Persistent activity in the brain is involved in working memory and motor planning. The ability of the brain to hold information ‘online’ long after an initiating stimulus is a hallmark of brain areas such as the prefrontal cortex. Recurrent network loops such as the thalamocortical loop and reciprocal loops in the cortex are potential substrates that can support such activity. However, native brain circuitry makes it difficult to study mechanisms underlying such persistent activity. Here we propose a platform to study synaptic mechanisms of such persistent activity by constraining neuronal networks to a recurrent loop like geometry. Using a polymer stamping technique, adhesive proteins are transferred onto glass substrates in a precise ring shape. Primary rat hippocampal cultures were capable of forming ring-shaped networks containing 40–60 neurons. Calcium imaging of these networks show evoked persistent activity in an all-or-none manner. Blocking inhibition with bicuculline methiodide (BMI) leads to an increase in the duration of persistent activity. These persistent phases were abolished by blockade of asynchronous neurotransmitter release by ethylene glycol tetraacetic acid (EGTA-AM).

1. Introduction

The ability of the brain to hold information online is a remarkable feat. Working memory involves holding traces of memory for short periods of time. It is believed that persistent activity in the cortex is responsible for retaining such information over short periods of time.1,2 Persistent activity from single unit recordings3 as well as fMRI imaging in working memory tasks4 show increased levels of activity sustained for short periods of time ranging from milliseconds to seconds. This ability of local brain circuits to maintain activity after cessation of the initiating input has been observed in various brain regions including the prefrontal cortex and the thalamus.5–8 It has been proposed that reverberating activity in recurrent neuronal networks underlies such observed persistent activity.9,10 Using a technique of defining the network geometry, in vitro systems have been used to study specific in vivo phenomena, such as persistent activity11 and information transfer.12

1.1. Neural adhesive molecules in defined geometries

During development cells are constantly migrating, responding to various extracellular molecules. These extracellular molecules are presented to cells usually in the form of a gradient. In vitro these conditions can be recreated by techniques where neurons are exposed to protein-coated beads or stripes of substrate-bound protein (e.g. bead assays and stripe assays). These assays are well suited for studying guidance molecules, adhesion molecules and are typically presented in the form of static gradients.13 Typically cells are grown on glass substrates or tissue culture polystyrene. To obtain optimum cell adhesion these surfaces are treated with adhesive proteins and or by changing the surface chemistry. By changing the surface chemistry, the areas over which adhesive proteins reside can be controlled. The advent of lithographic techniques has allowed the control of adhesion of such proteins up to a micron range, which coincides with that of a single cell. Patterning of adhesive proteins using lithography, typically involves creating a micron scale stamp with an elastomer, the stamp is brought in contact with the substrate to transfer adhesive protein in a defined geometric pattern.

Microcontact printing (μCP) has been used to restrict neuronal networks to specified geometries14–16 and also to investigate the role of axonal guidance molecules.17 Early demonstration of in vitro control over neuronal polarity at a single neuron level was demonstrated by Stenger and colleagues18 where they used microlithography to pattern growth permissive surfaces to control neuronal polarity. μCP techniques have since been applied to restrict dissociated neurons to defined geometrical patterns for various applications such as patterning, surface gradient formations as well for axonal guidance studies.19,20 Specific designs also restrict the locations of soma or cell bodies. By restricting soma to locations near electrodes on micro electrode arrays, improvements in recording quality have been shown.14,21–23 Microcontact printing is a powerful technique for restricting and defining the allowable geometry of a developing in vitro neuronal...
network. We utilize this technique to control the geometries of the networks used for the present work.

1.2. Persistent activity and information transfer

Recurrent networks are capable of generating rhythmic oscillations as the network activity continually propagates around and is fed back into the network. There are currently no robust techniques for generating persistent activity in in vitro networks. One study used small disc-shaped islands of neuronal networks, containing 50–100 neurons, to allow the formation of recurrent networks without confounding effects of a larger external network. These small networks of recurrently connected hippocampal neurons display a form of activity upon stimulation with a single suprathreshold pulse termed reverberation. The reverberation within the network allows for a persistent activity lasting long after (>2 s) cessation of the input stimulus. These networks allow the realization of persistent activity similar to that associated with early in vitro memory formation with easy access to external experimental variables, but are limited in their ability to know the activity pathway taken by the reverberations. While this geometry disallows the confounding effects from a larger external network, it does not mitigate the effects from smaller recurrent loops within the network.

Alternately, patterned culture geometries have been used to precisely constrain axonal and dendritic growth geometry to achieve more explicit knowledge of the network connectivity, unlike standard dissociated cultures where axons are allowed to project in a random manner. The desired geometry is often defined through the surface patterning of specific adhesive and/or repulsive proteins to denote regions of neuronal adhesion and process growth. The ability to control network geometry allows control over the network parameters of a cultured network, as opposed to the random connection map arising from traditional dissociated cultures. Along these lines, networks have been defined into long, narrow cultures, to approximate a 1-dimensional signal propagation pathway. These “linear” networks have been used to study the 1-dimensional transfer of information with relation to models involving information theory.

Feinerman et al. show an inverse relationship between inter-neuronal distance and the information exchanged between neuronal nodes. They also showed that the relative activity strength between two nodes was held constant regardless of absolute activity strength. In other words, increased activity at one node caused a proportionate increase in activity of neighboring nodes, suggesting the connection strength is dominated by network architecture, regardless of the strength of input. Their system allows for definition of an activity pathway of a network as well as observation of the transfer of “information” (as defined through activity parameters) between various nodes. By combining this linear approach with a recurrently-connected ‘ring’ design, we demonstrate a platform to more-precisely define the activity pathway of recurrent networks which will allow more in-depth exploration of the geometric parameters underlying persistent activity in small networks.

2. Materials and methods

Briefly, silicon wafers were coated with a layer of negative photoresist SU-8-3025 (Microchem) at 60–70 μm thickness and baked for 20 min. Stamps were created by exposing a high-resolution CAD mask (CADart services) to UV light for 20 s. The wafer was then developed until all features were well defined, washed in IPA and hard baked at 150 °C for 30 min. The masters were then washed to remove traces of un-crosslinked photoresist and other organic solvents in 5% TritonX-100 for 20 min, followed by two washes in water. Poly-dimethylsiloxane (PDMS) (DOWCorning) was mixed in a 1 : 10 ratio of hardner to resin, mixed thoroughly and degassed. The degassed mixture was then poured over the master and allowed to bake at 95 °C for 2 h followed by cooling. The height of the resulting ‘ring’ structures in PDMS were approximately 60–65 μm.

Stamping of proteins was performed as follows: PDMS stamps were cleaned by sonicating in DI water for 20 min then dried under a stream of nitrogen. The stamps were charged in an oxygen plasma machine (Harrick plasma, NY) for 90 s and immediately coated with a protein ink. The biomolecular ink was poly-l-lyine (PLL) in borate buffer, for the immunocytological studies, the protein ink was PLL conjugated to FITC. Coated stamps were briefly placed in contact with round glass coverslips (12 mm φ) and removed quickly. The stamped coverslips were then rinsed in culture water (Invitrogen/Gibco) and allowed to air dry before plating cells.

Medium-density cultures of dissociated embryonic rat hippocampal neurons were prepared according to a previously described protocol with minor modifications, and approved by University of Pittsburgh IACUC. Briefly, hippocampi were removed from E 18–19 embryonic rats and treated with trypsin for 15 min at 37 °C, followed by washing and gentle trituration. The dissociated cells were plated on PLL-coated glass coverslips in 35 mm petri dishes with 100 K ml −1 cells per dish. The culture medium was Dulbecco’s minimum essential medium (DMEM; BioWhittaker) supplemented with 10% heat-inactivated bovine calf serum (Hyclone), 10% Ham’s F12 with glutamine (BioWhittaker), 50 U ml −1 penicillin-streptomycin (Sigma) and 1 × B-27 (Invitrogen/Gibco). One day after plating a third of the culture medium was replaced with the same medium supplemented with 20 mM KCl. Cytosine arabinoside (AraC, Sigma) was added to the culture dish (final concentration, 5 μM) around 5–7 DIV (days in vitro) to prevent overgrowth of glial cells. The optimal period for these cultures to be used for the experiments was 10–14 DIV.

For immunostaining the glass coverslips were fixed in 4% paraformaldehyde with sucrose, permeabilized with 5% Triton X-100 and washed three times respectively in PBS. They were then blocked in 5% BSA for 1 h, following which they are probed with primary antibody anti-tubulin, anti-synapsin 1 at 1 : 500 for 1 h and secondary antibody Alexa-555, Alexa-488 (Invitrogen) at 1 : 1000 and washed in PBS. Before mounting the cells were incubated in Hoechst 33342 (Invitrogen) at 1 : 1000 for 5 min and washed in PBS.

The resulting networks were observed taking shape of the ring structure as well as the surrounding grid structure. The ring structures in themselves were isolated from the rest of the grid. In some cases it was observed that the neurons in the ring grow over the central part of the ring, these rings were not used for any of the analysis. Field stimulation of all ring structures was performed using a bipolar theta glass capillary electrode (Warner instruments). The electrode was placed <50 μm from the cell that...
was being stimulated. The cells were maintained in Hepes buffered-saline (HBS) containing 150 mM NaCl, 3 mM KCl, 3 mM CaCl₂, 2 mM MgCl₂, 10 mM Heps, and 5 mM glucose (pH 7.4). In all cases control stimuli refers to a single pulse 2 ms long. Paired pulse stimuli (PPS) are two or three pulses 200 ms apart and 2 ms long at 80 V applied using an external unit (Grass, SD-9). Stock solutions of bicuculline methiodide (BMI) and EGTA-AM (Sigma Research Bio-chemicals) were first prepared in water or DMSO and diluted (1 : 1000) in HBS before being used. Throughout the experiment the cells were perfused with fresh bath solution at a constant rate of $\frac{1}{\text{ml min}}$.

2.1. Ca²⁺ imaging

Stock solution of Calcium sensitive dye Fluo4-AM (Invitrogen) at 1.5 mM was prepared in DMSO. The above solution was diluted in HBS to give a final concentration of 2.5 μM. Cells were usually incubated for 45 min to 1 h at room temperature in the dark before the commencement of the experiment. All images were acquired on a Leica DMB microscope, equipped with an emCCD camera (Roper Scientific S12B) at 30 Hz. The images were analyzed offline using custom written Matlab (Mathworks) scripts. Changes in fluorescence were reported as 

$$\Delta F/F = \frac{F_0 - B_0 - (F_1 - B_1)}{(F_0 - B_0)}$$

where $F_0$ and $B_0$ are the intensities of the somas and the background in the first frame and $F_1$, $B_1$ are the intensities of the soma and the background that vary with time.²⁷

Analysis of all videos was perfomed offline on Matlab (Mathworks) on custom written scripts. For each culture before stimulation, baseline movies of the network were acquired. In no case did we find any spontaneous activity in the networks. All regions of interest (ROIs) were selected from brightfield images of the networks. Each network was stimulated multiple times and the average over all stimulated trials was used for further analysis. For each trace, the fluorescence change was determined as described above. The algorithm then found the first time point where the signal rose past 50% of the maximum, then found the last time point where the signal dropped below 50% of the maximum. The duration between these two time points was used as the half-width duration. The other metric that was used for measurements was the time take to reach maximum change in fluorescence intensity. All values are reported as average ± standard error of mean.

3. Results

Here we present a ring-shaped network of primary cultured neurons yielding a recurrently connected network capable of producing prolonged activity following a single stimulus. The purpose of this work is to create an in vitro platform capable of studying persistent activity in networks.

3.1. Ring design

The ring geometries were fabricated into raised stamp designs using a standard micromolding procedure.²⁸,²⁹ The ring geometry is defined by the outer radius and track width (Fig. 1A). Scanning electron micrographs of the resultant stamps are shown in Fig. 1B. Raised ring-shaped surface used to define the culture geometry can be seen.

3.2. Ring-shaped neuronal cultures

Ring-shaped protein stamps were fabricated to define neuro-adhesive regions for creating the desired neuronal culture patterns (Fig. 2A). Patterns of neuro-adhesive molecule (poly-λ-lysine conjugated to FITC) were transferred via stamping onto a glass substrate, forming a ring pattern (Fig. 2B). It was observed that the cells constrain themselves to the stamped locations. Inconsistencies in the transfer of PLL (Fig. 2B, dark voids) correspond to locations also absent of neuronal processes (Fig. 2D, dark voids).

Fig. 1  Schematic of ring geometries. (A) Ring geometry is defined by the outer radius (arrow) and the track width (width of region). Track width can be determined by subtracting the inner radius from the outer radius. The outer radius determines the maximum extent of the neuronal network and the track width helps determine the complexity of the encompassed network. (B) Scanning electron micrographs of stamps used to create protein rings for neuronal culture. (Top) The isometric view shows the defined ring shape and (bottom) the cross section shows the precise edges.
Different plating densities resulted in different numbers of neurons composing the ring networks. The current design forms small neuronal networks (~40–60 neurons). Low-density cultures have a lower survival rate so it was important to determine the network densities formed from initial plating densities. We restricted our assessment to three initial plating densities that allowed formation of ring-shaped networks. It was observed that neurons plated at 100 000 cells ml$^{-1}$ were able to form ring structures. Densities at or lower than 10 000 cells ml$^{-1}$ did not have the ability to reliably form ring structures (data not shown). Neurons plated at densities higher than 10$^6$ cells ml$^{-1}$ also did not form consistent ring structures. The high number of cells in these networks tended to depart from the defined protein stamp geometry as well as formed large clusters of neurons, making analysis of individual cells difficult. As seen in Fig. 2E, although plating densities varied by two orders of magnitude, final culture densities only varied by one order of magnitude.

3.3. Functional connectivity

We tested the ability of the ring networks to propagate a given signal along the ring network. Calcium-sensitive indicator dye Fluo4-AM was used to examine the onset of electrical activity in neurons within the network. External bipolar electrode was placed near the ring culture and minimal field potentials were used to excite the nearest neurons in the network. An emCCD camera was used for high-speed capture of fluorescent images of the network.

Prior to data analysis, circular ROIs were chosen from brightfield images of the network at regions appearing to correspond to neuronal soma. Changes in fluorescence observed for those locations were used to assess activity within the network. Fig. 3 top trace shows the response to a single stimulus for a randomly selected ROI. Since these ROIs were chosen without knowledge of their fluorescence baselines and responses, not all ROIs had the same observed response magnitudes (SFig 1†). Though magnitudes differed, temporal response of ROIs were similar. These responses were characterized by a quick rise in fluorescence (typically 2 frames of the movie, or ~60 ms) followed by a slow exponential decay (typically 2–4 s). There were no spontaneous responses observed above baseline. We further ensured the response was due to our stimulus by using multiple stimulus pulses. Fig. 3 middle and 3 bottom trace show the responses of the same ROI to two and three stimulating pulses respectively. Even the ROIs that barely rise above baseline (SFig 1B, bottom trace†) shows a definite response to two and three stimuli (SFig 1C and SFig 1D, bottom traces, respectively†). Note that magnitude scale is held constant for traces from a single stimulus, but the magnitude scale is different for each stimuli.

3.4. Persistent activity

Brief extracellular stimulation of one region of the network was capable of evoking activity lasting a few seconds, termed
persistent activity. Persistent activity was observed in ring structures upon single stimulation in some cases. This persistent activity was characterized by a large rise in fluorescence (6-fold increase) followed by a persistence phase of the activity signal (Fig. 4). Fig. 4B shows individual traces taken from 10 ROIs of a single ring network, corresponding to the 10 ROIs in Fig. 4A. One can see that although each trace is unique, they share similar characteristics that distinguish them from the quickly decaying responses seen in Fig. 3. These responses are characterized by a large increase in intensity over the first ~2 s followed by a persistence of the activity signal for several seconds before a gradual decay.

Although the majority of rings responded with a sharp rise in fluorescence followed by a slow decay, as described in the control cases, a small percentage (13%) of rings responded with a persistent phase of activity. In order to better study this persistent activity, we needed to increase the likelihood of persistent activity generation. We abolished inhibition in networks by addition of GABA_A antagonist BMI to increase this likelihood.\textsuperscript{11} As expected, the persistent activity with BMI in the medium (Fig. 4C, BMI) occurred in 100% of the cases and exhibited the same characteristics of the persistent activity from medium without BMI (Fig. 4B). When compared with the majority of control cases, there were differences seen in both the average increase in fluorescence intensity (0.20 ± 0.02 A.U. for control, 1.41 ± 0.14 A.U. for BMI) as well as the average half-width duration (0.43 ± 0.06 s for control, 1.35 ± 0.15 s for BMI) when BMI was added to the medium. Also, each ROI showed similar responses across multiple trials (Fig. 4C), regardless of treatment. The plots in Fig. 4C show the averaged responses from five trials of two individual ROIs (dark colored traces) along with the corresponding confidence intervals (pale colored regions) for each treatment.

We also examined whether the persistent activity observed was being caused by the same molecular mechanisms described by Lau et al.,\textsuperscript{11} by adding the calcium chelator EGTA-AM, to the medium. The addition of EGTA-AM suppresses persistent activity based on asynchronous neurotransmitter release. Addition of EGTA-AM to the medium abrogated the persistent activity seen previously. The average fluorescence traces become both qualitatively and quantitatively similar to the original control traces (no persistence). The EGTA-AM traces exhibited a fast small rise in intensity followed by a slow exponential decay (Fig. 4C, EGTA-AM). The EGTA-AM traces displayed an average increase in fluorescence of 0.12 ± 0.01 A.U. as well as an average half-width duration of 0.27 ± 0.03 s.

3.5. Network-dependent changes

Because each network is different, averaging each experimental treatment (control, BMI, EGTA-AM) across different networks yields a larger amount of variance than that seen within an individual network. In order to show the differences between treatments, we look at the cumulative distribution of 3 parameters; the time to reach maximum fluorescence intensity, the maximum fluorescence intensity, and the half-width duration. These parameters for all analyzed ROIs across all networks are shown as cumulative distributions in Fig. 5A–C, respectively. Insets show the average of each parameter. Average and standard deviation data for each ROI from a single network is shown for the change in fluorescence intensity, time to reach maximum

![Fig. 4](image)

Fig. 4 Persistent activity in ring network cultures. (A) Fluorescent micrograph of neuronal culture shows a neuronal ring culture with regions of interest (colored dots). (B) Individual traces of ROI fluorescence intensity following stimulation (arrowhead). Trace color corresponds to ROI color from part A. Scale bar is: 2.5 s X-axis, −ΔF/ΔF_Y-axis. (C) Change in fluorescence intensity at two ROIs upon stimulation in presence of media (control), change in fluorescence intensity after addition of BMI to the media and change in fluorescence intensity after addition of EGTA-AM to the media. Only synchronous phase of transmission is seen, all persistence of the signal is abolished in EGTA-AM case. Bright red trace is average of five trials bound by deviation (light red) and black trace is baseline from average of five trials. Scale bar: fluorescence image: 100 μm, 1.25 s X-axis, −0.5ΔF/ΔF_Y-axis for control, EGTA-AM and 1.25 s X-axis, −5ΔF/ΔF_Y-axis for BMI.
change in fluorescence intensity, and the half-width duration in SFig 2.

4. Discussion

Most in vitro neuron activity studies explore the electrical properties of either a single neuron or the connection between two neurons. In these cases, pharmacological methods are used to suppress network activity so that interactions from a randomly-connected network do not confound the data. Although our interest is specifically exploring network activity, we limit the confounding effects from extensive random populations by limiting the network size and better define the possible pathway lengths by constraining the inner-region of the network.

4.1. Ring design and neuronal networks

We have developed a method to culture sparse neuronal networks of a defined geometry that allows for the repeatable observation of persistent activity. The current geometry allows formation of recurrently-connected ring-shaped neuronal networks to allow exploration of activation parameters and maintenance characteristics of persistent activity arising from reverberations in these networks. The ring network defined through this geometry limits both the upper extent of the network as-well-as the lower extent. We use these limitations to not only better-define the range of possible pathway lengths, but also limit the likelihood of destructive interference by removing the possibility of smaller recurrent loops within the network. By limiting the upper extent we remove the effects from larger unknown network loops as well as other sources of activity. By limiting the lower extent (i.e. ring shape instead of a simple disc) we limit the possible variability in network characteristics as well as destructive interference arising from smaller network loops. This ring-geometry was used to form the experimental networks used throughout this experiment.

The ring geometry allows us to limit areas where pathways can form within the network and further limits the possible activity pathways created by the neuronal network. As seen in Fig. 1 and 2, the activity pathways created in the ring geometry can have an approximate minimum and maximum path-length (as determined by the circumferences associated with the internal and external radii of the ring design). The axons and dendrites do not travel in a perfect arc, so the actual path-length as a signal propagates around the ring will always be longer than a circumference determined by a circle’s radius. In the case of a ‘disc’ shaped geometry (i.e. inner radius = 0), the path-length will still have an approximate upper-bound, but any number of smaller network pathways can easily be formed within the disc. These smaller pathways cause two main problems for studying the activation parameters and maintenance characteristics of reverberatory activity in small neuronal networks. The first problem is that destructive interference of an activity signal can occur when a stimulus signal is split and then brought back together. When this occurs, no reverberations are generated in the network, making it impossible to study the reverberatory characteristics of that network. The second problem is that reverberations could be generated in smaller than expected pathways. This could confound attempts to study the effects of network geometry on activity properties. An example of such a problem could be data recorded from smaller than expected pathways could underestimate the persistence times of a specific geometry, increasing the experimental variability, and making it more difficult to determine the differences between different sized geometries (since the same small pathway could be formed in any number of larger discs, yielding the same activity dynamics).

We have demonstrated neuronal cultures in a ring-shaped geometry through microstamping adhesive proteins onto the substrate surface. Dissociated neurons plated onto the substrate will grow almost exclusively on the protein coating. Likewise, neuronal processes will comply with the geometry of the protein stamp through 2 weeks of growth and development (later time-points were not assessed in this study). One of the crucial factors to the quality of the network geometry is the quality of the protein stamp. Since the stamped protein promotes neuronal adhesion and growth, areas with more protein will be more likely to promote neuronal adhesion and growth (data not shown). The neurons were plated at a density of 100 000 cells ml⁻¹. This density created networks containing similar numbers of cells as...
those used in previous studies\(^1\) (Fig. 2E). Extended neuronal growth and recurrent network morphology were verified through immunocytochemistry to ensure suitable experimental networks (SFig 3†).

### 4.2. Functional connectivity

The ring-shaped networks created here were functionally connected in a recurrent fashion. We assessed this by stimulating networks at a single location along their circumference and observing activity changes around the entire network. Functional connectivity was verified through use of single, double and triple pulse stimuli (Fig. 3). The monitored ROIs were seen to increase their fluorescence immediately following each stimulus. This behavior is consistent with the actions of a functionally connected network. We further immunostained these ring networks for synaptic markers and verified for the formation of synapses throughout the network (SFig 3†).

### 4.3. Persistent activity

Although formation of a small, defined, functional neuronal network is useful, of particular interest is the establishment of a neuronal network capable of reliable production of persistent activity, similar to those seen in vivo during working-memory processes. A limited number of networks exhibited a drastically different response from that described earlier. This response was characterized by a large slow increase in fluorescence with a much longer raised fluorescence. Though this response occurred in a limited fashion under control conditions, we could induce this response regularly when the inhibitory inputs in the network were blocked by addition of BMI. Characterization of this response using time to maximum intensity, maximum fluorescence intensity and half-width duration demonstrated a clear difference between control and persistent activity responses.

An additional observation was that there was a consistent relationship between the maximum response of ROIs in a single network across multiple trials, regardless of activity strength. We found that the relative strength seen between ROIs during a short-duration signal was maintained for persistent phases also (SFig 4†). We observed a linear trend when we plot the relative strength at an ROI with and without persistent activity (e.g. + or − BMI). In other words, the stronger ROIs under control conditions are the stronger ROIs under BMI conditions. This result is consistent with previous studies that found signal strength is maintained throughout a network pathway.\(^1\)

Also, throughout this study we used two slightly different network geometries, 300 μm outer radius with track width of either 150 or 100 μm. The slight differences in measures seen between these two track widths showed no significance (STable 1†), but suggest those geometry parameters might effect activity.

We added EGTA-AM to the BMI medium to demonstrate that persistent activity was not simply an effect of the removal of inhibition. EGTA-AM is a slow calcium chelator. Excitatory neuronal inputs are transduced from one neuron to the next by pre-synaptic release of neurotransmitter that is then sensed on the post-synaptic side. In a typical “signal”, the release of neurotransmitter on the pre-synaptic side is synchronized, causing a large release of neurotransmitter for signaling. This release is due to a quick rise in calcium in the pre-synapse due to the incoming action potential. If enough inputs are synchronized an action potential is then generated in the post-synaptic neuron. In addition to synchronous release, neurotransmitter is spontaneously and randomly released from the pre-synapse (i.e. asynchronous release) at a much lower level than synchronous release, but forms the “background” noise of a neuron’s activity. The level of asynchronous release is based on the level of calcium in the pre-synapse. A higher level of asynchronous release will result in a neuron that is more likely to cause an action potential in the post-synaptic neuron. The decrease in inhibitory inputs, due to the added BMI, allows the signal to propagate around the network and re-activate the earlier synapses, resulting in a persistent activity.

Addition of EGTA-AM allows for the synchronous phase of transmission but not the slower asynchronous phase. In order to demonstrate that the activity persistence was due to the “recycling” of the initial activity signal around the network, we used EGTA-AM to abolish asynchronous transmission. A fast calcium chelator would be able to greatly reduce the effect of calcium as it was entering the pre-synapse. This would stop any signal before it got started by limiting the transmission of a signal from one neuron to the next, preventing any persistence of the signal. With the addition of EGTA-AM, we still see an activity signal propagate around the network (initial rise in activity in all locations around the network) but we see no large increase due to persistent activity (quick drop in signal, just as in the control case) as the calcium is buffered.

Two other parameters not explicitly explored in this study is the onset and termination of the persistent phase of activity. The onset of this persistent phase of activity, while initiated by the external electrical stimulus, can be enabled by changing the balance of inhibitory and excitatory connections. We ensure that imbalance here through the use of BMI. Of similar interest is the termination of the persistent phase of activity. Lau et al. showed asynchronous release of neurotransmitter from pre-synaptic terminals is important for maintenance of reverberatory activity.\(^1\) Activity-dependent increase in pre-synaptic residual calcium enhances asynchronous neurotransmitter release and such reverberations are terminated by a slow timescale synaptic depression.\(^1\) Suppression of this activity specifically abrogates the persistent phase without affecting the initial propagation around the network. Our EGTA-AM experiments show that the persistent activity observed here is of the same form demonstrated in earlier work.\(^1\)

### 5. Conclusions

We have demonstrated a small, well-defined, recurrently-connected neuronal network capable of producing a form of persistent activity. This activity, when highly inhibited was stronger, took longer to achieve maximal response, and lasted for a significantly longer duration compared to control cases. An earlier study of disc-shaped networks reported persistent activity in approximately 52% of cases with the addition of BMI,\(^1\) as opposed to approximately 6% of control cases. We observed persistent activity in 100% of cases after the addition of BMI. This demonstrates the ability of the current work to form a robust platform to study persistent activity in small neuronal
networks. The persistence of activity was due to the asynchronous release of neurotransmitter, which when inhibited, abolished all persistent phases of activity. We characterized a series of the persistent phase measures through high-speed imaging of calcium-sensitive dye. Future work could include further characterization of the current geometries using patch-clamp electrophysiology to visualize the cyclic activity, which happens at too high of a timescale to be resolved in detail by current calcium-sensitive dye techniques. Other work could include an extension of the geometries tested (using either electrophysiology or calcium-sensitive dye techniques) to determine the effects of these geometries on the persistent activity measure. In conclusion, we have shown an in vitro neuronal network platform capable of producing persistent activities for parameter studies in defined geometries.

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