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Development and characterization of a microfluidic chamber incorporating fluid ports with active suction for localized chemical stimulation of brain slices

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Abstract

We report a novel microfluidic chamber incorporating fluid ports with active suction to achieve localized chemical stimulation of brain slices. A two-level soft-lithography process is used to fabricate fluid ports with integrated injection and suction holes that are connected to underlying microchannels. Fluorescence imaging, particle tracking velocimetry, and cell staining are used to characterize flows around a fluid port with or without active suction to validate effective localization of injected chemicals. To demonstrate biological applicability of the chamber, we show an induction of cortical spreading depression (CSD) waves in mouse brain slices through controlled focal delivery of potassium chloride solution.

Introduction

In vitro neurobiological experiments using brain slices have played a key role in improving our understanding of the central nervous system. Unlike cell cultures, brain slices retain local neuronal connections and anatomy,¹ closely approximating intact brains. Furthermore, vascular and metabolic influences that exist in *in vivo* preparations are effectively suppressed,² enabling independent tests of biological hypotheses involving neurovascular coupling.

The brain is organized into different functional modules.³ The ability to induce or suppress neuronal activity in specific modules through localized delivery is of great interest to neuroscientists. This is especially true for spatio-temporally varying neural phenomena such as seizures and cortical spreading depression (CSD), which initiate and spread from a specific focus. Delivery of a chemical agent stimulating or suppressing such phenomena

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should be precisely confined within a particular area while the surrounding neurons get information only through the neuronal network.

Glass micropipettes have long been used to inject soluble chemicals into extracellular or intracellular spaces of brain tissues and study their impacts.^{4–6} Microfluidic devices have recently emerged as an elegant alternative platform to inject chemicals into extracellular spaces of brain slices. They are less invasive, offer potentially higher spatial resolution, and can be integrated with wide ranges of stimulation and sensing modalities.

Several research groups have developed microfluidic devices for cell cultures with localized stimulation capability.^{7–14} These devices, however, are not suited for tissue slices that require a continuous supply of nutrients and oxygen. Tissue slices also have finite thicknesses and healthy cells tend to be located away from the exposed surfaces.

Microfabricated probes^{15–18} have been developed to mimic conventional concentric dual glass micropipettes to locally release chemicals on biological samples. Queval *et al.*¹⁶ applied such microfluidic probes for localized chemical injection into artificially thinned cultured brain slices for bottom-up imaging using an inverted microscope.

Microfluidic probes, however, have limitations especially when applied to brain slices. These relatively large probes positioned above the slices block optical views and also impede the placements of electrodes and other transducers directly above regions that are chemically stimulated.

Planar microfluidic platforms placed underneath a brain slice can circumvent these difficulties and allow much greater flexibility in designing the geometry of ports and their relative arrangements. Planar devices can also be readily scaled up to incorporate injection ports on multiple discrete locations. Moreover, they allow for visualization, spectroscopic recording, and electrode placement from above the slice, avoiding modification of most commonly used slice physiology setups.

Several studies have shown that planar microfluidic devices can induce controlled changes in the chemical environments of brain slices.^{19–23} Blake *et al.*¹⁹ developed a three-layer slice chamber with multiple inlets. The chamber delivers chemicals along a line path as part of an overall perfusion flow. A focal delivery of chemicals was therefore not feasible. Mohammed *et al.*²⁰ incorporated a microfluidic port on the bottom surface of a perfusion chamber to locally inject chemicals into a slice pressed against the chamber surface. Other studies reported three-dimensional microdevices with penetrating micro-ports to deliver oxygen, nutrients, and possibly neuromodulators into thick brain slices.²¹ However, the impact of mechanical trauma and tissue damage must be assessed for each neurological phenomenon of interest.

A key challenge for planar microfluidic devices has been the difficulty in sealing slice–port interfaces for localized delivery of chemicals. Simply pressing a slice against fluidic ports is problematic as it may generate undesired mechanical stimulation or possibly damage the brain tissue. This motivates alternative designs to achieve localized chemical stimulation while circumventing deleterious effects associated with physical sealing.

In the present work, we report our progress in developing a microfluidic chamber where we employ active fluid suction, as opposed to physical sealing, to achieve localized injection of chemicals. We fabricate the chamber using a two-level soft-lithography process and perform flow and concentration visualization experiments to demonstrate effective confinement of injected chemicals. A successful localized induction of cortical spreading depression is then demonstrated by focal delivery of a potassium chloride solution.

Chamber design

The design of our microfluidic chamber is schematically illustrated in Fig. 1. The chamber incorporates a base layer containing an array of microposts over which a brain slice is placed. As demonstrated in a previous study,²⁴ a dense post array is effective in ensuring a laminar flow of an oxygenated artificial cerebrospinal fluid (ACSF) and hence maintaining slice viability. The array also mechanically supports the brain slice while minimizing mechanical trauma. In our design, the post array is denser near the side walls of the chamber to prevent the formation of recirculating flow patterns.

Select microposts are replaced with fluid ports, each consisting of a single injection hole surrounded by multiple suction holes. The suction holes actively confine an injected fluid and thereby eliminate the need for physical sealing. The port locations are selected to approximately follow the shape of the neocortical layer of mouse brain slices. The diameter of the injection holes is approximately 100 μm , comparable to the sizes of typical functional modules of rodent brains.²⁵

Materials and methods

Device fabrication

The microfluidic chamber is made of PDMS (poly-dimethylsiloxane) and fabricated using a soft lithography technique.²⁶ A set of two molds is made by patterning negative photoresist layers. The first mold is for fabricating the underlying supply and suction channels and the other for the post arrays, fluid ports, and through holes.

Fig. 2 illustrates the cross-sectional views of a fluid port after each major processing step. A thin layer of negative photoresist (SU-8 2007, MicroChem Inc.) is first spin coated at 3000 rpm to a thickness of approximately 10 μm on a clean dehydrated wafer to enhance the adhesion of a subsequent photoresist layer. For the first mold, a second layer of SU-8 (SU-8 2100, MicroChem Inc.) is next spin coated at 3300 rpm to a thickness of approximately 100 μm and soft baked at 95 $^{\circ}\text{C}$ on a levelled hot plate for 25 minutes. The hot plate temperature is ramped gradually from 65 $^{\circ}\text{C}$ to 95 $^{\circ}\text{C}$ over approximately five minutes to reduce thermal stress. The wafer is cooled down to room temperature before the next processing step. The photoresist layer undergoes exposure, post-exposure bake (PEB), development, and hard bake according to the supplier's recipe as illustrated in Fig. 2(a).

For the second mold, a two-level structure is fabricated using a procedure illustrated in Fig. 2(b). Alignment marks are first created in a PECVD (plasma enhanced chemical vapour deposition) silicon nitride layer using RIE (reactive ion etching). A 100 μm thick SU-8 layer

is then spin coated, soft baked, UV exposed, and baked again. This base layer is used to define the post array and the bottom surface of the perfusion chamber. A second 100 μm thick SU-8 layer is next spin-coated and subjected to soft bake, UV exposure, and post-exposure bake steps. This second SU-8 layer defines the suction holes and the through holes that will connect the injection holes to the underlying microchannels. The two resist layers are developed and rinsed in isopropanol and water before they are hard baked at 150 $^{\circ}\text{C}$.

We prime the photoresist mold in HMDS (hexamethyldisilazane) vapour overnight to facilitate the peeling process. A 10 : 1 mixture of PDMS base and curing agents (Sylgard 184; Dow Corning) is degassed and poured onto each of the two molds. The molds are placed in a vacuum chamber for 10 minutes until all the air bubbles trapped between the PDMS and SU-8 layers are released. A sheet of polyethylene is then placed against the PDMS layer to squeeze out any residual PDMS and open the through holes. A weight is put on the polyethylene sheet to ensure tight contact. The PDMS layers are cured on a hot plate at 75 $^{\circ}\text{C}$ for two hours.

The two PDMS layers are peeled off from their molds and placed in contact with each other after an oxygen plasma treatment (600 mTorr, 70 W for 10 seconds). Methanol is used as a lubricant between the layers to facilitate alignment. After the alignment is complete, the assembly is heated on a hot plate to evaporate away any residual solvent. The assembly is then baked at 95 $^{\circ}\text{C}$ overnight to achieve strong bonding between the PDMS layers. The final PDMS device is bonded to a custom-made PDMS slice chamber frame to complete the perfusion chamber.

Brain slice chamber characterization setup

The experimental setup we use to characterize our devices and perform CSD induction experiments is shown in Fig. 3. In order to maintain the viability of a brain slice, the assembled chamber incorporates an inlet and an outlet port to sustain continuous flows of an oxygenated artificial cerebrospinal fluid (ASCF) solution over and underneath the brain slice at 32 ± 1 $^{\circ}\text{C}$. This main ASCF solution is delivered to the chamber using gravity at 2 ml min^{-1} . A piece of filter paper (not shown) is placed at the outlet to minimize flow fluctuations. A platinum wire (300 μm in diameter) is placed on top of the slice, but away from the gray matter area of interest, to immobilize the slice at the beginning of each experiment.

Multiple Tygon tubings (ID: 0.02" and 1/8") are bonded to the inlets and outlets of the microchannels. Two separate pumps are used for independent control of injection and suction, respectively. A syringe pump (sp100i Pump; WPI, Sarasota, FL) is used to deliver a fluid into the injection port at mean velocities of the order of 0.001 m s^{-1} to 0.1 m s^{-1} . A vacuum pump is used to create suction flows at mean velocities of the order of 0.01 m s^{-1} to 0.1 m s^{-1} .

Our parametric simulation study indicates that the profile of a chemical plume is determined primarily by the ratio of injection to suction flow rates rather than their individual values under the present experimental conditions. At high flow rates, the flows may exert an appreciable shear stress on the brain slice surface. The lower limit to the injection flow rate

was set primarily by the stability of a syringe pump in our study and can be reduced by using a pressure-driven pumping scheme in combination with a flow resistor. We do not observe any mechanically induced CSD events for all flow rates used in the present study. More systematic studies, however, may be necessary to rigorously elucidate the upper limit on the acceptable flow rates for different types of slice experiments.

The brain slice is imaged using a 4× objective lens (UPlanSApo, Olympus America, Melville NY) coupled either directly to a high sensitivity camera (Watec 902H2 Ultimate, Tsuruoka, Japan) *via* a tube lens (for optical intrinsic signal imaging) or through a filter cube (Chroma Technology) for epifluorescence imaging.

Slice preparation

Experimental protocols are approved by the UCLA Chancellor's Animal Research Committee. Female C57Bl/6 mice (25–30 g) are housed at 21 °C on a 12 h light–dark cycle. Food and water are available without restriction. Deep anesthesia is achieved with isoflurane. The animal is decapitated and the brain is quickly removed and submerged in an ice-cold oxygenated ACSF solution (ionic composition: 125 mM NaCl, 3 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃ and 10 mM glucose) for 1 minute before being mounted on a slicer. Coronal slices of 400 μm thickness are then cut in ice-cold oxygenated ACSF solution with a standard vibratome (MA752 Motorised Advance Vibroslice, Campden Instruments). The slices are then incubated in an oxygenated ACSF solution at 28 °C for one hour before the experiments. For all the experiments, the slices are imaged within 1–3 hours after harvest.

Numerical simulations

3D multi-physics finite element (FEM) simulations are performed to predict the flow and concentration profiles for different combinations of the injection and suction flow rates. We solve the Navier–Stokes equations for fluid dynamics and the time-dependent convection–diffusion equation for mass transport. Two sets of simulation are conducted: one without a slice (Fig. 5 and 6) and the other with a brain slice placed on the port (Fig. 7). A finite gap of approximately 25 μm between the bottom surface of the brain slice and the top of the fluidic port is assumed. A parametric study indicates that the predicted concentration profiles do not depend sensitively on this gap.

The density and viscosity of ACSF are assumed to be those of water. The mass diffusivity of fluorescein molecules in ACSF is assumed to be comparable to that in water ($2 \times 10^9 \text{ m}^2 \text{ s}^{-1}$). A parametric study suggests that mass diffusion in ACSF is not significant since the Peclet number is much greater than 1 for all cases considered in the present work. The effective diffusivity within brain slices is set to be $0.2 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ based on previous experimental results.^{27,28}

We discuss the concentration (Fig. 5 and 7) and velocity profiles obtained from the numerical simulations and compare them with our experimental results in the next section.

Result and discussion

Demonstration of the confinement of injected chemicals through active suction

Fig. 4 shows fluorescence images we obtained while injecting an ACSF solution mixed with a fluorescent dye (fluorescein isothiocyanate; FITC; Sigma-Aldrich Inc.) into the port without (a and c) or with (b and d) active suction. In the absence of active suction, the injected fluorescent dye is convected downstream by the main perfusion flow, forming a broad lateral plume. Since the Peclet number is much greater than 1, the effect of mass diffusion is small. With a sufficiently strong suction, the fluorescence dye is effectively confined and no “leakage” of the fluorescence dye downstream of the port is observed.

Fig. 4(c) and (d) are obtained with a brain slice placed on top of the port whereas Fig. 4(a) and (b) are obtained without a brain slice. Since the slice has a finite optical thickness and scatters light, the images of the fluorescent plumes are more blurred in Fig. 4(c) and (d) compared with Fig. 4(a) and (b). However, the images do suggest that the active suction is effective in confining injected chemicals in actual brain slice experiments.

Concentration/velocity profile characterization

To further quantify the relevant characteristics of the device, we first use fluorescence microscopy to determine spatial concentration profiles of an injected chemical under different combinations of injection and suction rates. We perform these experiments in the absence of a brain slice to avoid complications associated with absorption and scattering of light within the brain slice.

A small amount of fluorescein (<0.1%) is added to a solution of ACSF and the mixture is injected through the injection hole. A high numerical aperture lens (20×, NA = 0.95 Olympus, XLUMPlanFI) is used to obtain optical sectioning of fluorescence intensity profiles in the depth (*z*) direction. We first set the lens focus at the bottom surface of the chamber. We then raised the lens incrementally using a micro-stepper while recording images at each *z*-location. A discrete composite 3D concentration profile of the fluorescein plume can be reconstructed from the recorded image stacks.

We further use 2D particle tracking velocimetry (PTV) to characterize the velocity field around the port. Seed microspheres (polystyrene bead, 1 μm, Polysciences, Inc.) are pre-dispersed in an ACSF solution introduced into the injection port. The locations of these seed particles are tracked using a high speed video camera (Fastcam MC2, Photron) operating at 10 000 frames per s. By analyzing the particle trajectories, one can construct the velocity fields around the fluid port.

We examine three different combinations of injection and suction rates: Case 1 (suction dominant), Case 2 (suction comparable to injection), and Case 3 (injection dominant). The predicted concentration profiles across a port cross-section are shown as colormaps in Fig. 5(a). Fig. 5(b) shows top views of the measured fluorescence intensity profiles at a plane right above the fluidic port. The mean injection and suction velocities for each case are: 0.025 and 0.099 m s⁻¹ (Case 1); 0.080 and 0.014 m s⁻¹ (Case 2); 0.080 and 0.099 m s⁻¹ (Case 3).

For Case 1 (suction dominant), the injected chemical forms a vertical plume that is confined below the top surface of the port. Under this condition, the injected chemical does not reach the brain slice. In the opposite condition, *i.e.* Case 3 (injection dominant), the injected chemical leaks out of the port forming a long lateral plume that merges with the main perfusion flow (directed perpendicular to the page). At optimal flow rate ratios (Case 2), the plume still exceeds the top surface of the port but is confined laterally and does not merge with the main perfusion flow. This is the condition we use in our later localized CSD stimulation experiments.

The experimentally determined plume boundary, defined as the locations of maximum slope in each fluorescence intensity profile, is marked with black symbols on the simulation results in Fig. 5(a). The experimental plume profiles agree well with the simulation results for all three cases.

The predicted velocity profiles are also consistent with the PTV results. Fig. 6 shows the top views of the port at two different z -levels (distance from the bottom chamber wall) with predicted velocity vectors shown in black arrows and measured velocity vectors in green arrows. For Case 3, the seed particles are mostly directed into the suction holes at a lower z -level (just above the top surface of the post). At a higher z -level, the particles follow the main perfusion flow rather than being directed into the suction holes, indicating unsuccessful confinement. When suction is comparable to injection (Case 2), leakage of the injected fluid is eliminated and the injected particles are directed to the suction holes even at the higher z -level. Under this condition, the plume is successfully localized around the port.

Cell staining experiments

Fluorescent microscopy is complemented by cell staining experiments to further demonstrate localized chemical injection in the presence of a brain slice. Fig. 7(c) shows the fluorescence images obtained from the top of brain slices for five different injection flow rates. The mean injection velocity increases from the left to the right images ($V_i = 0.0043$; $V_{ii} = 0.0210$; $V_{iii} = 0.0275$; $V_{iv} = 0.0325$; $V_v = 0.0415 \text{ m s}^{-1}$). The mean suction velocity is fixed at 0.0180 m s^{-1} . The right most image corresponds to a situation where the plume just starts to lose its confinement.

The fluorescence images do demonstrate systematic increases in the chemical plume size with increasing injection flow rates. Due to scattering of light across the thickness of a slice, however, it is not a trivial task to directly correlate a fluorescent intensity profile with the plume size.

To circumvent this difficulty, we inject an ACSF solution containing a cell-staining dye for a short period (30 s) and examine the spatial extent of cell staining. We perform two different sets of experiments using two different dyes: trypan blue (Sigma-Aldrich) and methylene blue (0.25% in potassium PBS, Sigma-Aldrich).

Trypan blue does not stain intact live cells but only cells with damaged membranes.²⁹ The bottom layers of brain slices mostly contain such dead or otherwise damaged cells due to

trauma experienced during slicing. Trypan blue staining experiments therefore allow us to gauge the spatial extent of a chemical plume in contact with the brain slice bottom surface.

Methylene blue, in contrast, stains intracellular structures (rough endoplasmic reticulum) of live cells³⁰ and allows us to locate healthy cells *within* the brain slice that come in contact with the injected chemical.

Fig. 7(a) shows the predicted concentration profiles at the bottom surface of the brain slice for the five different injection flow rates. The images of cells stained with trypan blue (discrete dots) are superposed on the numerical simulation results. The spatial distributions of the stains are consistent with the simulation results, exhibiting systematic increase in the areas populated by the stained cells with increasing injection-to-suction flow rates. Most stained cells are located within regions with predicted relative concentrations >0.7 . The present numerical model does not account for uptake of the dye molecules by cells. We note in passing that the stain imaging is performed after turning the slices over and, during this extra handling process, some of surface dead cell layers were lost due to mechanical abrasion.

Fig. 7(b) shows the predicted concentration profiles at $45\text{ }\mu\text{m}$ above the slice bottom surface. This location is chosen accounting for the fact that the thickness of the surface dead cell layers in rodent brain slices is estimated to be typically $15\text{--}70\text{ }\mu\text{m}$.^{31,32} Images from the methylene blue staining experiments, where the stained cells are represented once again as dots, are overlayed on top. Overall, the areas occupied by the stained cells are smaller than those for the trypan blue experiments. This is because methylene blue only stains intact viable cells deeper into the slice where the concentration profiles are narrower.

Local induction of cortical spreading depression (CSD)

Cortical spreading depression (CSD) is a self-propagating wave of cellular depolarization. CSD is implicated in migraine headache, stroke, and brain injury and has therefore been a subject of extensive study by the neuroscientific community.^{33,34} During a CSD event, neurons and glia undergo a massive regenerative depolarization.^{33,35} Because of the energetic expense of regenerating functional ionic gradients after such a depolarization, only a healthy brain slice will support multiple CSD inductions. The ability to induce multiple CSD events is therefore considered a biologically relevant measure of the viability of brain slices.

We chemically induce CSD in brain slices by locally injecting a 1 M solution of KCl through the fluidic port. The experimental condition is similar to that used for Case 2 (or condition i in Fig. 7): mean injection velocity: 0.0042 m s^{-1} ; mean suction velocity: 0.0180 m s^{-1} . The injection flow is sustained less than five minutes for each CSD event. The elevated extracellular $[K^+]$ triggers a concentric depolarization of neurons and glial cells originating from that locus. The spreading depolarization causes cells to swell, resulting in changes in their optical properties.^{36–38} The spreading can therefore be observed optically as a dark ring spreading from its onset point, as illustrated in Fig. 8.

Using the current slice chamber, we can elicit multiple CSD events in each slice, confirming excellent slice viability. The time interval between successive CSD inductions is approximately 10 minutes, consistent with previous observations.³³ To our knowledge this is the first demonstration of controlled, noninvasive, focal induction of CSD in brain slices. Other methods either require perfusion over large sections of a slice^{39,40} or invasive techniques such as glass micropipettes.^{4–7,39} Moreover, conventional noninvasive techniques cannot control the number of CSD events elicited.^{39,40} We are able to induce multiple CSD events with controlled timing, and do not observe any events other than the ones we trigger.

The threshold concentration of chemical stimulants for CSD induction can be used as a measure of brain excitability in migraine models.⁴¹ Because of precise control over the molarity of the K^+ solution, our preparation will allow more fine-grained determination of CSD thresholds than previously possible. The results of an on-going systematic CSD induction study will be reported in a future publication.

Conclusions

A novel microfluidic chamber is demonstrated with the ability to simultaneously inject and withdraw a fluid to stimulate a brain slice in a locally controlled manner. The device is compatible with existing brain slice physiology setups and can easily be implemented. The low-cost fabrication process allows users to design a fine-tuned device for their specific application. Fluorescence imaging, particle tracking velocimetry, and cell staining experiments are performed to demonstrate spatial confinement of injected chemicals. The experimental results compare well with numerical simulation results.

Our work establishes approaches to characterize brain slices under localized chemical stimulation and provides an initial demonstration of how these approaches improve the characterization of a neurophysiological event. It also motivates further work to implement microfluidic devices for *in vivo* studies and therapeutic treatments of neurological diseases.

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Fig. 1.

(a) Schematics of the perfusion chamber incorporating integrated fluid injection/suction ports for localized chemical stimulation of brain slices. The top view (b) and cut view (c) of the fluid port (open circle in (a)) with one injection hole surrounded by five suction holes.

Fig. 2.

Abbreviated process flow for fabricating the dual-layer PDMS brain slice chamber.

Fig. 3.
Experimental setup for the microfluidic chamber testing and brain slice experiments.

Fig. 4. Fluorescence images of the injected flows: (a and b) without a slice and (c and d) with a brain slice placed on the port.

Fig. 5.

Concentration profiles around the injection ports. (a) Side view: the experimentally measured profiles (black symbols) compare well with the simulation results. The profiles are taken on cross-sectional planes perpendicular to the main perfusion direction (the black arrow shows the horizontal location). (b) Top view: fluorescence images taken at the plane right above the port (black arrow in (a)). The blue arrow indicates the direction of the main perfusion flow.

Fig. 6. Particle tracking velocimetry results. (a) Field of view (the rectangular area). (b and c) Predicted (gray arrows) and experimentally obtained (green arrows) velocity fields for (b) Case 3 (injection dominant) and for (c) Case 2 (injection comparable to suction) at two different z -levels.

Fig. 7.

(a) Trypan blue staining experiment results (black dots) overlaid on the predicted concentration profiles (colormap) at the bottom of the brain slice for five different injection to suction flow rate ratios. (b) Methylene blue staining results overlaid on the predicted concentration profiles within the slices (at 45 μm from the bottom of the slice). (c) Fluorescent images taken from the top of the slices. The scale bar corresponds to 100 μm .

Fig. 8.

Temporal montage of a CSD wave initiated by local chemical stimulation using the present brain slice chamber (time from 0 second to 14 seconds). The lower inset shows the location of the cortex region on a brain slice.