Journal of Visualized Experiments

An enhanced green fluorescence protein-based assay for studying the neurite outgrowth in primary neurons

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60031R2
Full Title:	An enhanced green fluorescence protein-based assay for studying the neurite outgrowth in primary neurons
Keywords:	Enhanced green fluorescent protein; ImageJ; microscopy; neurite outgrowth; primary neurons; transfection
Corresponding Author:	Kwok-Fai Lau HONG KONG
Corresponding Author's Institution:	
Corresponding Author E-Mail:	kflau@cuhk.edu.hk
Order of Authors:	Wai Wa Ray Chan
	Wen Li
	Dik Long Dennis Chau
	Kwok-Fai Lau
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Hong Kong

TITLE:

An Enhanced Green Fluorescence Protein-Based Assay for Studying Neurite Outgrowth in Primary Neurons

AUTHORS AND AFFILIATIONS:

Wai Wa Ray Chan[1]*, Wen Li[1,2]*, Dik Long Dennis Chau[1]*, Kwok-Fai Lau[1]

[1]School of Life Sciences, The Chinese University of Hong Kong, Hong Kong Special Administrative Region, China

[2]Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, Guangdong Province, China

*These authors contributed equally.

Corresponding Author:

Kwok-Fai Lau (kflau@cuhk.edu.hk)

Email Addresses of Co-authors:

Wai Wa Ray Chan (raychan@link.cuhk.edu.hk)

Wen Li (wen.li1@siat.ac.cn)

Dik Long Dennis Chau (denniscdl@link.cuhk.edu.hk)

KEYWORDS:

Enhanced green fluorescent protein, ImageJ, microscopy, neurite outgrowth, primary neurons, transfection

SUMMARY:

In this report, we describe a simple protocol for studying neurite outgrowth in embryonic rat cortical neurons by co-transfecting with EGFP and the protein of interest.

ABSTRACT:

Neurite outgrowth is a fundamental event in the formation of the neural circuits during nervous system development. Severe neurite damage and synaptic dysfunction occur in various neurodegenerative diseases and age-related degeneration. Investigation of the mechanisms that regulate neurite outgrowth would not only shed valuable light on brain developmental processes but also on such neurological disorders. Due to the low transfection efficiency, it is currently challenging to study the effect of a specific protein on neurite outgrowth in primary mammalian neurons. Here, we describe a simple method for the investigation of neurite outgrowth by the co-transfection of primary rat cortical neurons with EGFP and a protein of interest (POI). This method allows the identification of POI transfected neurons through the EGFP signal, and thus the effect of the POI on neurite outgrowth can be determined precisely. This EGFP-based assay provides a convenient approach for the investigation of pathways regulating neurite outgrowth.

INTRODUCTION:

Neurites, including both axons and dendrites, are the projections from neurons involved in the establishment of the neural networks. The dynamic outgrowth of neurites is essential for

neurodevelopment. However, the underlying regulatory mechanisms underneath remain unclear. In particular, neurite damage is often observed in various neurodegenerative diseases and after brain injuries[1]. Therefore, investigation of the roles of putative molecules in various neurite outgrowth regulatory pathways would improve our understanding of the process. Moreover, it may reveal novel therapeutic targets for various neurological disorders. Neuronal cell lines are valuable models for studying neuronal processes including neurite outgrowth as they are easy to manipulate and transfect[2,3]. However, genetic drift has been reported to occur in some commonly used cell lines, which could lead to variations in their physiological responses[4]. Moreover, differential protein expression has been shown between neuronal cell lines and primary neurons. For instance, PC12, a neuronal cell line derived from rat adrenal gland that is widely used for studying neurite outgrowth[2,3], does not express NMDA receptors[5]. Furthermore, it has been proposed that the reduced responsiveness of the mouse neuroblastoma line neuro-2a to neurotoxins in comparison to primary neurons is due to the lack of expression of certain membrane receptors and ion channels[6]. Therefore, primary neurons are a more desirable and representative model for the investigation of neurite outgrowth. However, the use of primary neurons is hindered by their low transfection efficiency[7].

Here, we describe a method that involves the co-transfection of the protein of interest (POI) and EGFP to primary rat cortical neurons. The EGFP acts as a morphological marker for the identification of successfully transfected neurons and permits the measurement of neurites. We validated this method by using compounds/molecules that have been reported to modulate neurite outgrowth. Moreover, FE65, a neuronal adaptor protein that has been shown to stimulate neurite outgrowth, was used to illustrate this approach[8,9]. This protocol involves (1) the isolation of primary cortical neurons from embryonic day 18 (E18) rat embryos, (2) the co-transfection of neurons with EGFP and the POI (FE65 in this study) and (3) the imaging and analysis of the neurons by using the image processing software ImageJ with the NeuronJ plugin[10,11].

PROTOCOL:

All procedures followed were in accordance with the ethical standards of the animal experimentation ethics committee of the Chinese University of Hong Kong.

1. Preparation of coverslips

1.1. Place a sterile 18 mm circular coverslip into each well of a 12-well tissue culture plate.

1.2. Coat the coverslip with 5 µg/mL poly-D-lysine solution in a humidified 37 °C incubator for at least 1 h.

1.3. Aspirate the poly-D-lysine solution from the tissue culture plate and rinse the coated coverslips once with sterile water.

2. Rat embryonic neuron dissection

2.1. Sacrifice a timed-pregnant Sprague-Dawley rat at a gestational age of 18 days (E18) by

either cervical dislocation or CO_2 asphyxiation.

NOTE: Please check local regulations for the sacrifice of pregnant rats.

2.2. Open the abdominal cavity of the pregnant rat with dissecting scissors and transfer the uterus to a 10 cm Petri dish.

2.3. Open the uterus and the amniotic sac carefully with small dissecting scissors and remove the placenta from the rat embryo using small dissecting scissors. Transfer the whole embryo to a 10 cm Petri dish with pre-chilled phosphate buffered saline supplemented with glucose (PBS-glucose, 10 mM sodium phosphates, 2.68 mM potassium chloride, 140 mM sodium chloride and 3 g/L glucose) using a pair of small forceps.

2.4. Cut along the sagittal suture of the skull and open it carefully with a pair of small dissecting scissors. Transfer the embryonic brain with a small flat spatula to a 10 cm Petri dish with ice-cold PBS-glucose.

2.5. Separate the two cerebral hemispheres from the cerebellum and brain stem using two #5 tweezers under a dissection microscope.

NOTE: Please see reference[12] for the structure of the rat brain.

2.6. Remove the meninges using the #5 tweezers.

2.7. Isolate the cortex from the cerebral hemispheres with two straight #5 tweezers.

2.8. Transfer the isolated cortex to ice-cold PBS-glucose in a 15 mL centrifuge tube.

3. Primary cortical neuron culture

NOTE: All procedures in steps 3 and 4 are performed inside a Class II Biosafety cabinet.

3.1. Settle the isolated cortex by gravity at 4 °C for 5 min and aspirate the PBS-glucose.

3.2. Add 1 mL of 0.05% Trypsin-EDTA to the isolated cortex and mix gently by tapping and incubate the tissue in a 37 °C water bath for 10 min to allow enzymatic digestion. Tap the tube gently to mixing every 2 min.

3.3. Add 4 mL of maintenance medium (e.g., Neurobasal Medium) to the tissue/trypsin mixture.

NOTE: All the maintenance medium used in this protocol is supplemented with Penicillin-Streptomycin and B-27 supplement[13].

3.4. Dissociate the tissue gently by trituration using a 1 mL pipette.

3.5. Pellet the dissociated cells by centrifugation at 200 x *g* for 5 min. Aspirate the

supernatant.

3.6. Repeat steps 3.5 to 3.7 twice.

3.7. Resuspend the cell pellet in 1 mL of maintenance medium.

3.8. Add 10 µL of 0.4% Trypan Blue solution to 10 µL of cell suspension for counting of viable cells by a hemocytometer.

3.9. Plate the neurons at a density of 65,000/cm^2 (viable cells) in 1 mL of maintenance medium per well in a 12-well plate.

4. Cell transfection and fixation

4.1. At 2 days in vitro (DIV2), transfect 0.5 µg of EGFP construct (pEGFP-C1) to neurons together either with or without of 0.5 µg of POI by using 1 µL of transfection reagent (e.g., Lipofectamine 2000). Use manufacturer's instructions.

NOTE: Mammalian expression constructs were prepared by using an endotoxin free plasmid preparation kit. Treatment with chemicals/molecules (in this manuscript cytochalasin D (Cyto D) and nerve growth factor (NGF) were used) can be done at 6 h after transfection.

4.2. Aspirate the culture medium 24 h post-transfection and wash the transfected cells once with 37 °C PBS (10 mM sodium phosphates, 2.68 mM potassium chloride, 140 mM sodium chloride).

4.3. Fix the cells with 4% paraformaldehyde in PBS for 10 min in the dark at room temperature.

4.4. Wash the fixed cells three times with PBS.

4.5. Add a minimal amount of fluorescence mounting medium on a microscope glass slide. Carefully transfer the coverslip from the 12-well plate onto the mounting medium with the sample facing the glass slide.

NOTE: Seal the edge of the coverslip with nail polish if an aqueous mounting medium is used.

5. Measurement of neurite outgrowth

5.1. Use a 40x objective for capturing images using an epi-fluorescent microscope.

5.2. Capture images from at least 40 intact neurons with EGFP signal per transfection.

5.3. Open the captured image in ImageJ software with the NeuronJ plugin[11] to measure the length of the longest neurite, from the cell body to the tip of the growth cone, of each imaged neuron.

5.4. Analyze the data obtained with the software to determine the effect of the targeted proteins in neurite outgrowth.

REPRESENTATIVE RESULTS:

To test this methodology, we used Cyto D and nerve growth factor NGF, which have been shown to inhibit and stimulate neurite outgrowth respectively[14-16]. The neurite length of neurons transfected with EGFP were measured after treatment with Cyto D or NGF. The transfection efficiency of EGFP to the neurons was 2.7% (1,068 neurons counted). As shown in **Figure 1A**, Cyto D suppressed neurite extension in a dose-dependent manner. Conversely, neurite outgrowth was potentiated in the neurons treated with NGF (**Figure 1B**).

Next, we investigated the utility of this system by transfecting the neuronal adaptor FE65, which has been shown to promote neurite outgrowth. Primary rat cortical neurons were co-transfected with FE65 and EGFP. Despite the low transfection efficiency, immuno-fluorescence analysis revealed that over 80% of the neurons were successfully co-transfected with EGFP and FE65 (**Figure 2A**). Similar to previous reports[8,9], FE65 significantly stimulated the neurite outgrowth by 2x (**Figure 2B**). We also analyzed the expression of EGFP and FE65 at different time points by Western blot analysis. As shown in **Figure 2C**, EGFP and FE65 were detected 6 h and 12 h post-transfection, respectively. Similar expression levels of the proteins were observed in 1 d to 7 d post-transfection neurons. This indicates that the analysis of neurite outgrowth could be done as early as 6 h post-transfection or in more mature neurons. Together, this data suggest that the mentioned protocol is suitable for determining the role of putative neurite outgrowth regulatory proteins by classical transfection.

We also monitored the effect of gene dosage on neurite outgrowth by transfecting primary rat cortical neurons with different amounts of FE65 plasmid DNA. As shown in **Figure 2D**, a dose-dependent increase in neurite extension was observed from 0 – 0.5 μg of FE65 plasmid DNA. However, there was no significant difference between neurons transfected with either 0.5 μg or 1 μg of plasmid DNA (**Figure 2D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Neurite outgrowth is modulated by Cyto D and NGF. E18 rat cortical neurons were transfected on DIV2 with an EGFP expression vector. 6 h post-transfection, the cells were treated with (**A**) 0-0.5 μg/mL Cyto D or (**B**) 0-100 ng/mL NGF for 24 h. Then the neurons were fixed and imaged accordingly. Images were captured with 40x objective using an epi-fluorescence microscope and the length of the longest neurite from the cell body to the tip of the growth cone was measured by using ImageJ with the NeuronJ plugin. Three independent experiments were performed and at least 40 neurons were measured in each group. The bar chart showed the fold change in mean neurite length. Unpaired t-test was adopted for the statistical analysis. *$p<0.001$, **$p<0.05$ Error bars were S.E.M.

Figure 2: FE65 stimulates neurite outgrowth. E18 rat cortical neurons were transfected on DIV2 with either empty vector control (EV) or FE65 together with an EGFP expression vector. Cells were fixed and imaged 24 h after transfection. (**A**) The transfected neurons were counterstained with anti-FE65 antibody and the number of cells with EGFP or FE65 singly transfected and EGFP + FE65 co-transfected were counted. Three independent experiments were performed and at least 100 cells were counted in each experiment. Data were expressed

as the percentage of cells with EGFP and EGFP + FE65 signals. *p<0.001 Error bars were S.D. (**B**) The length of the longest neurite from the cell body to the tip of the growth cone was measured by using ImageJ with the NeuronJ plugin. Representative neuron images were shown in the right panel. FE65 was stained by a goat anti-FE65 antibody as described[8,17]. Three independent experiments were performed and at least 40 neurons were measured per transfection. The bar chart showed the fold change in mean neurite length. The data were analyzed by unpaired t-test. *p<0.001 Error bars were S.E.M. Scale bar was 10 µm. (**C**) Western blot analysis of the expression of levels of EGFP and FE65 at the post-transfection time points as indicated. EGFP and FE65 were detected by mouse anti-GFP and anti-myc (to FE65 C-terminal myc tag), respectively. (**D**) The average neurite length of neurons transfected with various amounts of FE65 plasmid DNA as indicated. Statistical analyses were performed using one-way ANOVA tests with Bonferroni post-hoc test. *p<0.001, **p<0.05. Error bars were S.E.M. Scale bar was 10 µm.

DISCUSSION:

As stated before, PC12 and its subclones are widely employed for studying neurite extension because they have excellent transfection efficiency[2,3]. In contrast, primary neurons have a low transfection rate, which is a major obstacle for studying neurite outgrowth regulators by transfection[7]. Here, we describe a convenient protocol for quantifying neurite outgrowth in primary neurons. Despite the low overall transfection efficiency, more than 80% of the transfected neurons were successfully co-transfected with the two proteins: FE65 and EGFP (**Figure 2A**). Therefore, by analyzing the EGFP labelled neurons, the effect of FE65 on neurite outgrowth could be precisely determined (**Figure 2B**).

Another advantage of the EGFP-based approach described is that immunofluorescence staining can be omitted. Immunofluorescence staining of Tuj 1, a neuron-specific class III β-tubulin, is widely employed as a morphological marker when studying neurite elongation[18-21]. In addition to Tuj 1, staining of the POI is required for the identification of transfected neurons[19,22,23]. It is known that the consistency of immunofluorescence staining, which is crucial for neurite outgrowth measurement, is affected by many factors including sample preparation and antibodies[24]. Hence, the overall simplicity of the EGFP-based method could improve the accuracy of the assay.

In our protocol, neurons are fixed for neurite measurement 24 h post-transfection. Thus, neurons are exposed to the transfection reagent for 24 h. It is long known that transfection reagents are toxic to cells[25]. If the effect of the POI on neurite extension needs to be determined beyond DIV3, fresh medium replenishment may help to minimize the toxicity. Moreover, alternative gene delivery methods may be considered such as Ca^{2+} phosphate co-precipitation and nucleofection, both of which are shown to have a less toxic effect to cells[26]. Additionally, we show here that the highest effect of FE65 on neurite outgrowth is observed in the neurons transfected with that 0.5 µg of FE65 and 0.5 µg of EGFP plasmids by using 1 µL of transfection reagent. For other POIs, the optimal amounts of plasmid DNA and transfection reagents should be determined as proteins have widely different turnover rates.

In addition to gene delivery method, cell density is another critical parameter to be considered. If the neuron density is too high, it becomes difficult to identify the origins of neurites as they would overlap with each other. Although plating cells at a low density may

resolve the issue, the survival of primary neurons would be significantly reduced at a low cell density[27]. Primary rat hippocampal neurons have been shown to grow healthily at the density between 40,000 to 100,000/cm^2 [28]. In this protocol, a density of 65,000/cm^2 of rat cortical neurons is used. Nevertheless, it is important to determine the appropriate cell density for different types of neurons under different experimental conditions.

The neurite measurement of developing rat primary neurons (i.e., DIV3 neurons) is described here. However, more mature neuronal phenotypes can be observed in neurons beyond DIV3[29]. As the effects of POIs or drugs on neurite extension in mature neurons may be different from the developing neurons, investigation by using neurons from different stages would provide a more comprehensive perspective. It is worth noting that we were still able to observe EGFP expression in the neurons 7 d post-transfection. This may facilitate the study of POIs or drugs on neurite outgrowth in mature neurons.

Human induced pluripotent stem cell (hiPSC)-derived neurons are valuable tools in identification of novel therapeutic approaches. For instance, investigation of neurite outgrowth in these cells could reveal novel targets in neuroregeneration as the use of hiPSC-derived neurons avoids the species differences. Similar to primary rodent neurons, hiPSC-derived neurons are difficult to transfect[30], which may reduce the co-transfection efficiency of EGFP and the POI. Hence, the use of polycistronic mammalian expression vectors could ensure that all the transfected cells express the entire set of transfected genes including EGFP and POIs. Additionally, alternative gene delivery methods, such as electroporation, may improve transfection efficiency. Again, cell viability is an issue which needs to be considered when using such gene delivery approaches.

It is known that the rat embryonic brain cortex contains different types of neurons including spiny stellate neurons, bipolar neurons and long-projecting neurons[31]. While this protocol stated here can reveal the general effect of POIs on neurite outgrowth, it is possible that the same POI may exhibit different responses in different types of neurons. For example, myostatin increases the number of sensory axons but not that of motor axons[32]. In such a scenario, modification of the protocol is necessary, such as prior isolation of specific types of neurons by flow cytometry. Alternatively, specific neuronal marker antibodies may be used for the identification of the required types of neurons during imaging.

ACKNOWLEDGMENTS:

This work was supported by funds from the Research Grants Council Hong Kong, Health and Medical Research Fund (Hong Kong), CUHK direct grant scheme, the United College endowment fund and the TUYF Charitable Trust.

DISCLOSURES:

The authors declare that they have no conflicts of interest with the contents of this article.

REFERENCES:

1 Kaplan, A., Bueno, M., Hua, L., Fournier, A. E. Maximizing functional axon repair in the injured central nervous system: Lessons from neuronal development. *Developmental Dynamics.* **247** (1), 18-23 (2018).

2 Harrill, J. A., Mundy, W. R. Quantitative assessment of neurite outgrowth in PC12 cells.

Methods in Molecular Biology. **758**, 331-348 (2011).
3 Yeyeodu, S. T., Witherspoon, S. M., Gilyazova, N., Ibeanu, G. C. A rapid, inexpensive high throughput screen method for neurite outgrowth. *Current Chemical Genomics.* **4**, 74-83 (2010).
4 Ben-David, U. et al. Genetic and transcriptional evolution alters cancer cell line drug response. *Nature.* **560** (7718), 325-330 (2018).
5 Edwards, M. A., Loxley, R. A., Williams, A. J., Connor, M., Phillips, J. K. Lack of functional expression of NMDA receptors in PC12 cells. *Neurotoxicology.* **28** (4), 876-885 (2007).
6 LePage, K. T., Dickey, R. W., Gerwick, W. H., Jester, E. L., Murray, T. F. On the use of neuro-2a neuroblastoma cells versus intact neurons in primary culture for neurotoxicity studies. *Critical Reviews in Neurobiology.* **17** (1), 27-50 (2005).
7 Karra, D., Dahm, R. Transfection techniques for neuronal cells. *Journal of Neuroscience.* **30** (18), 6171-6177 (2010).
8 Cheung, H. N. et al. FE65 interacts with ADP-ribosylation factor 6 to promote neurite outgrowth. *The FASEB Journal.* **28** (1), 337-349 (2014).
9 Li, W. et al. Neuronal adaptor FE65 stimulates Rac1-mediated neurite outgrowth by recruiting and activating ELMO1. *Journal of Biological Chemistry.* **293** (20), 7674-7688 (2018).
10 Schneider, C. A., Rasband, W. S., Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods.* **9** (7), 671-675 (2012).
11 Meijering, E. et al. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry. Part A: the Journal of the International Society for Analytical Cytology.* **58** (2), 167-176 (2004).
12 Swanson, L. W. Brain maps 4.0-Structure of the rat brain: An open access atlas with global nervous system nomenclature ontology and flatmaps. *The Journal of Comparative Neurology.* **526** (6), 935-943 (2018).
13 Brewer, G. J. Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. *Journal of Neuroscience Research.* **42** (5), 674-683 (1995).
14 Yamada, K. M., Spooner, B. S., Wessells, N. K. Axon growth: roles of microfilaments and microtubules. *Proceedings of the National Academy of Sciences of the United States of America.* **66** (4), 1206-1212 (1970).
15 Casella, J. F., Flanagan, M. D., Lin, S. Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change. *Nature.* **293** (5830), 302-305 (1981).
16 Calabrese, E. J. Enhancing and regulating neurite outgrowth. *Critical Reviews in Toxicology.* **38** (4), 391-418 (2008).
17 Lau, K. F. et al. Dexras1 Interacts with FE65 to Regulate FE65-Amyloid Precursor Protein-dependent Transcription. *Journal of Biological Chemistry.* **283** (50), 34728-34737 (2008).
18 Cui, X. et al. Niacin treatment of stroke increases synaptic plasticity and axon growth in rats. *Stroke.* **41** (9), 2044-2049 (2010).
19 Khodosevich, K., Monyer, H. Signaling involved in neurite outgrowth of postnatally born subventricular zone neurons in vitro. *BMC Neuroscience.* **11**, 18 (2010).
20 Tang, F. et al. Resveratrol Enhances Neurite Outgrowth and Synaptogenesis Via Sonic Hedgehog Signaling Following Oxygen-Glucose Deprivation/Reoxygenation Injury. *Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology.* **43** (2), 852-869 (2017).

21 He, W., Liu, Y., Tian, X. Rosuvastatin Improves Neurite Outgrowth of Cortical Neurons against Oxygen-Glucose Deprivation via Notch1-mediated Mitochondrial Biogenesis and Functional Improvement. *Frontiers in Cellular Neuroscience.* **12**, 6 (2018).
22 Tesarova, P. et al. Receptor for advanced glycation end products (RAGE)--soluble form (sRAGE) and gene polymorphisms in patients with breast cancer. *Cancer Investigation.* **25** (8), 720-725 (2007).
23 Park, S. Y. et al. Hippocalcin Promotes Neuronal Differentiation and Inhibits Astrocytic Differentiation in Neural Stem Cells. *Stem Cell Reports.* **8** (1), 95-111 (2017).
24 Radbruch, A. Immunofluorescence: Basic Considerations. *Flow Cytometry and Cell Sorting*. Springer Lab Manual Book Series. 38-52 (2000).
25 Wang, T., Larcher, L. M., Ma, L., Veedu, R. N. Systematic Screening of Commonly Used Commercial Transfection Reagents towards Efficient Transfection of Single-Stranded Oligonucleotides. *Molecules.* **23** (10) (2018).
26 Sariyer, I. K. Transfection of neuronal cultures. *Methods in Molecular Biology.* **1078** 133-139 (2013).
27 Banker, G. A., Cowan, W. M. Rat hippocampal neurons in dispersed cell culture. *Brain Research.* **126** (3), 397-342 (1977).
28 Banker, G. A., Cowan, W. M. Further observations on hippocampal neurons in dispersed cell culture. *The Journal of Comparative Neurology.* **187** (3), 469-493 (1979).
29 Biffi, E., Regalia, G., Menegon, A., Ferrigno, G., Pedrocchi, A. The influence of neuronal density and maturation on network activity of hippocampal cell cultures: a methodological study. *PLoS One.* **8** (12), e83899 (2013).
30 Hiragi, T. et al. Differentiation of Human Induced Pluripotent Stem Cell (hiPSC)-Derived Neurons in Mouse Hippocampal Slice Cultures. *Frontiers in Cellular Neuroscience.* **11**, 143 (2017).
31 Zeng, H., Sanes, J. R. Neuronal cell-type classification: challenges, opportunities and the path forward. *Nature Reviews. Neuroscience.* **18** (9), 530-546 (2017).
32 Jones, M. R., Villalon, E., Northcutt, A. J., Calcutt, N. A., Garcia, M. L. Differential effects of myostatin deficiency on motor and sensory axons. *Muscle & Nerve.* **56** (6), E100-E107 (2017).

Figure

Figure 1

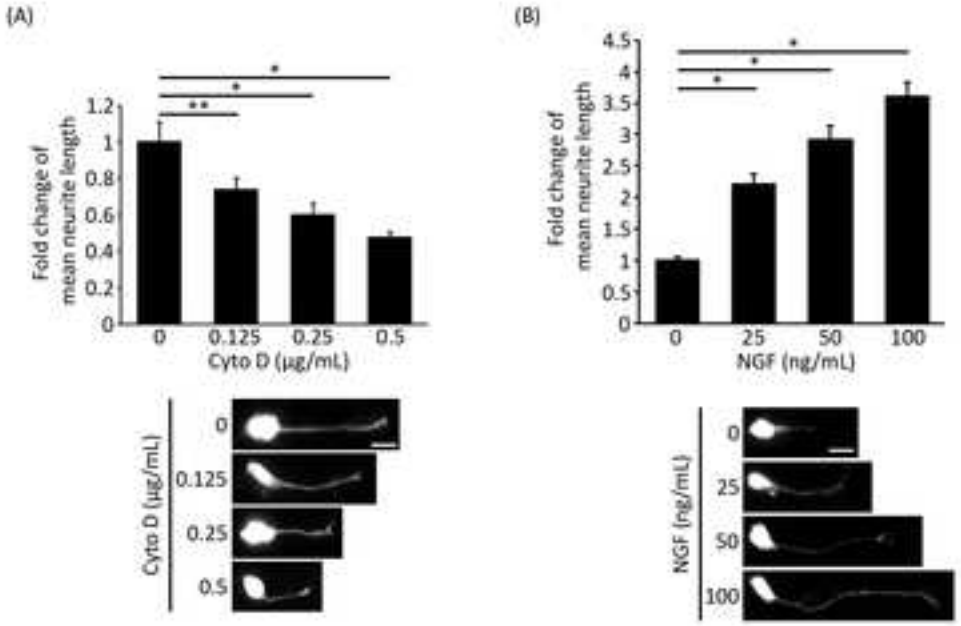
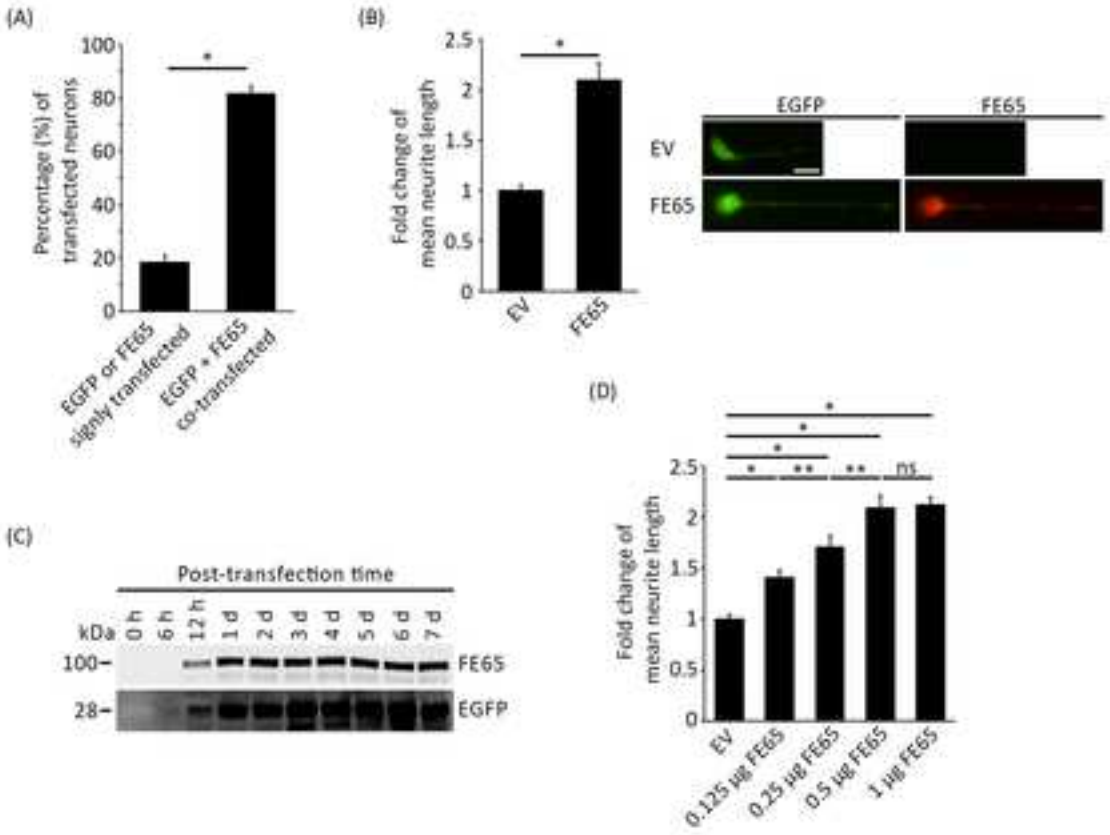


Figure 2



Name of Material/ Equipment	Company	Catalog Number
#5 tweezers	Regine	5-COB
18 mm Circle Cover Slips	Thermo Scientific	CB00180RA
B27 Supplement	Gibco	17504044
Cytochalasin D	Invitrogen	PHZ1063
D-(+)-Glucose	Sigma-Aldrich	G8270
Dimethyl Sulfoxide	Sigma-Aldrich	D2650
Dissecting Scissors, 10 cm	World Precision Instruments	14393
Dissecting Scissors, 12.5 cm	World Precision Instruments	15922
EndoFree Plasmid Maxi Kit	QIAGEN	12362
Fluorescence Mounting Medium	Dako	S302380
Lipofectamine 2000 Transfection Reagent	Invitrogen	11668019
Neurobasal Medium	Gibco	21103049
NGF 2.5S Native Mouse Protein	Gibco	13257019
Nugent Utility Forceps, 10mm, Straight Tip	World Precision Instruments	504489
Paraformaldehyde	Sigma-Aldrich	P6148
pEGFP-C1	Clontech	#6084-1
pCI FE65		
PBS Tablets	Gibco	18912014
Penicillin-Streptomycin	Gibco	15140122
Poly-D-lysine hydrobromide	Sigma-Aldrich	P7280
Spatula	Sigma-Aldrich	S4147
Trypsin-EDTA (0.05%), phenol red	Gibco	25300062
Trypan Blue Solution, 0.4%	Gibco	15250061

Comments/Description

Sterilize before use.

Dissolved in DMSO.

Please see references 8 and 15

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: An enhanced green fluorescence protein-based assay for studying neurite outgrowth in primary neurons

Author(s): Wai Wa Ray Chan, Wen Li, Dik Long Dennis Chau, Kwok-Fai Lau

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Kwok-Fai Lau
Department:	School of Life Sciences
Institution:	The Chinese University of Hong Kong
Title:	Associate Professor
Signature:	
Date:	21-3-2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

MS ID: JoVE60031R1

MS TITLE: An enhanced green fluorescence protein-based assay for studying the neurite outgrowth in primary neurons

Dear Dr. Nguyen,

Thank you for communicating with us about the situation of our manuscript. As suggested in your email dated 24 June 2019, we have revised our manuscript according to the comments from reviewers #5, #6 and #7. To facilitate the review process, our responses to the first review from reviewers #1, # 2, # 3 and #4 are also included.

Reviewer #5

Major Concerns:

1.The method of immuno-staining of FE65 must be described in PROTOCOL.

Thank you for the suggestion the immuno-staining protocol for FE65 has been described in our other publications. We have now included the references for the immuno-staining protocol for FE65.

2. The method of analysis how to trace the neurite and how to quantify the length of neurite by ImageJ and NeuronJ must be described in PROTOCOL.

Thank you for the suggestion. Similar concern is also raised by reviewer #2. As stated in our response to reviewer #2, we shall include a video during JoVE video preparation to illustrate how we perform neurite measurement by using ImageJ (with NeuronJ plugin).

3. Transfection rate (percentage in total neurons) of EGFP-expressing cells must be mentioned in REPRESENTATIVE RESULTS.

Thank you for the suggestion. We have now included the transfection rate information. Please refer to lines 197-199 of the manuscript.

Minor Concerns:

1. The information of plasmid vector must be described.

Thank you for the suggestion. We have now included the information of the plasmid vectors in the material list.

Reviewer #6

Major Concerns:

1. Authors raised the essential problem for neurite outgrowth analysis is low efficiency of transfection. However, this study does not deal with increasing neuronal transfection efficiency.

Similar concern is raised by reviewer #3. As stated in our response to reviewer #3, our protocol is not attempted to improve the transfection efficiency of primary neurons. Instead, it provides an easy way for the identification of the neurons which has been transfected with a protein of interest (POI) by co-transfecting with EGFP. Since our data has suggested that (Figure 2A) the co-transfection efficiency (not general transfection efficiency) of a POI (FE65) and EGFP is around 80%, most EGFP transfected primary

neurons should also be co-transfected with the POI. Therefore, the effect of POI on neurite outgrowth could be determined by measuring the neurite length of the EGFP labeled neurons.

2. If a protein of interest (POI) is intracellular one, its expression plasmid needs to be transfected. In this case a GFP or RFP plasmid can be co-transfected to identify transfected cells as was done here.

Thank you for the comment. We agree with reviewer #6 that the protocol stated in this manuscript can be modified to facilitate different applications in different laboratories.

However, this idea is not new. Also, it is far better to identify the transfected cells by staining with antibody against POI. EGFP affects neuronal health.

As suggested by the editor, novelty is not a requirement for publication in JoVE.

3. If a POI is extracellular, transfection is not necessary. One only needs to stain cells with neuron-specific antibodies.

Thank you for the comment. We agree with reviewer #6 that transfection is not necessary if a POI is extracellular. We are not attempted to provide a protocol to replace the classical approaches. Instead, our protocol provides an alternative way for studying neurite outgrowth in primary neurons in particular for intracellular proteins. We have compared our protocol and the classical staining approach for neurite outgrowth study. Please see discussion section paragraph 3.

Minor Concerns:

1. Neuronal images shown in the figures are unusual in that their differentiation is too slow. DIV 3 neurons (transfected on DIV 2) grown on a PDL-coated coverslip usually differentiate into stage II with axons and dendrites. However, the illustrated images show only one neurite.

Thank you for the comment. We agree with reviewer #6 dendrites are difficult to see from the neuronal images. As EGFP signal is strong in the cell bodies, we took images with relatively low exposure. This facilitates the identification of the cell body boundary which is crucial in the measurement of neurite length. Although they are difficult to see, some faint dendrites can be observed in the enlarged images.

2. Instead of WB (Fig. 2C), immunocytochemistry and densitometry should be employed, to track the temporal expression of proteins from transfected cells. As authors mentioned efficiency of neuronal transfection is very low. Therefore, WB results in relatively crude results than immunocytochemistry.

Thank you for the comment. The purpose of Western blots for EGFP and FE65 is to illustrate that the expression of the proteins at different time points. Therefore, the analysis of neurite outgrowth could be done as early as 6h post-transfection or in more mature neurons. We agree with reviewer #6 that immunocytochemistry and densitometry can also be employed to track temporal expression of proteins from transfected cells.

Reviewer #7

Minor Concerns:

1. If any, fluorescence images of cortical neurons should be added.

Thank you for the comment. Fluorescence images of cortical neurons have been shown in Figs 1A and 1B (EGFP) and Fig 2B (both EGFP and FE65)

2. Which enzymes were used to dissociate cortical neurons before trituration process, please you explain?

Thank you for the comment. Trypsin (0.05% Trypsin-EDTA solution), a widely employed proteolytic enzyme for cell and tissue dissociation, is used to dissociate cortical neurons before trituration process. We have now stated the information more clearly in protocol section 3.3.

3. The authors should add references to the protocol section.

Thank you for the comment. We have updated the references throughout the manuscript including the protocol section.

Below please see our responses to the comments from the editorial, reviewers #1, #2, #3 and #4 of the first review.

Editorial comments:

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. Please employ professional copyediting services if needed.

Thank you for the suggestion. We have carefully revised the manuscript.

2. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

An ethics statement is added accordingly. Please refer to lines 89-90 of the manuscript.

3. 2.2: Please describe how to isolate the embryos.

We have now included a section to describe rat embryo isolation. Please refer to lines 103-119 of the manuscript.

4. Please specify all surgical tools used throughout the protocol.

The surgical tools used are now included in both the protocol and the material list.

5. 4.4: At what temperature are the cells fixed?

The temperature for fixing the cells is now included. Please refer to lines 214-215 of the manuscript.

6. Discussion: As we are a methods-based journal, please discuss critical steps in the protocol, modifications and troubleshooting of the method, and limitations of the method.

Thank you for the suggestion. We have updated our manuscript accordingly. Please refer to the discussion section of the manuscript.

7. References: Please do not abbreviate journal titles; use full journal name.

We apology for the mistake. We have downloaded the most updated JoVE EndNote style for reference formatting in the revised manuscript.

Reviewer #1

Major Concerns:

The procedure has been extensively used in many other publications, and there are no improvements provided neither in efficiency nor in simplicity.

As novelty is not a requirement for publication, this comment is disregarded.

Reviewer #2

Major points

Protocol section

- The transfection protocol reported is quite general and lacks important information and/or tricks that could explain for example the high transfection efficiency claimed by the authors

We apologize for missing the information about transfection. We have now included the information. Please refer to lines 199-205 of the manuscript.

We also apologize for the improper presentation of data in Figure 2A. We aimed to illustrate the co-transfection efficiency of FE65 and EGFP in the neurons in the figure. In the same transfection, approximately 80% of transfected neurons contained both FE65 and EGFP. This suggest that the co-transfection efficiency of FE65 and EGFP to neurons is quite high (Not the general transfection efficiency). We have now labeled the Figure 2A more clearly.

- Section 5 "Measurement of neurite growth" needs to be expanded. This is probably the most important part of the protocol and image processing is usually complex. A figure/video guiding the researcher in this operation would be appropriate

Thank you for the suggestion. We shall include a video during JoVE video preparation to illustrate how we perform neurite measurement by using ImageJ.

- Please reports the composition of solutions

We apologize for missing some of the information. We have now updated our manuscript and the material list to include all the composition of solutions that were prepared in our lab.

- 3.4 paragraph. Dissociate the tissue gently by trituration using 1mL pipette. It is impossible to dissociate a tissue (even the brain) simply by pipetting. Trypsin is normally included in the step.

We apologize for missing such important information in our protocol. The protocol has been updated. Please refer to lines 143-145 of the manuscript.

- Lipofectamine 2000 can be toxic. It is not clear how long the cells have been incubated with lipofectamine. This information is quite critical and must be reported or at least discussed.

Thank you for the suggestion. We have now included a section in the discussion to discuss the toxicity issue regrading lipofection. Please refer to lines 359-370 of the manuscript.

Representative results

Fig.1. To give an idea of transfection efficiency, it would be better to include representative images of GFP-transfected neurons under control or after incubation with Cyto D and NGF.

Fig.2. To give an idea of co-transfection efficiency, it would be better to include a representative image of GFP-transfected neurons double stained with FE65

Thank you for the suggestions. We have updated both Figures 1 and 2 accordingly.

Discussion

The discussion is very limited. There are several points that authors should discuss:

- Cell density and transfection efficiency may be critical for detecting neurite outgrowth. In fact, if the cell density and/or transfection efficiency were high, it would be difficult to identify each single cell outline and thus correctly measure neurites extension.

- The observation time. Usually neurons acquire a fully mature phenotype after several days in culture, but the protocol reports neuron transfection and observation within 3 days from dissection. This may impact the effect of protein or drugs.

- the gene dosage

-The expression time of protein of interest and GFP

-The plasmid. Co-transfection could be quite critical. Indeed, only in case of high transfection efficiency reasonably every cell contains both plasmids and expresses both proteins. Polycystronic plasmids could be more efficient.

Thank you for the suggestions. We have performed new experiments to illustrate the expression of POI (i.e. FE65) and EGFP at different time points (Figure 2C) and the effect of gene dosage (Figure 2D). Moreover, we have also discussed the issues suggested by reviewer 2. Please refer to the discussion section (lines 372-398).

Minor Concerns:

- The text would benefit from English language and style revision. There are several typos and grammatical errors. Ex Row 77 resin o rinse?

We apologize for the mistakes. We have carefully revised the manuscript.

- The following paper should be cited. Yeyeodu et al, A Rapid, Inexpensive High Throughput Screen Method for Neurite Outgrowth. Current Chemical Genomics, 2010, 4, 74-83.

Thank you for the suggestion. We have now included the reference. Please refer to line 65 of the manuscript.

Reviewer #3

Major Concerns:

1. In the introduction the authors argue that PC12 cells are not a good model for primary CNS neurons as a rationale for their protocol for culturing primary rat cortical neurons. Since numerous papers have used primary neurons for studying axon growth and guidance mechanisms, I don't find this to be a strong rationale and the authors cite only a single publication in which PC12 cells were used to study neurite outgrowth. This is not a fair representation of the field.

We agree with reviewer 3 that stronger rationales are needed to justify the use of primary neurons over neuronal cell lines. We have revised our manuscript accordingly. Please refer to lines 64-75 of the manuscript.

2. The authors argue that the low transfection rate of primary neurons is a limitation for using them to study axon outgrowth mechanisms. However, their methodology does not seem to improve low transfection efficiency and the authors do not quantify this efficiency. This limits how precisely effects of FE65 on axon outgrowth can be determined.

Thank you for the comment. In fact, our protocol is not attempted to improve the transfection efficiency of primary neurons. Instead, it provides an easy way for the identification of the neurons which has been transfected with a protein of interest (POI) by co-transfecting with EGFP. Since our data has suggested that (Figure 2A) the co-transfection efficiency (not general transfection efficiency) of a POI (FE65) and EGFP is around 80%, most EGFP transfected primary neurons should also be co-transfected with the POI. Therefore, the effect of POI on neurite outgrowth could be determined by measuring the neurite length of the EGFP labeled neurons.

3. The authors use only a single method for transfection although other labs have used electroporation which is an improvement on transfection of primary neurons with Lipofectamine. In this respect the protocol is somewhat out of date.

Thank you for the comment. We agree with reviewer 3 that transfection efficiency would be improved by using electroporation for the extremely difficult transfect cells such as human iPSCs. Therefore, the use of electroporation and other gene delivery methods are discussed. Please refer to lines 359-370 and 389-398 of the manuscript.

4. While novelty is not a criterion for JOVE, this manuscript largely describes well established and widely used methods for culturing cortical neurons along with transfection approaches that do not improve upon transfection efficiency. Further, the authors fail to consider other techniques such as electroporation which may improve efficiency of transfection. The authors in the discussion suggest that their protocol for co-transfection will be useful for studying neurite outgrowth of human iPSC derived neurons. Since they show that primary rat cortical neurons are difficult to transfect and human iPSCs are even more.

Thank you for the comment. As stated in our response to reviewer 3's concerns 3, gene delivery for "extremely difficult transfect cell types" such as human iPSCs could be improved by using electroporation. We have therefore updated our manuscript. Please refer to lines 389-398 of the manuscript.

Minor Concerns:

The manuscript contains some lapses in English usage and grammar.

We apologize for the mistakes. We have carefully revised the manuscript.

Reviewer #4

Major Concerns:

The study is interesting. However, the authors did not provide any data to demonstrate why their method is superior to current methods for neuronal transfection with EGFP. These data are essential to support the authors' conclusion.

Thank you for the comment. As stated in the introduction section, neuronal cell lines are widely used for studying neuronal processes including neurite outgrowth as they are relatively easy to manipulate and transfect. However, the exact neurite outgrowth response may be affected by genetic drift and the lack of expression of certain neuronal molecules in these neuronal cell lines. Therefore, it is more desirable to use primary neurons to study neurite outgrowth. Our manuscript aims to provide a detailed protocol for studying neurite outgrowth in primary neurons which are known to have low transfection efficiency. In our protocol, the neurons transfected with a POI can be determined by co-transfecting with EGFP (the co-transfection efficiency of POI and EGFP is over 80%. Please see Figure 2A). Therefore, this is a major advantage of our protocol. We have now revised our manuscript with additional information. Moreover, we have included new data to illustrate the expressions of FE65 (i.e. the POI) and EGFP at different time points (Figure 2C). As the two proteins still expressed on day 7 post-transfection, this suggests that our protocol can be used in both developing and mature neurons. Additionally, immunofluorescence staining, which is used for classical neurite outgrowth study, could be omitted in our protocol. We have included a new section to discuss this advantage. Please refer to lines 350-357 of the manuscript.

Since we have positively addressed the comments presented by the 7 reviewers, we hope that you will now find our manuscript suitable for publication in the JoVE.

Yours sincerely,

Kwok-Fai Lau. PhD.