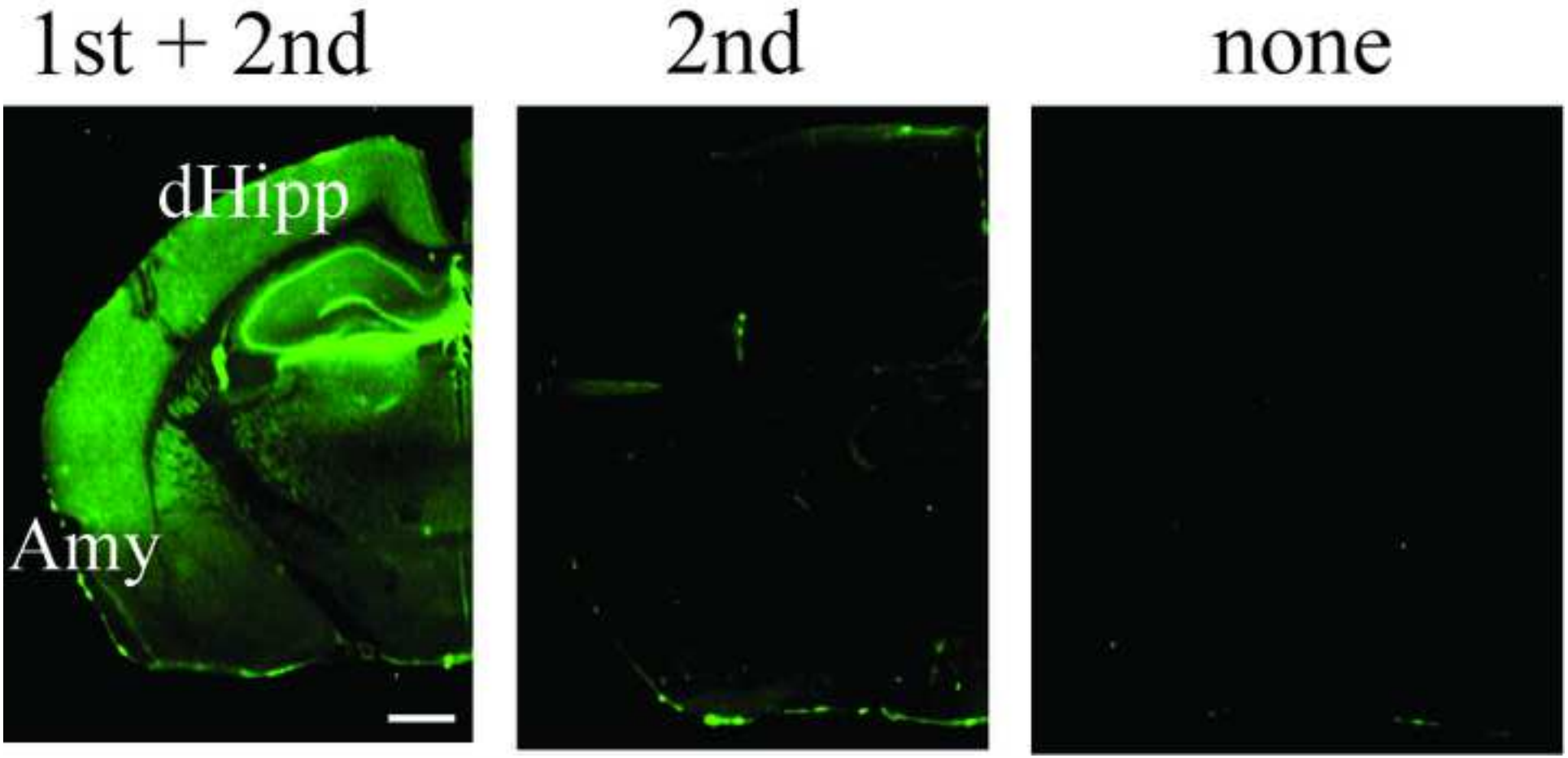


Figure 3

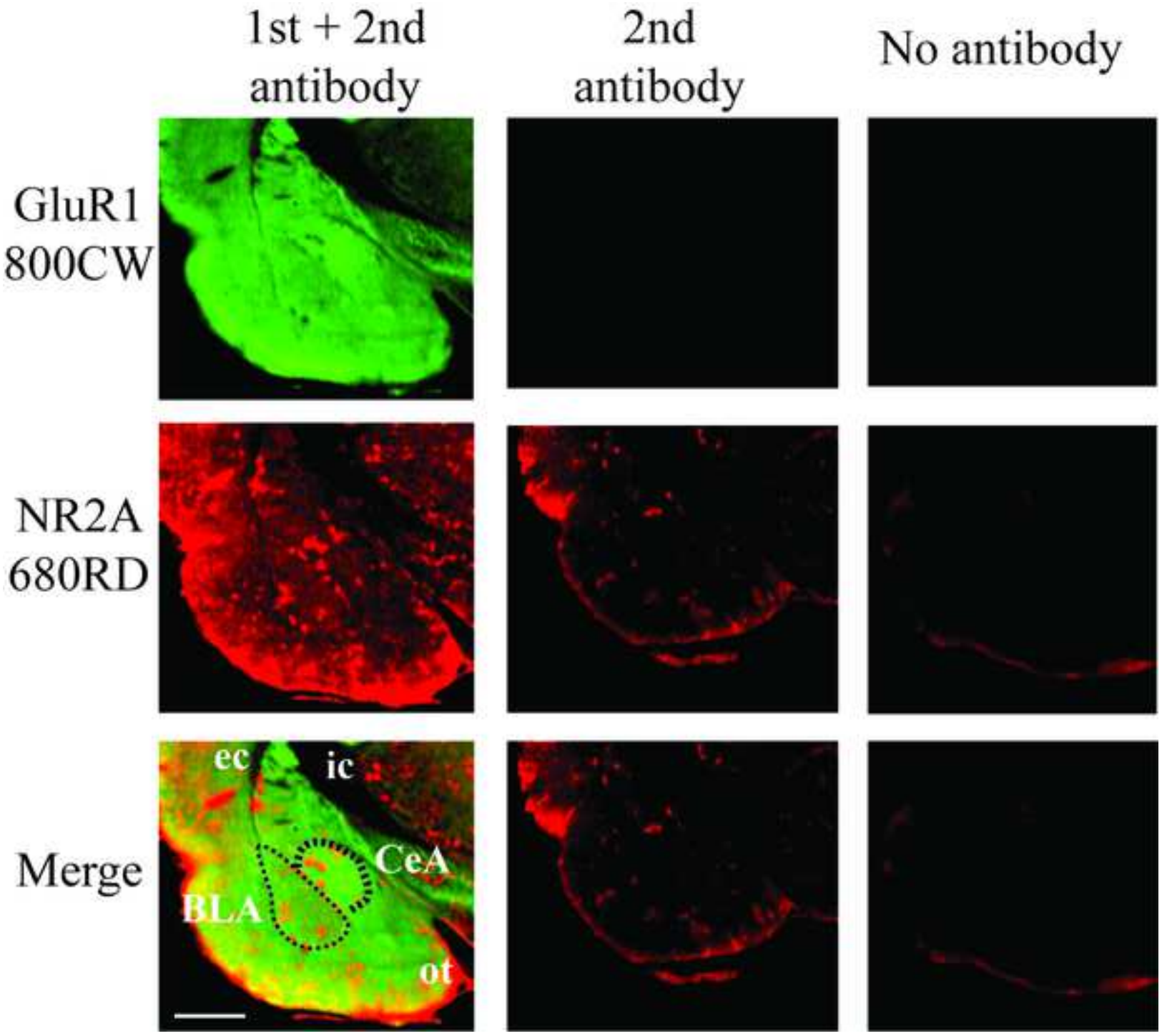
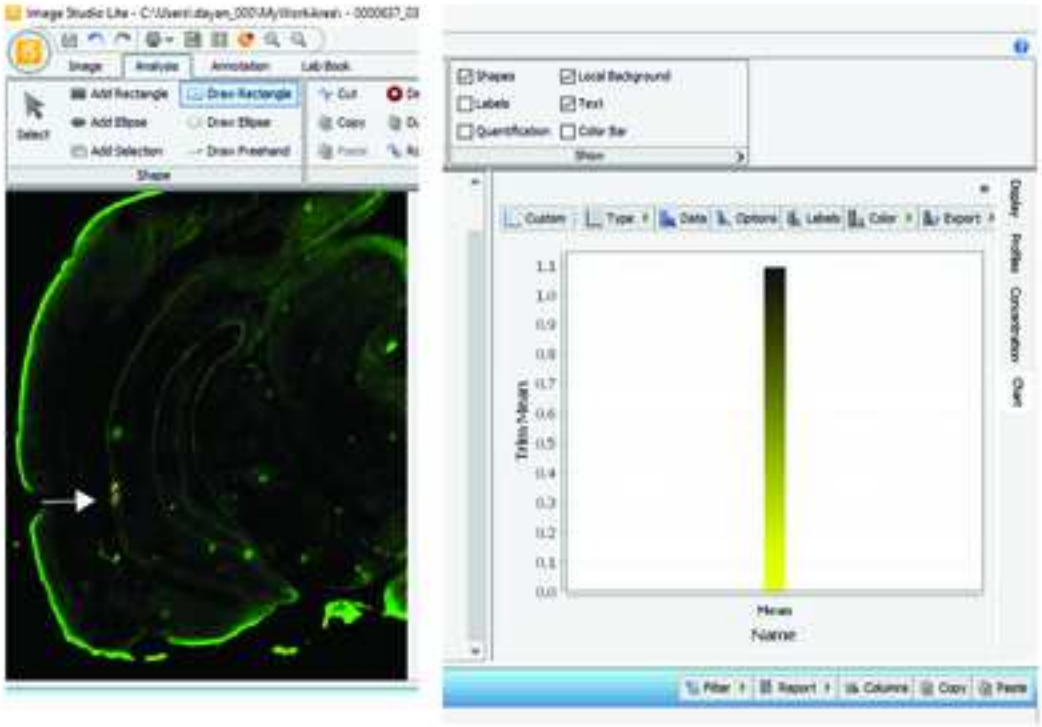
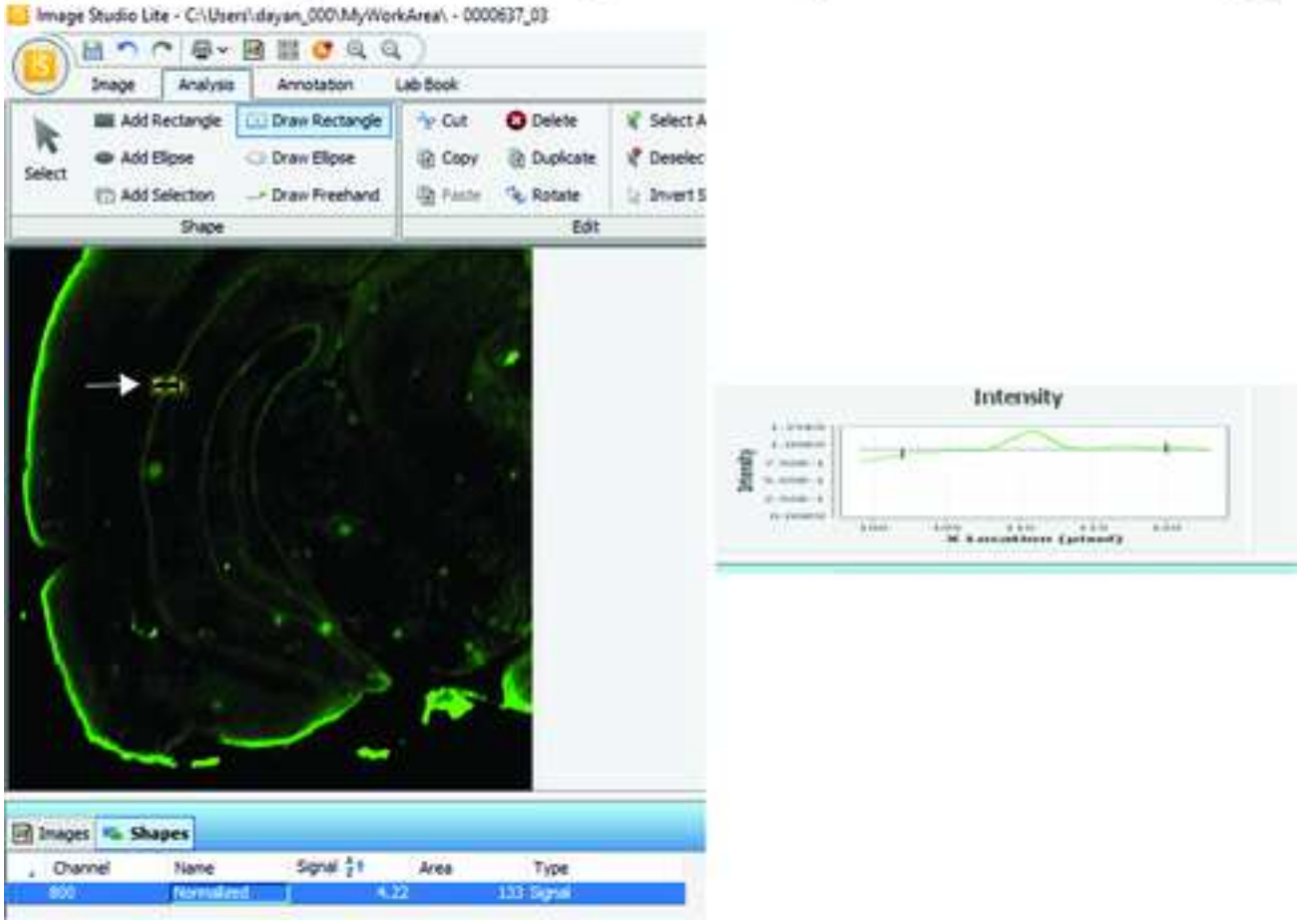


Figure 4

A) Measuring mean protein expression in the vHipp



B) Measuring normalized protein expression in the vHipp



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	Brain Extraction		
Anesthesia Induction Chamber	Kent Scientific	VetFlo-0530SM	
Kleine Guillotine	Harvard Apparatus	73-1920	
Friedman Rongeur	Fine Science Tools	16000-14	used to remove back of skull
Delicate Dissecting Scissors	Fischer Scientific	08-951-5	used to cut upward along midline of skull
Micro Spatula	Fischer Scientific	21-401-5	used to scoop out brain
Glass Microscope Slides	Fischer Scientific	12-549-6	
	Immunohistochemical Reaction		
Triton X-100			Used as a mild detergent to permeabilize cells after fixing in P
Tween-20			Used as a small amount of detergent added to TBS to procuce
Licor Odyssey scanner	Licor Biotechnology Inc.		
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araformaldehyde, also used as mild detergent in combination with host serum and secondary antibody
e TBS-T after coverslipping slides with primary antibody

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Date:	1/6/2019

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Editorial comments: Changes to be made by the author(s) regarding the manuscript:

Concern 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Response 1: We have read through the manuscript and corrected as many typos as we could find.

Concern 2. Authors and affiliations: Please provide an email address for each author.
Response 2: These have now been provided.

Concern 3. Please add a Summary section before the Abstract section to clearly describe the protocol and its applications in complete sentences between 10−50 words: "Here, we present a protocol to …"
Response 3: This has now been added.

Concern 4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Triton X-100, tween-80, Licor Biotechnology Inc., etc.
Response 4: We have addressed these issues in the revised manuscript

Concern 5. Protocol: Please revise it to be a numbered list following the JoVE Instructions for Authors; step 1 followed by 1.1, followed by 1.1.1, etc. Please refrain from using bullets, dashes, or indentations.
Response 5: This has been formatted as requested.

Concern 6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
Response 6: This has been added.

Concern 7. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Response 7: This has been revised as suggested.

Concern 8. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use

of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the
Response 8: Revised as suggested

Concern 9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.
Response 9: Revised as requested.

Concern 10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
Response 10: Revised as requested.

Concern 11. Line 86: Please specify the age, gender and strain of rats. Are rats anesthetized before brain extraction? Please specify all surgical tools used.
Response 11: Revised as requested.

Concern 12. Line 88: What are the regions of interest in this protocol?
Response 12: The hippocampus and amygdala. We have added this to the protocol.

Concern 13. Lines 92, 94: At what temperature is the tissue fixed/permeabilized?
Response 13: At room temperature. This has been added to the protocol.

Concern 14. Lines 110-116: Please describe how to perform the reaction in the imperative tense.
Response 14: Revised as requested.

Concern 15. In the Protocol, please describe how to obtain semi-quantitative measures of protein expression. Software steps must be explicitly explained ('click', 'select', etc.) with specific details (e.g., button clicks or menu selections for software actions, numerical values for settings, etc.).
Response 15: Revised as requested

Concern 16. Representative Results: Please revise to explain the results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However, for figures showing the experimental set-up, please reference them in the Protocol.
Response 16: The results show the validation assay first. In the context of the technique described this shows that fluorescence activity detected in the near-infrared range reflect primary-secondary antibody binding and not autofluorescence. This result validates the use of the technique. Figure 3 shows the same validation process for detection of two proteins in the

same brain region. In the context of the technique described this shows that fluorescence activity detected in the near-infrared range reflect detection of two distinct proteins using two different secondary antibodies to detect two different proteins. All figures are referenced in paragraph text. There are no figures showing experimental set up, but Figure 1 shows conceptually what immunohistochemistry involves. This figure is referenced in the introduction.

Concern 17. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Response 17: There is no one critical step using this technique (immunohistochemistry), but a critical process is the validation assay (lines 208-209), which is also where troubleshooting and modification of the technique would take place (e.g. change primary antibody). Limitations of the technique are mentioned in lines 201-208. The significance of this technique with respect to existing methods is provided in the first paragraph. The technique can be used to measure neural activity in multiple brain regions, combined with statistical techniques to examine functional connectivity, and measure AMPA/NMDA ratios, which is a neurobiological signature of learning and memory. For future applications, the technique could be used to label pan and phospho proteins in the same brain region (e.g. pAkt/Akt) to examine signaling within a molecular pathway in a specific brain region (e.g. PI3K/Akt signaling in the central nucleus of the amygdala).

Concern 18. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.
Response 18: None of the authors have any competing financial interests or other conflicts of interest. This section has been added.

Concern 19. Figure 2 and Figure 3: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.
Response 19: Scale bar has been added to the images.

Concern 20. Figure 1 legend: There is no brown circle; rather a filled black circle. Please revise the figure legend. Please also describe what the green squares represent.
Response 20: Addressed as requested

Concern 21. Each figure legend should include a title and a short description of the data presented in the figure and relevant symbols. Discussion of the figures should be placed in the

Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.
Response 21: We have tried to keep the legend for all figures to a minimum.

Concern 22. Figure 2 and Figure 3 legends: Please describe what different panels represent. Please move the discussion of the figures to the Representative Results section.
Response 22: The panels have been described. Any discussion of the figures is simply to explain what the figures mean.

Concern 23. Figure 4 legend: Please move details of the methodology to the Protocol section.
Response 24: Revised as requested.

Concern 24. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.
Response 24: Revised as requested.

Concern 25. Please use superscript arabic numerals to cite references in text. The superscript number is inserted immediately next to the word/group of words it applies to but before any punctuation.
Response 25: We have downloaded the EndNote template for JOVE and used this template to format all references.

Concern 26. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
Response 26: We have downloaded the EndNote template for JOVE and used this template to format all references.

Reviewers' comments:

Reviewer #1: This technical report describes an "autoflouresence" of sorts after use of a primary and secondary antibody on brain sections without being tagged with a fluorescent label. Using the red to near infrared range, the authors describe this somewhat bizarre auto-antibody labelling. I have certainly not come across the phenomenon before, and on that basis, this work is worthy of publication. My only comments would be that the authors should really make it clear that this labelling is WITHOUT a conjugated fluorophore. It took me several read overs to come to grips with what they were trying to say.
Response: The technical report describes how secondary antibody with attached near-infrared fluorophores can be combined with high resolution scanning to obtain semi-quantitative

measures of protein expression in brain tissue.

Reviewer #2: This work describes the possibility of combining immunocytochemistry with near-infrared high-resolution scanning for semi-quantitative measure of protein expression in different brain regions. This manuscript explains the way for reaching the results with detailed procedure, including the duration for each step, and the concentration of the chemicals needed for the sample preparation. I believe that this manuscript is a good fit for JoVE, and the paper is very well written. I would recommend publication of this work.
Overall response: We would like to thank the reviewer for their kind comments and describe how we address their concerns listed below.

Concern 1. Line 98, 101, 102: It should be "coverslips", instead of "coverslipps". Please check throughout.
Response 1: We have corrected this typo throughout

Concern 2. Line 147: Please define "IEGs".
Response 2: We have now defined this.

Concern 3. Line 164, 167: Please stick with either near-infrared immunocytochemistry or near infrared immunocytochemistry throughout.
Response 3: We have used the first phrase throughout the manuscript.

Concern 4. Line 208: Should be "brown circles" instead of "brown circle".
Response 4: Editors requested a different revision, which we made.

Concern 5. It seems that the figures are not at their proper resolution. Please double check if the figures got 300 dpi
Response 5: All images are at 300 dpi TIFF images.