High-Density Multilayer Graphene Microelectrode Arrays for Optogenetic Electrophysiology in Human Embryonic Kidney Cells

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Introduction: Optogenetic electrophysiology offers high precision cellular analysis by electrophysiological recording under optogenetic control [1, 2]. Such studies often use microelectrode arrays (MEA) to obtain massively parallel recording from densely packed cells. Among the MEA materials, graphene has been suggested to be well suited for optogenetic electrophysiology [1-3], enabling transparent, flexible, and low-noise MEAs for *in vivo* recording of the local field potential (LFP) [1]. To date, most graphene microelectrodes were 25-300 μm in size to achieve high signal-to-noise ratios, and placed in a 150-900 μm pitch for single-unit recording [1, 3]. Such device dimension however has limited spatial resolution compared to closely-packed silicon MEAs [4], and cannot offer spatial oversampling of the cell activity. Here we present a 28-μm pitched multilayer graphene MEA with 13-μm sized electrodes, the smallest in literature, for high-density optogenetic electrophysiology in human embryonic kidney (HEK) cells. Our MEA was made of CVD-grown multilayer graphene for its low sheet resistance, which was one-time transferred onto a Si/SiO_2 substrate, instead of layer-by-layer transfer steps (see [2]) that may increase the electrode impedance by contaminants. Our electrodes had 2 MΩ impedance at 1 kHz (2.38 $\Omega\cdot cm^2$) in electrochemical impedance spectroscopy (EIS), 6 times smaller than those made by layer-by-layer transfer steps if they were made in the same size [2]. Our MEA was able to record optogenetically evoked extracellular signals in HEK cells co-expressed with opsins (*ChR2*) and Ca^{2+} reporters (*jRCAMP1a*) [5]. The signal amplitude increased with the intensity (not the duration) of the optogenetic stimulus, and qualitatively matched the position of optogenetically responsive cells (confirmed by *jRCAMP1a* imaging). Our work suggests the possible use of multiplayer graphene MEA for optogenetic electrophysiology in HEK cells.

Methods: Our MEA was built on commercial CVD-graphene wafers with 3-5 layered graphene one-time transferred to a Si/SiO_2 substrate. 13 μm sized graphene electrodes were RIE patterned in a 28-μm pitch, contacted by ITO wires (30 nm) and Ti/Au pads (7/150 nm), and passivated by a 3 μm-thick SU8 layer (O_2-plasma treated to enhance its hydrophilicity) (Fig. 1a). The MEA was wire-bonded onto a PCB, which was then side-flipped and packaged (via PDMS) on a home-built lifting station with a lab jack (Fig. 1b). HEK cells were cultured on a PDMS piece, transferred to a Petri dish filled with cell imaging medium that contained 80 mM $CaCl_2$ [5], and placed on the stage of an inverted fluorescence microscope (Leica). We used this microscope to find responsive cells by *jRCAMP1a* imaging at 632/60 nm (0.1-s pulses of 575/22-nm excitation was applied at 0.5 fps to alleviate the photo-bleaching effect [6]), apply a 470/40-nm stimulus window (when the excitation was off) to activate *ChR2*, and form the MEA-cell contact by finetuning the lifting station (Fig. 1c). The recording data was sampled at 10-kHz and band-pass filtered at 1-7 kHz by an Intan chip (RHD 2164), with the cell medium being biased by one Ag/AgCl pseudo-reference electrode.

Results and Discussions: Configured in a 3-electrode EIS setup (Gamry) in 1 × PBS, 12 out of 13 electrodes showed uniform impedance and phase distribution, 2.06 ± 0.14 MΩ and $-87.2 \pm 1.8°$ at 1 kHz, across the array (Figs. 2a, 2b). This MEA was then applied for extracellular recording of *ChR2-jRCAMP1a* co-expressed HEK 293 cells. Under 470 nm stimulus at 22.6-90.4 mW/cm^2 intensity and 1-10 s duration, we observed signal oscillations at the start and the end of the stimulus, whose amplitudes are larger than the control data with no cells (*i.e.* light-induced artifact); the periodic noise outside the 470-nm window was believed to be caused by the pulsed 575-nm excitation (Fig. 2c). These oscillations increased their amplitudes (12-electrode average) with the 470-nm stimulus intensity (Fig. 3a), but weakly depended on its duration (Fig. 3b). Moreover, the spatial mapping of the oscillation amplitudes across the MEA qualitatively matched the optogenetically evoked Ca^{2+} transients [5], $\Delta F/F_0$, from the *jRCAMP1a* imaging data at each electrode site (Fig. 3c). This result suggests that multiplayer graphene MEA may serve as a candidate for high-density optogenetic electrophysiology in dense cell populations. Our choice of multiplayer graphene over mono-layer graphene here lowered the electrode impedance and benefited the signal-to-noise ratio, however at the expense of increased light-induced artifact. This limitation may be overcome in the future by coating 2-3 layered graphene with transparent and conductive materials to achieve low impedance and low light-induced artifact simultaneously.

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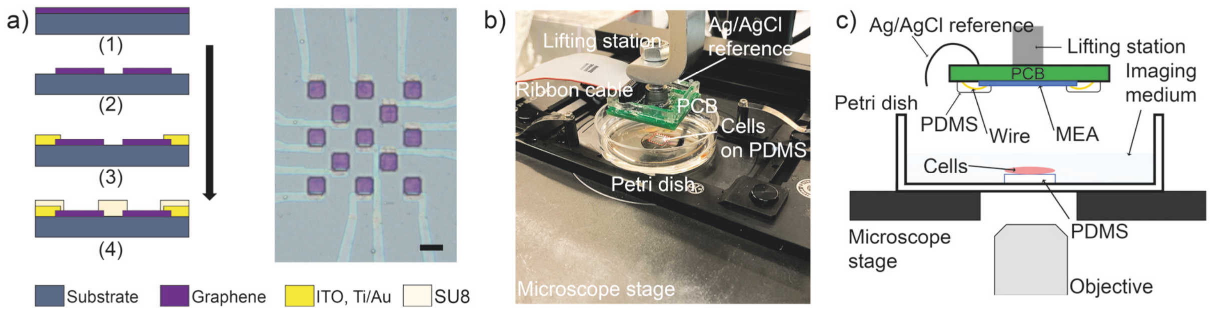

Fig. 1. MEA fabrication and the experimental setup. a) Fabrication steps (left) of the multiplayer graphene MEA (right), including (1) CVD-graphene wafer, (2) graphene electrode patterning, (3) electrode contacts formed by ITO wires and Ti/Au pads, and (4) SU8 passivation. Scale bar, 20 µm. b) Experimental setup: a MEA was wire-bonded on a PCB, which was then side flipped, packaged to a home-built lifting station, and accessed by an Intan chip via one ribbon cable. HEK 293 cells were cultured on a PDMS piece and transferred to a Petri dish filled with the cell imaging medium, which was later biased by one Ag/AgCl reference electrode. c) Illustration of the experimental setup. The MEA was lowered and aligned to contact HEK cells by finetuning the lifting station.

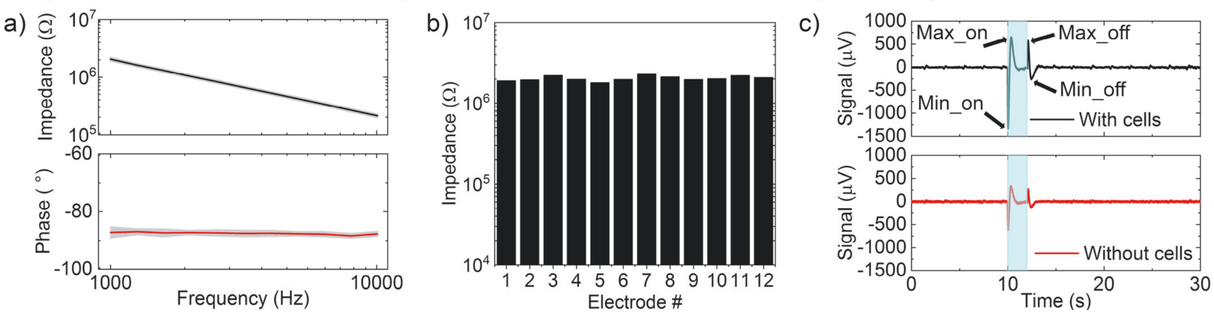

Fig. 2. Electrochemical characterization and optogenetic electrophysiology traces. a) EIS data across the MEA (12-electrode averaged), which showed uniform distributions of the impedance (top) and phase (bottom). Shaded areas represent ±1 SD. b) EIS impedance at 1 kHz from 12 electrodes. c) Typical 30 s-recording traces at one electrode with (top, 3-time averaged) and without (bottom, *i.e.* light-induced artifact) *ChR2-jRCAMP1a* co-expressed HEK cells, applied with a constant 470-nm stimulus (blue window) at 2 s duration and 90.4 mW/cm^2 intensity. Max_on/off and Min_on/off are defined as the maximum and minimum values of signal oscillation when the 470-nm stimulus is on/off, respectively.

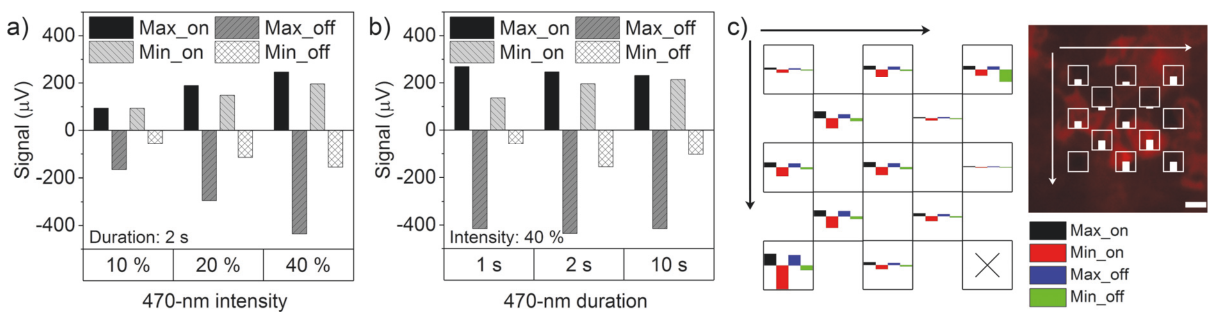

Fig. 3. Quantification of the electrophysiology data. a) Oscillation amplitudes (12-electrode averaged Max_on/off and Min_on/off values) versus the 470-nm stimulus intensity (10-40 % corresponds to 22.6-90.4 mW/cm^2) at 2 s duration. b) Oscillation amplitudes (12-electrode averaged Max_on/off and Min_on/off values) versus the 470-nm stimulus duration (1-10 s) at 90.4 mW/cm^2 intensity. c) Spatial mapping of the oscillation amplitudes across the MEA with the 470-nm stimulus at 90.4 mW/cm^2 intensity and 2 s duration (left, the half-height of each illustrative square is 500 µV; the crossed electrode failed during MEA fabrication). The *jRCAMP1a* image of *ChR2-jRCAMP1a* co-expressed HEK cells was overlaid with the MEA image (right) to compare the electrophysiology data with the *jRCAMP1a* imaging data collected simultaneously. Bar graphs overlaid on the MEA image represent the optogenetically evoked $\Delta F/F_0$ at each electrode site; the height of each illustrative square is 5 %. Scale bar, 20 µm.

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