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Title: Local Chemical Stimulation of Neurons with the FluidFM

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Local Chemical Stimulation of Neurons with the FluidFM


Abstract: Physiological communication between neurons is dependent on the exchange of neurotransmitters at the synapses. Although such chemical signal transmission targets specific receptors and allows for subtle adaptation of the action potential, in vitro neuroscience typically relies on electrical currents and potentials to stimulate neurons. The electric stimulus is unspecific and the confinement of the stimuli within the media is technically difficult to control and introduces large artifacts in electric recordings of the activity. Here, we present a local chemical stimulation platform that resembles the in vivo physiological conditions and can be used to target specific receptors of synapses. Neurotransmitters are dispensed using the force-controlled FluidFM nanopipette, which provides an exact positioning and precise liquid delivery. We show that controlled release of the excitatory neurotransmitter glutamate induces spiking activity in primary rat hippocampal neurons as measured by concurrent electrical multielectrode array and optical recordings. Furthermore, we characterize the glutamate dose-response of neurons by applying stimulation pulses of glutamate with concentrations from 0 to 0.5 mM. This new stimulation approach, which combines the FluidFM for gentle and precise positioning with microelectrode array read-out, makes it possible to chemically modulate the activity of individual neurons while simultaneously record their induced activity in the entire neuronal network. The presented platform not only offers a more physiological alternative compared to electrical stimulation, but also provides the possibility to study the effect of local application of neuromodulators and other drugs.

Introduction

Unraveling how billions of nerve cells in the human brain work together and carry out their functions ranging from processing sensory signals to storing information is one of the key challenges of this century.[1] The brain is predominately studied either as a whole in top-down fashion, or bottom-up on the level of single cells and small networks. Basic functions of the brain such as perception, processing and storage of information are carried out by networks of tens to hundreds of cells. However, despite focused efforts in theoretical and experimental neuroscience we still know very little about how these basic functions of the brain are realized.

The developing nervous system starts to exhibit spontaneous electrophysiological activity during maturation.[2] This is exploited when culturing dissociated neuronal tissue in vitro as a model system of the central nervous system as they show similar electrical activity.[3] Such networks offer a much more controlled environment and easier access, compared to their counterparts in a living animal. They have been fundamental to unraveling basic properties of individual neurons and assemblies of neurons. The spontaneous activity patterns exhibited by such neuronal networks are very diverse, which requires the recording and analysis of large datasets to establish a baseline to which activity changes within the neuron network can be compared to.

Investigating the response of a network to external inputs allows for a more insightful statistical analysis compared to that of spontaneous activity recordings, in particular with the commonly used large randomly connected networks.[4] By this means, one can study learning effects in the network, which are induced by reoccurring external stimuli mimicking a sensory input.[5] Such repeated stimulation can also be useful for investigating dose responses and toxicity levels.[6]

Neuronal population activity is typically monitored by electrically recording the changes in the extracellular potential, or using fluorescent calcium indicators. Patch-clamp recordings provide the most accurate measurement of nerve activity down to the level of single ion channels, but the technique is difficult to scale up for the measurement of neuronal networks. So far, reliable multi-cell patch clamp recordings could only be achieved by a limited number of laboratories, with a maximum of around 10 parallel cell patch recordings.[7,8]

Microelectrode arrays (MEAs), which we also used here, provide a popular alternative to patch-clamp because they allow for recording the extracellular activity of neurons at every electrode over a time period of up to many months[9]. Such extracellular recordings have an excellent time resolution but due to the complicated field propagation within the culture, high spatial resolution can only be achieved by sufficiently dense electrode arrays.[10] To overcome this problem MEAs can be combined with calcium imaging to improve the spatial resolution. The method is based on monitoring the intracellular calcium concentration as a proxy of neuronal activity with a fluorescent indicator. The excellent spatial resolution of modern imaging systems makes it possible to image the activity of even subcellular compartments; but the temporal resolution is lower compared to electrical recordings due to the kinetics of the calcium indicator.[11]

Investigating how a network reacts to external stimuli is essential for understanding the network behavior. A number of methods have been developed to manipulate the activity of in vitro cultures...
Neurotransmitters play a crucial role in the functioning of neurons. In addition to recording neural activity, MEAs can also be used to stimulate neurons by delivering short voltage or current pulses. Such electrical stimulation was for example used to induce spike timing-dependent plasticity. While convenient, stimulation with MEAs has a number of difficult practical issues. It is especially difficult to precisely and reproducibly confine electrical stimulation because the electrodes are fairly large and the extent of the stimulated area depends on the location of axons and cell bodies on the stimulating electrodes and around them. Moreover, electrical stimulation is not able to provide neuromodulator-specific activation of the neurons, which would be rather important for the induction of plasticity in synapses.

Local dispensing of stimulants over the neurons with glass pipettes resembles more closely the physiological conditions of how signals between the neurons are transmitted by the exchange of neurotransmitters. The neurotransmitter glutamate was found to interact with neurons found all over the brain. Mainly three different types of receptors are activated by glutamate, the ligand-gated AMPA/kainate and NMDA ionotropic receptors, and the G-protein coupled metabotropic receptors. These receptors are mostly clustered at the post synaptic site, but are thought to dynamically relocate upon chemical stimulation playing an important role in the plasticity of the synapses. Especially NMDA receptors have shown to be linked with many different processes in the brain.

Glass micropipettes are often used to dispense stimulants in combination with patch clamp recordings, e.g. to characterize the response to local application of glutamate. However, glutamate can only be released locally and in small doses because a global application to glutamate to the entire culture can be toxic and cause neuronal degeneration. Furthermore, the precise confinement of the dispensed stimulant required to investigate the effect of subcellular areas such as individual dendritic spines requires micromanipulators and a skilled operator. The amount of dispensed stimulant can be more precisely controlled by using microiontophoresis, where ions are propelled by the electric current. However, as only charged compounds can be delivered using microiontophoresis, this often requires adaptation of the pH in the case of neurotransmitters. In addition, the concentration of the dispensed compound depends on the distance to the manually positioned pipette, and also on the applied voltage, which makes it more complex to estimate the concentration profile compared to pressure-based systems.

Scanning ion conductance microscopy (SICM) is a scanning probe method that is particularly suited to image surfaces of soft biological samples. Once the SICM pipette approached the surface, the ion flux through the pipette aperture is restricted, which results in a detectable resistance change. With the help of piezo actuators, the positioning of the pipette becomes more precise compared to conventional manual positioning. This technique has been used in combination with microiontophoresis to map receptors on living cells and to examine subcellular features of cardiomyocytes. Quantification of the concentration of the dispensed solution can be estimated using numerical simulations or recently also measured by electrochemistry. Due to their small size, neurotransmitters diffuse very rapidly when dispensed in vitro, which makes it difficult to control the stimulation area. This is in contrast to in vivo stimulation with neural prostheses, such as in Jonsson et al., where the localization of the stimulant can be achieved more easily due to the restricted diffusion in the dense brain tissue.

Optically, neurons can be stimulated following two main approaches, either activating light-gated ion channels or uncaging compounds of the stimulants. The former approach, optogenetics, allows precisely timed and localized activation of light-sensitive molecules in systems ranging from in vitro cell cultures to live behaving animals. Light-gated ion-channels can be used to elicit action potentials when neurons are exposed to light. Although this method has already been shown to have versatile applications in neuroscience, it does not allow for the local release of compounds. Uncaging on the other hand is a method to locally provide stimulants by optically removing the photolabile protecting groups of the caged compounds and hereby rendering them active, while localizing the interaction to the illuminated areas with a resolution of >1 µm. Using two-photon systems in combination with specifically designed caged compounds an even better spatial confinement of the excitation volume has been achieved. Furthermore, the infrared wavelength of the two-photon system allowed for better tissue penetration of the laser light, making it also suitable for in vivo applications. Quantification of the released compounds is complicated though, since the laser intensity needs to be calibrated with radiometric dyes. Caged versions of several neurotransmitters have been published, many of them are, however, not yet commercially available and have to be synthesized on site.

The advances in microfluidics have also made a significant impact in neuroscience. Microfluidic devices have given researchers the possibility to control the position of neurons, how they interconnect, and to manipulate individual compartments. A microfluidic chip was, for example, used to simultaneously record and chemically stimulate brain tissue slices and an on-chip fluid delivery system was used to locally apply neurotransmitters to a patterned network of neurons while performing patch clamp recordings. Such microfluidic systems are well suited for automated measurements. The site of stimulation, however, depends on the design of the microfluidic chip and cannot be modified during the experiments. On the other hand, open space microfluidic systems such as the microfluidic probe or the fluidic force microscope (FluidFM), a force-controlled nanopipette, are not limited to pre-defined positions and can supply chemical solutions to any point of a culture dish.

Here, we present an experimental platform for stimulating and recording neuronal networks based on the integration of a MEA and a FluidFM system. The FluidFM combines an atomic force microscope (AFM) with nanofluidics, and its force-control allows for exact positioning of the nanopipette in relation to the cell membrane. As an open-space microfluidic system, it can deliver chemical stimuli with precise spatio-temporal control, which is a drastic improvement over traditional glass pipettes. The platform is fully compatible with the commonly used MEA systems and calcium imaging techniques. We show that individual neurons can be targeted for stimulation using an inverted microscope. Using the AFM force-control, after a gentle approach of the top of the
cell membrane by the cantilever, neurons were stimulated by dispensing glutamate from a 2 μm aperture at the tip of the cantilever. A pressure controller was used to control the exact amount of the dispensed solution. The 2 μm aperture and the precise force-controlled positioning improved the localization of the chemical stimulation by one order of magnitude compared to a recently published retinal stimulation platform by Rountree et al.\textsuperscript{[21]}

We show the capabilities of the platform to reliably and repeatedly stimulate neurons by locally releasing the excitatory neurotransmitter glutamate. Furthermore, we also demonstrate that precisely controlling the distance between the aperture and the cell can be used to apply the desired glutamate concentration to the target neuron. This high level of control over the stimulation allows for investigating the effects of long-term stimulation on the behavior of the stimulated neuron and its network. These capabilities make the presented platform a promising tool to investigate neuronal networks by locally providing neurotransmitters as inputs for basic neuroscience research, but also to investigate the effect of neuromodulators on the behavior of such networks in pharmacological applications.

Results

Repeated Local Chemical Stimulation

Chemical stimulation of neurons to study their behavior and their role within their network is usually achieved by using manually controlled glass pipettes. More recently, scanning probe techniques were introduced to precisely control the stimulation position in the horizontal, and in particular the crucial but challenging vertical direction.\textsuperscript{[26]} Here, we introduce the use of a force feedback allows a precise positioning of the cantilever in the z-direction, and a gentle approach of the cell surface. Once the cantilever is in position, the stimulation solution inside the hollow cantilever can be applied to the target cell. Depending on the aperture size, constant flow rates down to aL/s are obtainable.\textsuperscript{[44]}

Here, we used cantilevers with an aperture of 2 μm diameter (see Figure 1B for a close-up view) with a minimum reproducible flow rate in the range of 10 fL/s.\textsuperscript{[45]}

The cantilever was positioned on top of the target cell with the help of the optical microscope, as illustrated in Figure 1C. After an approach with a setpoint of 12 nN, the cantilever was immediately retracted by 0.5 μm to avoid any further contact with the cell. We did not observe any change in spiking activity after the approach compared to the baseline recording. The activity induced by 0.4 Hz stimulation consisting of 300 ms long pulses of 50 mBar with 0.5 mM glutamate was characterized by analyzing the simultaneous MEA and calcium recordings presented in Figure 2A-B and Figure 2C respectively. This combined analysis made it possible to study the increased firing rate after the individual stimulation pulses on both the network (Figure 2B) and single cell level (Figure 2C). The spiking activity before stimulation consisted predominantly of synchronized culture-wide spikes followed by little to no activity for 5-10 s. When the target cell was stimulated with glutamate, we observed more frequent spiking activity that coincided with the stimulation pulses. The applied stimulation solution of 0.5 mM glutamate corresponds to an estimated glutamate concentration at the synapse upon fusion of a glutamate-filled vesicle with the presynaptic membrane.\textsuperscript{[46]}

While the electrical recording of the directly stimulated neuron showed a high direct response rate of 92% as expected (see Figure 2A for a representative example), the response of the indirectly stimulated neighboring electrodes was very diverse with direct response rates ranging from 0 to 76 % in a session of 50 stimulation pulses. Only spikes occurring within 700 ms of the stimulus onset were classified as direct responses to minimize false positive spikes caused by secondary responses or
spontaneous activity. A decrease in culture-wide response activity was visible in both the MEA recordings (Figure 2A and B) and calcium imaging results (Figure 2C). However, the long-term MEA measurements (Figure 2B) showed a recovery in spiking activity 150 s after stimulation.

In summary, the experimental results show how neurons could reliably and repeatedly be stimulated while continuously measuring the activity of individual cells and the whole network. The hollow cantilever filled with the stimulation solution can target any cell in the dish by positioning the cantilever on top of the target cell without disturbing it thanks to the force-controlled approach of the cantilever.

### Controlling stimulation dose

Action potentials are transmitted between neurons by the release of neurotransmitters from the presynaptic neuron, which bind to receptors at the postsynaptic neuron. Here, we attempted to characterize the single-cell glutamate dose-response by applying stimulation pulses of glutamate concentrations from 0 to 500 µM. The number of postsynaptic glutamate receptors in the synaptic cleft varies greatly between neurons and individual receptors, but is in the order of a few hundreds.\(^{47-49}\) The affinity of glutamate receptors to its target is 1-50 µM,\(^{50}\) which means that when glutamate levels locally reach this concentration the receptors saturate causing a depolarization in the postsynaptic cell. This depolarization is achieved by releasing the content of glutamate-filled presynaptic vesicles into the synaptic cleft, increasing the local glutamate concentration up to even 5 mM,\(^{46}\) which helps to minimize the synaptic transmission time delay due to diffusion and binding kinetics.

![Figure 2](image-url)

**Figure 2.** Extracellular recordings of activity induced by local stimulation with glutamate. **A**) The local chemical stimulation reliably and repeatedly induced responses as visible in the extracellular recording. Glutamate was repeatedly dispensed from the FluidFM cantilever on top of the target neuron, while the neuronal activity was measured by the MEA electrode below the cell. The onsets of the 50 stimulation pulses are indicated with red vertical lines. **B**) The raster plots show the activity of the network as simultaneously measured by the 60 MEA electrodes and calcium imaging. The directly stimulated electrode/region of interest is highlighted in green, and the duration of the individual stimulation pulses are indicated in red. **D**) The percentage of direct responses to the stimulation pulses are shown on a map of the MEA. The directly stimulated Electrode 24 showed a response rate of 92 %, while the neighboring electrodes, which showed comparable spontaneous activity, responded diversely from almost no response up to 76 %. The green rectangle indicates the area recorded with calcium imaging as shown in E, with the electrode positions indicated in red, and the cantilever in white.
We tried to mimic this process by locally and temporally controlling the glutamate concentration around the cell membrane using the FluidFM. Unfortunately, it was not possible to estimate the local concentration of glutamate with a fluorescent tracer, because fluorophores with a molecular mass similar to that of glutamate does not exist at the moment, and as such, their diffusive behavior are considerably different. Therefore, the expected local concentrations of glutamate during stimulation were calculated using numerical simulations (Figure 3C-F). See the Materials and Methods section for further details and the validation of this approach. The local concentration of glutamate quickly drops with the distance from the cantilever aperture (see Figure 3F), which we used to control the glutamate exposure of individual cells according to Figure 3C. The precise positioning of the cantilever allowed us to change the concentration easily and reproducibly in the relevant µM range.

The time-course of the simulated glutamate concentration at the target cell during a stimulation session is shown in Figure 3D. The

Figure 3. Controlling the glutamate stimulation dose by adjusting the cantilever position. A) The probability of obtaining a direct response upon chemical stimulation depended on the glutamate concentration in a dose-response-type fashion. The curve was generated by stimulating the target from different z-distances in three trials in two cultures. B) The delay between stimulation onset and induced response decreased as the cantilever was brought closer to the target cell and the glutamate concentration increased. The concentration of glutamate at the cell surface was characterized using numerical simulations shown in C-F. D) Time course of glutamate at the target cell from simulations for different cantilever-cell distances. While the first pulses of 50 mbar for 300 ms show a smaller dose due to dilution of the stimulation solution inside the cantilever, the remaining pulses show a rapid increase in glutamate concentration when the cantilever is close to the target. E) This is clearly visible when magnifying the grey shaded area in D. F) Local concentration profile of glutamate around the stimulation site during stimulation with 50 mbar. As the cantilever is withdrawn from the target cell, the concentration profile around the target is broadened due to diffusion. C) The simulated peak glutamate concentration at the target strongly depends on the distance to the cantilever aperture. This distance dependency of the concentration was exploited with the precise positioning of the FluidFM to stimulate neurons with different concentrations of glutamate in the range of 0 to 0.5 mM during the experiments shown in A and B.
glutamate concentration rapidly increased at the onset of the pressure pulses when the cantilever was close to the target as depicted in detail in Figure 3E. Figure 3F displays how glutamate was distributed around the stimulation site at the end of a stimulation pulse, when the concentration was the highest. As summarized in Figure 3C, the local glutamate concentration could be adjusted between 0 and the nominal glutamate solution concentration (500 µM) by approaching the target with the FluidFM cantilever. Utilizing this glutamate concentration control, we could investigate the response of neurons to chemical stimulation as a function of stimulant concentration. As presented in Figure 3A, the probability of obtaining a direct response from a neuron directly below the cantilever showed a clear dose-response-like behavior as a function of local glutamate concentration at the cell surface. The dose-response curve was obtained in three trials using two cultures, each consisting of a stimulation protocol of at least 40 pulses at 0.4 Hz at each z-height ranging from 1 to 100 µm. For this, in each case a cell on top of an electrode was selected for stimulation and responses were measured with the MEA electrode directly below the stimulation site. In the first two trials in Culture 1, the target neurons were stimulated at logarithmically increasing heights from 5 to 100 µm, which resulted in peak glutamate concentrations up to 200 µM. As clearly visible in Figure 3A, the spiking responses drastically decreased between 16 µM and 3.5 µM peak glutamate concentration, which correspond to stimulation heights of 25 and 50 µm, respectively. We thus investigated this region in more detail in Culture 2 to find the dose-response curve displayed in Figure 3A. The half-maximal enhancement (EC50) obtained from fitting the three trials to the Hill equation was 8.7 µM. While this concentration is much lower than the estimated 0.5-5 mM in the synaptic cleft,[46] it is in the expected range of the glutamate affinity to its receptor.[50] Therefore, as soon as the local concentration exceeds this value in the synaptic cleft, we expect the activation of the receptors and therefore a response at the postsynaptic side. However, with our external chemical stimulation approach, glutamate had to diffuse into the synaptic cleft, which is expected to be much slower than the natural process of synaptic vesicles releasing their content directly into the synaptic cleft. This should manifest in longer delay times in a concentration dependent manner, which we have also observed in our experiments (see Figure 3B).

These results demonstrate how crucial it is to control the distance between the aperture and the targeted cell. An offset of only 4 µm from the target already decreases the effective concentration by half. The force-controlled positioning of the cantilever with the FluidFM made it possible to exploit this property to investigate the receptor-ligand interactions at the synapses of neurons.

Long-Term Stimulation Changes Network Response

Earlier studies investigating how stimulation changes network behavior were limited to electrical stimulation as a convenient way of stimulating cultures over longer time ranges.[5,52] Here, we investigated if repeated chemical stimulation could induce changes in the network over time. The peri-stimulus timing histograms (PSTHs) of the stimulated electrode for 3 separate sessions in Figure 4A demonstrate the change in induced activity by repeated stimulation. Throughout the stimulation sessions, the sharp response at 170 ms following the stimulus onset in the first session changed into a much broader response distribution.

![Figure 4](image-url)

Figure 4. Network response to repeated stimulation in 15 stimulation sessions lasting over an hour. A) Peri-stimulus timing histogram (PSTH) of the stimulated electrode during 3 selected stimulation sessions and a negative control using a solution without glutamate. The amount of spikes increased throughout the sessions and the spike response time distribution was broadened. The spiking activity was unaffected when stimulated with buffer only. B and C) The PSTH of two non-stimulated electrodes in the surrounding of the stimulation site, as indicated in E), initially showed no direct response. In trial 15 however, a clear increase in spikes 430 ms after stimulus onset was visible in Electrode 36 indicating a change in network behavior due to repeated stimulation. D) Local chemical stimulation reliably induced direct responses at the stimulated position, whereas the average of direct responses at the non-stimulated sites was significantly lower. The spiking activity at the stimulation site was unaffected during the negative control using a solution lacking glutamate.
broader distribution of spike response times with an overall increase in the number of induced spikes.

The PSTHs of Electrode 21 (Figure 4B) as a representative example of a non-stimulated electrode distant to the stimulation site did not show considerable changes during the course of 15 stimulation sessions stimulating at the same site. Electrode 36 in Figure 4C, however, showed a reproducible increase in activity peaking at 430 ms after stimulus onset in session 15, indicating that stimulation has not only altered the behavior of the directly stimulated electrode but also influenced the activity pattern of the network. We can exclude that this increase of activity is due to crosstalk from the dispersed glutamate because the increase in concentration at this neighboring electrode is in the order of 0.1 µM as shown in Supplementary Figure S3.

The negative control with a solution lacking glutamate showed no effect compared to the baseline of spontaneous activity, as demonstrated within the last row of Figure 4A-C and summarized in Figure 4D. As another control, dispensing glutamate solution on an empty MEA chip without cells also showed no response in the MEA recordings. Throughout the experiments, a general decrease in network-wide bursting activity was observed (Supplementary Figure S4). A 30 min long-term exposure to high glutamate concentrations (2-5 µM) has shown to be toxic. In our case, the volume of 0.5 mM glutamate solution released during a single stimulation pulse was about 1 µL (using a flow rate of 64 µL s⁻¹ mbar⁻¹) and a total volume per experiment of 1 nL dispersed over the 1000 stimulation pulses. With an overall medium volume of 1.5 mL, this resulted in a final glutamate concentration increase of ~0.3 nM.

Such a low concentration is not expected to change the activity pattern of the neuron network, instead, we assume that a more complex adaptation process caused by the repeated stimulation pattern should be responsible for the decrease in the network-wide spontaneous bursts. While the stimulation with 0.5 mM glutamate increased the local glutamate concentration around the target neuron above the long-term toxicity threshold as characterized by the previously shown numerical simulations, such transient bursts of high glutamate should also take place during regular synaptic transmission, where the local glutamate concentration is expected to rise up to 5 mM when a glutamate-filled vesicle fuses with the pre-synaptic membrane. After stimulation, in our experiments the local glutamate concentration rapidly decreased due to diffusion and glutamate uptake by the neighboring cells; this is probably why we have not observed any cell death during the stimulation experiments.

The experiments and investigation with numerical simulations show the capabilities of this platform to locally deliver molecules with a controlled dose at any point in the culture, while continuously monitoring extracellular activity. The pressure-based dispensing is compatible with any aqueous solutions and the stability and precision of FluidFM-based operation enables long term measurements.

Discussions

MEA recording and calcium imaging are often used to examine the activity of neuronal networks. Typically, spontaneous activity is recorded, or, in the case of MEA, responses to electrical stimulation are investigated. Here, we present a platform for chemical stimulation, which is compatible with both MEA-based activity detection and fluorescent calcium imaging. Electrical stimulation of neurons using MEAs is simple to implement, however, the localization of the stimulation is difficult to achieve and control. In contrast, the platform presented within this work can apply local chemical stimulation in in vitro cultures of neurons at any arbitrary position. Local stimulation with glutamate induced spiking activity in neurons, as the repeated responses in Figure 2A demonstrate. Out of the 50 individual stimuli applied to the target neuron per session, in average over 90 % elicited a spiking response as measured by the electrode below the stimulation site. The responses of the non-stimulated electrodes presented in Figure 2B-C were much more varied, with some electrodes showing no direct responses during any of the 50 stimulation pulses, while others responded with rates up to 76 %. This was in contrast to the spontaneous activity, which was comparable in the majority of electrodes. It thus appears that the diversity in response rates was due to the network connectivity causing various degrees of indirect activation by local stimulation. Overall, the local stimulation had a temporary, excitatory effect on the directly stimulated neurons, followed by an inhibitory effect on the network-wide spontaneous activity that lasted up to 150 s. The effective area of stimulation can be determined by numerical simulations, and only depends on the position of the cantilever and flow parameters in contrast to electrical stimulation using MEA electrodes, where the spread of the stimulation pulse within the cell culture is poorly understood and can be hardly controlled due to the various and unknown dielectric properties of the cell cultures.

Moreover, to our best knowledge, this is the first study that shows the integration of a scanning-probe-based stimulation setup with the widely used MEA technology. The advantage of delivering the stimulating solution by an AFM-based system is the fast and gentle targeting and positioning of the pipette, which does not require the manual control of an operator. This combination opens up the possibility for automated probing of in vitro networks of neurons by repeatedly stimulating selected nodes of the network, while continuously recording the response of not only the stimulated neuron but also the whole network. Previous studies investigating neurotransmitter dose responses were performed using either glass pipettes or microfluidic chips. In both cases a range of solutions with different concentrations were manually prepared and loaded into individual pipettes or channels, respectively. This inherent limitation to a priori selected concentrations does not apply to the setup presented here, as diffusion is exploited to obtain the desired concentrations by the dilution of the solution as it is dispensed from the microfluidic channel of the cantilever. This new approach together with the characterization using numerical simulations was used to obtain the dose response curve presented in Figure 3A. The experiment illustrates the potential of the presented platform for pharmacological testing. Interestingly, a similar approach was used in a microfluidic device by Tybrandt et
al.\textsuperscript{[66]}, which controlled the stimulation dose of the neurotransmitter acetylcholine through modulation of the stimulation pulse duration. While the site of stimulation is fixed on the chip and cannot be moved, the dispensing using an Organic Electronic Ion Pump has the advantage of being practically leak-free, in contrast to the FluidFM stimulation, where leakage from the aperture due to diffusion is unavoidable. The upper limit for the resulting concentration at the substrate is 10 µM when the cantilever is positioned 1 µm above the substrate as determined using numerical simulations. Using glass pipettes, the different concentrations could only be achieved by using a separate pipette for each of the 10 glutamate concentrations. However, it would be impractical if not impossible to precisely position one pipette after the other on top of the cell membrane without damaging it. The predominantly excitatory effect of glutamate on the postsynaptic current by the activation of receptors at the postsynaptic side has been well characterized since the early days of neurophysiology.\textsuperscript{[57]} However, whether or not the stimulation amplitude is enough to trigger an action potential at the neuron is more complex than the activation of a single receptor. Such responses rely on the temporal and spatial integration of excitatory and inhibitory signals from all the neurons that connect to it. Here, we determined the dose-response relationship for glutamate-stimulation-induced action potentials from three trials in two cultures as presented in Figure 3A. In these trials, a drastic increase in induced responses to the stimulation was observed at an effective glutamate concentration of 8.7 µM. The range of this value is in good agreement with the 3 µM glutamate concentration reported in the literature as the characteristic concentration for activating the receptor-ligand interactions at the single cell level.\textsuperscript{[58]} Based on our purely mechanistic simulation of glutamate diffusion and convection, the local glutamate concentration did not drop below the stimulation threshold in between glutamate pulses for the stimulation closest to the substrate. Therefore, we would expect a continuous elevated spiking activity from the neurons for these cases. However, we observe that neuron firing stops shortly after the stimulation pulse, which hints that our simple model might overestimate the concentration of active glutamate. For example, glutamate is known to have interaction with serum proteins\textsuperscript{[61]}, which might result in inactivation of available glutamate.

Besides this short-term activation effect, we also investigated single cell and network-wide responses over longer time scales and repeated stimulation sessions as summarized in Figure 4. At the target neuron (Figure 4A), an increase in the number of induced spikes with a wider spread was observed throughout the stimulation sessions. It is unclear however, whether this increase is due to an adaptation of the target neuron or due to an activation of additional neurons in the close proximity of the electrode. In comparison, electrodes further from the stimulation site did not show such clear response pattern during the first session (Figure 4B and C). After repeated stimulation, however, the neighboring Electrode 36 showed signs of increased activity timed with the stimulation. This could indicate that with several repetitions the local stimulation slowly influenced also more distant areas.

Conclusions

The results of the experimental platform presented here show the potential of chemical stimulation as a precise and more physiological stimulation technique compared to traditional electrical stimulation. The high level of control over stimulation position and dose makes this platform a versatile tool for investigating the behavior of networks of neurons by presenting chemical stimuli to individual nodes within the network.

Since the pressure-based dispensing system is compatible with any soluble stimulants, the platform could also be used to investigate the response to other neurotransmitters or neuromodulators. With the recent availability of cantilevers with smaller apertures, future efforts will target the effects of stimulation on a submicrometer target area exploring the possibility of chemical stimulation of subcellular features such as dendritic spines. While the 60-electrode MEA chips used here are ideal for long-term measurements, their spatial resolution is poor. The lack of spatial resolution was compensated by the simultaneous calcium image recordings, which are on the other hand subject to phototoxicity and bleaching, making long-term measurements challenging. The recent advances in CMOS-based high-density MEAs\textsuperscript{[52]} could provide an ideal solution to combine the advantages of the MEA and calcium imaging techniques used here.

In summary, the integration of the FluidFM with MEA- and calcium-imaging-based activity recordings showed the potential of this combined setup as a new tool to interact with individual neurons in a culture dish. In future, the platform developed here should not only present a powerful tool to probe in vitro neuronal networks mimicking an “artificial synapse”, but also to use and study the effects of neuromodulators in such networks.

Experimental Section

Cell Culture

The experiments were performed with primary rat hippocampal neurons harvested from E17 embryos of time-mated pregnant Wistar rats (Harlan Laboratories, Netherlands). The cells were plated and cultured in serum-free medium (Neurobasal medium supplemented with 2 % B-27, 1 % penicillin-streptomycin and 1 % GlutaMAX all from Thermo Fisher, Switzerland) in order to suppress astrogia proliferation and optimize cell survival.\textsuperscript{[56]} Cells were plated at a concentration of 75000 cells per cm\textsuperscript{2}, calculated for the bottom surface area of the multielectrode array chips, and cultured at 37 °C and 5 % CO\textsubscript{2}, exchanging half of the medium with fresh medium once a week.

MEA Chip Preparation

Before cell seeding, the chips were plasma treated (nitrogen plasma for 2 min at 18 W with a PDC-32G; Harrick Plasma, USA) and subsequently covered in 100 µg/mL Poly-D-Lysine (PDL, P7280, Sigma-Aldrich, Switzerland) in phosphate-buffered saline (PBS, pH 7.4; Thermo Fisher) for 45 min. After coating, the chips were repeatedly rinsed with purified Milli-Q water (from an Elx 5 water purification system, Merck Millipore, USA), covered with culture medium and placed into an incubator to adjust the pH and temperature before cell seeding.
Adaption of MEA System

A 64-electrode MEA2100-System (Multichannel Systems, Germany) was adapted to accommodate the FluidFM headstage by using a custom-made adapter to the MEA headstage, which connected the electrodes of the MEA chips to the MEA2100 amplifier (see Supplementary Material S1). The used custom-made glass-based MEA chips were produced by Qwane (Switzerland). The 40 µm diameter, optically transparent ITO electrodes were arranged in an 8 x 8 grid with inter-electrode distance of 200 µm, and an SU-8 layer on the areas between the electrodes served as isolation. Around the electrode area, a custom-made ring-shaped reservoir for cell medium allowed access to the FluidFM to reach the entire surface of the electrode array, while as reference, an external Ag/Ag-Cl pellet electrode was immersed into the medium. The stress on the cultures during long-term recordings was limited by introducing a custom telescopic chamber (okolab, Italy) between the headstage and the FluidFM, which created a humidified microenvironment with a controlled level of 5% CO₂. The temperature of the entire setup consisting of microscope stage, MEA, and FluidFM was controlled at 37 °C using an incubator box and a MEA cooling system adapted to the MEA2100 amplifier according to Dermutz et al.[60] (see Figure S1B).

Fluidic Force Microscope (FluidFM)

The FluidFM system used in this study consisted of a Nanosurf FlexAFM-NIR scan head, a ±200 mbar pressure controller with a resolution of ±0.2 mbar, and FluidFM micropipette cantilevers with a 2 µm diameter aperture (all purchased from Cytosurge, Switzerland). The spring constant of the individual cantilevers used were between 1.9 and 2.1 Nm⁻¹ as determined using Sader’s method[61] prior to the experiments. Automation of the approach and the timed delivery of pressure pulses were performed with the ARYA (Cytosurge, Switzerland) and EasyScan2 (Nanosurf, Switzerland) software.

The FluidFM and the MEA headstages were mounted on a moving stage. The stage was part of an Axio Observer Z1 inverted microscope equipped with a Colibri LED light source (Carl Zeiss, Switzerland) and a temperature controlled incubation chamber. Microscopy images and calcium recordings were obtained with Micro-Manager[62] using an electron multiplier CCD camera (Hamamatsu C9100-13; Hamamatsu Photonics K. K., Switzerland).

Stimulation Solution

For the stimulation experiments, L-glutamic acid (Sigma-Aldrich, Switzerland) was dissolved in culture medium at a final concentration of 0.5 mM. Fluorescein isothiocyanate (FITC) labeled mPEG (MW 2000, PG1-FC-2k, Nanos, Chemie-Brunschtwig, Switzerland) was used as a fluorescent tracer to visualize the dispensing process from the FluidFM. After dilution, the pH was checked to a pH value of 7.4. All solutions were filtered through 0.22 µm pore-size filters before use.

Stimulation Protocol

Each session consisted of at least 40 stimulation pulses with a pressure of 50 mbar for 300 ms. The stimulation frequency was set to 0.4 Hz. The stimulation pulses were synchronized with the MEA recording by connecting the pressure controller output signal to the digital input of the MEA controller after signal conditioning. Due to the soft-walled tubing system used to connect the pressure controller to the FluidFM cantilevers, a time delay between the onset of the pressure pulse and the start of dispensing at the cantilever aperture was present. This delay was characterized by dispensing a copper sulfate solution (50 mM CuSO₄ at pH 2) on top of a MEA electrode, where the dispensed solution created an electrical potential change measured by the MEA setup. The characteristic time delay measured using this method was 22 ms (see Figure S1D). The measured neuronal activity delay times presented further in this work were not corrected for this time delay. Cultures 1 and 2 were stimulated at 14 and 21 days in vitro (DIV), respectively.

Stimulations at Different Distances from the Cell Membrane

All the neuron stimulation distances presented in this manuscript were measured from the top of the cell membrane as assessed by the FluidFM approach using 12 nN as the approach force threshold. At each distance, a stimulation protocol of minimum 40 stimulations was applied. The stimulation frequency was kept at 0.4 Hz and each pressure pulse lasted for 300 ms. The direct response probability plotted in Figure 3A indicates the amount of successfully induced direct responses compared to the number of triggered stimulation pulses. In Figure 3B, each data point represents the average delay time of one stimulation protocol, while error bars correspond to the standard deviation.

MEA Data Analysis

The electrical activity was recorded with the MC_Rack software (Multichannel Systems, Germany) and processed with custom scripts. Slow fluctuations in the raw data were suppressed by applying a Butterworth high-pass filter with a cutoff frequency of 20 Hz. An adapted wavelet-based spike detection algorithm [63] was used for spike detection. The algorithm was validated with a differential threshold algorithm adapted from Macconnie et al.[64] and an algorithm using a simple threshold based on the standard deviation.

Calcium Imaging

For the experiments where calcium imaging was performed in addition to the electrical recordings, cells were transduced with a neuron specific AAV GCaMP6f vector using a synapsin promoter (AAV1.Syn.GCaMP6f.WPRE.SV40; Penn Vector Core, USA).[65] On the day of transduction, the culture medium was replaced with 500 µL fresh medium per MEA chip complemented with a 1:1000 dilution of the AAV stock solution (batch number CS0473DL). The cells were incubated with the virus for 3 days, after which the whole medium was replaced with fresh medium and the cells were cultured normally. Change in calcium intensities was recorded with the EM-CCD camera at a frame rate of 32 Hz. The recorded data was processed using a custom-developed Python library available at GitHub.[66] Electrical and optical recordings were manually synchronized during data analysis.

Activity Analysis

Spiking activity was classified as a direct response if a spike occurred within 700 ms of the stimulation onset. The cells above the electrodes were considered directly responding to a stimulation pulse if a detected spike occurred within this time window. Electrodes in which the standard deviation of the recorded signal exceeded 90 µV (60% of a typical spike peak-to-peak amplitude) were not considered for further analysis.

Peri-Stimulus Time Histogram

The timing of induced spikes of each stimulation session was binned with 5 ms bin size. To calculate the time-dependent firing rate, the total number of spikes in each bin was divided by the bin size and the number of stimulations within that particular session. For the characterization of the network responses in Figure 4, each session consisted of 50 300 ms long
glutamate stimulation pulses with 0.4 Hz frequency, applying 50 mbar pressure while keeping the tip aperture at 5 μm above the cell membrane.

**Numerical Simulation of Glutamate Concentration**

Measurement of the glutamate concentration e.g. by simultaneously dispensing a fluorescent tracer was not possible because the diffusive behavior of commonly used fluorescent molecules is considerably different to that of glutamate due to the much lower molecular weight of glutamate. Therefore, the dispensing of glutamate from the FluidFM cantilever aperture was modeled using COMSOL Multiphysics 5.2 (COMSOL, Burlington, USA) utilizing a time-dependent three-dimensional model of the cantilever and the surrounding liquid.

The applied numerical model solves the Navier-Stokes equation for incompressible fluids coupled to the convection-diffusion equation of dilute solutions. The walls of the cantilever and the substrate were modeled using no-slip boundary conditions, while the remaining boundaries were considered open boundaries. The inlet at the beginning of the channel inside of the cantilever was set to a fixed concentration, with a laminar inflow boundary taking into account the pressure drop along the non-simulated part of the channel. The fluid was modeled as a dilute solution in water with diffusion coefficients of 7.9 x 10^-10 m^2/s for glutamate[67] and 1.91 x 10^-10 m^2/s for mPEG-FITC.[68]

In order to validate the numerical simulations of dispensing glutamate, the dispensing of a fluorescent tracer (mPEG-FITC, MW 2000, Nanocs, Chemie Brunschwig AG, Switzerland) was experimentally characterized using three-dimensional laser scanning confocal microscopy (ZEISS LSM 510, Zeiss AG, Switzerland), and the results were compared to that of the numerical simulation. For more information, please refer to Figure S2.

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