

Contents lists available at [ScienceDirect](#)

MethodsX

journal homepage: www.elsevier.com/locate/mex



Method Article

Immunohistochemical amplification of mCherry fusion protein is necessary for proper visualization



B.A. Falcy*, M.A. Mohr, P.E. Micevych

UCLA DGSOM Dept of Neurobiology, 650 Charles E Young Dr. S, Los Angeles, CA 90095, Laboratory of Neuroendocrinology of the Brain Research Institute, United States of America

ABSTRACT

Fluorescent reporter proteins are a powerful tool being increasingly integrated into biological experiments. Their utility spans techniques such as live-cell imaging, validating transgene expression, and studying cell-type specific anatomy. As these reporters become more widely used, it is necessary to fully understand their benefits and limitations. One such recently developed red fluorescent protein, mCherry, has been well utilized due to its stability, brightness, and pH resistance. In the course of an experiment using the fluorescent reporter protein mCherry fused to a G-protein coupled receptor (mCherry fusion protein), our lab discovered a notable inability for the fusion protein to faithfully produce fluorescent signal representative of its expression in fixed tissue. Here, we demonstrate the importance of immunohistochemical amplification in tissue injected with various adeno-associated viruses (AAVs), containing mCherry fusion protein as a reporter. Our findings demonstrate that antibody amplification consistently provides a stronger signal when mCherry fusion protein is used as a reporter protein.

© 2020 The Authors. Published by Elsevier B.V.
This is an open access article under the CC BY-NC-ND license.
(<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

ARTICLE INFO

Method name: Immunohistochemical Amplification of Virally Expressed Fluorescent mCherry Fusion Protein

Keywords: Reporter protein, Immunohistochemistry, Adeno-associated virus, mCherry fusion protein, Fluorescence microscopy, DREADD

Article history: Received 4 October 2019; Accepted 28 May 2020; Available online 2 June 2020

* Corresponding author.

E-mail address: bfalcy@mednet.ucla.edu (B.A. Falcy).

Specifications table

Subject Area	Neuroscience
More specific subject area	Viral Vectors and Immunohistochemistry
Method name	Immunohistochemical Amplification of Virally Expressed Fluorescent mCherry Fusion Protein
Name and reference of original method	Visualization of virally expressed mCherry without amplification [1]. Direct modulation of GFAP-expressing glia in the arcuate nucleus bi-directionally regulates feeding.
Resource availability	All viruses used: addgene Primary antibody for mCherry: Abcam Secondary antibody (Goat x Rabbit): Jackson Immunoresearch All other reagents: Fisher scientific, Southern Biotech

Method details

Animals, AAV injections, and experimental design

This study was carried out in accordance with the principles and procedures of the National Institute of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles. Animals were given ad libitum access to food and water and were kept on 12:12 light cycles. All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

12 animals (all males, unless otherwise stated) were injected with one of 4 viruses ($n = 3/\text{virus}$). Viral injections were targeted to either the anteroventral periventricular nucleus of the hypothalamus (AVPV) or posterior hypothalamic nucleus (PH) and performed between 1–6 months of age. Glial fibrillary acidic protein (GFAP)-Cre animals (female, $n = 3$, available from JAX, Stock No. 024,098) were injected with pAAV-hSyn-DIO-hM3D(Gq)-mCherry (Addgene, cat. #44,361-AAV5) targeted to the AVPV. POMC-Cre animals ($n = 6$; 3 per virus, available from JAX, Stock No. 005,965) were injected with either pAAV-GFAP-hM3D(Gq)-mCherry (Addgene, cat. #50,478-AAV5) or pAAV-GFAP-hM4D(Gi)-mCherry (Addgene, cat. #50,479-AAV5), both targeted to the AVPV. WT animals were injected with pAAV-GFAP-hM3D(Gq)-mCherry (Addgene, cat. #50,478-AAV5), targeted to the PH. The coordinates for injection into the AVPV, relative to bregma, were as follows: A/P: + 0.5 mm, M/L: +/- 0.3 mm, D/V: - 5.5 mm. The coordinates for injection into the PH, relative to bregma, were as follows: A/P: -1.6 mm, M/L: +/- 0.4 mm, D/V: -5.0 mm. 300nL of virus was injected at a rate of 30 nL/ minute. After injection was complete, the needle remained in place for 10 min before being withdrawn to avoid drawing out excess virus. The virus was allowed a 20–29 day period of expression.

It should be noted that the GFAP-Cre group was injected with a Cre-dependent virus under the control of the synapsin promoter. Expression is nevertheless seen in this group (Fig. 1), likely because some astrocytes can express synapsin [2].

Tissue fixation

Animals were anesthetized and decapitated. Brains were quickly removed and placed whole into fresh 4% paraformaldehyde overnight (~18 hrs) at 4 °C. The brain was then transferred to a 30% sucrose solution in phosphate buffer at 4 °C until it sunk, at which point it was considered cryoprotected and ready to be sectioned (approximately two days). Sections (35 μm thick) were cut in a cryostat (-20 °C), and stored at -20 °C in cryoprotectant solution until used for immunohistochemistry.

Immunohistochemistry

Fluorescent immunohistochemistry was performed on brain sections to amplify endogenous mCherry fluorescence. The following protocol was used. Note that a prior titration of the primary antibody was conducted and showed no difference in signal when using the mCherry primary antibody at a dilution of between 1:1000 and 1:5000.

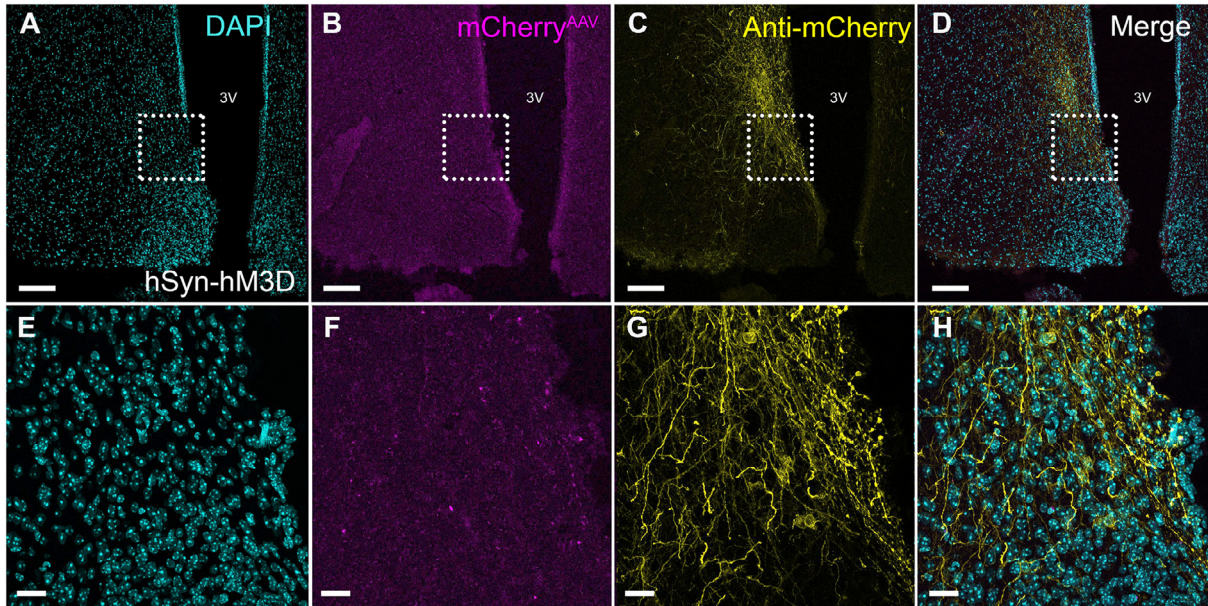


Fig. 1. Confocal z-stacks collected from an animal injected with AAV5-hSyn-hM3D-mCherry into the AVPV. Dashed boxes in low power images (A-D; 21 μm stacks, 1 μm step) are enlarged in higher power maximum intensity projections (E-H; 19 μm stacks, 0.5 μm step). DAPI is pseudocolored cyan (A, E), virally expressed mCherry is pseudocolored magenta (B, F), immunohistochemical amplification of virally expressed mCherry is pseudocolored yellow (C, G), and a merged image of all three channels is shown in D & H. Scale bars = 100 μm (A-D); 20 μm (E-H). 3v = third ventricle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Day 1

- (1) Wash in TBS 3 times, for 5 min each.
- (2) Block in TBS-Plus Goat containing 3% normal goat serum and 0.3% Triton-x in 1X TBS for 30 min at room temperature.
- (3) Incubate in mCherry primary antibody, rabbit polyclonal anti-mCherry at a dilution between 1:1000 and 1:5000 in 0.3% Triton-X in 1X TBS for 24 h at 4 °C.

Day 2

- (4) Wash in TBS 3 times, for 5 min each.
- (5) Incubate in secondary antibody Alexafluor 488 Goat anti Rabbit at a dilution of 1:1000 for 2 h at room temperature.
- (6) Wash in TBS 3 times, for 5 min each.
- (7) Mount on subbed superfrost/plus slides.
- (8) Coverslip with DAPI Fluoromount-G

Reagent List:

- Normal Goat Serum (Equitech-Bio, Cat# SG30-0500)
- Rabbit polyclonal anti-mCherry antibody (Abcam, Cat# ab167453)
- AlexaFluor 488-Conjugated Affinipure Goat anti-Rabbit IgG (Jackson ImmunoResearch, Cat# 111-545-144; diluted 1:1 in glycerol and stored at -20 °C)
- Superfrost/Plus Microscope Slides (Fisher Scientific, Cat# 12-550-15)
- (4',6-diamidino-2-phenylindole) DAPI Fluoromount-G (Southern Biotech, Cat# 0100-20)

Microscopy

Tissue was visualized with a Zeiss LSM 880 confocal laser scanning microscope using Zen 2012 software. High power images were comprised of Z-stacks, acquired using C-APOCHROMAT 40x - 1.2 W objective, with blue diode (405 nm), argon (488 nm), and helium neon (HeNe; 594 nm) lasers across sections ranging from 19 μ m to 35 μ m in thickness, imaged at steps of 0.5 μ m. Low power images were taken using Plan-APOCHROMAT 10x/ 0.8 M27 objective and were taken across sections ranging from 21 μ m to 39 μ m in thickness, imaged at steps of 1.0 μ m.

Method Validation

Insufficient Virally Expressed mCherry Fusion Protein Fluorescence Was Improved Through Immunohistochemical Amplification

Initial observation that the mCherry reporter protein required immunohistochemical amplification for faithful visualization was replicated in tissue from animals injected with AAV5-hSyn-hM3D-mCherry, AAV5-GFAP-hM3D-mCherry, and AAV5-GFAP-hM4D-mCherry. AAV5-hSyn-hM3D-mCherry (Fig. 1) and AAV5-GFAP-hM3D-mCherry (Fig. 2) shared similarities in mCherry expression; in both groups, virally expressed mCherry (mCherry^{AAV}) failed to adequately represent mCherry presence in the tissue, however visualization of mCherry was dramatically improved through immunohistochemical labeling of the reporter protein (mCherry^{amp}). At high power, mCherry^{AAV} in these groups did not robustly exceed basal autofluorescence seen in non-injected control tissue (Fig. 5F). In tissue from AAV5-GFAP-hM4D-mCherry injected animals (Fig. 3), mCherry^{AAV} was not detected at low power and even at high power was very weak. Immunohistochemical amplification of mCherry resulted in dramatically improved, albeit diffuse, labeling at low and high power.

Each of the three animal groups injected with different viral constructs consistently benefited from immunohistochemical amplification of mCherry. Amplified signal varied, depending on the virus, from strictly cellular (Fig. 1) to more dispersed labeling (Figs. 3 and 4). In most cases, virally expressed mCherry signal was indistinguishable from background autofluorescence.

These findings indicate the necessity for investigators to independently verify their reporter protein-dependent findings through a secondary method, such as immunohistochemical amplification.

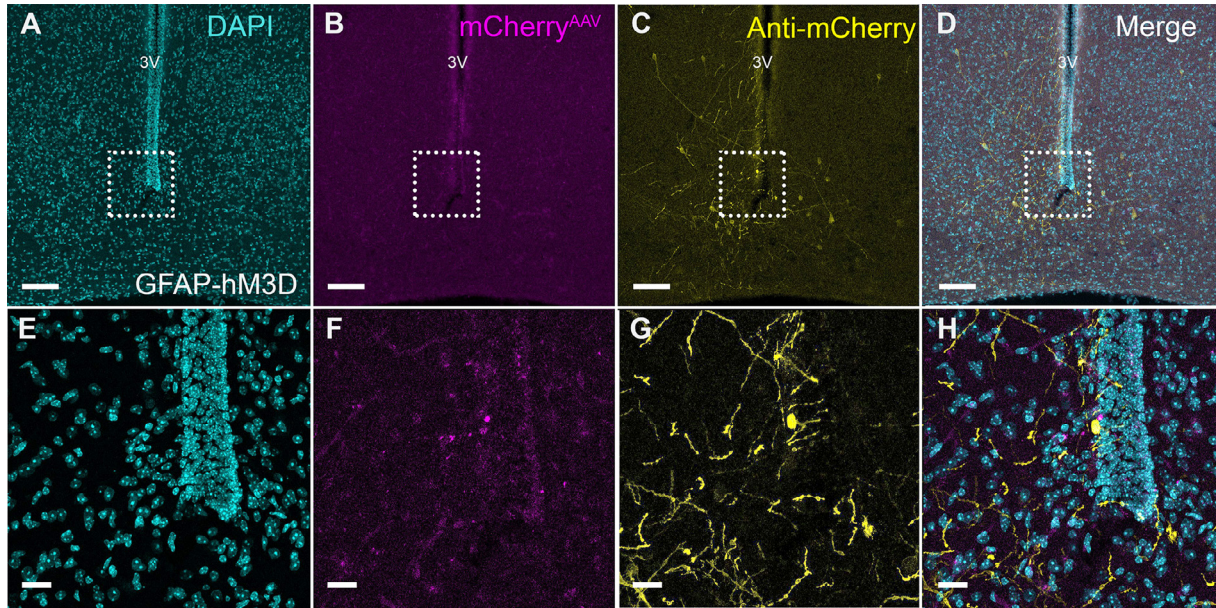


Fig. 2. Confocal z-stacks collected from an animal injected with AAV5-GFAP-hM3D-mCherry into the AVPV. Dashed boxes in low power images (A-D; 25 μm stacks, 1 μm step) are enlarged in higher power maximum intensity projections (E-H; 30.5 μm stacks, 0.5 μm step). DAPI is pseudocolored cyan (A, E), virally expressed mCherry is pseudocolored magenta (B, F), immunohistochemical amplification of virally expressed mCherry is pseudocolored yellow (C, G), and a merged image of all three channels is shown in D & H. Scale bars = 100 μm (A-D); 20 μm (E-H). 3v = third ventricle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

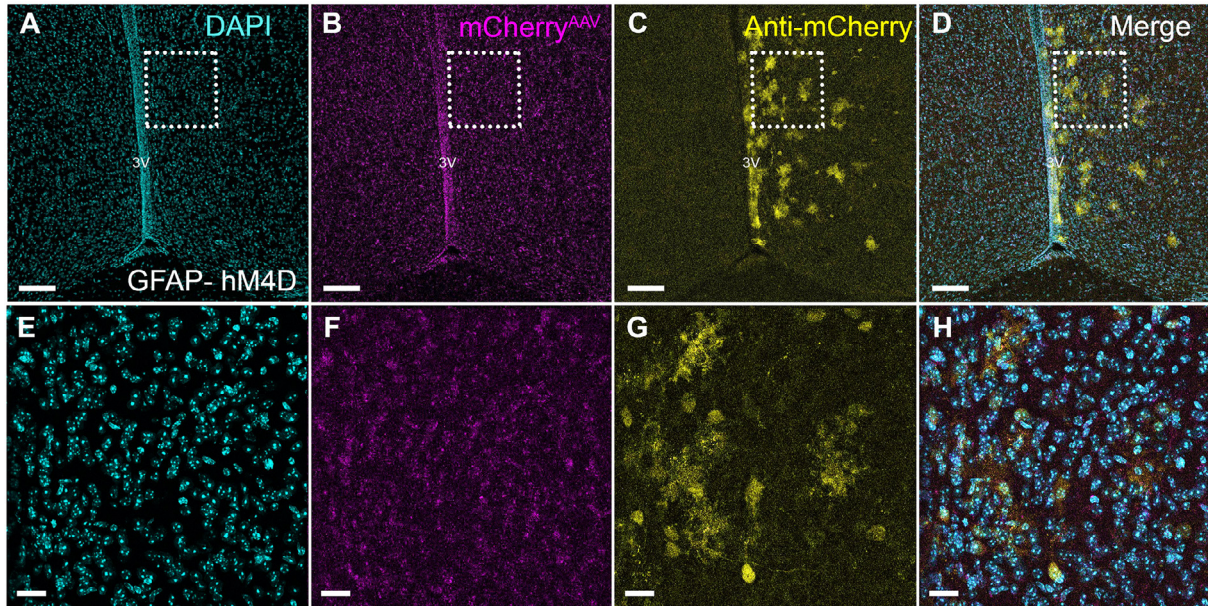


Fig. 3. Confocal z-stacks collected from an animal injected with AAV5-GFAP-hM4D-mCherry into the AVPV. Dashed boxes in low power images (A-D; 31 μm stacks, 1 μm step) are enlarged in higher power maximum intensity projections (E-H; 22 μm stacks, 0.5 μm step). DAPI is pseudocolored cyan (A, E), virally expressed mCherry is pseudocolored magenta (B, F), immunohistochemical amplification of virally expressed mCherry is pseudocolored yellow (C, G), and a merged image of all three channels is shown in D & H. Scale bars = 100 μm (A-D); 20 μm (E-H). 3v = third ventricle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

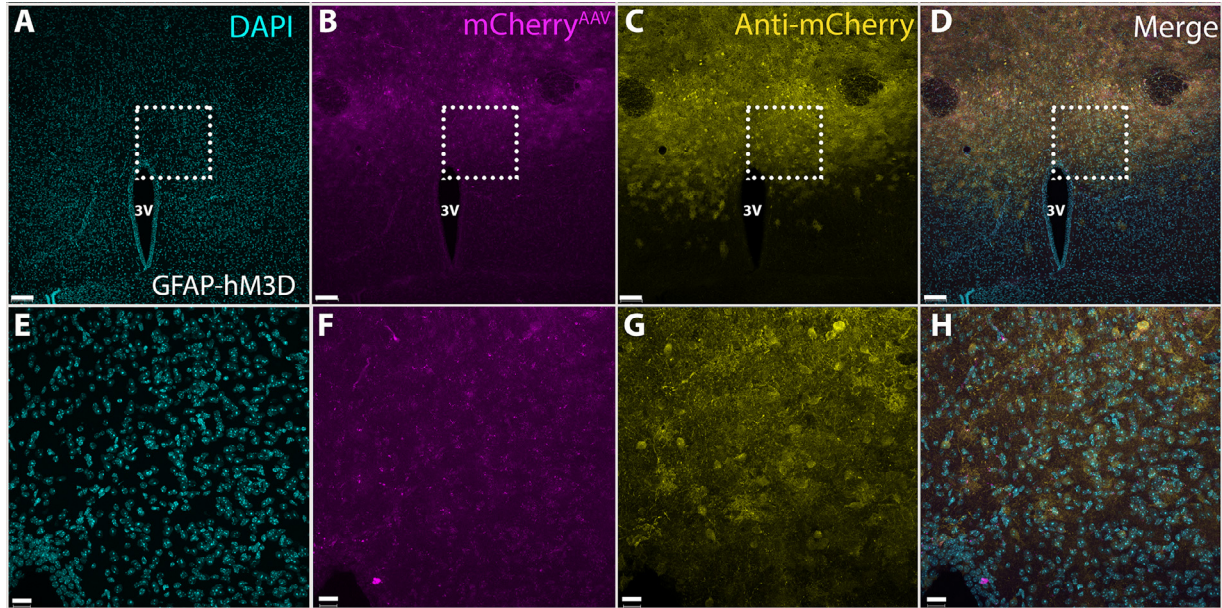


Fig. 4. Confocal z-stacks collected from an animal AAV5-GFAP-hM3D-mCherry into the PH. Dashed boxes in low power images (A-D; 24 μm stacks, 1 μm step) are enlarged in higher power maximum intensity projections (E-H; 24 μm stacks, 0.5 μm step). DAPI is pseudocolored cyan (A, E), virally expressed mCherry is pseudocolored magenta (B, F), immunohistochemical amplification of virally expressed mCherry is pseudocolored yellow (C, G), and a merged image of all three channels is shown in D & H. Scale bars = 100 μm (A-D); 20 μm (E-H). 3v = third ventricle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

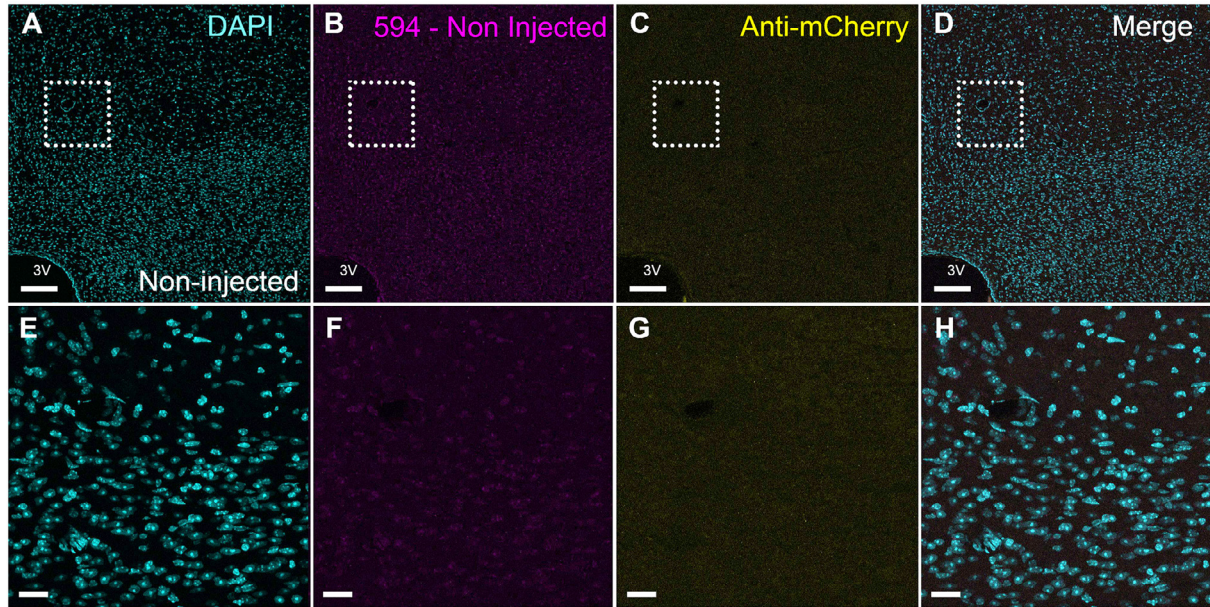


Fig. 5. Confocal z-stacks collected from an animal that was not injected with mCherry. Dashed boxes in low power images (A-D; 39 μm stacks, 1 μm step) are enlarged in higher power maximum intensity projections (E-H; 34.5 μm stacks, 0.5 μm step). DAPI is pseudocolored cyan (A, E), the 594 nm excitation is pseudocolored magenta (B, F), immunohistochemical amplification and 488 nm excitation is pseudocolored yellow (C, G), and a merged image of all three channels is shown in D & H. Scale bars = 100 μm (A-D); 20 μm (E-H). 3v = third ventricle.

We used fluorescence microscopy to evaluate the ability for virally expressed mCherry fusion protein, delivered into live animals using three different AAVs, to produce a fluorescent signal representative of its presence in tissue and compared this to an immunohistochemically amplified signal of mCherry expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

It appears that reporter protein capability may vary from one viral construct to another, suggesting that initial pilot studies using viral constructs may be able to establish a static understanding of the reporter protein's fidelity. It should be noted that the above experiments utilized mCherry as a fusion protein with a G-protein coupled receptor (GPCR; either hM3D or hM4D). It is thus possible that its fusion to these GPCRs may limit its endogenous fluorescence capacity. Moreover, other factors may also determine the detectability of the fusion protein, such as length of viral incubation, amount of virus injected, and location of viral injection. Thus, the need exists for consistent and continuous validation of mCherry's reporting ability.

Declaration of Competing Interest

The authors declare no competing financial interests.

Acknowledgments

National Center for Advancing Translational Sciences UCLA CTSI Grant [UL1TR001881](#) (MAM), Eunice Kennedy Shriver National Institute of Child Health & Human Development Grant Number HD097965-01 (MAM), Eunice Kennedy Shriver National Institute of Child Health & Human Development Grant Number HD042635 (PEM).

References

- [1] N. Chen, et al., Direct modulation of GFAP-expressing glia in the arcuate nucleus bi-directionally regulates feeding, *eLife* 5 (2016) e18716.
- [2] Shiwei Wang, et al., Synapsin I is an Oligomannose-Carrying Glycoprotein, Acts as an Oligomannose-Binding Lectin, and Promotes Neurite Outgrowth and Neuronal Survival When Released via Glia-Derived Exosomes, *J. Neurosci.* 31 (20) (2011) 7275–7290.