Photonic Nanojet-Mediated Optogenetics

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Optogenetics has become a widely used technique in neuroscience research, capable of controlling neuronal activity with high spatiotemporal precision and cell-type specificity. Expressing exogenous opsins in the selected cells can induce neuronal activation upon light irradiation, and the activation depends on the power of incident light. However, high optical power can also lead to off-target neuronal activation or even cell damage. Limiting the incident power, but enhancing power distribution to the targeted neurons, can improve optogenetic efficiency and reduce off-target effects. Here, the use of optical lenses made of polystyrene microspheres is demonstrated to achieve effective focusing of the incident light of relatively low power to neighboring neurons via photonic jets. The presence of microspheres significantly localizes and enhances the power density to the target neurons both in vitro and ex vivo, resulting in increased inward current and evoked action potentials. In vivo results show optogenetic stimulation with microspheres that can evoke significantly more motor behavior and neuronal activation at lowered power density. In all, a proof-of-concept of a strategy is demonstrated to increase the efficacy of optogenetic neuromodulation using pulses of reduced optical power.

1. Introduction

Optogenetics has been widely recognized as perhaps the most powerful tool for neuromodulation in the past decade, providing a significant boost to neuroscience research. Optogenetics allows for millisecond temporal resolution in regulating neuronal activity and potential single-cell spatial resolution, optogenetics could not only further our understanding of brain function but also reveal possible therapies for neuropsychiatric disease. Neuronal activity modulated by optogenetics mainly depends on optical gating of opsin channels that have been exogenously expressed in specific types of neurons. The opening of opsin channels by millisecond-long optical stimulation enables the generation of action potential or neuronal inhibition, making optogenetics a versatile and adaptable neuromodulation approach. However, there remains an important issue that opsin non-expressing cells are often activated at high optical intensity through other mechanisms (e.g., photothermal effects), although only opsin-expressing cells are expected to be the target. Therefore, the accuracy and specificity of optogenetics are not only a result of genetic modification of opsins, but also good control of optical power density.

The delivery of light in optogenetics often requires the implantation of an optical fiber located within 100 μm of the target neuron populations. Optical attenuation by tissue scattering and absorption, at the most commonly utilized wavelengths of 400–600 nm, causes an exponential decay of power density in brain tissue and results in quick power decrease. Power drops by 50% at a depth of 100 μm and 90% at 200 μm have been reported, thus limits the possible stimulation area only close to the tip of the optical fiber with sufficient photons. This complicates the balance between specificity and efficacy, because a higher optical power density is often applied to compensate for the power loss, which could lead to nontargeted cells being affected. Therefore, a strategy that could enable effective stimulation of neurons with lower optical power density could be highly desirable for the optogenetic paradigm.

A photonic nanojet (PNJ) is a highly focused light beam generated close to the shallow side of an illuminated lossless dielectric microcylinder or microsphere. It features subwave-
Figure 1. Dielectric microsphere enhanced optogenetics. A) Schematic illustration of optogenetics without a microsphere. The diverging laser beam is ineffective opening the ChR2 channel. B) When a PS microsphere is placed near the ChR2 protein, the beam is focused by the PS microsphere and can successfully open the channel. C) Simulated optical field distribution showing the input light diverging and attenuating along the propagation direction. D) The input light is focused into a small region at the shadowed surface of the microsphere. Inset shows that the waist radius of the focal spot is 600 nm. E) Power density of the focal spot of the PS microsphere as the function of its diameter. F) Power density collected by an optical fiber as a function of the distance between the fiber tip and microsphere. PS(+) pink line, PS(−) black line. Inset shows a schematic of the optical setup for the power measurement. G) Experimental optical field distribution indicates the input light diverges without the microsphere. H) When the microsphere is placed in the optical axis, the input light is focused because the microsphere acts as a microlens. Inset shows a SEM image of the PS microsphere. I) Power density collected by an optical fiber as a function of the input power. PS(+) red line; PS(−) black line. Inset shows the optical setup for the power measurement.

2. Results

2.1. Optogenetics Enhancement Scheme and Light Focusing by PS Microspheres

In this study, we used a transparent PS microsphere in vitro and in vivo to focus optical power locally. Our basic idea was that without a PS microsphere (PS(−)), the diverging light beam is ineffective in opening the ChR2 ion channel (Figure 1A). When a PS microsphere (PS(+)) is placed near the cell membrane, the light beam is strongly focused by the microsphere, and the increase in power density is enough to open the channel, allowing cations into the neuron, leading to activation (Figure 1B). A simulation was performed to investigate the light focusing property of PS microspheres using Comsol Multiphysics. In the simulation, the incident light was set as a Gaussian beam at a wavelength of 488 nm launched from a tapered fiber and the refractive index...
of PS microsphere was set as 1.6. Simulated optical field distribution shows the incident light diverging and attenuating along the propagation direction in the condition of PS(−) (Figure 1C). By placing a PS microsphere at the optical axis, the incident light was focused into a PNJ with a waist radius of 600 nm (Figure 1D). The focusing performance of the PNJ was related to the sizes of PS microspheres. For the 488 nm incident laser beam, the highest power density of the PNJ occurred at a microsphere diameter of 2.5 μm (Figure 1E). The calculated enhancement factor, which was defined as the power density of PS(+)/PS(−), decreased with the increase of the distance between the input fiber and microsphere (Figure 1F). The enhancement factor was calculated as 2–5 when the distance was less than 200 μm. Therefore, the distance between the fiber and microsphere was controlled within 200 μm for subsequent optogenetic experiments. The focusing property of PS microspheres was then experimentally investigated via Tyndall effect[^21] of nanoparticles suspended in the PS solution (Figure 1G,H). The PS microspheres were found capable of generating PNJs with the highest power density at microsphere diameter of 3.0 μm. The experimental results indicated a slight deviation with the simulation (2.5 μm) because the numerical model of the PS microsphere was totally homogeneous and transparent in the simulation. The enhancement factor was measured as ≈2 at a 150 μm distance between the fiber and microsphere with different incident optical powers (Figure 1I). Based on these data, we chose PS microspheres with a diameter of 3.0 μm for converging light and optical stimulation in subsequent in vitro and in vivo experiments.

### 2.2. Enhancement in Activation of ChR2 by PS Microspheres In Vitro

The ability to enhance neural activation by the PS microsphere-generated PNJs was first investigated in cells in vitro. Broadly, ChR2-expressing cells were stimulated with 488 nm light with or without PS present, and whole cell patch clamp recording was used to record both channel and neuronal activity (Figure 2A). To show the effect of the PNJs in opsin activation, ChR2 was overexpressed in 293T cells with lipofectamine-mediated plas-
mid transfection, in which very few ion channels are expressed endogenously. With pulsed light stimulation (wavelength: 488 nm; pulse duration: 100 ms), ChR2-293T cells showed inward currents dependent on the optical power density (Figure 2B), indicating the successful expression of ChR2. However, in the presence of microspheres (PS(+)−), inward current intensity was significantly increased compared with that in the absence of the microspheres (PS(−)) at the same optical power density (Figure 2B,C). The enhancement by PS ascended to a maximum of ≈150% at an optical power density of 0.5 mW mm\(^{-2}\) and decreased at higher densities due to the saturation of ChR2 channel opening (Figure 2D). In addition, to prove our concept that PS works as microlenses but did not depend on its own material characteristics, TiO\(_2\) was applied to investigate its effect of enhancing the activation of the ChR2-293T cells, and the results showed the similar effects with that of PS microspheres (Figure S1, Supporting Information). We next investigated whether the microspheres could enhance the activation of primary neurons expressing ChR2. Primary cultured hippocampal neurons were transduced by AAV9-hSyn-ChR2-mCherry, and channel expression was verified by neurons showing red fluorescence (Figure 2E) Notably, PS microspheres are visible near the neurons using brightfield microscopy. Pulsed optical stimulation (pulse duration: 20 ms) induced power-density-dependent inward currents, and PS(+) neurons showed significantly high inward current intensity at all tested power densities (Figure 2F,G). Interestingly, the enhancement by PS reached a peak of ≈121% at 1.4 mW mm\(^{-2}\) and then dropped between 1.4 and 2.6 mW mm\(^{-2}\), but ascended further at higher power densities (>2.6 mW mm\(^{-2}\)), which is not consistent with the case of ChR2-293T cells (Figure 2H). This is because neurons express other voltage-dependent cation channels which contribute to inward currents following by the activation of ChR2 at high power. Current clamp recording revealed that the rate of generated action potentials in primary neurons (power density: 2.0 mW mm\(^{-2}\); pulse duration: 10 ms) was significantly greater in PS(+) cells at lower power densities than PS(−) cells (Figure 2J). PS(+) and PS(−) showed a similar action potential rate at 3.1 mW mm\(^{-2}\), suggesting that ChR2 opening was saturated at this density (Figure 2J).

2.3. Regulating Neuron Activity with PS Microspheres Ex Vivo

We next evaluated the enhancement effect of our proposed strategy ex vivo. In order to exclude the effects of inhibitory neuron, AAVs coding for CamKII-ChR2-mCherry but not hsyn promoter used in the in vitro experiment were injected into the lateral ventricles of neonatal mice. The mice were sacrificed 14 days later with their brains were recovered and sliced for calcium imaging, microelectrode array (MEA) recording as well as membrane potential recording with patch clamp (Figure 3A). ChR2 was demonstrated to be successfully expressed in the hippocampus and partially cortex (Figure 3B). X-Rhod-1 was used as the intracellular calcium indicator in living brain slices. Fifty neurons expressing mCherry were selected, and the changes of their intracellular calcium stimulated with blue light for 1 min were recorded (Figure 3C). Optogenetic stimulation at 2.2 mW mm\(^{-2}\) could not increase the intracellular calcium in most neurons, even in PS(+) cells, but 3.5 mW mm\(^{-2}\) stimulation was significantly enhanced in PS(+) by ≈30% (Figure 3B). MEA recording was also performed to detect extracellular electric signals in brain tissue. The PS(+) showed significantly more spikes after both tested power densities, whereas PS(−) only showed a few spikes at 3.5 mW mm\(^{-2}\) (Figure 3D). Moreover, membrane potential recording with patch clamp was also applied to investigate the effect of PS on the neural firing after blue light stimulation. The results showed that neurons in the presence of PS exhibited more spikes than without PS after 1 min blue light stimulation (Figure 3E,F). These results were consistent with those from calcium imaging and MEA experiments. Thus, ex vivo experiments also confirmed the ability of PS microspheres to significantly enhance the efficiency of optogenetic stimulation at the same power density.

2.4. Enhanced Optogenetics by PS Microspheres In Vivo

As a further test of the system’s efficacy, we tried to apply our PNI-enhanced optogenetics scheme in vivo. The optical neural interface developed by the Deisseroth et al. is widely used for optogenetic neuromodulation but blue light used to gate ChR2 undergoes significant scattering and absorption in tissue leading to attenuation of power density. To demonstrate that the PS microsphere-based optical neural interface utilization of photons for neuromodulation in vivo, we used a locomotion-related behavior assay. AAVs coding for CamKII-ChR2-mCherry, with or without PS microspheres, were injected into M1 region, which controls motor activity (Figure 4A). Optical fibers were implanted in the M1 regions (depth: 50 μm) of a C57BL/6 mice 21 days post-transduction. To confirm the spatial distribution of the PS microspheres, some mice were injected with green fluorescent-labeled PS along with the virus. After implantation of optical fibers, we observed that ChR2-mCherry-labeled neurons were surrounded by PS with green fluorescence, below the optical fibers, representing the location of the microspheres (Figure 4A). An open-field behavior test was conducted during which blue light was switched from 1 min ON to 1 min OFF, and the motion trail of the mice was recorded (Figure 4B). At a relatively low optical power density (1.4 mW mm\(^{-2}\)), PS(−) mice showed the same activity as when the light was off, but the PS(+) mice obviously increased their locomotor behavior (Figure 4C) Notably, when the power density was increased to 5.6 mW mm\(^{-2}\) both PS(−) and PS(+) obviously increased locomotion, indicating the successful gating of ChR2 in both. PS(+) mice covered significantly increased distances and significantly increased their speed when the 1.4 mW mm\(^{-2}\) light was switched on at (Figure 4D–F). Both groups showed significant increases in locomotion at 5.6 mW mm\(^{-2}\). The M1 regions of mice treated with 1.4 mW mm\(^{-2}\) light were later stained for the activation marker c-Fos. PS(+) mice showed significantly greater c-Fos expression in the region, indicating effective enhancement of neuronal activation consistent with the locomotion data (Figure 4G, H). Crucially, we observed ChR2 saturation at higher power density (5.6 mW mm\(^{-2}\)), such that there was no significant difference between the PS(−) and PS(+) groups. This confirms that the ChR2 channels were successfully encoded and were functioning as expected. These results show that the optical power threshold for gating ChR2 in vivo was considerably reduced (≈75% reduction) by the effect of...
Figure 3. Microsphere-based optogenetics in modulating neuron activity in brain slice. A) Schematic illustration of ex vivo experiments for testing microsphere-based optogenetics. Newborn mice were injected in lateral ventricle with AAV9/CamKII-hChR2-mCherry, and brain slice were collected for calcium imaging, MEA recording as well as membrane potential patch-clamp recording after 14 days virus injection. B) Representative images of ChR2-mCherry expression in the brain after 14 days virus infection. (Scale bar in large field image: 100 μm; scale bar in enlarged image: 25 μm.) C) Bar chart shows the mean ± SEM of fifty neurons’ Ca²⁺ fluorescence changes induced by light stimulation. n = 50, ***p < 0.001, unpaired two-tailed t-test. D) Representative traces of light-induced neuronal activity in brain slice with ChR2 in with or without PS, obtained by MEA recording as well bar chart of the mean ± SEM that show the firing number, n = 3, *p < 0.05 unpaired two-tailed t-test. E) Membrane potential recording e after 1 min light stimulation in the brain slice with ChR2 in with or without PS. F) Summary bar graph showing the frequency of neuronal firing after 1 min of light stimulation with or without PS. n = 6–9, *p < 0.05, ** p < 0.01, unpaired two-tailed t-test.

PNJs, confirming that the optogenetic stimulation was indeed enhanced by the presence of PS microspheres.

2.5. Discussion and Conclusions

This study shows the application of PS microspheres as the microlenses to enhance the localized power density and reduce the optical power threshold for enhanced optogenetics with better efficacy and less off-target effect through in vitro, ex vivo, and in vivo experiments. These experiments demonstrate that PS microspheres can significantly enhance cellular calcium dynamics, inward current, electrical activity and signaling, and motor behavior in animals. Crucially, the PNJ effect of the PS microspheres greatly boosted the activation of neurons in vivo, helping to circumvent the issue of optical attenuation and scattering in brain tissues. The proposed PNJ-enhanced optogenetics could enable even greater spatial accuracy in optogenetic neuromodulation due to the reduction of off-target effects and the concentration of optical power around the microspheres. This research provides the first documentation of the microsphere-aided optogenetic enhancement.

Although optogenetics has become a powerful technique in facilitating the study of physiological functions of specific brain regions and even single cells, researchers always have concerns that nontargeted neurons could be inhibited by high-intensity optical stimulation, which may lead to false interpretation of the phenotypes by optogenetics not only because of the targeted neurons.
but also off-targeted neurons due to the photonic effect. Much effort has been made to develop new opsins, activatable by longer wavelength light to avoid thermal and photonic toxicity. For example, redshifted opsin channels have been applied in optogenetics, enabling lower-energy photons to regulate neuron activity directly compared with those at shorter wavelengths.\textsuperscript{[26,27]} In parallel, improving the photonic distribution is another possible strategy. Our study used microspheres as lenses to generate PNJs that concentrate the optical intensity in a localized area, thereby allowing light of lower power density to successfully stimulate targeted cells. That said, we consider the PS microspheres representative of the enhancement that may be achieved by microsphere lensing in general, not specifically microspheres of this material. Indeed, being made of a foreign material may lead to some undesirable biocompatibility issues, especially in the brain. Notably, PS microsphere has been shown biocompatible with the brain tissue.\textsuperscript{[28]} In a previous study by the authors of this paper, we have demonstrated that endogenous microstructures (e.g., red blood cells) existing in the body can also be applied as microlenses.\textsuperscript{[29]} Since PS microspheres were used in this work to generate PNJs,
our study can be viewed as a proof-of-concept example for the microlens-based enhancement strategy for optogenetics. In spite that microlens-based optogenetics enabled precise regulation of targeted neuronal activity, we have to confess the requirement of invasive optical fiber implantation still cannot be avoided. Therefore, other strategies for light delivery, such as focused ultrasound to guide the propagation and emission of light in the local brain tissue noninvasively can become complementary to the nanoprojector based optogenetics in the future studies.\[30,31]\n
Using microspheres for the formation of PNJs offer the possibility of tailoring their physical properties depending upon the intended application. Opsins that have been applied in optogenetics can be activated by light at different wavelengths. For example, ChR2, the most popular excitatory opsin, is gated by wavelengths from 460 to 490 nm, while NpHR, a popular inhibitory opsin, is sensitive to yellow light at 560 nm. Given the diversity of wavelengths required to stimulate commonly used opsins, the refractive index and size of the microspheres can be optimized for better performance. For example, a longer wavelength generally results in a larger focal length at the same microsphere size. Thus, when a longer wavelength is required for stimulation, a higher refractive material can be used to maintain the size and the depth of the focus, and vice-versa, in which TiO2 (a higher refractive index \( n_s = 2.73, n_i = 3.06 \)) may be the better choice that works as the microlenses. We consider this study as a guide for the future development and applications of microsphere-enhanced optogenetics.

To conclude, we have applied PS microspheres as the microlenses to enhance the localized field intensity and reduce the optical power threshold for optogenetics. The PNJ effect of the PS microspheres greatly boosted the inward currents of the target neuron at a relatively low optical power density, circumventing the optical attenuation and scattering issue in brain tissues. The proposed PNJ-enhanced optogenetics, which enables a more spatially refined modulating of the neuronal activity and avoids the side effects on nontarget neurons, may provide new light and thought in the development of the highly precise optogenetics.

3. Experimental Section

**Preparation of PS Microsphere Solution:** PS microspheres (Huge Biotechnology Co., Ltd, Shanghai, China) with an average diameter of 3 μm and a refractive index of 1.6 were used in the experiments. To improve the biocompatibility, the surfaces of the PS microspheres were modified with amino acids. PS microsphere solution was diluted to a suitable concentration of \( n_s = 5.0 \times 10^3 \) μL \(^{-1}\) with PBS buffer solution. The solution was then treated with an ultrasonic vibrator to prevent the aggregation of microspheres. Finally, the microspheres were injected into cell suspension or tissues with a microinjection syringe.

**Animals:** Eight-week-old C57BL/6J mice were used for ex vivo and in vivo experiments. Standard housing conditions, with 12 h light/dark alternation and food and water available ad libitum, were provided for all animals. All animal experiments were conducted in accordance with the university on animal experimentation, and approval by the Animal Ethics Committee and The Hong Kong Polytechnic University was obtained for all related procedures.

**Cell Culture:** All cells were incubated in a humidified incubator maintained at 37 °C with 5% CO\(_2\). Primary neurons were obtained surgically from mouse embryos at day 16 as described previously.\[33\] Cortices were then collected and sectioned in ice-cold Neurobasal medium and then incubated for 15 min in 0.25% trypsin–EDTA in a water bath at 37 °C. Cells were then collected by centrifuging and washing with Neurobasal medium, mechanically triturated with a pipette and then allowed to settle. The supernatant was discarded, cells were resuspended in medium containing 10% FBS + 2% B27 serum-free supplement + 0.25% l-glutamine, and seeded in 35 mm dishes containing poly-l-lysine (PLL)-coated coverslips at \( 5 \times 10^6 \) cells per dish. The medium was changed after 24 h to Neurobasal + 0.25% l-glutamine + 2% B27 + 1% penicillin–streptomycin and half exchanged every 72 h.

**Intracerebroventricular Viral Injection:** High-titer viruses (pAAV-CaMKIIa-hChR2(H134R-mCherry)) and (pAAV-hsyn-hChR2(H134R-mCherry)) were obtained commercially and stored at −80 °C prior to use. The ChR2-H134R gene was fused with that of the fluorescent reporter mCherry. The CaMKIIa promoter was used to preferentially transduce only excitatory neurons. Virus diluted 1:100 in PBS was added to primary neuron cultures at DIV7 at room temperature. For each dish with \( 5 \times 10^6 \) cell density, \( 10^6 \) genome copies were directly added into the cell medium. The plates were gently shaken and placed in the incubator for 3–5 days and the cell condition and fluorescence were closely monitored. Plates showing cells with successful transduction (evaluated by bright fluorescence) were used in further experiments.

**Patch Clamp:** Whole cell patch clamp was used for recording the inward currents and membrane potential in vitro. Borosilicate glass patch pipettes (Vitrex, Modulorh A/S, Herlev) were pulled by using a micropipette puller (P-97, Sutter Instrument Co.) and filled with pipette solution to have a resistance of 5–7 MΩ. Current clamp was applied for measuring membrane potential and voltage clamp was applied for measuring inward current. Transduction was confirmed by fluorescence imaging. The cells were put in bath solution containing (mM): NaCl 130, KCl 5, MgCl\(_2\) 1, CaCl\(_2\) 2.5, Hepes 20, and glucose 10 (pH 7.4). The pipettes filling solution contained \( 1 \times 10^{-3} \) M MgCl\(_2\), \( 138 \times 10^{-3} \) M KCl, \( 10 \times 10^{-3} \) M HEPES, \( 10 \times 10^{-3} \) M NaCl, and 2-mannitol compensated for osm 290 (pH 7.2). Digidata 1440B (Axon Instruments) and amplifier (Axopatch-700A, Axon Instruments) together with pClamp Version 9 software were used for data recording. The data were analyzed by using Clampfit 10.0. Neuronal membrane potential in acute brain slice recording was conducted with EPC10 patch clamp amplifier (HEKA) equipped with upright microscope with fluorescence (Olympus BX51W1). The pipette solution was the same with cultured cells and the live brain slice was bathed with gas-filled bubbled artificial cerebral spinal fluid (ACSF) containing (mM): NaCl 124, Glucose 10, KCl 3, NaHCO\(_3\) 26, CaCl\(_2\) 1, NaH\(_2\)PO\(_4\) 1.25, and MgSO\(_4\) 2. The gas comprised of 95% O\(_2\) and 5% CO\(_2\) to maintain a pH of 7.4 in ACSF.

**Acute brain slice preparation and calcium imaging:** Two weeks after intracerebroventricular viral injection, mice were anesthetized with isoflurane 5% in a carbon monoxide mixture delivered to an anesthesia induction chamber.
ber at 2 L min$^{-1}$. No reflexes in response to foot and paws pinch was ob-
served and then the mice were decapitated. Brains were taken out quickly
and sliced in ice-cold and gas-bubbled artificial cerebral spinal fluid (ACSF)
containing (mM): NaCl 124, Glucose 10, KCl 3, NaHCO$_3$ 26, CaCl$_2$ 2,
NaH$_2$PO$_4$ 1.25, and MgSO$_4$ 2. The gas comprised of 95% O$_2$ and 5%
CO$_2$ to maintain a pH of 7.4 in ACSF. Coronal sections 300 μm thick were
sliced by using a vibrating microtome (Leica VT1200S) and recovered in
37 °C gas bubbled ACSF for 1 h before experiments. The prepared acute
brain slices were stained with a fluorescent calcium indicator X-Rhod-1,
AM (X-14210, Invitrogen) at 4 × 10$^{-6}$ m final concentration in the rACSF
with gas bubbling at room temperature. A stained slice was then placed
into a confocal dish for microscopy.

The stimulation and imaging system consists of an inverted fluores-
cence microscope (IX73, Olympus) with Cy3 filter and a sCMOS cam-
era (ORCA-Flash4.0 LT Plus C11440-42U30, Hamamatsu) and 488
nm light shed from top down. Slices were imaged for 2 min before stim-
ulation for baseline calculation, then stimulated with only one shot (10
ms) blue pulse and imaged for another 5 min. The stimulation period
(10 ms) was not imaged. During imaging, a perfusion pump continuously
pumped fresh bathing solution (gas-bubbled rACSF) to keep the brain slice
in good condition.

**MEA Recording:** Multielectrode array (MEA) recording was performed
by USB-MEA256-System, which included an amplifier, four-channel stim-
ulus generator and two temperature-control units maintaining solution
and base plate at 33 °C. The MEA chamber was sterilized by UV for 2 h.
Mouse brain slices were presoaked in artificial cerebrospinal fluid (ACSF)
and centered on the MEA perfused with 3 mL min$^{-1}$ flowing ACSF. Slices
were stabilized for 1 h at 33 °C before recording. After stabilization, slices
were transferred onto the MEA chamber.[32] Fluorescent microscope was
used to determine which channels the regions with mCherry expressed
were located. The channels with target regions of data were sample at 25
kHz. Neuronal firing with larger than 50 μV was defined as the significant
event in the data analysis.

**Stereotaxic Injection and Fiber Insertion:** C57BL/6 mice were anesthetized
with xylazine (10 mg kg$^{-1}$) and ketamine (100 mg kg$^{-1}$) in PBS. Hairs above the operation location were shaved and a
portion of scalp was excised allowing exposure of the skull. Mice were then
fixed onto the stereotactic apparatus and a hole was drilled on the skull with coordinates AP 0.25 mm, ML
−1 speed, followed by holding the injection pipette for 10 min at the
injection site. The pipette was then withdrawn slowly with another
5 min pause halfway. The surgery site was disinfected and sutured and mice
were allowed to reduce the acclimation noise. Illumination was powered
by small screws on the skull. Open-field behavioral recordings were
performed using the Inpersoftware with 10 ms pulse width at 20 Hz of light delivery.

**Immunohistochemical Fluorescent Staining:** 60 min after optogenetic
stimulation, mice were perfused with PBS followed by 4% paraformalde-
hyde (PFA) (cat. no. P1110, Solarbio) in PBS Brains slices 10 μm thick were
dissected in paraffin. Slices were then blocked by using 10% normal goat
serum + 0.3% Triton in PBS +5% BSA for 2 h at room temperature. Slices
were then placed in primary antibody solution (c-Fos (9F6) Rabbit mAb,
Cell Signaling Technology) diluted (1:500) in blocking buffer at 4 °C for
16–18 h. After washes with PBS, slices were incubated with secondary anti-
obody (Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody,
Alexa Fluor 488, Invitrogen) diluted (1:1000) in the blocking buffer for 2 h
at room temperature, followed by washes with PBS. Slices were allowed to
dry and mounted on glass slides with Prolong Diamond Antifade Mount-
tant with DAPI, capped with a coverslip and then imaged. Samples were
imaged by using an upright confocal microscope, in the UBSN facilities of
The Hong Kong Polytechnic University. Cells showing both c-Fos and
mCherry signals were counted manually in five FOVs in each brain slice,
and the percentage of c-Fos$^+$ cells was counted per slice imaged.

**Quantification and Statistical Analysis:** All statistical tests in this study
were performed using GraphPad Prism 8 for Windows. Details of the sta-
tistical analyses performed for each figure are provided in the figure leg-
ends. A p value of <0.05 or below was considered statistically significant
for all experiments.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from
the author.

**Acknowledgements**

This work was supported by Key-Area Research and Development Pro-
gram of Guangdong Province (2018B0303300101), the Hong Kong Re-
search Grants Council General Research Fund (15104520, 15102417, and
15326416), Hong Kong Innovation Technology Fund (MRF/018/18X and
MHP/014/19), internal funding from the Hong Kong Polytechnic Univer-
sity (1-ZE1K and 1-ZVW8), Research Institute of Smart Ageing (1-CD76),
and the Fundamental Research Funds for the Central Universities (nos.
11621107 and 21619331) in Jinan University and the Science and Techno-
logy Program of Guangzhou (no. 2020002030179). The authors would like
to thank the facility and technical support from University Research Facility
in Life Sciences (ULS) and University Research Facility in Behavioral and
Systems Neuroscience (UBSN) of The Hong Kong Polytechnic University.

**Conflict of Interest**

The authors declare no conflict of interest.

**Author Contributions**

J.G. and Y.W. contributed equally to this work. J.H.G., Y.Z.H., Y.C.L., and
L.S. assisted in conceptualization and design; J.H.G., Y.W., Z.Y.G., F.C.,
S.K., Z.H.Q., X.Y.Z.H., J.-J.C.H., D.M.H., T.H.C.H., R.Z., J.J.Z.H., K.F.W.,
Q.X.X., T.ZH., X.D.H., and Y.C.R. assisted in experiments and/or data anal-
ysis; and J.H.G., Y.Z.H., Y.C.L., F.C., S.K., and L.S. assisted in writing—
review and editing.

**Data Availability Statement**

The data that support the findings of this study are available from the cor-
responding author upon reasonable request.

**Keywords**

light focusing, microspheres, optogenetics, photonic nanojets

Received: September 16, 2021
Revised: February 4, 2022
Published online: February 20, 2022
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