Remote nongenetic optical modulation of neuronal activity using fuzzy graphene

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The ability to modulate cellular electrophysiology is fundamental to the investigation of development, function, and disease. Currently, there is a need for remote, nongenetic, light-induced control of cellular activity in two-dimensional (2D) and three-dimensional (3D) platforms. Here, we report a breakthrough hybrid nanomaterial for remote, nongenetic, photothermal stimulation of 2D and 3D neural cellular systems. We combine one-dimensional (1D) nanowires (NWs) and 2D graphene flakes grown out-of-plane for highly controlled photothermal stimulation at subcellular precision without the need for genetic modification, with laser energies lower than a hundred nanojoules, one to two orders of magnitude lower than Au- or Si-based nanomaterials. Photothermal stimulation using NW-templated 3D fuzzy graphene (NT-3DFG) is flexible due to its broadband absorption and does not generate cellular stress. Therefore, it serves as a powerful toolset for studies of cell signaling within and between tissues and can enable therapeutic interventions.

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Cell–cell communication is fundamental to the emergence of higher-order function in tissue. Since Hodgkin and Huxley’s (1) seminal work on the electrical activity of squid giant axons, input/output interfaces with electroactive cells and tissues have steadily improved through a variety of techniques and materials, enabling increased spatial and temporal control (2). The development of optogenetics (3) has revolutionized our ability to control the activity in excitable cells and tissues and fundamentally changed the questions that we can ask and answer. Clinical translation potential of optogenetics is limited, since it requires genetic modification of the cells or organisms to introduce the light-sensitive proteins (3, 4). Similarly, magnetogenetics, which uses superparamagnetic nanoparticles for localized heating and stimulation (5, 6), has enhanced our ability to modulate cellular activity, but it also requires genetic modification, that is, expression of the heat-sensitive transient receptor potential ion channel (TRPV1). It also is limited by a relatively long latency of up to 5 s between the magnetic field application and the onset of neural activity (5). As an alternative to genetic modification, direct infrared (IR) laser pulses are used to induce membrane depolarization (7, 8). However, direct IR stimulation does not provide cell specificity, spatial stimulation precision, or subcellular resolution, and suffers from a narrow energy density threshold for stimulation vs. damage to the tissue (9, 10). An emerging alternative that can provide nongenetic, instantaneous, and spatial control is photothermal stimulation using Au- and Si-based nanomaterials (11–14). Although this approach can provide light-based control similar to optogenetics (with subcellular precision), it has limitations, including the need for relatively high laser power, low photothermal conversion efficiency, low absorption cross-section, and limited long-term stability when a stable interface is needed (15, 16).

Two-dimensional (2D) cell culture has been foundational for in vitro research of cellular communication (17, 18). However, its use for disease modeling and designing of new therapeutic interventions has been limited due to its challenge in portraying a native 3D tissue microenvironment (19–22). The 3D tissues, such as spheroids and organoids, have opened new opportunities to develop in vitro models that better mimic in vivo physiology and have enabled new ways to study disease pathology (23, 24). New technologies are needed in order to understand the mechanisms underlying cell–cell communication and the interplay between different cell populations in health and disease. Therefore, there is a need to develop a remote, nongenetic, and minimally invasive modulation of neuronal activity in 3D tissues that will enable understanding of cellular physiology in both healthy and diseased states to develop new therapeutic platforms.

Here, we resolve the limitations of the current state-of-the-art platforms with a complementary approach based on a hybrid nanomaterial composed of 3D out-of-plane fuzzy graphene grown on an intrinsic Si nanowire (i-SiNW) template. The high

Significance

Modulation of cellular electrophysiology helps develop an understanding of cellular development and function in healthy and diseased states. We modulate the electrophysiology of neuronal cells in two-dimensional (2D) and 3D assemblies with subcellular precision via photothermal stimulation using a multiscale fuzzy graphene nanostructure. Nanowire (NW)-templated 3D fuzzy graphene (NT-3DFG) nanostructures enable remote, nongenetic photothermal stimulation with laser energies as low as subhundred nanojoules without generating cellular stress. NT-3DFG serves as a powerful toolset for studies of cell signaling within and between in vitro 3D models (human-based organoids and spheroids) and can enable therapeutic interventions.

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photothermal efficiency and subcellular dimensions of nanowire-template 3D fuzzy graphene (NT-3DFG) (25) enable instantaneous, remote, and nongenetic photothermal stimulation of cells at subcellular spatial resolution using significantly lower laser energies, that is, one to two orders of magnitude lower than other optical platforms. NT-3DFG’s high broadband absorbance spectrum provides flexibility to modulate neuronal behavior with different excitation wavelengths. The case with which the surface chemistry of NT-3DFG can be tuned via chemical or peptide-based functionalization enables stable biointerfaces. This allows us to modulate the neuronal activity in both 2D neuronal cultures and 3D neural spheroids, toward an understanding of cellular physiology, with the long-term goal of applications in intact tissues.

**Results**

**Highly Controlled Out-of-Plane Growth of Single- to Few-Layer 3D Graphene on a SiNW.** Previously, we demonstrated synthesis of NT-3DFG mesh composed of 3D out-of-plane single- to few-layer graphene flakes grown on an interconnected SiNW mesh template (25). Here, we isolate individual NT-3DFG to achieve subcellular interfaces with neurons (Fig. 1A). The out-of-plane morphology of graphene flakes from the i-SiNW core, illustrated in Fig. 1A, was confirmed by scanning electron microscopy (SEM) and high-resolution transmission electron microscopy (HR-TEM) imaging (Fig. 1B and C and SI Appendix, Figs. S1 and S2). The presence of single- to few-layer graphene flakes was confirmed by HR-TEM (Fig. 1D and SI Appendix, Fig. S2) and Raman spectroscopy (for detailed Raman characterization and analysis, see SI Appendix). The intricate architecture of out-of-plane graphene flakes forms a polycrystalline network (Fig. 1D, Inset) (26). The interplanar distances for the first and second nearest C–C neighbors are experimentally derived to be 0.123 and 0.212 nm, respectively (SI Appendix, Table S3). These values agree with the expected interplanar spacing for the (1120) plane (d_{1120} = 0.123 nm) and the (1010) plane (d_{1010} = 0.213 nm) (25, 27, 28). The distance between individual graphene layers, calculated using the (0002) reflection in the diffraction pattern (SI Appendix, Table S3) and interplanar fringe intensities in the HR-TEM image (SI Appendix, Fig. S2), was determined to be 0.36 and 0.37 nm, respectively. These values are greater than the interlayer distance of bulk graphite (0.344 nm), corroborating the presence of turbostratic graphene (25, 26, 28, 29).

**NT-3DFG Exposed Graphene Surface as a Platform for Chemical Modifications.** We have previously demonstrated that the out-of-plane synthesis of graphene flakes results in an enormous available surface area (25). In addition, graphene flakes provide the opportunity for surface functionalization via n–π interactions or covalent bonding (30, 31). Directed self-assembly of a polypeptide (31) can control the surface charge of the graphene, hence maximizing NT-3DFG electrostatic attachment to the cell membrane. To create stable aqueous suspensions and increase cell membrane adhesion, graphene flakes were treated with HNO₃ and functionalized with lysine-phenylalanine based peptide (KF₄) (30, 31), as confirmed by X-ray photoelectron spectroscopy (XPS) characterization (for detailed XPS characterization, see SI Appendix).

**High Photothermal Efficiency of NT-3DFG.** Bottom-up synthesis of NT-3DFG allows us to tailor the 3DFG flake size and density by changing the synthesis time (SI Appendix, Fig. S5A). Changing the physical architecture of NT-3DFG by increasing the 3DFG flake size and density leads to an increase in the absorbance in the ultraviolet visible (UV-Vis) regime (SI Appendix, Fig. S5 B and C). The UV-Vis spectrum for i-SiNW agrees well with previously reported results (32–34). The enhanced broadband absorption by NT-3DFG can be attributed to the increased light trapping by the densely packed out-of-plane 3DFG flakes, as this structure is synonymous to that of nanostructured silicon and carbon materials (35–37). Due to its high broadband absorption, that is, UV to near-IR (NIR), of ca. 95%, NT-3DFG synthesized for 90 min was selected for subsequent characterization. At a single-nanowire level, we observe that the addition of out-of-plane 3D graphene architecture along an i-SiNW leads to a fourfold to fivefold increase in the extinction spectrum (Fig. 2A). We note that the full width at half maximum (FWHM) of the elliptical laser spot used for the single-nanowire extinction measurements (5 μm × 8 μm) is much larger than the diameter of NT-3DFG (see Materials and Methods for details regarding the UV-Vis and single-nanowire extinction spectroscopy). This results in the measured extinction magnitude being a lower limit to the actual material property. The broadband absorption across visible and NIR spectrum by single NT-3DFG is further corroborated by the extinction measurements. The ability to use NIR is crucial for translation to in vivo studies because it enables an increase in tissue penetration depth due to reduced absorption by water and hemoglobin (38).

Absorption of light energy by materials can lead to the generation of thermal energy (via photothermal effects) (11, 12, 39–41) and/or the accumulation of charge carriers (via photoelectric effects) (14, 42). In the case of NT-3DFG, we observe a dominant photothermal effect. This was quantified by measuring the effective local temperature change in the vicinity of isolated NT-3DFG under laser illumination using a microoptics resistance method (39), as illustrated in SI Appendix, Fig. S7 (for more details, see Materials and Methods). For a representative isolated NT-3DFG, the local temperature change increases from 1.86 ± 0.03 K to 6.74 ± 0.07 K with increase in laser power from 2 mW to 10 mW (635-nm laser with a 20-μm spot size and 1-ms pulse width) (Fig. 2B). NT-3DFG is a broadband absorber; thus any laser wavelength pulses can be used to induce a local temperature change. We demonstrate this by measuring the local...
The local temperature change can be further regulated by modulating the duration of the illuminating laser pulses at constant power. Under constant laser intensity (10 mW), reducing the laser pulse duration from 10.0 ms to 0.1 ms reduces the maximal temperature change (ΔT) from 8.89 ± 0.07 K to 2.61 ± 0.04 K (Fig. 2C). However, the average rate of change in the temperature (ΔT/Δt) drastically increases from 5,000 K/s to 20,000 K/s (for details on calculations, see Materials and Methods). The increase in temperature of NT-3DFG, when a 1-ms pulse of 2-mW or 10-mW laser is applied, is ~140- and 260-fold, respectively, higher than that observed with the application of the same laser pulses to bare i-SiNWs (Fig. 2D and SI Appendix, Fig. S9 and Tables S6-S10). We attribute the enhanced photothermal performance of NT-3DFG to its high UV-Vis absorbance; it will necessitate future studies to elucidate the mechanism for this behavior. Similar temperature changes have been demonstrated for either Au- or Si-based nanomaterials, but only with laser power densities one to two orders of magnitude higher than those used on NT-3DFG [e.g., 240 kW/cm² for Si-based nanomaterials (12, 14), and 31 kW/cm² for Au nanoparticles (11) as compared to 1 kW/cm² to 3 kW/cm² for NT-3DFG].

**Neuron and NT-3DFG Interaction.** Previously, photothermal effects have been demonstrated to induce changes in cell membrane capacitance, eliciting an action potential in excitable cells (11, 12, 14, 39-41). The high photothermal efficiency of NT-3DFG makes it a promising nanomaterial to enable stimulation with significantly lower laser energies. Prior to employing NT-3DFG for photothermal stimulation of neurons, it is critical to assess the cytotoxicity of NT-3DFG as well as identify the interface between NT-3DFG and target neurons. We synthesized NT-3DFG suspensions with an average length of ca. 7.32 μm (SI Appendix, Fig. S10) and incubated varying concentrations of the suspension with primary rat dorsal root ganglion (DRG) neurons (for details, see Materials and Methods). As the NT-3DFG concentration is increased from 0.15 ± 0.01 μg/mL to 0.75 ± 0.05 μg/mL, the density of NT-3DFG also increases from 257 ± 56 wires/mm² to 565 ± 104 wires/mm² (SI Appendix, Fig. S11 and Table S12). Consequently, the density of cells interfaced with NT-3DFG also increased from 104 ± 19 cells/mm² (i.e., 19 ± 5% of total cells) to 179 ± 20 cells/mm² (i.e., 45 ± 14% of total cells) (SI Appendix, Fig. S11 and Table S12). We note that NT-3DFG concentration of 0.15 ± 0.01 μg/mL has no significant effect on the viability of neurons after 24 h of incubation (SI Appendix, Fig. S12). Increasing the NT-3DFG concentration to 0.75 ± 0.05 μg/mL leads to a decrease in viability from 94 ± 4% to 88 ± 5% (SI Appendix, Fig. S12). This observation is in line with the effect of increasing nanoparticle concentration on cell viability for various nanoparticles (43-45).

After incubating DRG neuron cell cultures with NT-3DFG for 24 h, we observe that NT-3DFG adhere to the plasma membrane rather than getting internalized by the cells. This is highlighted by the 3D reconstruction, orthogonal sections, and SEM images of the interface between DRG neurons and NT-3DFG presented in Fig. 3 and SI Appendix, Fig. S13. We attribute the adhesion between plasma membrane and NT-3DFG to the polypeptide surface functionalization of NT-3DFG. Thus, the high photothermal performance, minimal cytotoxicity, and noninvasiveness...
of NT-3DFG establish it as an ideal candidate for targeted photothermal stimulation of neuronal cells.

**Modulation of Neuronal Electrical Activity Using NT-3DFG.** To investigate the remote, nongenetic, optical modulation of neuronal activity capabilities of NT-3DFG, the interface between NT-3DFG and DRG neuron plasma membrane was illuminated with laser pulses, as illustrated in Fig. 4A (for details, see Materials and Methods). The significantly smaller diameter of NT-3DFG (1.4 ± 0.1 μm) compared to the neuronal body (10 μm to 50 μm) enables high precision and subcellular spatial resolution (Fig. 4B). Illuminating the cell–NT-3DFG interface with laser
pulses leads to depolarization of the cell membrane, as observed in the patch clamp measurements (Fig. 4C and D). The membrane potential was progressively depolarized with increasing laser energy, either by increasing the laser power from 1.45 mW to 3.02 mW while keeping pulse duration fixed at 1.2 ms (Fig. 4C) or by increasing the laser pulse duration from 0.4 ms to 1.6 ms while keeping the laser power fixed at 2.28 mW (Fig. 4D). Laser energy of ca. 3.6 μJ per pulse induced enough depolarization of the cell membrane to reach the action potential threshold. The absence of any depolarization upon illumination of the cell away from the NT-3DFG confirms that the stimulation occurred due to photothermal effect of the NT-3DFG (SI Appendix, Fig. S15). The high photothermal efficiency of NT-3DFG enables stimulation of neurons with laser energies fivefold to 60-fold lower than what has been reported previously using other nanomaterials (11–14).

The mechanism of photothermal stimulation via NT-3DFG follows optocapacitive stimulation, that is, photothermal stimulation of cells due to changes in membrane capacitance. The rate of change in temperature at the cell membrane \( \frac{dT}{dt} \) is proportional to the rate of change of membrane capacitance \( \frac{dC_m}{dt} \), resulting in a capacitive current \( I_c \) (46). The greater the magnitude of \( I_c \), the more easily an action potential is evoked. As long as the magnitude of instantaneous \( \frac{dT}{dt} \) is large

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**Fig. 5.** Highly controlled photothermal stimulation of neurons. (A) Representative recorded membrane potential of a repetitively stimulated DRG neuron (DRG neuron was patch-clamped in whole-cell configuration and current clamp mode). Electrical stimulation was performed by injecting a pulse of 100 nA for 1 ms. Photothermal stimulation was performed with 405-nm laser with pulse of 2.28-mW power and 0.6-ms pulse duration (1.37 μJ). (B) Representative recorded membrane potential of a DRG neuron exposed to trains of laser pulses at different frequencies (n = 3 DRG neurons). A 405-nm laser with pulse of 3.02-mW power and 0.7-ms pulse duration (2.11 μJ) was used (DRG neuron was patch-clamped in whole-cell configuration and current clamp mode). Purple and orange arrows indicate an action potential and a subthreshold depolarization, respectively.

**Fig. 6.** Photothermal stimulation with NT-3DFG does not affect cellular viability. (A) Schematic illustrating the experimental design of the viability assay performed on the Calcein loaded DRG neurons before and after the application of laser pulse. Blue arrows indicate illumination by 635-nm laser with 1-ms pulse duration and varying powers of 4 mW (4 μJ), 6 mW (6 μJ), 8 mW (8 μJ), 10 mW (10 μJ), and 22 mW (22 μJ), at varying frequencies (1, 5, and 10 Hz). Orange arrows indicate pulse train of 2,500 pulses of 1-ms pulse duration at 500 Hz. Green arrows indicate acquisition of images using blue laser and GFP filter. (B) Bright-field and fluorescence images of Calcein loaded DRG neurons before and after laser pulses. White arrow indicates the NT-3DFG illuminated by the laser. Red arrow indicates a neighboring cell without laser illumination. (Scale bars, 50 μm.) (C) Box plot presenting the change in fluorescence of targeted cells (green) and neighboring cells (red) as a function of laser pulse condition (n = 10 DRG neurons). Empty circles indicate outliers in the respective data sets.
enough to generate $I$, greater than the necessary threshold, an action potential will be elicited irrespective of the total duration of the laser pulse. Therefore, shorter pulses are more energetically efficient at triggering an action potential. The thermal energy output of longer pulses beyond the threshold point does not contribute toward triggering an action potential (Fig. 2C) (40). By applying laser pulses of sub-10-μs pulse duration, we were able to further reduce the energy required to reach the action potential threshold to sub-100 nJ per pulse. With NT-3DFG, we were able to reduce the pulse duration from 1 ms (2.7-mW laser power, 2.7-μJ laser energy) to 2 μs (10-mW laser power, 20-nJ laser energy) and still elicit an action potential (Fig. 4E and SI Appendix, Fig. S16 and Table S13). Although the 500-fold decrease in laser pulse duration required a fourfold increase in laser power to reach the threshold energy, the effective energy input to elicit an action potential reduced by more than 100-fold (Fig. 4E and SI Appendix, Table S13).

For optocapacitive stimulation, the energy needed to reach the action potential threshold ($E_{Tb}$) and the laser pulse duration ($\Delta t$) follow a power-law relationship (40). We observe a similar power-law relationship for NT-3DFG–based photothermal stimulation (Fig. 4E), corroborating the role of an optocapacitive mechanism in photothermal stimulation via NT-3DFG. The magnitude of the prefactor coefficient (10.66) of the $E_{Tb}$ vs. $\Delta t$ fit reveals a 2.5- to 10-fold lower energy requirement to reach threshold membrane potential to stimulate a cell in comparison to Au, carbon nanotube mesh, and other graphite-based nanomaterials (40). Additional membrane voltage measurements were performed with holding potentials ≤ −110 mV to prevent the activation of voltage-gated ion channels. The generation of transient inward currents upon laser illumination of the NT-3DFG–neuron interface (Fig. 4F) further corroborates the optocapacitive nature of the stimulation (41).

To investigate the reproducibility of the photothermal stimulation technique, the NT-3DFG–cell interface was repetitively illuminated with laser pulses. Fig. 5A demonstrates the response of a patch-clamped neuron upon electrical stimulation (left spikes) followed by photothermal stimulation using a 405-nm laser (right spikes). The neuron elicited an action potential in response to every stimulus application, showing high reproducibility. Furthermore, the laser pulses were applied with varying frequencies of 1, 10, and 20 Hz for 10 s each. Neurons were able to reliably follow pulse trains with frequencies up to 10 Hz (Fig. 5B). However, there were action potential failures at 20-Hz stimulation (Fig. 5B, orange arrow). This is likely to reflect the intrinsic properties of DRG neurons (i.e., the ion channels underlying spike initiation), given that nociceptive neurons, those with action potential shapes similar to those shown in Fig. 5B (47, 48), rarely fire at frequencies above 3 Hz (49). This assay confirms that NT-3DFG enables highly controlled and reproducible remote modulation of neuronal activity at subcellular precision.

**Effect of Photothermal Stimulation on Cell Viability.** To evaluate the effect of photothermal stimulation on cellular viability, we investigated the power threshold at which viability was impacted. DRG neurons, loaded with Calcein, a live cell indicator dye, were interfaced with NT-3DFG. Then 1-ms laser pulses of varying laser power and pulse frequency ranging from 4 mW to 22 mW and 1 Hz to 10 Hz, respectively, were applied to the cell–nanowire interface (Fig. 6A). The cells were imaged before and after the laser pulses (Fig. 6B) (details can be found in Materials and Methods). No significant change in fluorescence intensity was observed for laser pulses applied at 1 Hz to 10 Hz for 10 s with laser powers of 4 mW (4 μJ per pulse) to 22 mW (22 μJ per pulse) (Fig. 6C). Cells that were exposed to 500-Hz pulse trains of 1-ms pulses for 5 s with 22-mW laser power (2,500 μs per pulse) exhibited a significant loss in fluorescence (Fig. 6 B and C). At higher laser powers, the local change in temperature induced by NT-3DFG is higher. Under the extreme stimulation conditions of 22 mW and 500 Hz, the change in temperature experienced by the plasma membrane will be much greater than that under safe photothermal stimulation conditions. Extreme change in local temperature is expected to damage the plasma membrane. We note that neighboring cells did not show a change in fluorescence, indicating that the fluorescence change of the cell under stimulation was not due to photobleaching of the dye. The maximum energy used for photothermal stimulation of neurons was ca. 3 μJ, significantly lower than the energy needed to damage the cells, thus confirming that there were no toxic effects of the NT-3DFG–based photothermal stimulation platform.

**Multicell Dynamics via Calcium Imaging.** To demonstrate the capability of modulating neuronal activity at a network scale with subcellular precision, NT-3DFGs were interfaced with neuronal networks, and multicell dynamics were investigated upon photothermal stimulation using calcium imaging. Calcium is a ubiquitous secondary messenger that plays an essential role in the signal transduction of excitable cells (51). Calcium ions enter the excitable cells during an action potential firing; therefore, changes in intracellular calcium can be used as a proxy for...
electrical activity (51). To evaluate the effect of photothermal stimulation on the calcium dynamics of DRG neurons, NT-3DFGs were interfaced with cells loaded with calcium indicator dye, Cal 520. Illumination of the NT-3DFG with a laser pulse of 4-mW power and 1-ms pulse width led to an influx of calcium, as evident from the increase in fluorescence (Fig. 7 and SI Appendix, Fig. S17). Furthermore, the stimulation led to the propagation of the calcium transient from the stimulated cell to the neighboring cells as observed by the delay in the fluorescence change response of the neighboring cells (Fig. 7 and Movie S1). The propagation of the calcium transients is attributed to the presence of intercellular junctions and diffusion of chemical messengers (12, 14, 52–54). As a control experiment, we illuminated cells away from the NT-3DFG site using the same conditions for the laser pulses and observed no change in fluorescence, confirming the high spatial precision of the photothermal stimulation (SI Appendix, Fig. S18).

Our remote nongenetic photothermal stimulation can be easily implemented in a 3D cellular organization such as cellular spheroids or organoids. The inability of 2D cell culture models to recapitulate in vivo-like cytoarchitectural organization and intercellular connections has impeded their use, especially for disease modeling and designing new therapeutic interventions (19, 20, 55). Complex 3D arrangement of cells, such as spheroids and organoids, better represents in vivo-like behavior in terms of morphology (56), cell survival (57), and electrophysiological characteristics (58). However, there is a need to develop a non-genetic, remote, minimally invasive, and biocompatible platform that can allow precise modulation of the neuronal activity in 3D systems to better understand cellular physiology of both healthy and diseased states, for developing new therapeutics.

As a proof of concept, we demonstrate the capability to interface NT-3DFGs with 3D cortical neural spheroids. NT-3DFG can be conformally distributed throughout the periphery and bulk of cortical spheroids simply by incubating the target spheroid(s) with NT-3DFG suspension (SI Appendix, Figs. S19 and S20). Our simple approach of interfacing NT-3DFG with 3D neural spheroids allows us to interface isolated NT-3DFG with individual neuronal cells. Illuminating the interface between individual NT-3DFG and cortical neurons in the spheroids with laser pulses of 4- to 6-mW power and 1-ms pulse width leads to an influx of calcium in the neuronal networks, as observed by changes in the fluorescence intensity of the cells (Fig. 8, SI Appendix, Fig. S21, and Movie S2). The duration and shape of the calcium transients observed in the neural spheroids upon photothermal stimulation are in line with reported literature (59, 60). Illumination of cells away from the NT-3DFG result in no observable change in the fluorescence emission, further confirming the spatial precision of the photothermal stimulation in a 3D cellular arrangement (SI Appendix, Fig. S22). This work demonstrates the ability to stimulate and modulate activity of 3D neural spheroids noninvasively with high precision.

Discussion
In this study, we demonstrate a hybrid nanomaterial composed of 3D fuzzy graphene grown on an i-SiNW template for remote, nongenetic optical modulation of neuronal activity in both in two and three dimensions, at a single-cell level. Presence of graphene allows straightforward surface modifications using both chemical treatments and peptide coatings. The unique structure of this hybrid nanomaterial leads to extraordinary optical and photothermal properties that enable stimulation of neurons with laser energies as low as subhundred nanojoules, one to two orders of magnitude lower than Au-, C-, and Si-based photostimulation (11–14). The <1.5-μm diameter of NT-3DFG allows stimulation with high precision and subcellular spatial resolution. Moreover, the ability of NT-3DFG to absorb light in the NIR regime will be crucial for modulation of neuronal activity in an in vivo model (38). The NT-3DFG platform will greatly impact our basic understanding of cellular communication across various electrically active cells (such as neurons and myocytes) and tissues in both health and disease. In addition to basic research, this technology will allow development of therapeutic interventions for a variety of diseases resulting in problems such as muscle loss, chronic pain, and neurodegeneration. The ability to modulate activity of neural spheroids opens up unique opportunities to use spheroids and organoids as model systems for therapy development for various neurodegenerative disorders, such as Parkinson’s disease. Moreover, this platform can be adapted to address challenges in tissue engineering by enabling noninvasive high-precision stimulation control of engineered tissues (61).

Materials and Methods

Synthesis of NT-3DFG. NT-3DFG is synthesized via a two-step process: i-SiNWs synthesis, followed by 3DFG synthesis. The i-SiNWs were synthesized through Au nanoparticle (AuNP)-catalyzed vapor–liquid–solid growth process (25). A 2.0 cm × 2.0 cm (100) Si substrate with 600-nm thick thermal oxide (p-type, ≥0.005 Ω cm, Nova Electronic Materials Ltd., catalog no. CP02 11208-0X) or 1.5 cm × 1.5 cm fused silica (University Wafer, catalog no. 1013) substrates were cleaned with acetone and isopropry alcohol (IPA) in an ultrasonic bath for 5 min each followed by N2 blow dry. The substrate was then treated with UV ozone (PSD Pro series digital UV-Ozone, Novascan) at 150 °C for 10 min. Cleaned substrates were then functionalized with 500 μL of 4:1 deionized (DI) water:poly-l-lysine (PLL) (0.1% wt/vol, Sigma-Aldrich, catalog no. P8920) for 8 min. They were then gently cleaned three times in DI water and N2 blow-dried. Then, 500 μL of 9:1 DI water:30 nm AuNP solution (Ted Pella, catalog no. 15706-1) was dispersed onto the PLL-coated substrate for 8 min. The substrate was then gently washed three times in DI water and N2 blow-dried. AuNP functionalized substrates were then introduced into a custom-built chemical vapor deposition (CVD) setup. Once a baseline pressure of 1 × 10−5 Torr was reached, the temperature was ramped up to 450 °C in 8 min, which was followed by a 5-min temperature stabilization step. Nucleation was conducted at 450 °C for 15 min under standard cubic centimeters per minute (sccm) H2 (Matheson Gas) and 20 sccm SiH4 (10% in H2, Matheson Gas) at a controlled pressure of 40 Torr. The synthesis step was performed at 450 °C for 100 min under 80 sccm H2 and 20 sccm SiH4 at a controlled pressure of 40 Torr. The sample was cooled down to room temperature at base pressure post-synthesis.

The 3DFG was synthesized through plasma-enhanced CVD (PECVD) process (25). Post i-SiNW synthesis, the sample was introduced into a custom-built PECVD setup. The synthesis process was carried out at 800 °C and at a total pressure of 0.5 Torr. The SiNWs sample was placed 4.0 cm from the edge of the radio frequency (RF) coil and our oxygen pressure was 0.005 Torr. The temperature of the temperature was ramped up to the synthesis temperature at 55 °C min−1, which was followed by a temperature stabilization step at the synthesis temperature for 5 min, under a flow of 100 sccm Ar (Matheson Gas). Prior to the synthesis step, inductively coupled plasma was generated using a 13.56-MHz RF power supply (AG 0313 Generator and AIT-600 RF, power supply and auto tuner, respectively, T&C Power Conversion, Inc.) with the plasma power constant at 50 W; the furnace was moved over the sample following plasma ignition. The synthesis step was conducted under 50 sccm CH4 precursor (5% CH4 in Ar, Airgas) for 5, 10, 30, or 90 min. The plasma was turned off after the synthesis step, and the sample was rapidly cooled from growth temperature to 100 °C in 30 min under the flow of 100 sccm Ar (62).

NT-3DFG Sample Preparation for Structural, Optical, and Photothermal Characterizations. A 5 mm × 5 mm sample of NT-3DFG was cleaved from the center of the growth substrate and placed in a clean 2-mL scintillation vial. Then, 100 μL of IPA was added to the vial. The vial was placed in an ultrasonic bath (Crest Ultrasonics, catalog no. P230D) at 45 kHz and 32 W for 2 s. Then 5 μL of the suspension was drop-casted onto Si/SiO2 or glass substrates, and the IPA was allowed to dry in the clean hood. The samples were kept on the hot plate at 60 °C for 15 min.

SEM. SEM imaging was performed using an FEI Quanta 600 field emission gun SEM. Images were acquired at accelerating voltages of 2 kV to 20 kV with a working distance of 5 mm. For imaging of isolated NT-3DFGs, NT-3DFGs were drop-casted on indium tin oxide-coated polyethylene terephthalate films. No additional conductive coating was applied to any samples to assist with imaging.
NT-3DFG diameter was quantified from the high-resolution (2,048 × 1,768 pixels) SEM images using ImageJ (Java-based open-source image processing software, NIH). Quantification was performed on 150 wires across three independent samples.

HR-TEM. HR-TEM imaging was performed using an FEI Titan G2 80-300 Cs corrected TEM fitted with a high-resolution Gatan Imaging Filter Tridem energy filter. A lacey carbon, 300-mesh Cu TEM grid (Ted Pella, catalog no. 01895) was gently rubbed over an NT-3DFG growth substrate. HR-TEM and selected area electron diffraction (SAED) images were acquired at an accelerating voltage of 300 kV with 2,048 × 2,048 slow-scan charge coupled device (CCD) camera. SAED plane spacing analysis and fringe intensity cross-section profiles were quantified from corresponding HR-TEM images using ImageJ (25, 62).

Single-Nanowire Extinction Measurements. Extinction was measured with a supercontinuum laser (NKT Photonics, SuperK Extreme EXB-8) after passing through a Thorlabs monochromator (Princeton Instruments, Acton SP2300) to output a single wavelength with < 2 nm bandwidth. The laser was collimated into a beam size of 1 mm and split evenly into a reference and probe beam for balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlabs, PMM-40 balanced detection with Nirvana balanced photoreceiver (Newport, Nirvana Auto-Balanced Photoreceiver 2007). The probe beam was directed to one side of the back aperture of a reflective objective (Thorlas...
from the vial was removed and placed aside for a later step. The tissues were treated with 0.25% trypsin (ethylenedinitrilo)tetraacetic acid (EDTA) solution (ThermoFisher, catalog no. 25200056) and incubated for 5 min. Post incubation, the tissues were treated with 0.25% trypsin EDTA solution supplemented with 0.1% DNase (ThermoFisher, catalog no. EN0521) and incubated for another 5 min. The solution was replaced by the previously removed media, and the tissue was dissociated by physical trituration five times using a sterile fire-polished glass Pasteur pipette ~1 mm in diameter, followed by another five times with a pipette smaller in diameter, that is, ~1/2 mm to 3/4 mm in diameter. The dissociated cells were then spun down at 1,100 rpm for 1 min and resuspended in 1 mL of fresh Neurobasal media (ThermoFisher, catalog no. A14170-1) supplemented with 1× B27 (ThermoFisher, catalog no. 25200056) and incubated for 5 min. Cortical tissues were dissociated as previously established protocol (64). Briefly, sterile 10 g/L solution of agarose (Invitrogen, catalog no. 16500) was prepared in 0.9% (wt/vol) NaCl (Sigma, catalog no. S5886) in DI water. The agarose spherical microwells were equilibrated twice in Neurobasal medium for 30 min in 37 °C chamber with 5% CO2. The agarose microwells were equilibrated twice in Neurobasal medium for 30 min in 37 °C chamber with 5% CO2. The agarose microwells were equilibrated twice in Neurobasal medium for 30 min in 37 °C chamber with 5% CO2. Cortical tissues were dissociated as mentioned above, and neurons were seeded at a density of 500,000 cells per mold in 190 μL of Neurobasal medium supplemented with 1× B27, 1% Glutamax, and 1% Penicillin/Streptomycin (ThermoFisher, catalog no. 15140122). The neurons were used to form cortical spheroids for photo thermal-stimulation (as explained below) and incubated in a 37 °C chamber with 5% CO2 until used for experiments. Fifty percent of the cell culture media was replaced with fresh media every 3 d.

Cortical Spheroid Formation. The spheroids were formed following a previously established protocol (64). Briefly, sterile 10 g/L solution of agarose (Invitrogen, catalog no. 16500) was prepared in 0.9% (wt/vol) NaCl (Sigma, catalog no. S5886) in DI water. The agarose spherical microwells were equilibrated twice in Neurobasal medium for 30 min in 37 °C chamber with 5% CO2. Cortical tissues were dissociated as mentioned above, and neurons were seeded at a density of 500,000 cells per mold in 190 μL of Neurobasal medium supplemented with 1× B27, 1% Glutamax, and 1% Penicillin/Streptomycin. The spheroids were cultured for up to 60 d, and 50% of the cell culture media was replaced with fresh media every 3 d.

NT-3DFG Suspension Preparation for NT-3DFG Concentration Viability Study. NT-3DFG were synthesized on 15 mm × 15 mm Si/SiO2 chips with 2 μm of PECVD SiO2 following the synthesis protocol described above. The mass of synthesized NT-3DFG was measured to be 140 ± 5 μg with a mass per unit area of 0.640 ± 0.0225 μg/mm2. The NT-3DFG samples were cleaved into 5 mm × 5 mm samples, and the effective mass of each 5 mm × 5 mm was calculated by measuring the geometric area of each chip using ImageJ (SI Appendix, Table S11). The cleaved NT-3DFG samples were treated with 70% w/vt HNO3 (CMOS Grade, J.T. Baker, catalog no. JT9606-3) for 2 h (Fig. 3A). The samples were rinsed three times with sterile DI water and treated overnight with 100 μg/mL lysine-phenylalanine based peptide (KF4) on a shaker (31). This was followed by washing three times with DI water. NT-3DFG were released from the Si/SiO2 substrate by etching the SiO2 layer in buffered HF (Alfa Aesar, catalog no. 44627) for 10 h. The released NT-3DFG were rinsed three times with sterile DI water and transferred to siliconization vials with 500 μL of sterile 1× PBS. In order to prepare the suspension, the samples were sonicated at 20 kHz and 36 W for 1 min using a probe sonicator (FB120, Fisher Scientific).

NT-3DFG Concentration Viability Assay. NT-3DFG suspensions were sterilized via UV treatment for 2 h prior to concentration preparation. NT-3DFG suspension preparations were concentrated in sterile 1× PBS as described in SI Appendix, Table S12. DRG neurons were cultured in glass-bottomed dishes following the protocol described above. The mass of synthesized NT-3DFG was measured to be 140 ± 5 μg with a mass per unit area of 0.640 ± 0.0225 μg/mm2. The NT-3DFG samples were cleaved into 5 mm × 5 mm samples, and the effective mass of each 5 mm × 5 mm was calculated by measuring the geometric area of each chip using ImageJ (SI Appendix, Table S11). The cleaved NT-3DFG samples were treated with 70% w/vt HNO3 (CMOS Grade, J.T. Baker, catalog no. JT9606-3) for 2 h (Fig. 3A). The samples were rinsed three times with sterile DI water and treated overnight with 100 μg/mL lysine-phenylalanine based peptide (KF4) on a shaker (31). This was followed by washing three times with DI water. NT-3DFG were released from the Si/SiO2 substrate by etching the SiO2 layer in buffered HF (Alfa Aesar, catalog no. 44627) for 10 h. The released NT-3DFG were rinsed three times with sterile DI water and transferred to siliconization vials with 500 μL of sterile 1× PBS. In order to prepare the suspension, the samples were sonicated at 20 kHz and 36 W for 1 min using a probe sonicator (FB120, Fisher Scientific).

NT-3DFG Sample Preparation for Photothermal Stimulation. Si/SiO2 chips with as-grown NT-3DFG were cleaved in 1 cm × 1 cm samples, and treated with 70% w/vt HNO3 (CMOS Grade, J.T. Baker, catalog no. JT9606-3) for 2 h, followed by washing three times with DI water, and then transferred to siliconization vials. The samples were sterilized for 2 h in 70% ethanol and air-dried in the cell culture hood for 15 min, followed by 2-h UV treatment. The samples were rinsed three times with sterile DI water and treated with 100 μg/mL lysine-phenylalanine based peptide (KF4) overnight on a shaker. The peptide solution was replaced with 500 μL of sterile 1× PBS and sonicated for 5 to 10 s.

Photothermal Stimulation—Patch Clamp Measurements. The NT-3DFG suspension was added to the DRG culture dish and incubated for 2 h to 16 h at 37 °C with 5% CO2. Following the incubation of peptide modified NT-3DFGs withDRG neurons, the cell culture dish was rinsed three times with recording solution (NaCl 132 mM, KCl 4 mM, MgCl2 1.2 mM, CaCl2 1.8 mM, Hepes 10 mM, glucose 5.5 mM; pH 7.4) and mounted on a microscope and viewed through 40×/0.65NA water immersion objective lens (Fig. S14). The 405-nm diode laser (Hangzhou NaKu Technology) was mounted and aligned to the central axis of the microscope objective. The 405-nm laser line was TTL-modulated. Laser power was adjusted by density filters (Thorlabs). Patch pipettes were pulled on a Sutter Instruments P-2000 CO2 laser micropipette puller (Novata) to produce 2- to 5-MΩ resistances when filled with internal pipette solution (NaCl 10 mM, KF 130 mM, MgCl2 4.5 mM, Hepes 10 mM, EGTA 9 mM, ATP 2 mM, pH 7.4). The analog waveform from a high-speed universal serial bus (USB) data acquisition device (Measurement Computing Corp., USB-1604) drove the amplifier (Axon Instruments, Axopatch 200B) to clamp the cell membrane current or voltage. Membrane potential recordings were made in current clamp mode using patch clamp amplifier and current injection with 1-ms pulse width, and 400-ms interpulse interval. Current recordings were made in a voltage clamp mode, and the voltage was held at −110 mV to −150 mV. Current clamp and voltage clamp measurements were performed across 12 and 3 DRG neurons, respectively.

The density of NT-3DFG as a function of NT-3DFG concentrations was measured using differential interference contrast (DIC) images of DRG study and Matlab (n = 4 for each concentration, 10 independent DIC images per sample). The density of cells interfaced with NT-3DFG was measured using ImageJ (n = 4 for each concentration, five independent DIC images per samples).

DRG and NT-3DFG Interface. DRG neurons were cultured in glass-bottomed dishes following the protocol described above. One hundred μL of NT-3DFG suspension was added to the cell cultures 24 h after initial cell seeding, and the cell cultures were incubated for another 24 h at 37 °C with 5% CO2. CellMask orange plasma membrane stain (ThermoFisher, catalog no. C10045) was used to stain the cell membrane. The cell cultures were incubated with a final concentration of 5 μg/mL of the dye for 15 min at 37 °C with 5% CO2. The samples were gently rinsed with Tyrode’s buffer. Live-cell imaging was performed using upright confocal microscope and a 20×/0.50 NA water immersion objective. The 3D reconstruction and orthogonal sections were generated using maximum intensity projection protocol in Imaris viewer (Oxford Instruments).

For SEM imaging for DRG neurons interfaced with NT-3DFG, the samples were fixed using 4% paraformaldehyde (Electro Microscopy Sciences, catalog no. 15710) in 1× PBS for 20 min at room temperature. The cell cultures were then gently rinsed three times with 1× PBS for 5 min each on a shaker at room temperature. The 1× PBS was replaced with DI water. DI water was then serially replaced (25%, 50%, 75%, 90%, and 100%) with 200-proof ethanol (Pharmco-Aaper, catalog no. 111000220) with 5-min incubation at room temperature under each ethanol concentration. Critical point drying (CPD) of the samples were performed using a CPD system (Autosamdi-B15, Tousimis).
Photothermal Stimulation—Calcium Imaging. For DRG cultures, the NT-3DFGs were drop-cast on the cells. For cortical spheroids, the spheroids were matured with 5% CO2 and 95% air for 24 h. The sample was then incubated at 37 °C for 2 h. The cells were then incubated with 2 μM live cell indicator, Calcein AM dye, for 10 min at 37 °C with 5% CO2. Post incubation, the sample was gently rinsed with carboxygenated Tyrode’s buffer solution and loaded on the microscope setup. Then 1-ms laser pulses of varying powers (0.4, 2.8, 10, and 22 mW) were applied on the cell–wire interface for 10 s each at 1, 5, and 10 Hz. Bright-field and fluorescent images (obtained using blue laser and GFP filter) were obtained before and after laser pulses. As a control, 1-ms laser pulses of 25-mW power at 500 Hz were applied on the cell–wire interface for 5 s, to induce cell death, indicated by the loss in fluorescence due to leakage of the Calcein AM dye. Quantiﬁcation was performed on 10 DRG neurons,

\[
\Delta F/F_0 = \frac{F - F_0}{F_0} \times 100,
\]

where \(F_0\) and \(F\) are fluorescence values of the cell (evaluated using Matlab) before and after laser pulses, respectively.

Data Availability. All data related to the presented ﬁgures are available through Carnegie Mellon University research repository (KiltHub) at doi.org/10.1184/R1/4943241.v1.

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