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Optical Stimulation and Imaging of Functional Brain Circuitry in a Segmented Laminar Flow Chamber

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Abstract

Microfluidic technology is emerging as a useful tool for the study of brain slices, offering precise delivery of chemical factors along with robust oxygen and nutrient transport. However, continued reliance upon electrode-based physiological recording poses inherent limitations in terms of physical access as well as the number of sites that can be sampled simultaneously. In the present study, we combine a microfluidic laminar flow chamber with fast voltage-sensitive dye imaging and laser photostimulation via caged glutamate to map neural network activity across large cortical regions in living brain slices. We find that the closed microfluidic chamber results in greatly improved signal-to-noise performance for optical measurements of neural signaling. These optical tools are also leveraged to characterize laminar flow interfaces within the device, demonstrating a functional boundary width of less than 100 μm . Finally, we utilize this integrated platform to investigate the mechanism of signal propagation for spontaneous neural activity in the developing mouse hippocampus. Through the use of localized Ca^{2+} depletion, we provide evidence for Ca^{2+} -dependent synaptic transmission.

Introduction

Physiological recordings of explanted brain slices are a powerful method for understanding neuronal circuit activity.¹ Brain slices preserve the complex neuronal connectivity that is not present in simple cultures of neural cells. At the same time, they allow much more direct access than intact brains. This technique was pioneered by Yamamoto and McIlwain, who succeeded in measuring the first elicited synaptic and cellular activity in a brain slice.² Typically, living slices are maintained in open recording chambers with nutrient and waste exchange provided by a flow of oxygenated artificial cerebrospinal fluid (ACSF). The slice sits either at the air-liquid interface (Haas chamber) or submerged under ACSF flow, with the latter providing more rapid chemical exchange and better preservation of slice morphology.³

Recently, microfluidic devices have emerged as useful tools for the modulation and control of chemical microenvironments around the brain slice. Blake et al.⁴ leveraged non-mixing laminar flow to focus a stream of Na^+ -free solution on one half of a medullary brain slice, abolishing spontaneous neural activity in that half of the brain slice while not affecting the

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other half. Other examples include an array of microfabricated nozzles for selective neurotransmitter delivery,⁵ a microfluidic probe that simultaneously dispenses and aspirates reagents to achieve highly localized delivery,⁶ and an array of dispensing/aspirating nozzles for creating complex chemical patterns.⁷ While microfluidic platforms have been successful for localized spatiotemporal control of the brain slice chemical environment, the recording of neural activity has continued to rely on the use of physical electrodes. This approach limits measurement to a small number of specific points on the brain slice, thus precluding the observation of coordinated network activity within complex neural circuits.

Recently, optical methods have been developed for monitoring and manipulating neuronal activity across large cortical regions with high spatiotemporal resolution. Fast voltage-sensitive dye (VSD) imaging of membrane potential changes in neuronal ensembles has enabled the visualization of complex neuronal signaling patterns across large two-dimensional regions with millisecond temporal resolution. Further, laser photostimulation by release of caged glutamate neurotransmitters has allowed signaling to be initiated at any point on a brain slice.⁸ In this report, we combine these powerful optical techniques with a microfluidic laminar flow chamber that allows selective chemical delivery to different regions on a brain slice. The integration of microfluidics and optics results in improved signal-to-noise characteristics for the imaging of neural signals and enables previously difficult or impossible experiments in the study of brain function.

Materials and methods

Device fabrication

Device masters were formed by using a laser cutting tool (VersaLaser VLS-2.3, Universal Laser Systems, Scottsdale, AZ) to pattern layers of tape (936 Transparent Packing Tape, Bazic Products, Vernon, CA) on a glass slide, as described previously.⁹ Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) parts were cast from these masters to create two device layers. We employed an X-shaped channel that has been shown to achieve a sharp interface along the entire boundary between two flows, in contrast to the typical Y-shaped channel configuration in which the sharpness of the interface decreases as a function of distance from the inlets.¹⁰ As shown in Fig. 1B, the bottom layer contains a circular chamber 10 mm in diameter and 400 μm in depth, where the brain slice is secured. The top layer contains fluidic channels 150 μm in depth. Prior to assembly, an 8-mm diameter cap was punched out of the top device layer using a biopsy punch to facilitate loading of brain slices into the device. After forming fluidic inlets and outlets, the device layers were permanently bonded to each other by oxygen plasma treatment.

Slice preparation and experimental setup

Living hippocampal or other cortical slices, 400 μm in thickness, were prepared from neonatal mice at 4–6 days postnatal (P4–P6). Slice preparation has been previously described in detail⁸ and was similar to preparation for electrophysiology experiments, but with the addition of an incubation step in ACSF supplemented with 0.2 mg/ml NK360 absorbance voltage-sensitive dye (Nippon Kankoh-Shikiso Kenkyusho, Japan) for 1 hour.

Brain slices were transilluminated with 705-nm light and voltage-dependent changes in the light absorbance of the dye were captured by a MiCAM02 fast imaging system (SciMedia USA Ltd., Costa Mesa, CA) as diagrammed in Fig. 1A. Data images were captured at a rate of 4.4 ms per frame, covering a field of view of $1.28 \times 1.07 \text{ mm}^2$, with a spatial resolution of $14.6 \times 17.9 \mu\text{m}^2/\text{pixel}$. VSD imaging data was visualized by calculating the percent change in pixel intensity, $\Delta I/I\%$, and plotting this value as a color-coded heat map.

The slice chamber was perfused by ACSF using a pressure-driven flow system (AutoMate Scientific, Berkeley, CA) pressurized by carbogen (95% O₂ + 5% CO₂). Flow rates through the tubing were manually controlled by inline intravenous (IV) flow regulators and were maintained at approximately 0.3 μ L/s, or 1.08 mL/hr, for each of the two device inlets. Air bubbles in the microfluidic chamber could be prevented by careful recapping of the device after brain slice loading and by inspecting the tubing for trapped gas prior to connection to the device.

For photostimulation experiments, ACSF perfusate was supplemented with 0.2 mM MNI-caged-L-glutamate (4-Methoxy-7-nitroindolinyL-caged-L-glutamate, Tocris Bioscience, Ellisville, MO). Glutamate uncaging was accomplished by a short focused laser pulse (355 nm, 1 ms, 20 mW), resulting in evoked neuronal activity at the point of exposure. The focal diameter of the laser beam was previously estimated at 150 μ m.⁸

Results and discussion

Slice viability and spontaneous network activity

Spontaneous network activity (SNA) has been described in many developing neural circuits including the hippocampus.¹¹ Recurring SNA events were observed in our neonatal hippocampal slices with a period of roughly 2 minutes (Fig. 2). This neural activity persisted in experimental sessions lasting up to 6 hours with no sign of abatement, demonstrating the viability of explanted brain slices in the microfluidic perfusion chamber. The transparent PDMS chamber provided good compatibility with the optical system for both image acquisition and laser stimulation. Through VSD imaging, the system was able to measure the spatial propagation of SNA signals at a level of detail that had not previously been possible using electrode-based electrophysiology. SNA events originated in CA3 and propagated bidirectionally, both in the forward direction towards CA1 as well as in the reverse direction towards the dentate gyrus (DG). Importantly, reverse propagation is not present in the mature hippocampus, and thus this phenomenon merited additional investigation.

Enhanced signal-to-noise performance

The microfluidic chamber was found to provide an optimal, low-noise environment for VSD imaging. Noise levels were characterized by taking VSD measurements of inactive brain slices. Noise in the perfused conventional chamber was unacceptably high, with the noise signal often matching or exceeding the magnitude of signals from real neural activity. The noise in the conventional chamber dropped to acceptable levels when perfusion was temporarily halted, but the lowest noise levels were measured in the microfluidic chamber. In fact, the perfused microfluidic chamber exhibited significantly better signal-to-noise ratio (SNR) than the non-perfused conventional chamber (19.5 dB vs. 12.9 dB), as illustrated in Fig. 3. In terms of slice physiology, it is preferable to maintain active perfusion in order to maintain steady transport of nutrients, stimulants, and waste, hence the microfluidic chamber is advantageous. The improved noise characteristics of the microfluidic device probably stem from the closed chamber and the low flow rate, which reduce turbulence and contaminants.

Characterization of segmented flow

Segmented laminar flow was demonstrated in the microfluidic chamber by perfusing two fluids, with fluorescein added to one. Visualization was performed by fluorescent microscopy and quantified by ImageJ software (NIH). The width of the fluid interface was then quantified by measuring the transition in fluorescence intensity (Fig. 4). The sharpness of the boundary was found to decrease when flowing over a brain slice. For example, a

boundary width of 190 μm in an empty chamber degraded to 480 μm with a slice in the chamber, likely due to flow disruption by features on the slice surface.

Our purpose in creating segmented flows was to create two distinct chemical environments in neighboring regions on a single brain slice. While fluorescence intensity measurements showed a fairly broad interface between regions, it remained possible that the boundary was actually sharper when considering biological function, due to thresholding effects, for example. Thus, we also probed the laminar flow interface by investigating laser stimulation of neural activity. Caged glutamate was selectively delivered to a brain slice by segmented laminar flow, and laser pulses were applied at a series of points spanning the interface (Fig. 5). Robust neuronal signaling responses were evoked when the laser pulse was delivered at positions where caged glutamate was present at adequate concentrations. As the laser pulses moved across the laminar flow interface, the evoked response dropped off abruptly as the pulses entered the region with lower concentration of caged glutamate. Each laser pulse was separated by 100 μm , and there was a sharp difference in evoked response between pulses spaced just 100 μm apart, indicating that the width of the boundary between regions of differing biological function can be constrained to less than 100 μm . Similar results have been achieved in four different experiments involving both cortical and hippocampal slices.

The limitations of the flow control apparatus resulted in substantial drift ($\sim 100\text{s}$ μm) in the boundary position over the course of 10 min. However, neuronal propagation measurements were completed in less than 1 second, during which time the boundary did not shift by a detectable amount. In practice, the flow was manually adjusted to place the boundary in a desired position, followed immediately by a stimulation and propagation measurement.

Probing the mechanism of reverse neuronal propagation

At this point, we returned to examine the reverse signal propagation that was earlier observed. The adult hippocampus exhibits a strongly feed-forward circuit organization with unidirectional information flow from DG to CA3 to CA1. Reverse propagation in the developing hippocampus is therefore unexpected and intriguing. Specifically, we wished to examine whether this reverse propagation utilized a synaptic mechanism, as with forward propagation in the mature hippocampus, or if in fact another form of transmission was responsible, such as direct coupling through gap junctions. Synaptic signaling is dependent on the presence of extracellular Ca^{2+} , and hence we proceeded to examine this question by the use of segmented delivery of Ca^{2+} .

With Ca^{2+} present across the entire P4 mouse hippocampus, photostimulation in CA3 evoked bidirectional signal propagation towards both CA1 (forward) and DG (reverse), similar to the pattern of SNA. Next, the chamber was switched to a segmented flow, in which the DG region was depleted of Ca^{2+} ions. Photostimulation in CA3 again evoked reverse propagation towards DG, however the signal propagation halted abruptly at the boundary of the Ca^{2+} -depleted region (Fig. 6). Switching back to global perfusion of Ca^{2+} ions restored reverse propagation down to the DG (not shown). The experiment was repeated on three slices with similar results. This result clearly rules out a major role for gap junctions in activity propagation and supports Ca^{2+} -dependent synaptic transmission as the mechanism for reverse neuronal propagation in the developing hippocampus. Importantly, segmented delivery of calcium ions allowed the initiation of neuronal signaling to be decoupled from propagation. Initiation and propagation would remain convoluted in an experiment where calcium ions were simply depleted from the entire brain slice. Thus, as demonstrated here, microfluidic modulation via segmented flow enables unique slice experiments that shed new light on neuronal circuit mechanisms.

Conclusions

We have demonstrated the successful integration of microfluidics with advanced optical tools from neuroscience, enabling spatial control of the chemical microenvironment, broad visualization of neural network dynamics, and command of signal initiation all to be achieved simultaneously in a living brain slice. The combination of these techniques provides improved sensitivity through noise reduction and the ability to perform novel mechanistic studies through unprecedented experimental control. Specifically, the spatial control of both chemical agents and neuronal signal initiation allowed for the well-controlled investigation of synaptic transmission in the reverse neuronal propagation observed in the early developing hippocampus. This platform is adaptable to many different chemical agents and various regions of the brain besides the hippocampus, hence it should prove useful in a number of future neuroscience studies. Additionally, the successful integration of microfluidics and photonics for neuroscience suggests that similar approaches may be successful for the study of other organ and tissue explants or cultures.

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Fig. 1. Integration of a segmented-flow microfluidic chamber with optical imaging and stimulation of neural signaling. (A) A living brain slice is maintained under perfusion by supplemented ACSF solutions fed by pressure-driven flow. The VSD-stained slice is transilluminated with 705 nm light and voltage-dependent changes in the light absorbance of the dye are captured by a MiCAM02 fast imaging system (up to 1 ms/frame). A dichroic mirror in the compound microscope allows simultaneous laser excitation at 355 nm. (B) Non-mixing laminar flow in the microfluidic chamber bathes the brain slice in two different chemical environments, separated by a sharp boundary. The chamber has a removable cap through which slices may be loaded. (C) A commercial brain slice perfusion chamber (top, Warner Instruments), a custom-machined conventional perfusion chamber (middle), and the microfluidic chamber used in this work (bottom). Segmented flow is illustrated using red and blue dyes in the microfluidic chamber.

Fig. 2.

Spontaneous network activity (SNA) demonstrates slice viability. Fast voltage-sensitive dye allows dynamic 2D imaging of neural propagation at a level of detail that is not possible by electrophysiology. Events originated in CA3 and propagated towards both CA1 (forward) and DG (reverse). SNA events occurred once every 2 minutes and persisted for the full duration of experimental sessions lasting up to 6 hours, demonstrating the viability and neural activity of the brain slice in the microfluidic perfusion chamber. The color scale codes response strength, with warmer colors indicating greater excitation.

Fig. 3.

Microfluidic flow chamber provides improved signal-to-noise characteristics. VSD noise from inactive brain slices was compared to signals from actual brain activity. Noise in the perfused conventional chamber (A) was the worst, with noise magnitude often matching or exceeding signals from actual brain activity (D). With perfusion halted, noise in the conventional chamber (B) dropped to usable levels, however noise was the lowest in the microfluidic chamber, even with perfusion (C). The traces below the images correspond to time-dependent signals acquired from within the white squares (5×5 pixels) on the images above. The position of each square was chosen in order to be representative. Signal-to-noise ratio (SNR) was calculated by comparing the peak levels (arrowheads) from the noise traces (A–C) with the peak level of SNA signal (D). Color levels represent the relative magnitude of changes in VSD optical signal.

Fig. 4.

Laminar flow creates segmented chemical environments over a brain slice. (A) The laminar flow boundary was visualized by fluorescent labeling of an ACSF stream in a device chamber without a brain slice. The image intensity was traced from X to X' and plotted in (B), showing a boundary width of 190 μm, measured from the 90% intensity point to the 10% point. (C–D) The boundary is considerably less sharp when a brain slice is placed in the device chamber, increasing to 480 μm.

Fig. 5.

Spatial compartmentalization of biological function. (A) Laminar flow was used to deliver caged glutamate (visualized by fluorescein tracer) selectively to part of a brain slice. The bright region contained caged glutamate and fluorescein. Laser pulses were delivered at a series of points (1–7) spanning the fluid interface. Positions that evoked a neural response were labeled as filled green circles, while positions that did not evoke a response were labeled as empty red circles. It can be seen that the responsiveness of each position correlated to the presence of caged glutamate. Laser spots were separated by 100 μm , hence the difference in response between positions 3 and 4 indicates that the width of the boundary between regions of differing biological function can be less than 100 μm . This is the same boundary as shown in Fig. 4C–D. (B–D) Evoked neural activity from laser stimulation at sites 1–3 evoked a robust response in neural activity. (E–F) Laser stimulation at sites 4–6 failed to evoke neural activity. Arrowheads indicate noise.

Fig. 6.

Reverse neuronal propagation requires Ca^{2+} -dependent synaptic transmission. (A) Fluorescent image showing global perfusion of Ca^{2+} ions, labeled by a fluorescein tracer. (B) Under global Ca^{2+} perfusion, laser stimulation in CA3 evokes reverse propagation that reaches the DG. (C) Fluorescent image showing segmented delivery of Ca^{2+} by non-mixing laminar flows. The dashed line along the boundary is reproduced in the other panels as a reference. (D) Under segmented delivery of Ca^{2+} , reverse propagation is initiated at CA3 but halts abruptly at the edge of the Ca^{2+} interface.