Culturing of Neurons on Graphene Transistors for Higher Resolution Scanning of Processes

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BRIEF. This work presents support for using graphene for electrochemical scanning of neurons, as well as the optimal neuronal condition for growth on graphene.

ABSTRACT. Like transistors on a microchip, trillions of synapses are the computational units of the brain. Synaptic dysfunction has been widely vindicated in many neurological disorders like Schizophrenia and Alzheimer's disease. A single neuron can make thousands of synaptic connections with other neurons. Due to their high density and micrometer size, existing measurements such as optical imaging and patch clamp recording do not have sufficient spatiotemporal resolutions to study how many synaptic inputs are integrated into one neuron. Recently, graphene, a single layer allotrope of carbon, has emerged to high regard within fields of electrochemistry and physics. Thanks to graphene's exotic electric properties, it has been shown that optoelectric scanning of neuronal processes growing on graphene is an adequate means to study neuronal circuitry at synapse level. A variety of different culture methods were conducted and neurons with matured synaptic connections have successfully been grown directly on graphene. The normality of those neurons was verified by electrophysiology and optical imaging. This graphene based super-resolution and high throughput screening platform will bring us further understanding into the neuronal coding in our brain.

INTRODUCTION.

The brain is one of the most complex networks ever created by nature. Consisting of billions of neurons with trillions of connections, or synapses, it is the reason for the high functioning capabilities of many organisms. Neurons are the basic unit of the nervous system, communicating with each other across a vast network of synapses. The glial cells, of which the astrocyte is a primary example, are responsible for providing nourishment and structural support to the neuron. Modern neurobiological methodologies are largely inefficient in certain regards, especially for decoding signal processing in the extremely complicated neuronal network. The major drawback is either an inadequate resolution or insufficient speed of testing, both of which are necessary for surveying a vast neural network bonded by trillions of microscopic synapses [1].

For instance, electrophysiological recording has excellent sensitivity and temporal resolution, but only samples very limited number of neurons. In contrast, optical imaging is capable of imaging a large number of neurons but suffers from insufficient sensitivity and temporal resolution [1]. Tremendous efforts have been made to surmount these problems. For example, high-density microelectronics has been utilized to simultaneously record electrical signals from neural networks down to exceedingly small spatial resolutions [2]. Therefore, it is highly desirable to develop an ultrasensitive method to account for the minuscule size but astronomic number of synapses in the brain [1].

It has been set about to develop a nano-platform using graphene, a single layer of carbon benzene rings arranged in a honeycomb crystal lattice. The desirability of graphene in electroscanning experiments arises from its unique electrochemical properties, stemming from the unique electron dispersion at the six corners of each hexagonal cell.

A previous study [1] began comparing the electrophysiologies of neurons on graphene with those in normal culture condition. Building upon this study, morphologies of neurons also needed to be compared. Various culture conditions were tested, including the novel astrocyte banking culture (also known as a sandwich culture) (Figure S2). Tests were performed to determine whether the astrocyte banking culture offers a significant improvement in neuronal survival rates as compared to neurons cultured on graphene in other culture conditions. The specific research question at hand is whether or not neural networks grown on graphene are structurally and functionally similar to those grown by traditional culture procedure.

This innovation bares great potential in the medicinal field. For example, it can be employed as a high throughput screening platform for drugs targeting neurological diseases involving network dysfunction (e.g. epilepsy). Also, it may be modified for clinical diagnosis of neurological disorders (e.g. Alzheimer's disease).

MATERIALS AND METHODS.

Cell Culturing to Obtain Neurons.

The hippocampus and cortex were dissected and removed from a P1 rat pup brain. The hippocampi and the cortex were placed separately in dissection medium H+20. The contents were then washed three times with H+20 and three times with Hank's solution (Sigma, H2387). All liquid was aspirated, and dissociating solution containing DNase was added to each. Triturating with a Pasteur pipette ensured that the tissues were broken down into individual cells. The suspensions were centrifuged at 50 times gravity for 5 minutes and neural plating medium was added. The hippocampal suspension was then deposited on differentially treated glass coverslips (0.17 mm in thickness and 12 mm in diameter) while the cortical suspension was transferred to matrigel-treated culture flasks. Both were kept in the 37°C 5% CO_2 incubator. Two days later, 4Ara-C (4-cytosine arabinoside) was added to the hippocampal culture to inhibit glial growth.

Glial Cell Extraction.

When the glial cells within the cortical culture had reached confluence (about 7 days later), the culture was vigorously rinsed three times with phosphate buffer solution to remove neurons, oligodendrocytes, and microglia. Trypsin was added to the solution to detach astrocytes from flasks. Glial plating medium was added to the culture and the resulting solution was centrifuged at 50 times gravity for 5 minutes at 4°C. The astrocytes were then resuspended in glial medium, plated onto coverslips in a 24 well plate pre-treated with matrigel, and stored in the incubator. One day later, 1 mL of plating medium and 1 mL of 4-ARAC containing neuronal medium was added to each well.

Astrocyte Conditioned Medium.

After 2 more days of incubation, 1 mL of medium was removed from glial cell extraction, and 1 mL fresh medium added. The removed 1 mL was filtered to remove cell debris and designated as the conditioned medium, stored at -80°C for future use. The above process was repeated every day until the desired volume of conditioned medium was obtained.

Testing of Different Neuronal Conditions.

Hippocampal neurons were plated on each of 24 graphene-coated coverslips as described above, except that the coverslips were not treated with matrigel, as that would interfere with electrochemical scanning. The neurons were placed in the culture incubator for 1-2 days to allow for attachment to the graphene. Afterwards, each of a group of 8 coverslips was exposed to a different treatment. The first group consisted of neurons lacking astrocyte support. The second group consisted of neurons growing in astrocyte conditioned medium. The third group consisted of neurons exposed to the astrocyte banking culture. After two weeks, these three cultures underwent cell counting, fluorescence immunocytochemistry, patch-clamp recording (e.g. evoked postsynaptic current,

EPSC), and Raman microscopy scanning. Patch clamp recording was used to compare the electrophysiology of neurons on graphene with neurons in normal cell culture, while Raman scanning was used to identify the electrophysiological relationship between neurons and the graphene.

Measurement of EPSCs.

After obtaining high quality graphene, scanning photocurrent measurements were performed to locate the graphene transistor precisely. A small external current was applied to ensure that the transistor was in the "metastable" state, such that it would be extremely sensitive to any further electrical activity. The neuron was cultured on the graphene and the glass coverslip with the washer "foot" was placed on top, such that the astrocytes hung upside down over the neurons. Subsequently, electrical stimulation mimicking synaptic firing was delivered to excite all neurons in the region, and network output (i.e. EPSC) was monitored by patch-clamp recording at neuronal soma. Simultaneously, a piezo-controlled mirror with nanometer scale spatial resolution was used to fast locate the local action potential changes in the full neural circuit by detecting the local photocurrent signal changes of graphene, resulting from the gating effect from the local action potential changes. Then the scanning mirror was swapped between these recorded positions, and the related local action potential changes were monitored with sub-millisecond temporal resolution. Direct readout of neural integration from the somas of neurons was still needed to verify the reliability of the graphene-based measurement. To accomplish this, patch clamp studies were performed. Due to the easy access of neurons on this imaging system, it was possible to position patch-clamp electrodes on top of the chamber and perform recording using conventional methods.

RESULTS.

Figure 1 shows the results of the initial immunostaining with neurons of 6 days outside of the body. In these images, the red structures are neurons, the green structures are astrocytes, and the blue structures are nuclei of the cells. It is clear that the neurons in 1(a) and 1(b), although present at a much lower concentration than the neurons in 1(c), are morphologically similar to those in 1(c). In addition, there are fewer astrocytes in 1(a) and 1(b) than in 1(c). In comparing 1(a) and 1(b), it is clear that there are fewer astrocytes in 1(a), although they are more spread out than the astrocytes in 1(b), which seem to be situating themselves into dense, localized areas. The astrocytes in 1(a) and 1(b) are either not directly under the neurons, or under the neurons at such a low rate that scanning will not be affected. These results show that neurons are able to grow directly on graphene, and that neurons grown on graphene were morphologically similar to neurons grown on matrigel-coated coverslips.

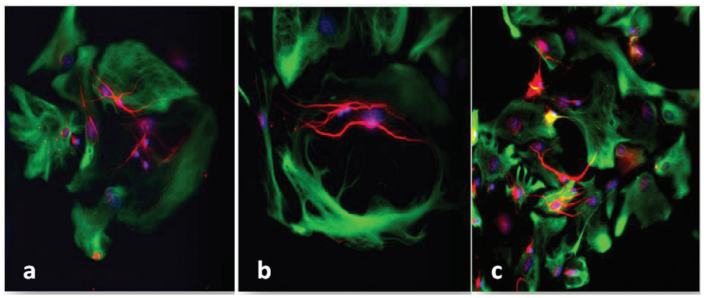

Figure 1. Immunostaining of (a) Neurons on graphene with astrocyte conditioned medium. (b) Neurons on graphene with low astrocyte interference. (c) Neurons on matrigel under normal culture conditions. The red structures are neurons (stained with 1:100 GFAP), the green structures are astrocytes (stained with 1:100 Tuj1), and the blue structures are the nuclei of the cells (stained with 1:50 DAPI). These show that neurons grown on graphene are morphologically similar to those grown on matrigel. All antibodies are from Sigma-Aldrich.

A second immunostaining (not shown) compared the individual neuronal culture conditions with each other, and showed that the astrocyte banking culture was the optimal condition. This was clear due to the obvious differences in the morphology between the conditions. Figure 2(a) and (b) show the results of Raman mapping of graphene G' peak in a 40x40 μm area in which a neuron cells resides in the middle of the images on a flat graphene sheet. Figure 2(a) displays a lower contrast to show the whole cell, whereas Figure 2(b) displays a higher contrast in recognition of the strongest signals. Figure 2(c) shows the fluorescence emission of the neuron cell. Figure 2(d) shows phase-contrast view of the neuron. The excitation wavelength for both Raman and fluorescence imaging in Figure 2 is 785 nm. The fluorescence emission is collected in 850 – 1700 nm range.

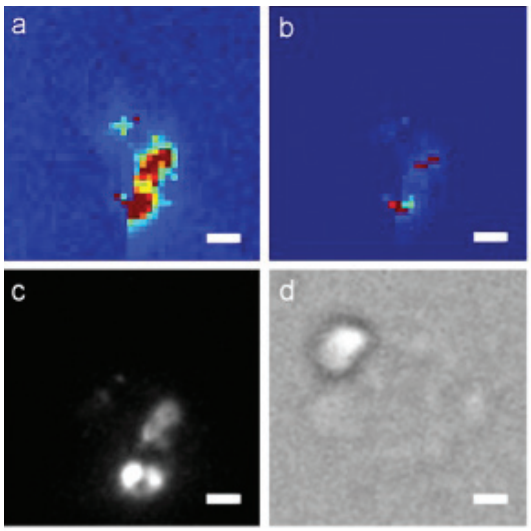

Figure 2. Raman mapping of a neuron cell with (a) high and (b) low contrast. (c) Fluorescence emission from the neuron cell. (d) Phase contrast view of the neuron cell. The scale bar is 5 um.

Figure 3 shows the Raman spectrum pulled out from the Raman mapping of a neuron cultured with astrocyte banking culture on graphene. Raman spectroscopy is used to determine whether the graphene is affected by the presence of the neuron. Graphene typically displays a G' peak around 2700 cm-1 and a G peak around 1600 cm^{-1}. Here, the G' peak from graphene Raman spectrum is displayed in Figure 3. The Lorentzian fitting shows the G' peak is at 2686 cm^{-1}.

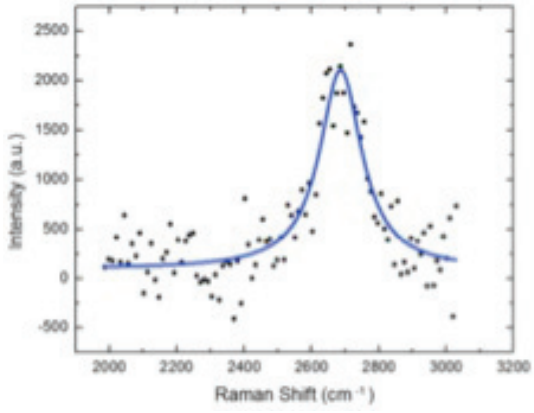

Figure 3. Raman spectrum from the neuron cell Raman mapping. Graphene Raman G' peak around 2700 cm^{-1} is shown.

DISCUSSION.

The results in Figure 1 show that neurons cultured on graphene tend to possess similar morphological properties with the neurons grown on matrigel coated coverslips under normal conditions. These results indicate that neurons are able to survive on substrates that are not specifically treated to support neurons, as matrigel is. This cannot be a universal claim, however, given that the neurons grown on graphene tend to develop into cultures of a much lower neuronal density than neurons grown on matrigel coated coverslips under normal condi

tions. In addition, comparisons between the neurons grown on graphene show that after treated for astrocyte removal, the astrocytes that remain are in such a low concentration that they have a low chance of interfering with graphene transistor scanning. These results ultimately allowed for proceeding with the knowledge both that neurons could grow with sufficient morphological development on graphene and that all neuronal conditions on graphene resulted in low astrocyte concentrations directly under the neurons.

The Raman mapping of graphene in Figure 2 and the corresponding Raman spectrum in Figure 3 indicate that neurons directly grown on graphene were able to interact with graphene. However, by comparing the four sub-figures in Figure 2, we notice that the Raman mapping result very much resembles the shape of the neuronal soma in direct contact with graphene. It is possible that the surface biomolecules (sugar, protein, and lipids) on the soma electrically and mechanically interact with graphene, an attractive feature that can be explored for electrochemical scanning or other detection methods. Moreover, the fluorescence emission in the near-infrared region excited by the 785 nm laser also bears resemblance to the graphene Raman mapping result. It is likely that in some regions, the strong fluorescence emission interacts with the Raman signals, significantly raising the background noise level.

Given the novelty of this method, very limited literature exists with which to compare these results, however, previous results [1] are used in conjunction with these results to lend ample support to the theory that graphene transistor can be used to develop a long desired tool to study synaptic transmission across neural network. Noticeably, the banking culture indeed performed optimally in maintain neuronal health compared to other conditions [2].

CONCLUSION AND FUTURE DIRECTION.

This research has shown that neurons cultured on graphene are morphologically similar to neurons cultured on matrigel-coated coverslips under normal conditions; therefore, graphene may be used as a high resolution scanning mechanism for neuronal processes at the synaptic level. Further, it has been shown that neurons grown on graphene are able to develop synaptic connections without astrocyte support. Finally, it has been shown that neurons cultured specifically under the astrocyte banking culture yielded higher survival rates and healthier development than neurons grown in other graphene culture conditions. Given that results relied mostly on immunostaining, which is generally considered to be a reliable process in biochemical analysis, the first and third conclusions are supported solely by results within this report. The second conclusion is supported by results within this report in conjunction with previous literature [1]. The fourth conclusion is supported solely by results within this report, with the idea stemming from previous literature [3].

In order to refine and strengthen these conclusions, further electrophysiological analysis is required. With more time, electrophysiological analysis of neurons under the astrocyte banking culture could be conducted, and these results would be compared to the results from [1] to see whether morphological similarity is correlated with electrophysiological similarity between neurons on graphene and those in traditional culture. In addition, patch clamp recordings and optical imaging tests could be conducted under the same conditions as the graphene scanning to demonstrate that graphene could act as a better method for sensitivity, resolution and throughput than either of the other methods. Finally, further steps could be taken to refine the Raman spectroscopy in both sensitivity and accuracy. These steps may include ensuring that cellular debris are accounted for by filtering all medium used, as well as Raman scanning within a plasma chamber to remove air particulates affecting spectroscopy. Also, a photocurrent microscope with fluorescence detection could be used. The cells need to be photo-bleached before Raman spectroscopy to minimize the background signal.

Future experiments include using the neuron graphene apparatus to deliver stimulations to specific synapses of a neuronal network to identify how local or global changes of synaptic inputs alter dendritic integration and thus network output. The high throughput and high resolution graphene apparatus will allow for unprecedented power to address many hitherto not understood questions about and beyond dendritic integration. Moreover, LED based mechanisms in conjunction with optogentics can be used to selectively stimulate specific types of neurons in the network. Overall, the combination of highly sensitive and high throughput of graphene transistors with matching optogenetic manipulation would allow neuroscientists for the first time to intentionally perturb and simultaneously register neural network signaling with sufficient spatiotemporal accuracy down to single synapses and throughput matching the astronomic numbers of synaptic connections.

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SUPPORTING INFORMATION.

Figure S1. Preliminary Results
Figure S2. Astrocyte Banking Culture Schematic

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