Probing electrical signals in the retina via graphene-integrated microfluidic platforms

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Graphene has attracted extensive attention in biological and biomedical fields due to its unique physical properties and excellent biocompatibility. We combine graphene field-effect transistors and scanning photocurrent microscopy with microfluidic platforms to investigate electrical signals in mouse retina. Remarkable photocurrent signals were detected from the graphene underneath the optic nerve head (ONH) of the retina, where the electrical activity from this region can modulate the carrier concentration of the graphene and induce local potential gradients. These built-in electrical potential gradients can efficiently separate photo-excited electron–hole pairs, leading to strong photocurrent responses in the graphene underneath the ONH. We also show that no significant photocurrent signal was observed in the graphene underneath either dehydrated or fixed retinal tissues, verifying that the photocurrent responses generated in the graphene underneath the ONH were indeed induced by the electrical activity in living retina. This method not only provides a way to investigate electrical processes in living retinal tissues, but also offers opportunities to study many other cellular systems involving cell–cell interactions through electrical signaling.

The retina is a window to the brain and as a part of the central nervous system (CNS), it shares similar properties with the brain, making it an ideal candidate for studying physiological and pathological activities in the CNS. Three cellular layers are connected by two layers of synapses formed by the axons and dendrites of neurons in the retina. The outer nuclear layer of the retina contains the cell bodies of light receptors, such as the rods and cones, which are responsible for dim light and precise color vision, respectively. Photoreceptor responses to light stimuli are transmitted as a graded potential through center layers to retinal ganglion cells (RGCs). RGCs, in turn, transmit visual information to the brain via produced action potentials conducted along their axons in the optic nerve. The optic nerve head (ONH) is the region of the retina where RGC axons are bundled together prior to exiting the globe of the eye as the optic nerve proper. This area does not contain neuronal soma and is associated with the “blind spot” in visual perception. However, it is an area of concentrated electrical activity due to the tight bundling of unmyelinated RGC axons.

Various techniques have been developed to study the neural activity in the retina and other neural networks. Patch-clamp recording can record voltage or current signals of a single cell with high electrical sensitivity and has been widely used to investigate neural activity in vitro and in vivo. Nevertheless, the sizeable probe tips and bulky micromanipulators involved in this technique make it challenging to investigate a large population of cells in an entire neural network. Recently, various kinds of fluorescence sensors, such as voltage sensitive dyes and calcium indicators, have been utilized to optically record electrical activities in neural networks with high throughput. However, the electrical sensitivity of optical imaging is significantly lower than that of patch-clamp recording. With high-density electrode arrays, microelectrode arrays (MEAs) can stimulate and record a large population of neurons simultaneously, making it a powerful technique to study the functions and connections of neural networks. Although field effect transistors (FETs) have been applied to improve the electrical sensitivity, electrode density and physical contact problems are still limiting the capability of MEAs in studying neural networks. The limitations of these currently available neurotechnologies thus call for new approaches to further improve our capability to probe and understand complex neural networks.

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After groundbreaking experiments toward the micromechanical exfoliation of graphene, it has become one of the most promising candidates for future electronics and optoelectronics because of its extraordinary electrical and optical properties.\textsuperscript{17–20} Absorbing merely 2.3% of incident light makes graphene compatible with high-resolution optical imaging. As a two-dimensional material, graphene exposes its whole structure to the environment, making it extremely sensitive to local electrochemical environment changes.\textsuperscript{21} Furthermore, due to its strength, flexibility, and biocompatibility, graphene can attach to cells and tissues to achieve high electrical sensitivity for a long-term experiment, making it an excellent material for biosensing.\textsuperscript{22–24}

Here, we combine graphene FETs with scanning photocurrent microscopy to detect the neural activity of mouse retina, which is placed on microfluidic platforms that can keep whole mouse retina healthy for multiple days.\textsuperscript{25} Electrical activity in living retinal tissues can modulate the carrier concentration of graphene, resulting in potential gradients that can separate photo-excited electron–hole pairs (EHPs) and produce photocurrent signals. In our experiments, strong photocurrent responses were detected from the graphene underneath the ONH, suggesting that the electrical activity of the ONH is sufficient to alter the local electrical properties of graphene. We also found that no remarkable photocurrent response was detected from the graphene underneath both dehydrated and fixed retina, which further confirms that the photocurrent signals in graphene that we observed were indeed from the electrical activity in living tissue.

Results and discussion

Fig. 1A shows the components of our graphene-based microfluidic platform: a glass cylinder, a slice of agar gel, a polydimethylsiloxane (PDMS) ring, and graphene transistors. First, graphene was synthesized via a standard chemical vapor deposition (CVD) method and transferred onto 170 µm thick coverslips with pre-patterned gold electrodes.\textsuperscript{26} We performed Raman spectroscopy measurements to inspect the quality and thickness of graphene on the coverslips with a 532 nm laser. As shown in Fig. 1B, the 2D peak has a symmetric shape and the 2D-to-G intensity ratio is about 2, indicating that the as-grown graphene is a monolayer structure. We also characterized the electrical transport properties of graphene transistors. In our experiment, a PDMS ring was placed on top of the chip with graphene transistors and filled with 1× phosphate-buffered saline (PBS) solution. A gold wire was inserted into the solution and used as a gate to set its electrochemical potential.

Fig. 1C shows the ambipolar behavior of a typical graphene transistor with the Dirac point close to 0.7 V, displaying p-type characteristics at zero gate bias.

After fabricating graphene transistors on a thin coverslip, we placed a slice of agar gel at the bottom of a glass cylinder, which serves as a supporting substrate to hold the retina in place as well as a porous pad that allows fresh media to slowly perfuse through to supply nutrients to the retina from the cylinder. Living retina was dissected and mounted on top of the agar gel. The cylinder was then inverted and inserted into the large hole of the PDMS ring, such that the retina came in contact with graphene transistors. Serum-free culture media containing neurotrophic factors were then added to the glass cylinder to maintain the retina health.\textsuperscript{25} Moreover, the media weight on top of the agar gel helps to hold the cylinder in place on top of the retina to restrict curling. Three days prior to enucleation and retina dissection, mice received an intravitreal injection of fluorophore-conjugated cholera toxin subunit B (CTB). CTB is an active uptake and active transport neural tracer that is preferentially endocytosed by RGCs and anterogradely transported along their axons to the axon terminal in the brain. We utilized the tracer to visualize RGCs and the unmyelinated segment of their axons in the retina and ONH during photocurrent recording.\textsuperscript{27} As demonstrated in Fig. 1D, a CTB-labeled (red) whole retina was placed on top of graphene transistors, where the dark regions are opaque gold electrode arrays. The fluorescence image presents the structure of the RGC layer. All the axons of RGCs from different parts of the retina extend from cell soma, group into bundles, and coalesce at the ONH in the center of the retina to exit the globe of the eye. During our experiments, heating gel pads were used to maintain an external environment of 36 °C. With culture media perfusion through the agar gel continuously, the microfluidic platform can sustain whole mouse retina for over 96 hours, as illustrated in our previous report.\textsuperscript{25}

To explore electrical activity in the retina, we performed spatially-resolved photocurrent measurements through a graphene-integrated microfluidic platform. A continuous-wave laser source with a wavelength of 785 nm was selected to prevent the retina from responding to the laser based on the negligible spectral sensitivity of mouse retina to the light of wavelength above 700 nm.\textsuperscript{28} The laser beam was deflected by a nanometer-resolution scanning mirror and then focused by a 40× objective (N.A. = 0.6) into a diffraction-limited spot (∼1 μm) on graphene transistors (bottom panel of Fig. 1A). The photocurrent signals were collected using a preamplifier and the corresponding reflection image was recorded by using a silicon detector. By overlapping the reflection image with the photocurrent image, the corresponding position of the photoresponse on the sample could be located.

Fig. 2A–D show the fluorescence, optical, reflection, and photocurrent images of a living retina in a graphene-integrated microfluidic platform, respectively. Pronounced photocurrent responses were observed at graphene–metal junction areas with opposite polarity for drain and source electrodes, because the Schottky-like barriers between metals and graphene can efficiently separate photo-excited EHPs (detailed photocurrent generation mechanisms will be discussed later). More importantly, remarkable photocurrent signals were detected in the ONH region of the living retinal tissue (Fig. 2D), indicating that the electrical activity of the ONH can modulate the carrier concentration of graphene and induce photocurrent signals. The photocurrent responses around the bottom electrode are
much weaker than those around the upper electrode, which is likely due to the electrical double layer formed between the retina and graphene that can screen the electrical signals from the retina and reduce the photocurrent responses in graphene. As shown in Fig. 2A, the fluorescence image in the region close to the bottom electrode is out-of-focus, suggesting that the ONH did not contact the graphene substrate around the bottom electrode region well. This mainly results from the naturally curved shape of the retina, which is not ideally compatible with the planar nature of graphene-integrated microfluidic platforms. If we can fabricate graphene transistors on flexible substrates (such as PDMS membranes), this issue is likely to be addressed. We also investigated electrical signals in the retina after it was dehydrated for 24 hours.
dehydration, the retinal tissue shrunk, slid, and made a good contact with the graphene substrate (Fig. 2E and F). However, the electrical properties associated with living retina were eradicated. No significant photocurrent response was observed from the entire dehydrated retina, except for the graphene–metal junction areas. As shown in Fig. 2H, the strong photocurrent signals induced by the living ONH shown in Fig. 2D disappeared when the ONH was dehydrated and moved away from its previous location, confirming that the photocurrent responses underneath the living ONH were induced by its electrical activity.

To further validate our result, we studied the electrical signals in a paraformaldehyde-fixed retina through scanning photocurrent measurements. Note that release cuts extending to the ONH in the retina were necessary to relieve the stiffness after fixation and successfully flatten the retina for contact with the graphene (Fig. 3A). During the fixation process, the tissue structure was preserved but biochemical reactions were terminated. As we expected, there is no electrical activity in the fixed retina and thus no photocurrent signal was detected in the ONH region (Fig. 3B–D). Comparing the photocurrent results from a living retina with those from dehydrated and fixed retina, we verify that our graphene-based scanning photocurrent microscopy is a promising tool to detect electrical signals in living retina.

For better understanding of the photocurrent generation mechanisms in graphene transistors underneath living retina, relative band diagrams across graphene transistors were extracted by numerical integration of the scanning photocurrent profiles along the dashed lines shown in Fig. 2D and H. The results shown in Fig. 4A reveal that for the case with dehydrated retina, potential gradients of graphene only exist near the gold electrodes, which are induced by two back-to-back Schottky-like barriers at graphene–metal junction areas. These local electric fields can efficiently separate photo-excited EHPs and lead to strong photocurrent signals at the graphene–metal junction areas as shown in Fig. 2H. The polarity of photocurrent responses depends on the direction in which the band bends. For example, with the assistance of the local electric field near the left electrode, photo-induced electrons and holes will travel to the left and right directions, respectively and lead to a current along the right direction. However, excess electrons and holes will travel to opposite directions near the right electrode and generate a photocurrent along the left direction. In addition, a flat band region is formed in the middle of graphene between two electrodes, where the photo-excited EHPs quickly recombine and thus no strong photocurrent response is detected. In contrast, when a living retina is placed on top of a graphene transistor, the cell bodies of RGCs that have neural activity initiated action potentials along their axons can change the local electrochemical environments of the underlying graphene. As a result, the band structure of the graphene will be bent in the areas with neural activities since charged molecules and local potentials can modulate...
the carrier concentration of graphene and induce local potential gradients (Fig. 4B), which can promote separation of photo-excited EHPs and thus photocurrent generation. Different polarities of photocurrent signals are directly related to the bending directions of local potentials. Moreover, the existence of the electrical double layer between graphene and the retina may shield the charged molecules when the layer thickness is beyond the Debye screening length. Therefore, only the electrical activity in the regions (such as the ONH), which directly contact or are extremely close to the graphene transistors, can induce strong photocurrent signals in graphene.

**Fig. 3** (A) Fluorescence image of a fixed retina. Scale bar is 200 µm. (B) Enlarged fluorescence, (C) optical, and (D) scanning photocurrent images of the retina in the ONH region, respectively. The edge-to-edge distance between two electrodes is 180 µm. Scale bar is 30 µm in B–D. ‘12’ and ‘0’ near electrodes are metal markers for location.

**Fig. 4** Schematic diagrams of band structures of a graphene transistor. (A) Photocurrent signals generate at graphene–metal junction areas due to the band structure bending. (B) A retina can modulate the carrier concentration of graphene underneath it. Black dashed lines denote the Fermi levels. Green solid lines show band diagrams extracted by numerical integration of the scanning photocurrent profiles along the dashed lines in Fig. 2H and D, respectively.
Conclusions

In conclusion, we develop a graphene-integrated microfluidic platform to investigate the electrical activity in mouse retinal tissues through spatially-resolved scanning photocurrent measurements. Remarkable photocurrent signals are detected in the ONH, suggesting that the electrical activity of RGC axons in the ONH can modulate the carrier concentration, induce local potential gradients, and thus produce photocurrent signals in underlying graphene transistors. Importantly, as control experiments, we find that no significant photocurrent response is observed in graphene underneath both dehydrated and fixed retinal tissues, which further confirms that graphene-based scanning photocurrent microscopy is a promising technique to investigate electrical processes in living retina. This method also opens the door for investigating cellular interactions in other neural networks and biological systems.

Methods

Graphene synthesis

Copper foils (Alfa Aesar 0.025 mm, 99.8% pure, annealed, uncoated) with a grain size of ~100 µm were cut into strips of 1 cm in width and then rinsed with 5% nitric acid (5 min) to eliminate contamination. We then loaded the copper foils onto a quartz boat, which was transferred to a horizontal furnace system. After the system was pumped down to 10 mTorr, the copper foil was annealed at 980 °C with 100 sccm hydrogen flowing through the tube for 1 hour. A mixed gas of 100 sccm H₂ and 20 sccm CH₄ was then introduced into the furnace for the growth of graphene at 980 °C for 30 minutes.

Device fabrication

The gold electrode arrays were patterned on 170 µm thick coverslips via photolithography, and deposited by e-beam evaporation with 5 nm Ti and 40 nm Au. 6 × 6 mm squares were then cut from a graphene/copper-foil. After spin-coating with an ultrathin poly(methyl methacrylate) (PMMA) layer, a wet etching process was performed to remove the copper foil. The PMMA/graphene film was then transferred to the coverslip with pre-patterned gold electrodes. To remove PMMA and clean the transistors, acetone (overnight), isopropyl alcohol (10 min), and deionized water baths were used. For microfluidic platforms, a liquid PDMS base polymer was mixed with a curing agent at a 10:1 mass ratio and poured over a blank wafer in a Petri dish, which was degassed in a vacuum chamber for 1 hour and cured at 70 °C for 3 hours. The solid PDMS layer was then cut into a 15 × 15 mm square with a 10 mm punched hole in the center and attached to the coverslip with pre-fabricated graphene transistors. A slice of agar gel was placed inside an 8 mm diameter glass cylinder. Once the retina was excised, it was placed and flattened on top of the agar gel. The cylinder was then inverted and placed into the hole of the PDMS layer on the coverslip. Culture media were added into the cylinder to keep the retina healthy.

Animal procurement and ethics statement

This study was conducted in accordance with the regulations set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animal protocols were approved by the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center. B6129SF2/J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and colonies were established in the animal facility at Vanderbilt University Medical Center. Mice utilized in this study were obtained from these in-house colonies.

Neural tracing

Neural tracing was performed as previously described. Briefly, anesthetized (2.5% isoflurane) adult mice (2-4 months) received a 1.5-2 µl intravitreal injection of CTB (10 µg µl⁻¹ in sterile PBS; Life Technologies) using a 33 gauge needle attached to a Hamilton syringe. Three days after CTB injection, mice were sacrificed by cervical dislocation and decapitation for live or dehydrated retina recordings or by transcardial perfusion with 4% paraformaldehyde for fixed retina recordings.

Organotypic retina culture

Eyes were enucleated and the retina was dissected for mounting in the microfluidic platform. Whole retina was maintained for 5 hours in the microfluidic device with serum-free, B27-supplemented medium (NeuroBasal; Gibco, Carlsbad, CA), as previously described. Media also contained 2 mM glutamine, 0.1% gentamicin, 1% N2 supplement (500 µg mL⁻¹ insulin, 10 mg mL⁻¹ transferrin, 630 ng mL⁻¹ progesterone, 1.6 mg mL⁻¹ putrescine, 520 mg mL⁻¹ selenite; Gibco), 50 ng mL⁻¹ brain-derived neurotrophic factor (Invitrogen, Carlsbad, CA), 20 ng mL⁻¹ ciliary neurotrophic factor (Invitrogen) and 100 µM inosine (Sigma, St Louis, MO).

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