

Video Article

Spectral Confocal Imaging of Fluorescently tagged Nicotinic Receptors in Knock-in Mice with Chronic Nicotine Administration

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URL: <https://www.jove.com/video/3516>

DOI: [doi:10.3791/3516](https://doi.org/10.3791/3516)

Keywords: Neuroscience, Issue 60, nicotine addiction, knock-in mice, spectral confocal imaging, yellow fluorescent protein, nicotinic acetylcholine receptors

Date Published: 2/10/2012

Citation: Renda, A., Nashmi, R. Spectral Confocal Imaging of Fluorescently tagged Nicotinic Receptors in Knock-in Mice with Chronic Nicotine Administration. *J. Vis. Exp.* (60), e3516, doi:10.3791/3516 (2012).

Abstract

Ligand-gated ion channels in the central nervous system (CNS) are implicated in numerous conditions with serious medical and social consequences. For instance, addiction to nicotine via tobacco smoking is a leading cause of premature death worldwide (World Health Organization) and is likely caused by an alteration of ion channel distribution in the brain¹. Chronic nicotine exposure in both rodents and humans results in increased numbers of nicotinic acetylcholine receptors (nAChRs) in brain tissue¹⁻³. Similarly, alterations in the glutamatergic GluN1 or GluA1 channels have been implicated in triggering sensitization to other addictive drugs such as cocaine, amphetamines and opiates⁴⁻⁶.

Consequently, the ability to map and quantify distribution and expression patterns of specific ion channels is critically important to understanding the mechanisms of addiction. The study of brain region-specific effects of individual drugs was advanced by the advent of techniques such as radioactive ligands. However, the low spatial resolution of radioactive ligand binding prevents the ability to quantify ligand-gated ion channels in specific subtypes of neurons.

Genetically encoded fluorescent reporters, such as green fluorescent protein (GFP) and its many color variants, have revolutionized the field of biology⁷. By genetically tagging a fluorescent reporter to an endogenous protein one can visualize proteins *in vivo*⁷⁻¹⁰. One advantage of fluorescently tagging proteins with a probe is the elimination of antibody use, which have issues of nonspecificity and accessibility to the target protein. We have used this strategy to fluorescently label nAChRs, which enabled the study of receptor assembly using Förster Resonance Energy Transfer (FRET) in transfected cultured cells¹¹. More recently, we have used the knock-in approach to engineer mice with yellow fluorescent protein tagged $\alpha 4$ nAChR subunits ($\alpha 4$ YFP), enabling precise quantification of the receptor *ex vivo* at submicrometer resolution in CNS neurons via spectral confocal microscopy¹². The targeted fluorescent knock-in mutation is incorporated in the endogenous locus and under control of its native promoter, producing normal levels of expression and regulation of the receptor when compared to untagged receptors in wildtype mice. This knock-in approach can be extended to fluorescently tag other ion channels and offers a powerful approach of visualizing and quantifying receptors in the CNS.

In this paper we describe a methodology to quantify changes in nAChR expression in specific CNS neurons after exposure to chronic nicotine. Our methods include mini-osmotic pump implantation, intracardiac perfusion fixation, imaging and analysis of fluorescently tagged nicotinic receptor subunits from $\alpha 4$ YFP knock-in mice (Fig. 1). We have optimized the fixation technique to minimize autofluorescence from fixed brain tissue. We describe in detail our imaging methodology using a spectral confocal microscope in conjunction with a linear spectral unmixing algorithm to subtract autofluorescent signal in order to accurately obtain $\alpha 4$ YFP fluorescence signal. Finally, we show results of chronic nicotine-induced upregulation of $\alpha 4$ YFP receptors in the medial perforant path of the hippocampus.

Video Link

The video component of this article can be found at <https://www.jove.com/video/3516/>

Protocol

1. Pump implantation

1. Before pump implantation, fill and prepare the Alzet mini-osmotic pumps (Alzet, Model 2002, Cupertino, USA) being careful not to introduce air bubbles. This model of mini-osmotic pump delivers solution at a rate of 0.5 μ l/hr for 14 days. Ensure sterile conditions. Weigh empty and filled pumps. At the conclusion of experiment (10 days after implantation), the remaining liquid in the pump can be removed with a syringe and needle and weighed to calculate the volume pumped.
2. Pumps with control solution contain saline (0.9% w/v, Teknova, S5819, Hollister, USA). To prepare nicotine solution, a 1 M stock solution of (-)-nicotine hydrogen tartrate salt (Sigma, cat# N5260) is diluted in saline (0.9% w/v) and sterilized by filtering through a 0.22 μ m syringe-end filter. We have previously administered nicotine at 0.4 and 2 mg/kg/hr (calculated as free base of nicotine) for 10 days.

3. Prepare three saline baths (three 10 cm saline filled petri dishes). Once the pump has been filled and capped, wash pump thoroughly in each successive saline bath to remove any traces of the drug on the outer shell. Immerse filled pumps in saline solution for storage until surgery, keeping the control and nicotine pumps in separate saline containers.
4. To encourage a healthy recovery and reduce the risk of post-surgical infection, have clean cages prepared for long-term recovery.
5. For short-term recovery immediately following surgery, prepare a cage containing a heating pad and a heat lamp.
6. To examine chronic nicotine effects we have 5 to 6 homozygous α 4YFP knock-in mice (2-3 months old) in each group (control or nicotine). The α 4YFP knock-in mouse line have been backcrossed for 10 generations to the C57BL/6J mouse strain. We make sure the age and sex of all mice are the same for the study and that all surgeries are performed on the same day to minimize variability. To prevent hypothermia to the mice during and after surgery, equip surgical table with a heating pad covered in sterile surgical drape.
7. Induce the α 4YFP knock-in mouse with 3 L/min oxygen and 3% isoflurane and then maintain anesthesia at 2.5 L/min oxygen and 1% isoflurane. We prefer isoflurane anesthesia because on completion of the surgery isoflurane clears the system rapidly and the mice are conscious and mobile within ~2 min. Apply eye drops immediately (Tear-Gel, Novartis) to avoid corneal damage.
8. Pumps are implanted subcutaneously through a skin incision on the back of the neck and the pump is pushed caudally down the dorsal aspect of the back. Wipe the area on the back between the forelimbs with 95% ethanol to matte the fur. Pinch the skin with 0.8mm Graefe forceps (Fine Science Tools, 11050-10) and make a 1 cm central lateral cut using iris scissors (Fine Science Tools, 14060-10).
9. To create a subcutaneous space for the pump, insert standard pattern scissors (Fine Science Tools, 14101-14) into the incision and push them carefully toward the caudal end of the animal.
10. Using 1.0 mm Graefe forceps (Fine Science Tools, 11650-10), remove the pump from saline. Hold the incision open using forceps and insert the pump into the incision with the osmotic pump cap facing the caudal end of the animal and push the pump to the caudal end.
11. Pinch wound shut using 0.8mm forceps and apply an adequate amount of Vetbond (3M, cat# 1469SB) glue. Hold until wound is sealed.
12. Remove the animal from isoflurane mask, inject with the analgesic, meloxicam (0.1 mg/kg s.c.), and place into a short-term recovery cage until conscious and mobile. Then place in a long-term recovery cage with food and water available *ad libitum*.

2. α 4YFP knock-in mouse fixation by intracardiac perfusion

1. Make solutions one day prior to procedure and leave at 4° C. To minimize variability all mice will be perfused on the same day and with the same batch of solutions.
2. Perform perfusion fixations in a well ventilated area. Flush line of peristaltic pump (Masterflex Easy Load, 7518-00; Masterflex Pump Controller, 60648) with ddH₂O.
3. Add ~0.0015g of heparin (Sigma, cat# H4784), an anticoagulant, to 20 ml PBS pH 7.6 (Invitrogen, cat# 70011).
4. Anesthetize α 4YFP knock-in mouse by injecting intramuscularly a mixture of ketamine (25 mg/kg, Wyeth Animal Health) and medetomidine hydrochloride (0.25 mg/kg, Pfizer) into the hind leg muscle and immediately place the animal back into its home cage.
5. Pin animal to a styrofoam lid inserted into a metal tray. Wipe thorax with 95% ethanol. Pinch skin with adson forceps (Fine Science Tools, 91106-12) and snip open the thoracic cavity with iris scissors. Clamp the ribcage using an ultra fine hemostat (Fine Science Tools, 13021-12) and expose the heart.
6. Begin pumping PBS at 4 ml/min and insert a 23G butterfly needle (Becton Dickinson, 367253) into the left ventricle of the animal. Immediately snip the right atrium to allow blood and perfusate to escape.
7. Perfuse 20 ml of PBS (pH 7.6), then 30 ml of 4% paraformaldehyde (pH 7.6, diluted with PBS from a 16% PFA stock, Electron Microscopy Sciences, cat# 15710), then 20ml of 5% sucrose (pH 7.6). We found that over fixation increases autofluorescence. Perfusion with 5% sucrose rinses residual PFA from the brain, which lowers autofluorescence.
8. Remove brain and store in 30% sucrose for 3 days.
9. To freeze brains for coronal sectioning, cut off the cerebellum with a razor blade and place brain in a plastic embedding mold (VWR, cat# 18986-1) rostral side up and submerge in O.C.T. Mounting Compound (Tissue-Tek, cat# 4583). Freeze in dry ice and store at -20 °C before slicing.
10. Section brains (30 μ m thick) on a cryostat and then transfer to coated slides. Store the brain sections at -20°C in slide boxes containing one anhydrous calcium sulphate stone. The slide box should be in a zip lock sealed bag to avoid moisture and subsequent freezer burn when stored at -20°C.

3. Imaging fluorescent nAChRs using spectral confocal microscopy

1. Ensure slides have minimal exposure to any light source to minimize photobleaching.
2. Coverslip the brain section slides with a mounting medium that does not inhibit fluorescent protein fluorescence. We have good success with Mowiol 4-88 (pH 8.5 in Tris-HCl and glycerol, EMD-Calbiochem, cat# 475904), which hardens after a few hours. Make sure the Mowiol equilibrates to room temperature before cover slipping in order to avoid air bubbles. Do not use nail polish when cover slipping as it will quench YFP fluorescence.
3. Images are acquired using a Nikon C1si spectral confocal microscope system. Details on the method of spectral confocal imaging and linear unmixing are provided elsewhere¹³⁻¹⁵. The rationale for using a spectral confocal microscope is that fixed brain tissue has inherent autofluorescence. Spectral confocal imaging on the Nikon C1si uses an array of 32 photomultiplier tube detectors that sample a specified range of wavelengths of fluorescent emitted light, refracted spatially into its different wavelengths via a grating dispersive element just like a prism refracting white light into a rainbow of colors¹⁶. What is collected is a lambda stack of images -- images collected at different wavelengths of light so that an emission spectrum is collected for each pixel of a lambda stack of images. Since YFP and tissue autofluorescence each have characteristic spectral signatures, the lambda stack can be deconvolved using a linear unmixing algebraic algorithm into separate YFP and autofluorescent signals (Fig. 2). Thus, very accurate quantification of YFP fluorescence can be determined even in tissue with significant levels of autofluorescence.
4. Various settings can be adjusted to optimize image quality and fluorescence collection efficiency. We will report settings we typically use but these settings can be adjusted depending on the sample and the confocal microscope. To accurately quantify changes in α 4YFP subunit expression with chronic nicotine we ensure that the grey scale level intensity of the signals of all pixels are below saturating value (< 4095 for 12 bit grey scale). Moreover, we accommodate for potential increase in signal due to receptor upregulation by adjusting the confocal settings

so that pixels are around one third of saturating value (~1300-1400) or less. Once the settings are optimized, the settings are maintained identical for all imaging sessions and samples.

5. We use the following settings for imaging α 4YFP using a 60X oil CFI Plan Apo VC objective (1.40 N.A., 0.13 mm working distance): 488 nm laser line at 15% maximal transmission of a 40 mW Argon laser, spectral detector gain at 220, spectral range imaged from 496.5 nm to 656.5 nm at 5 nm resolution, 512 x 512 pixels over a 50 μ m x 50 μ m area, a medium pinhole size (60 μ m diam), a 4.08 pixel dwell time, averaging over two scans and 12 bits of grey scale.

4. Linear unmixing of spectral confocal images and image analysis

1. To perform linear spectral unmixing on a sample image taken with a spectral confocal microscope, one must first acquire a reference spectrum for YFP and a reference spectrum for tissue autofluorescence.
2. An important requisite for any reference spectrum is to obtain a high signal to noise fluorescence spectrum imaged with the same laser line used for imaging of your samples. Although the 514 nm laser line of Argon has a higher excitation efficiency to excite YFP we prefer to image YFP with the 488 nm laser line because there is better separation from the peak emission of YFP and the 488 nm line is less likely to distort the peak and one can obtain the entire YFP emission spectrum including the rise to peak. We image soluble YFP transfected in a cell line so that a strong YFP signal is obtained and the resultant YFP spectrum is stored in our library of reference spectra. Transfected α 4YFP over expressed in cell lines may also be used since it will also provide a high signal to background spectrum.
3. To obtain a reference spectrum of autofluorescence we image autofluorescence from a wildtype mouse brain section from the various brain regions that we intend to image from the α 4YFP mouse and obtain their corresponding spectra. We use the same laser line, 488 nm, and image using nearly identical parameters, although we may alter the detector gain, laser intensity or perform average imaging to maximize signal to noise.
4. After obtaining a spectral confocal image from a brain region of the α 4YFP mouse, the image is then deconvolved into its YFP and autofluorescence signals by applying a linear spectral unmixing algorithm using the reference spectrum of YFP and the reference spectrum of wildtype mouse autofluorescence from the same brain region.
5. The unmixed α 4YFP image can then be opened in an image analysis software such as ImageJ (<http://rsbweb.nih.gov/ij/>) and then the mean pixel intensity is calculated for the region of interest. A plugin for ImageJ called "loci_tools.jar" (<http://www.loci.wisc.edu/bio-formats/imagej>) can be used to import Nikon ics/ids confocal files.
6. We repeat the same linear spectral unmixing and analysis to wildtype mice in the same brain region to obtain a background residual value.
7. Then one obtains the corrected mean α 4YFP intensity by subtracting the background residual value (4.6) from the mean uncorrected α 4YFP value (4.5).

5. Representative Results

We show a representative true color projection of a lambda stack of images of the medial habenula from a homozygous α 4YFP mouse (Fig. 3A) taken with a spectral confocal microscope. We also show the emission spectrum from a region of interest containing α 4YFP positive neurons from the same lambda stack image (Fig. 3B). A distinct emission peak is evident at ~527 nm, which is the peak fluorescence emission of YFP. The region neighbouring the medial habenula shows an emission spectrum lacking a spectral peak at 527 nm, indicating the absence of α 4YFP nAChR subunits. Following linear spectral unmixing using reference spectra of YFP and mouse brain autofluorescence with significant overlap of emission (Fig. 4), separation of YFP and autofluorescent signal is possible yielding an α 4YFP unmixed image, an autofluorescent unmixed image and a remainder channel. Clear localization of α 4YFP fluorescence can be identified in the tightly packed soma of the medial habenula (Fig. 4).

In the hippocampus α 4YFP is localized mainly in the medial perforant path, the temporo-ammonic path and the alveus¹². These are all glutamatergic innervation of the hippocampus. We examined the effects of chronic nicotine on α 4YFP expression in the hippocampal perforant path (Fig. 5). Chronic administration of nicotine (2 mg/kg/hr for 10 days) resulted in a significant increase ($69 \pm 14\%$) in α 4YFP fluorescence from control saline treated mice to chronic nicotine treated mice ($p = 0.001$, Wilcoxon rank sum test) (Fig. 5).

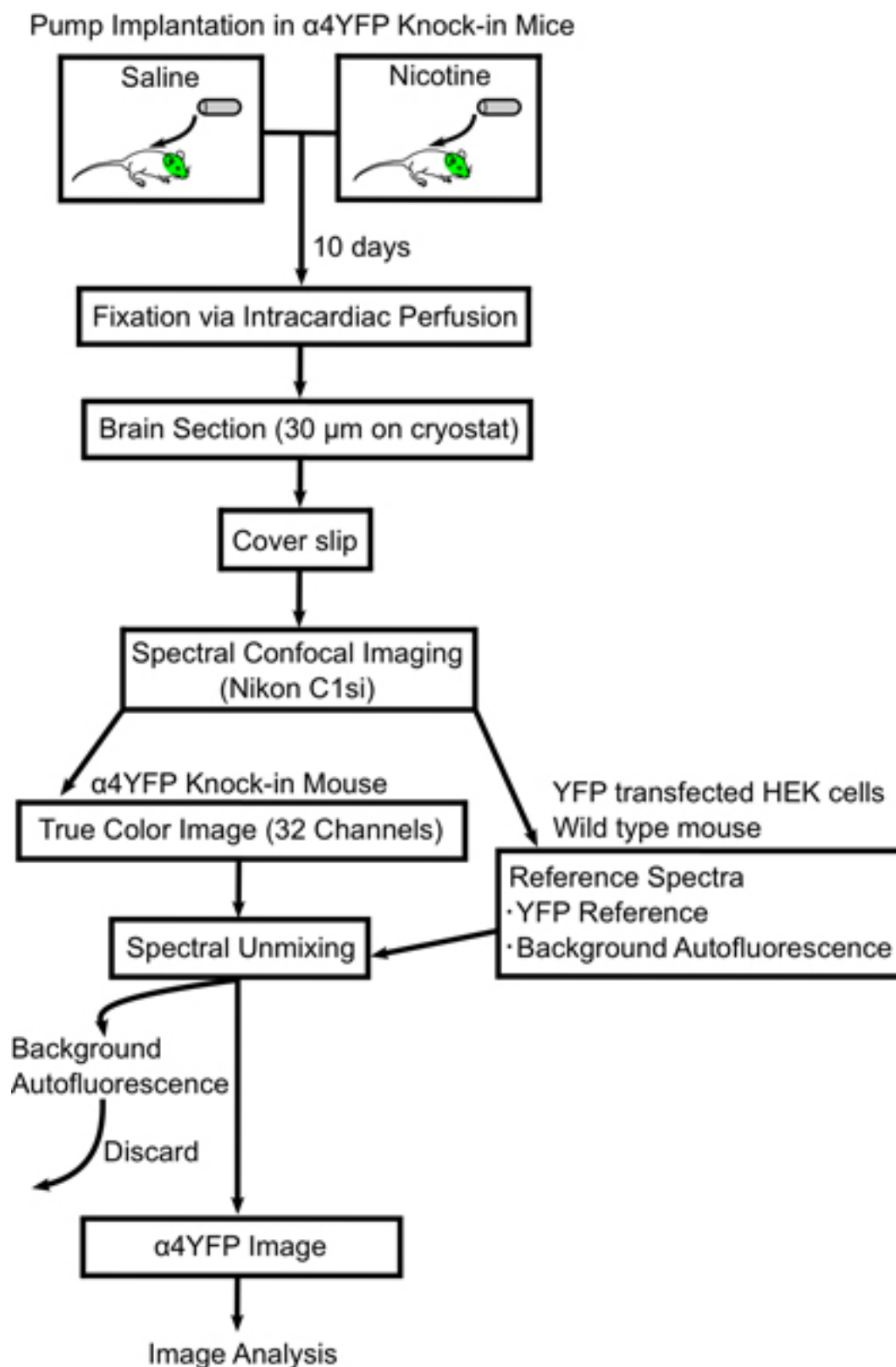


Figure 1. Flow chart showing procedure to image changes in α 4YFP nAChR subunits with chronic nicotine. Mini-osmotic pumps are filled with either saline or nicotine and implanted subcutaneously in α 4YFP homozygous mice. After 10 days mice are perfused and fixed with 4% paraformaldehyde and the mouse brains are sectioned (30 μ m thick) on slides. The brain section is imaged on a spectral confocal microscope (Nikon C1si) and spectrally unmixed into YFP and autofluorescent images. Then the α 4YFP images are analyzed further with ImageJ software.

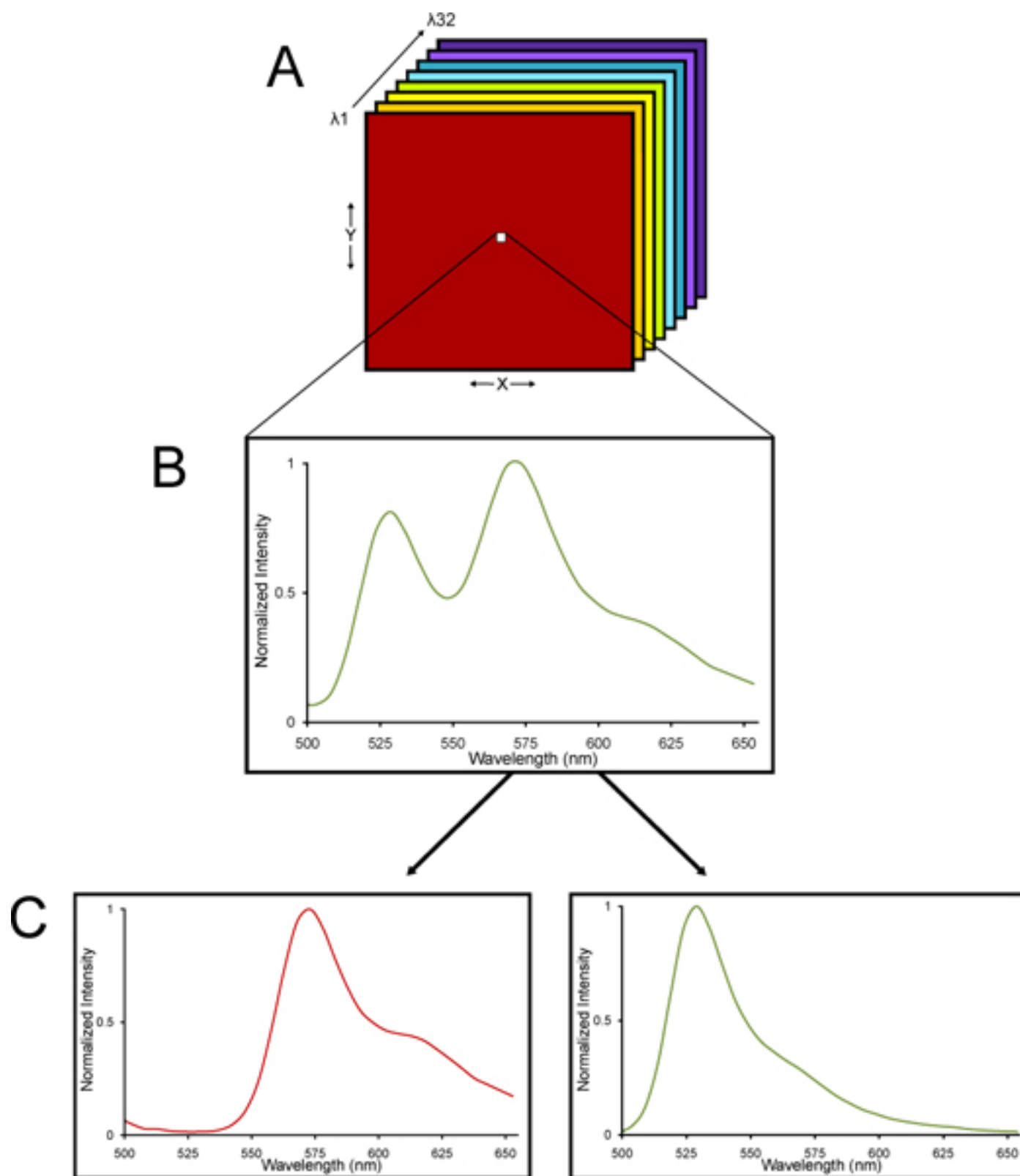


Figure 2. A schematic diagram of a lambda stack imaged from a spectral confocal microscope and linearly unmixed into its spectral components. (A) A lambda stack of images is collected. (B) A lambda stack consists of images collected at different wavelengths of light so that an emission spectrum is collected for each pixel across the entire stack. (C) Since YFP and tissue autofluorescence each have characteristic spectral signatures, the lambda stack can be deconvolved using a linear unmixing algebraic algorithm into separate YFP and autofluorescent signals. Thus, very accurate quantification of YFP fluorescence can be determined even in tissue with high levels of autofluorescence.

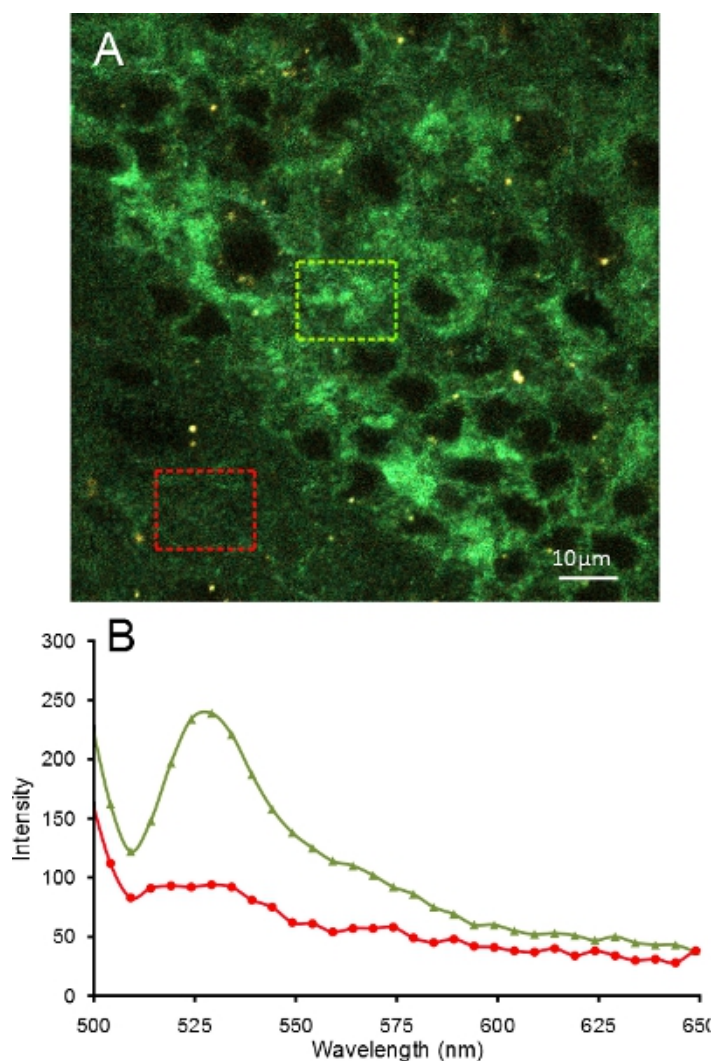


Figure 3. Spectral confocal image of a brain region expressing $\alpha 4$ YFP nAChRs. (A) A true color projection of a lambda stack of images of the medial habenula from an $\alpha 4$ YFP mouse taken with a Nikon C1si spectral confocal microscope. (B) Plots of spectra from a region of interest, which includes $\alpha 4$ YFP containing neurons (green), and a region of interest outside of the medial habenula (red).

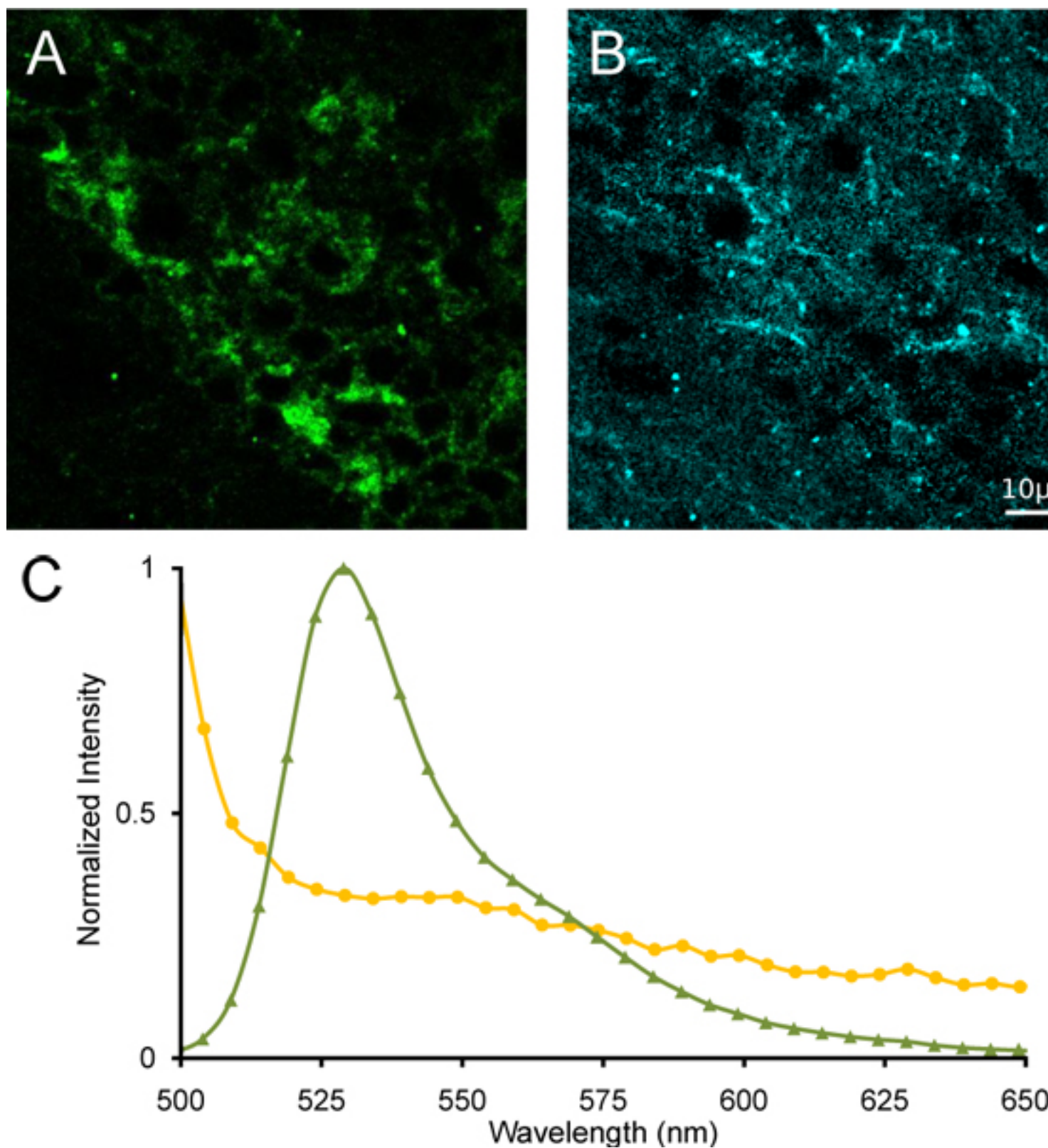


Figure 4. Linear spectral unmixing of the medial habenula. (A) Images of unmixed α 4YFP and (B) autofluorescence following linear spectral unmixing. (C) Reference spectra of YFP (green triangles) and autofluorescence (yellow circles) used for spectral unmixing.

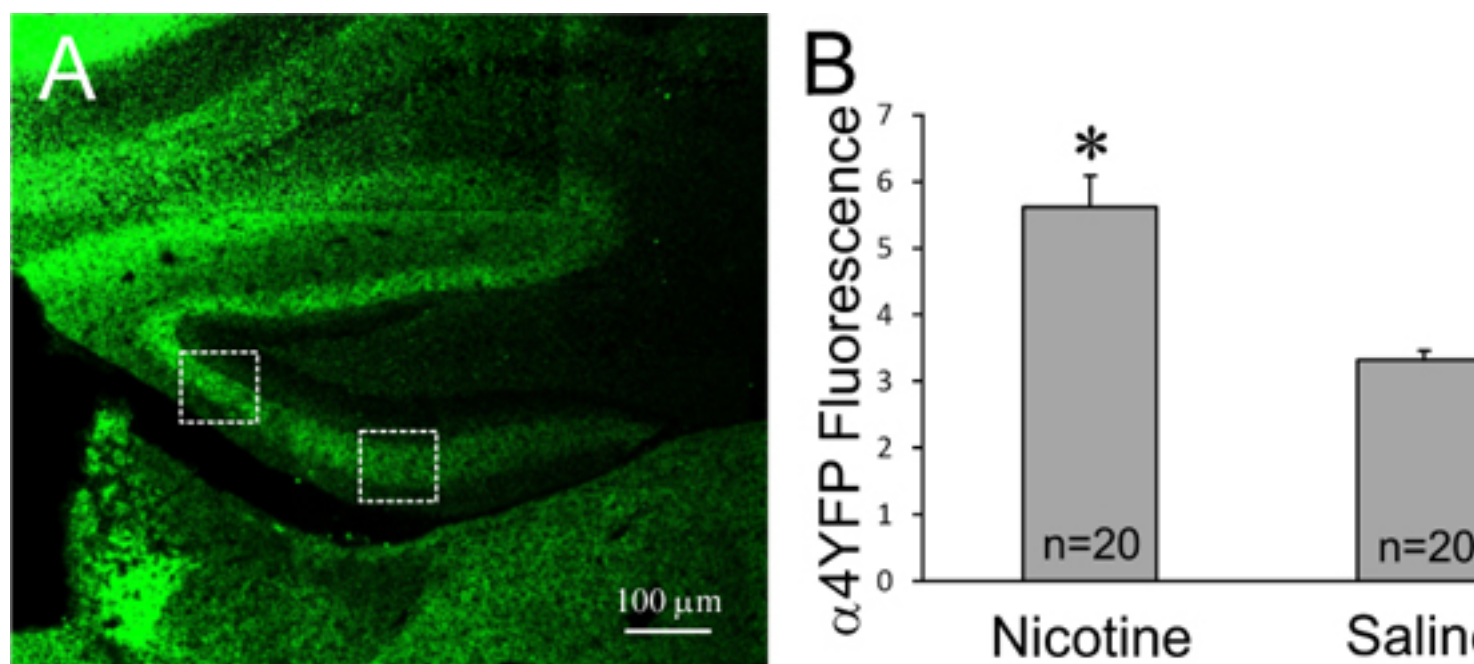


Figure 5. Upregulation of $\alpha 4$ nAChRs in the hippocampus of $\alpha 4$ YFP knock-in mice exposed to chronic nicotine. (A) A tiled montage of $\alpha 4$ YFP fluorescence from the hippocampus. The two dashed selection areas are the approximate locations on the inferior limb of the perforant path of the hippocampus where analyses were performed for each mouse. (B) $\alpha 4$ YFP fluorescence was significantly higher in the perforant path of mice exposed to chronic nicotine than chronic saline (*, $p = 0.001$, Wilcoxon rank sum test). Results represent mean $\pm$ SEM from $n = 20$ measurements for both saline and chronic nicotine treated mice (5 mice for each treatment group).

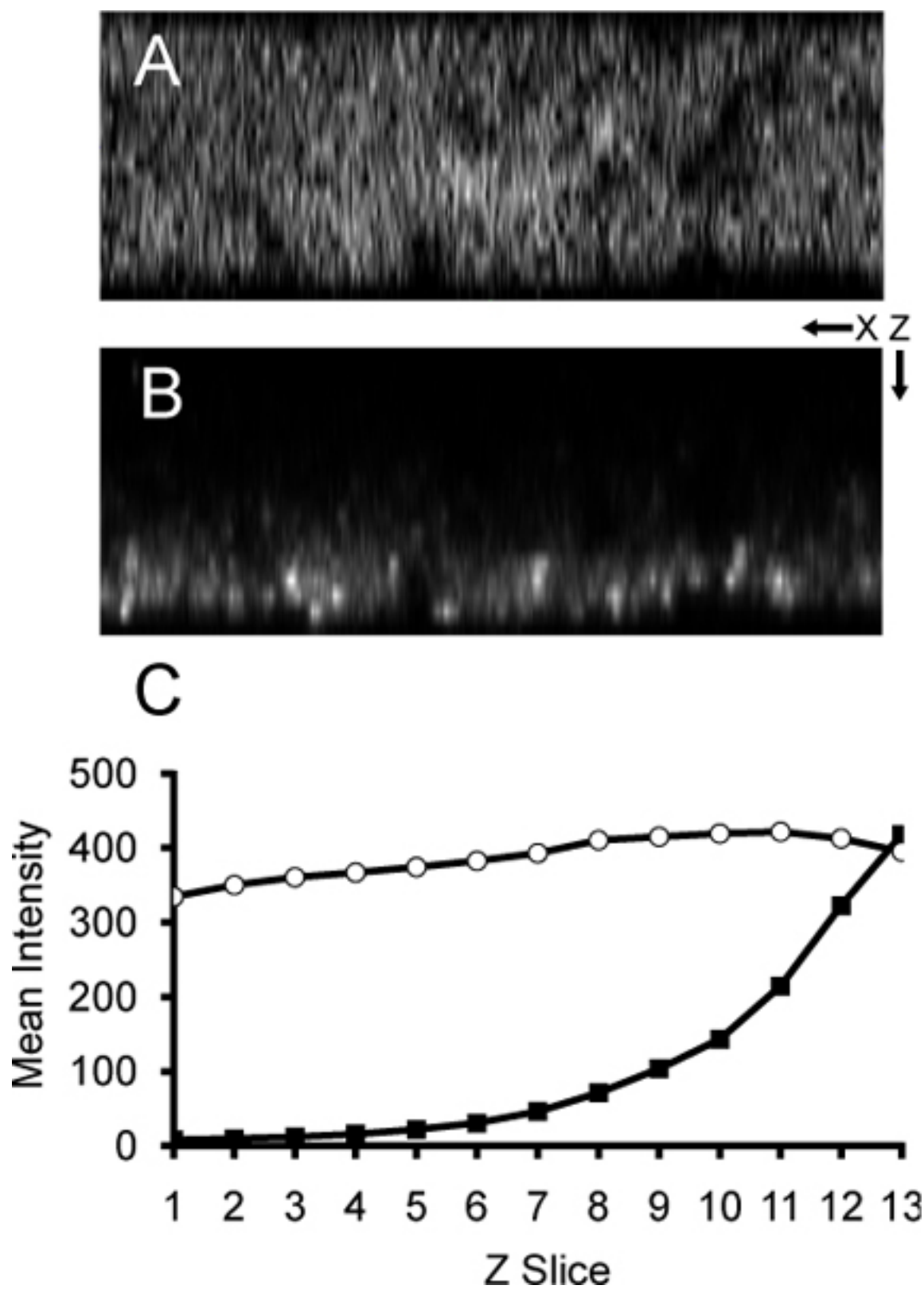


Figure 6. Better depth expression of α 4YFP as compared to antibody labeling. X-Z orthogonal views of α 4YFP fluorescence (A) and VGlut2 antibody with Cy5 as a secondary label (B). (C) Plots showing greater fluorescence signal intensity degradation over depth for antibody staining (black squares) as compared to α 4YFP (open circles).

Discussion

The use of a fluorescent receptor in a knock-in mouse model to determine quantity and localization of a specific ion channel provides a number of advantages. In contrast to proteins such as actin, which is ubiquitously expressed in all cells, ion channels are present in far fewer numbers and their expression varies between neuronal subtypes making accurate analysis via traditional immunohistochemical techniques challenging. The $\alpha 4$ YFP gene product is expressed at WT levels, being under control of the same promoters, enhancers and trafficking mechanisms¹². Fluorescent receptors also show the same function and sensitivity as the WT ion channel¹². Traditional methods of immunohistochemistry unfortunately show incomplete antibody penetration using a number of common antibodies on fixed rodent neural tissue slices¹⁷, leading to skewed results. A knock-in model provides consistent intensity levels throughout the tissue (Fig. 6). Although some commercial antibodies may have issues with nonspecificity^{18, 19}, with careful selection and concentration of antibodies and appropriate controls, one can obtain immunohistochemical localization of nicotinic receptors in the CNS^{20, 21}. Changes in $\alpha 4$ YFP fluorescence represents alterations in receptor subunit protein expression that can be quantified at subcellular resolution in specific subtypes of neurons. The results can be complemented with Western blots to quantify protein expression and RT-PCR to quantify for mRNA transcript level changes. These two techniques do not have cellular resolution to quantify changes in specific neuronal subtypes, except for RT-PCR when it is performed in conjunction with laser microcapture dissection.

The use of mini-osmotic pumps to deliver nicotine allows certain advantages to study the mechanisms of nicotine addiction – more specifically, the biochemical effects of nicotine on the regulation of $\alpha 4$ YFP nAChR subunit expression. The ease of surgical implantation and high reliability of mini-osmotic pump performance in continuous delivery of a calibrated rate of nicotine, are advantageous features of using mini-osmotic pumps. Another advantage is that drug delivery does not require restraint or handling of the animal, thus minimizing stress. A study of continuous chronic nicotine administration in mice have provided a detailed “calibration curve” showing the relations between continuous nicotine dose delivery, blood concentrations of nicotine and levels of nicotinic receptor upregulation in the mouse brain²². This study was instrumental in choosing a nicotine dose that produces significant levels of receptor upregulation. However, there are limitations to the chronic continuous delivery of nicotine. Other nicotine exposure methods such as oral administration²³⁻²⁵ and intravenous self-administration²⁶⁻²⁹ more closely mimic the pulsatile delivery of nicotine in smoking and the clearance of nicotine over the duration of sleep. Furthermore, intravenous self-administration of nicotine allows one to correlate drug dependence behaviour to biochemical alterations in the brain, including nicotinic receptor levels. Although there is evidence of nicotine intravenous self-administration in mice, in general there are far fewer studies showing nicotine intravenous self-administration in mice as compared to rats due to the fact that nicotine self-administration is more difficult to achieve in mice³⁰. Nevertheless, future work in nicotine self-administration models with the $\alpha 4$ YFP knock-in mice would further advance the field.

One of the significant advantages of using knock-in mice with fluorescently-tagged nicotinic receptors is the improved resolution of quantifying receptor expression at submicron scale inside specific subtypes of neurons as compared to traditional methods of radioactively labelled ligand binding. With the recent advent of super-resolution microscopy imaging, this opens exciting possibilities of imaging receptors at nanometer resolution inside CNS neurons³¹. Our methodology can readily be extended to the study of other ion channels and receptors.

Disclosures

We have nothing to disclose.

Acknowledgements

Anthony Renda was supported by a University of Victoria Graduate Fellowship Award. This research was supported by a Natural Sciences and Engineering Research Council of Canada Discovery Grant, a NARSAD Young investigator Award (to R.N.), a Victoria Foundation - Myre and Winifred Sim Fund, a Canadian Foundation for Innovation grant, a British Columbia Knowledge Development Fund and a Natural Sciences and Engineering Research Council of Canada Research Tools and Instrumentation Grant. We thank Jillian McKay, Christina Barnes, Ariel Sullivan, Jennifer MacDonald and Daniel Morgado for excellent mouse husbandry.

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