Near-Infrared Light-Controlled Gene Expression and Protein Targeting in Neurons and Non-neuronal Cells

Taras A. Redchuk, Maksim M. Karasev, Evgeniya S. Omelina and Vladislav V. Verkhusha

Near-infrared (NIR) light-inducible binding of bacterial phytochrome BphP1 to its engineered partner, QPAS1, is used for optical protein regulation in mammalian cells. However, there are no data on the application of the BphP1–QPAS1 pair in cells derived from various mammalian tissues. Here, we tested the functionality of two BphP1–QPAS1-based optogenetic tools—an NIR- and blue-light-sensing system for control of protein localization (iRIS) and an NIR light-sensing system for transcription activation (TA)—in several cell types, including cortical neurons. We found that the performance of these optogenetic tools often relied on physiological properties of a specific cell type, such as nuclear transport, which could limit the applicability of the blue-light-sensitive component of iRIS. In contrast, the NIR-light-sensing component of iRIS performed well in all tested cell types. The TA system showed the best performance in cervical cancer (HeLa), bone cancer (U-2 OS), and human embryonic kidney (HEK-293) cells. The small size of the QPAS1 component allowed the design of adeno-associated virus (AAV) particles, which were applied to deliver the TA system to neurons.

Precise control of cell physiology with high spatial and temporal resolution is in high demand in basic studies and biomedical applications. One of the several existing approaches to this problem—optical control of protein activity and protein–protein interactions with genetically encoded constructs (also referred as non-opsin optogenetics)—looks promising because of robust performance and high resulting precision. A variety of proteins naturally sensitive to light was used to develop the tools for such optical manipulation. Small and structurally well-characterized light-oxygen-voltage-sensing (LOV) domains were widely used for the design of optogenetic tools based on homodimerization, heterodimerization, and structural rearrangements (caging–uncaging). Examples of biological applications of LOV domain-based tools include regulation of protein localization, transcription activation in cells and animals, and control of receptor activity. Similar schemes were implemented by using blue-light-using FAD (BLUF) domains and cryptochromes. For instance, cryptochrome-derived heterodimerizers Cry2 and CIB were used for Cre recombinase-regulated gene expression. Although successfully used in many promising applications, all of these tools sense blue light, thus limiting their compatibility with popular probes for visualization and optical control excited within the same blue-green spectral range.

Unlike blue-light-sensing LOV and BLUF domains and cryptochromes, phytochromes are proteins absorbing light within the far red to near-infrared (NIR) spectral regions. Phytochrome from Arabidopsis, PhyB, along with its interacting partners, was used for the development of optogenetic tools based on light-controlled heterodimerization. It was applied for transcription activation, manipulation of cell morphology, and precise protein targeting in living fish embryos. As it is derived from a plant, PhyB requires phycocyanobilin for functioning, which is not produced by animal cells and therefore must be added exogenously or synthesized within the cells by additionally overexpressed enzymes.

Recently, several optogenetic systems were developed by using bacterial phytochromes as templates. Bacterial phytochromes incorporate biliverdin (BV) as a chromophore. BV is found in mammalian cells as a product of heme degradation, and, therefore, bacterial phytochromes work in most animal cells without additional non-native chemical compounds. A bacterial phytochrome from Rhodopsseudomonas palustris, RpBphP1 (hereafter BphP1), was used for light-controlled cell signaling manipulation and transcription activation. Subsequently, the engineered version of its interacting partner, PpsR2 (termed QPAS1), was used to design the dual-color (NIR and blue) light-sensing system for control of protein localization (iRIS).

Although a vast number of non-opsin optogenetic tools exist, with applications in mammalian cells and whole body applications, their performance can vary dramatically in different cell types. At the same time, the applicability of the...
tool to a certain experimental model relies strongly on specific features of cell physiology.

To explore this issue, in this study, we tested the performance of a BphP1–QPAS1 optogenetic system in mammalian cells of different origins, including neuroblasts and primary neurons. To analyze the protein transport machinery, which is crucial for the functionality of intracellular localization of optical controllers, we utilized a tool combining BphP1 and AsLOV2, called iRIS. Then we tested the BphP1–QPAS1 system for the ability to activate transcription in the same cells. Lastly, we took advantage of the small size of QPAS1 to address the problem of optogenetic system delivery to the neuronal cells. For this, we used adeno-associated viruses (AAVs) to deliver the BphP1–QPAS1 system for gene regulation to activate the transcription in primary neurons.

Results

Optical control of protein localization

To assess the functionality of a BphP1–QPAS1-based system in protein targeting applications, a series of mammalian cells of different origins were transfected with an iRIS construct (Figure 1). In the NIR-light-controlled component of iRIS, a CAAX motif (-CVIM) is utilized to target the protein of interest to the plasma membrane. In the blue-light-controlled component, nuclear localization of the protein of interest is achieved by the balanced action of nuclear localization (c-Myc NLS) and nuclear exclusion signals (Smad4 NES). An mCherry tag is used for visualization.

As observed by epifluorescence microscopy, in darkness, the iRIS-controlled protein was localized in the cytoplasm, showing slightly lower fluorescence intensity in the nucleus (Figure 2). Under NIR light of 740 nm (1 mW cm$^{-2}$), all tested cell types showed robust protein relocalization from the cytoplasm to the plasma membrane, where it decorated cell periphery and filopodia (Figure 2, Figure S1 in the Supporting Information). Under blue light of 460 nm (1 mW cm$^{-2}$), the nuclear accumulation of mCherry was observed in all cell types studied, except neurons and neuroblastoma-derived Neuro-2a cells. In Neuro-2a cells, under blue illumination, the protein of interest was observed on the plasma membrane, decorating the neurites, and in juxtanuclear compartments, which presumably represented aggresomes. Interestingly, another neuroblastoma-derived cell line, SH-SY5Y, showed normal nuclear mCherry localization under blue light. In rat primary cortical neurons, similarly to Neuro-2a, mCherry fluorescence decreased in the cytoplasm under blue light, but no nuclear fluorescence was observed. In some cell types, such as HEK-293 human embryonic kidney cells (Figure 2, bottom panel) under 460 nm illumination, a considerable amount of protein was observed in the plasma membrane. Likely, this was caused by a rather high iRIS

![Figure 1](image1.png)

**Figure 1.** Schematic representation of light-controllable protein targeting by using the NIR blue-light-inducible shuttle (iRIS). In cells transfected with iRIS, under 740 nm light, NES-mCherry-QPAS1-AsLOV2cNLS is relocalized to plasma membrane-bound BphP1-mVenus (NIR light-controlled component). Under 460 nm light, the same construct is relocalized to nucleus, due to uncaged nuclear localization signal in AsLOV2 (blue-light-controlled component). Localization of protein of interest is shown in red.

![Figure 2](image2.png)

**Figure 2.** Tri-directional protein targeting in living mammalian cells transfected with iRIS, controlled with 740 nm and 460 nm light. Under 740 nm illumination, the protein is observed on the plasma membrane; under 460 nm light, the protein is expected to be localized in the nucleus. Scale bar: 10 μm.
expression level and should be corrected either by reducing the amount of plasmid used in transfection or by using a weaker promoter for iRIS expression.

Quantification of fluorescence intensity showed a 35–50% signal decrease in the cytoplasm upon 740 nm light in all studied cell types (Figure 3A), which reflects the cytoplasm-to-membrane relocalization. In accordance with visual observations, quantitative analysis of mCherry distribution under 460 nm showed no significant increase in the nucleus-to-cytoplasm ratio in Neuro-2a neuroblasts and an insignificant increase (up to a 1.1:1 ratio) in primary neurons, whereas the other cell types showed a 1.3–1.5-fold increase (Figure 3B).

To further characterize the system for light-controlled subcellular protein targeting, we tested how long iRIS could continue to cause protein relocalization. As the reversibility of the LOV-based part of iRIS was previously characterized, we performed time-lapse imaging under NIR illumination, followed by thermal relaxation in the darkness, in sequential cycles. To achieve the maximal number of cycles within a time-lapse imaging session of reasonable duration, duration of 740 nm illumination was reduced to 5 min, which led to about a 20% decrease in the fluorescence level in the cytoplasm, whereas longer incubation caused a 40% decrease in epifluorescence microscopy. We found that at least seven cycles of iRIS relocalization from cytoplasm to the plasma membrane could be performed (Figure 3C).

In addition to epifluorescence microscopy, we employed confocal imaging to better characterize iRIS protein targeting of the plasma membrane. When imaged by epifluorescence microscopy, signal from the membrane is often mixed with the unspecific signal from the neighboring cellular environment. Moreover, blurred signal from the membrane, especially from filopodia, can appear in the cytoplasm, sometimes barely distinguishable from protein aggregates. Confocal microscopy enables avoiding of these effects, providing clear images of a particular optical section. In confocal images of HeLa cells expressing iRIS, after 10 min of light of 740 nm, a cell perimeter was clearly visible and highlighted with signals from filopodia (Figure 4A). The cytoplasm showed an even distribution of mCherry signal, which was approximately 60% lower than signal

Figure 3. Change in mCherry fluorescence levels of iRIS in different compartments under 740 nm and 460 nm illumination. A) mCherry fluorescence levels of iRIS as measured in the cytoplasm in the dark (gray) and after 10 min of illumination at 740 nm (pink). Error bars represent S.E.M., n = 5 cells for each bar. B) Nucleus-to-cytoplasm ratio of mCherry fluorescence of iRIS in the dark (gray) and after 10 min illumination at 460 nm (cyan). Error bars represent S.E.M., n = 5 cells for each bar. C) mCherry fluorescence levels of iRIS as measured in the cytoplasm of HeLa cells in cycles of 5 min illumination at 740 nm, followed by 20 min incubation in the dark. Error bars represent S.E.M., n = 5 cells for each bar. Images were acquired with an epifluorescence microscope.

Figure 4. NIR-light-driven relocalization of the iRIS protein, imaged with confocal microscopy. A) HeLa cells expressing iRIS. Scale bar: 10 μm. Dashed line marks the region used for profile plotting. B) Intensity profiles of mCherry fluorescence in cells shown in panel A. C) Cortical neuron expressing iRIS. An asterisk marks the neuron body, and the inset shows the body enlarged. Scale bar: 10 μm. Dashed line marks the region used for profile plotting. D) Intensity profiles of mCherry fluorescence in cell shown in panel C.
from the plasma membrane after illumination (Figure 4B). Similarly, in primary cortical neurons expressing iRIS, under 740 nm light, mCherry signal was localized at the cell perimeter (Figure 4C). In fluorescence intensity profiles, the plasma membrane appeared as sharp peaks, with a decrease in cytoplasmic fluorescence of about 50% compared to the intensity at the plasma membrane (Figure 4D).

Together, these data suggest that the BphP1–QPAS1 pair can be used in all tested cell types, including neurons. However, further optimization of the blue-light-sensing part of the iRIS system is needed to efficiently work in neuronal cells.

Light-controlled transcription regulation

We further analyzed the performance of the BphP1–QPAS1 optogenetic pair as applied to transcription control in different mammalian cell types. For this, a set of cells corresponding to those tested with iRIS was cotransfected with pQP-T2A plasmid and luciferase reporter, controlled by GAL4 upstream activation signal sequences (GAL4-UAS) (Figure 5). The pQP-T2A plasmid encodes BphP1 fused to a VP16 transactivator and QPAS1 fused to a GAL4 DNA-binding domain and tagged with SV40 NLS (PKKKRK). Under NIR illumination, the GAL4 DNA-binding domain and VP16 transactivator associate due to light-induced BphP1–QPAS1 interaction. Being in the nucleus, reconstituted transcription factor drives the expression of the GAL4-UAS-controlled reporter gene.

Most of the tested cell lines showed several-fold elevation in luciferase signal under NIR light, as compared to signal in darkness (Figure 6). The highest light-to-dark contrast was observed in HeLa, HEK-293, and U2 OS cells (28.0-, 8.9-, and 6.1-fold, respectively). Lower light-to-dark contrast was observed in COS-7, SH-SY5Y, and Neuro-2A cells, which was in accordance with data on optical control of protein targeting (Figure 3B), as transcription activation partly depends on nuclear localization of the BphP1–VP16 and NLS–GAL4–QPAS1 complexes, driven by nuclear localization signal.

Viral gene delivery of the BphP1–QPAS1 system to neurons

Primary cell cultures are often considered hard to transfect, and primary hippocampal and cortical neurons are sometimes considered some of the most difficult targets for gene transfer. Electrical transfection methods are reported to irreversibly affect cellular physiology and reduce neuron viability; chemical transfection methods (such as calcium phosphate transfection and lipofection) usually provide low transfection efficiency, especially in non-dividing cultures. To overcome these limitations, we decided to use a viral system for the delivery of the BphP1–QPAS1 pair to primary neuronal cultures.

We chose adeno-associated virus (AAV) serotype 9 because it was shown that this serotype effectively and specifically transduces neurons in mice and rats. As mammalian promoters are advantageous in some cases compared to viral promoters, we decided to use the CaMKII promoter to drive BphP1–QPAS1 expression in neurons. The shortened WPRE–PolyA expression cassette, the small size (519 bp) of QPAS1, and the general optimization of vector architecture allowed us to pack the whole BphP1–QPAS1 system (3684 bp) for transcription activation into a single AAV vector (Figure 7A).

Figure 5. Schematic representation of light-controllable gene transcription activation. The BphP1–VP16 fusion is localized in the cytoplasm in the dark. Under NIR light, it interacts with QPAS1 fused to the GAL4 DNA-binding domain. The complex is transported to the nucleus because of the presence of nuclear localization signal in the QPAS1–GAL4 construct, leading to activation of reporter gene expression (highlighted in green).

Figure 6. Change in luciferase expression under NIR light in mammalian cells of different origin cotransfected with pQP-T2A and luciferase reporter plasmids. Cells were kept in darkness or under 740 nm illumination. For each cell type, the luciferase induction level was normalized to the level of cells kept in darkness. Error bars represent S.E.M., n = 3. Luciferase levels for cells kept in darkness, cells illuminated with light at 740 nm, and cells transfected with reporter construct only can be found in Figure S2.

Figure 7. Viral gene delivery of the BphP1–QPAS1 system for transcription activation to primary cortical neurons. A) Constructs used for AAV production. B) Luciferase expression level in neurons transduced with AAV carrying constructs shown in A) and kept in darkness and under 740 nm light. Error bars represent S.E.M., n = 3.
UAS-driven luciferase reporter, with junk DNA added to reach the optimal length for AAV packaging, was delivered in separate AAV particles. In transduced primary rat cortical neurons, the luciferase signal was 2.5-fold higher under NIR light than in darkness (Figure 7B). Thus, we were able to apply, for the first time, an AAV gene transfer system for delivery of a bacterial phytochrome-based optogenetic system to primary neurons. Among viral gene delivery approaches, AAV-based systems are generally regarded as superior for neuronal cultures because of low toxicity, high efficiency, and safety.[21]

**Discussion**

In this study, we tested the applicability of optogenetic systems for protein targeting (iRIS) and transcription activation (TA), developed from a bacterial phytochrome, to mammalian cells of different origins. We found that the performance of BphP1–QPAS1-based systems varied significantly in different cells (Table 1). Particularly, in experiments with iRIS, poor efficiency of cytoplasm-to-nucleus relocalization was observed under blue light in Neuro-2A cells and in neurons. This shifted NLS/NES equilibrium in neuroblasts and neurons can be explained by differences in the active nuclear transport mechanisms between cells of neuronal and non-neuronal origins. In non-neuronal cells, in the cytoplasm, proteins carrying an NLS sequence bind importin-β and importin-α complexes. After transition through the nuclear pore, NLS-tagged proteins are released in the nucleus, and the importins are recycled.[20] In contrast, in cells of neuronal origin, due to their polar phenotype, importins are involved not only in cytoplasm-to-nucleus transport but also in synapse-to-nucleus signaling.[22] Thus, nuclear import machinery components are distributed in the cell in a different way, being localized not only in somal parts and the nucleus but also in neurites and partly immobilized by cytoskeleton.[25,26]

The TA system also showed remarkably varied results among the tested cells; this was in accordance with studies of other chemically[27] and optically[7,26] controlled systems tested in different cell types. Notably, the performance of the TA system depended not only on transcription factor cellular localization, controlled by nuclear import machinery, but also on the BphP1–QPAS1 interaction, which added an additional level of control to the system. In the dark, dissociated GAL4 and VP16 do not activate transcription. This might explain the available increase in reporter expression, despite the reduced relocalization functionality in certain cell types. Performance of the BphP1–QPAS1 pair in both described applications could be improved by further engineering of BphP1, aimed at reducing undesirable binding of QPAS1 in the dark. For this, similar to other light-sensing proteins,[13] directed molecular evolution and structure-based protein engineering of BphP1 could be used.

Our data suggested that the NIR protein targeting and TA systems could be used in various cell types; however, they require additional adjustment for neuroblastoma cells and neurons. Although a considerable number of optically controlled tools have been proposed to study neuronal functions,[29] the NIR-light-controlled tools are represented rather poorly in neuroscience. We anticipate that our results will be a starting point for further development and use of BphP1-based systems in neuronal tissues. Together with the NIR fluorescent proteins used for advanced imaging in neurons,[30–32] NIR optogenetic tools should be superior for multiplexing with blue/green-light-sensing constructs, including opsin-based actuators and inhibitors.

### Experimental Section

**Mammalian cell culture:** Human epithelioid cervix carcinoma (HeLa) cells, human bone osteosarcoma epithelial cells (U-2 OS), human embryonic kidney cells (HEK-293), African green monkey fibroblast-like cells (COS-7), human bone marrow neuroblastoma cells (SH-SY5Y), mouse neuroblastoma cells (Neuro-2a), and mouse embryonic fibroblast cells (NIH/3T3) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotic-antimycotic (Gibco). Effectene transfection reagent (Qiagen) was used for transfection. The culture medium was changed 6 h after transfection with a new one containing biliverdin (BV; 25 μM).

**Cell light activation and imaging:** Epifluorescence microscopy was performed by using an Olympus IX83 equipped with a 200 W

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**Table 1. Performance of the BphP1–QPAS1-based iRIS and TA optogenetic systems in cells of different origin.**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Organism</th>
<th>Tissue</th>
<th>Disease</th>
<th>Change under 740 nm [%][31]</th>
<th>iRIS Change in nucleus/cytoplasm ratio under 460 nm [19]</th>
<th>TA Light-to-dark signal ratio under 740 nm [12]</th>
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</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>Homo sapiens</td>
<td>cervix</td>
<td>adenocarcinoma</td>
<td>39</td>
<td>0.48</td>
<td>28.0</td>
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<td>U-2 OS</td>
<td>H. sapiens</td>
<td>bone</td>
<td>osteosarcoma</td>
<td>49</td>
<td>0.43</td>
<td>6.1</td>
</tr>
<tr>
<td>HEK-293</td>
<td>H. sapiens</td>
<td>kidney</td>
<td>normal condition</td>
<td>45</td>
<td>0.49</td>
<td>8.9</td>
</tr>
<tr>
<td>COS-7</td>
<td>Cercopithecus aethiops</td>
<td>kidney</td>
<td>SV40 transformed</td>
<td>46</td>
<td>0.43</td>
<td>2.6</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>H. sapiens</td>
<td>bone</td>
<td>neuroblastoma</td>
<td>36</td>
<td>0.33</td>
<td>3.0</td>
</tr>
<tr>
<td>Neuro-2a</td>
<td>Mus musculus</td>
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<td>neuroblastoma</td>
<td>29</td>
<td>0.05</td>
<td>2.5</td>
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<tr>
<td>Primary cortical</td>
<td>Rattus norvegicus</td>
<td>brain, cortex</td>
<td>normal condition</td>
<td>53</td>
<td>0.29</td>
<td>2.5</td>
</tr>
</tbody>
</table>

[a] Percent decrease in mCherry fluorescence in the cytoplasm as a result of protein relocalization from cytoplasm to plasma membrane; indicates the performance of NIR-sensitive component of iRIS. [b] Change in nucleus-to-cytoplasm signal ratio as a result of protein relocalization from cytoplasm to nucleus; indicates the performance of blue-sensitive component of iRIS. [c] Light-to-dark signal ratio in cells transfected with pQP-T2A plasmid for TA, measured by luciferase assay. [d] Detected in primary neurons transduced with AAV particles carrying the TA system.
metal halide arc lamp (Lumen 220PRO, Prior) and an optiMOS sCMOS camera (QImaging). Cells transfected with pQ-P-iris[3](Addgene #102584) were imaged by using a 60×, 1.35 NA oil objective lens (UPlanSapo, Olympus). During imaging, cells were kept in live cell imaging solution (Life Technologies–Invitrogen) at 37 °C. The data were analyzed by using SlideBook (v.6.0.8; 3λ, and ImageJ [v.1.50b] software.[33] For the relocation assay, blue and NIR illumination was applied by using the 460/20 nm and 740/25 nm custom-assembled LED arrays (LED Engin), respectively. First, cells were illuminated for 10 min with 740/25 nm LED (1 mW/cm²), then, after 30 min in darkness for relaxation, the dish was illuminated with 460/20 nm LED (1 mW/cm²). Focusing of the microscope was performed in an mCherry excitation channel to prevent unspecified activation of blue- and NIR-light-sensing components. CELView glass-bottomed dishes (Greiner Bio-One) were used for imaging. Confocal imaging was performed by using a Leica TCS SP8 X microscope equipped with a 63×, NA 1.4 and HC PL APO CS2 objective and a white light laser (470–670 nm).

After image acquisition, the images were analyzed by using Fiji software (v.1.50b).[34] First, the background fluorescence was subtracted. Then, five circular regions of interest (ROI) were placed randomly in the cytoplasm and nuclei of the imaged cells while avoiding aggregates, filopodia, and nucleoli. The mean fluorescence intensity was calculated for each ROI. After compensation for photobleaching, the mean values were calculated for 5–10 cells in each experimental group. The nucleus-to-cytoplasm ratio was calculated for cells illuminated with 460 nm light, and normalization to levels in the dark was performed for cells kept under 740 nm illumination. Data were plotted by using OriginPro (Origin Labs; v.8.6).

**Firefly luciferase (Fluc) assay:** For light-controlled transcription activation, cells were cotransfected with the constructs pQP-T2A[31] (Addgene #102583) and pFR-Luc (Agilent Technologies) in a 3:1 ratio in 24-well plates (Greiner Bio-One). Illumination of plates was performed directly in a CO₂ incubator with 740/25 nm light (0.2 mW cm⁻²) in alternating cycles of 30 s light and 180 s darkness. To measure Fluc activity, transfected cells were lysed 48 h after transfection. Cells were washed with phosphate-buffered saline (PBS), and lysis buffer (100 µL; 20 mM Tris-HCl, pH 8.0, 10 % glycerol, 0.1 % β-mercaptoethanol, 0.1 % Triton X-100, 1 mM PMSF) was added to each well of a 24-well plate and incubated on ice for 30 min. Cell lysates (10 µL) were mixed with firefly luciferase assay reagent (20 µL, NanoLight Technology) in 96-well half-area white plates (Costar). Bioluminescence signals were measured by using a Victor X3 multilabel plate reader (PerkinElmer).

**Neuronal culture and transfection:** Primary rat neuronal cultures were prepared at the Neuronal Cell Culture Unit at the University of Helsinki. All animal work was performed in accordance with the ethical guidelines of the European Convention and regulations of the Ethics Committee for Animal Research of the University of Helsinki. Dissociated cortical neurons were plated to 24-well plate at a density of 10⁵ cells per well, coated with poly-L-lysine (0.1 mg mL⁻¹, Sigma–Aldrich) in a neurobasal medium (Gibco), supplemented with B27 (Life Technologies/Invitrogen), L-glutamine (Invitrogen), and penicillin–streptomycin (Lonza). Neurons were transduced after three days in vitro (DIV3); each AAV was used at a concentration of 10⁶ genome copies per well. 740/25 nm illumination (0.2 mW cm⁻²) in alternating cycles of 30 s light and 180 s darkness started on DIV6, and cells were lysed on DIV9 for the Fluc assay. BV (25 µm) was added to the culture medium on DIV3. The Fluc assay was performed as described above.

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**Conflict of Interest**

The authors declare no conflict of interest.

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