An Electrically-Stimulate Optically-Record Microsystem Based on Active CMOS Multi-Electrode Array for Dissociated Cell Cultures

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***Abstract*—Calcium fluorescence-based optical recording combined with patch-clamp stimulation has become the standard technique for analyzing neural network behavior. At best, stimulation is limited to only a few channels in this case. Passive multielectrode arrays for two-dimensional electrophysiology only offer electrode densities of 60 electrodes per mm^2. Here, we report an active multielectrode array, constructed with a standard complementary metal-oxide-semiconductor (CMOS) technology, to perform localized extracellular stimulation of dispersed cell cultures. A 256x256 array integrated with in-pixel stimulators on a 4-by-4 mm^2 CMOS chip noninvasively stimulate hippocampal cells cultured on chip at cellular resolution. Combined with calcium imaging using high-affinity indicators, we demonstrate the ability to observe spatiotemporal dynamics of neural activity.**

I. Introduction

Calcium imaging is well suited for monitoring action potentials and neural network activity due to the ease of dye loading and the ability to simultaneously monitor multiple cells with high signal-to-noise ratio [1]. Optical stimulation techniques such as glutamate uncaging have advanced significantly, but still provide relatively few stimulation channels [2]. Patch-clamping stimulation allows only one-cell-per-trial manipulation. In the two-dimensional environment of cultures and brain slices, passive multi-electrode arrays (MEAs) can also be employed. Spatial resolution ($\sim$60 electrodes per mm^2) is limited by electrical connections to off-MEA electronics. In this work, we have designed and fabricated a 65,000-electrode MEA integrated on an active complementary metal-oxide-semiconductor (CMOS) chip to produce high-throughput high-resolution stimulation patterns. Combined with optical techniques based on calcium-sensitive dyes, the constructed microsystem is designed to offer high throughput and the ability to study spatiotemporal dynamics in neural networks.

We choose to implement extracellular stimulation using electrodes passivated with a thin (<20-nm) layer of the high-κ dielectric hafnium oxide (HfO_2), resulting in a capacitive stimulation interface (Fig. 1) [3]. This eliminates Faradaic reactions and prevents undesired effects such as electrolysis and tissue damage [4] at the cost of lower current stimulation levels. In this work we also explore the limits of non-Faradaic stimulation and optimize the interface design.

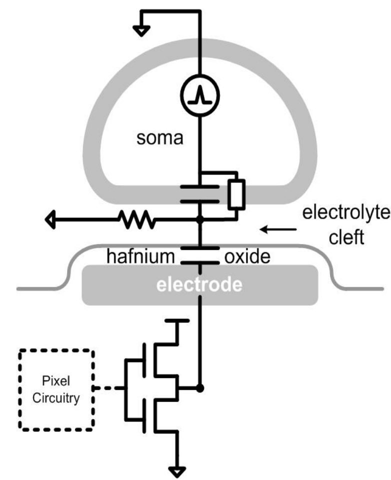

Fig. 1. Lumped circuit model of cross section interface between neuron soma and stimulating capacitor electrode.

II. Methods

A. Fully-Integrated Stimulation Array Design

The stimulation chip is fabricated in a commercial 0.25 μm CMOS foundry. The 4 x 4 mm^2 chip consists of a 256 x 256 array of square electrodes. Each electrode has an edge length of 11.4 μm with a pitch of 12.2 μm, giving rise to a total active stimulation array area of 3 x 3 mm^2 (Fig. 2a). The stimulation MEA is designed to activate any random combination of electrodes within the array with each electrode producing a unique stimulation waveform. Individual electrodes are driven by an in-pixel stimulator shown in Fig. 2b. Stimulation data bits are first written into the desired pixels sequentially and preceded by execution. During execution, the stored stimulus signals are driven onto the electrode at the stimulator output. A bit-0 holds the electrode potential at bulk electrolyte potential, i.e. ground. A bit-1 drives the electrode potential to the desired stimulation amplitude specified externally, allowing stimulation voltage levels between 0.6 to 5 V. Stimuli between

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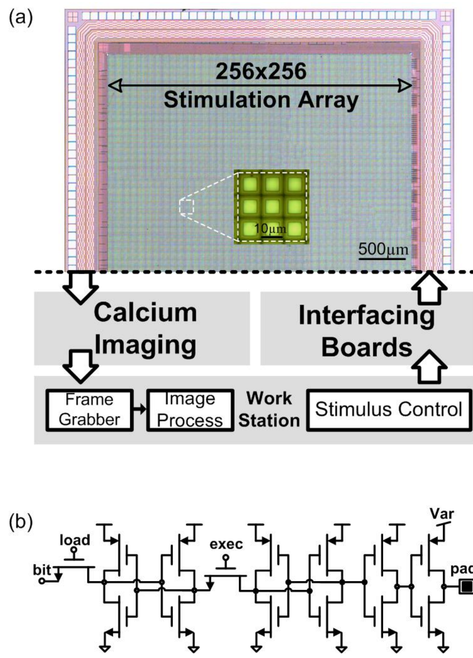

Fig. 2. (a) Chip micrograph with stimulation-record microsystem setup. Insertion shows microscope capture of the electrodes. (b) In-pixel stimulation pulse generator circuit schematics.

any two electrodes in the array can be synchronized and aligned within 45.7 psec with 125 nsec pulse resolution.

B. MEA Post-Fabrication and Package Assembly

The commercial CMOS foundry process used to fabricate the chip puts a Si_3N_4-SiO_2 dielectric stack on top of each electrode, leaving the top-metal electrodes 1.6 μm below the chip surface. To expose the electrodes, this layer is removed using a dry-etch process with a composite gas of C_4F_8 and O_2. A low-temperature atomic-layer-deposition (ALD) process is then used to directly deposit a 15 nm HfO_2 layer onto the planarized electrodes to make the interface capacitive.

To enable external interfacing, the chip is wire bonded to a 272-pin ball-grid-array (BGA) package. The peripheral circuits and bond pads are donut epoxy encapsulated, leaving the electrode area exposed. A polypropylene well is attached to the chip and sealed with polydimethylsiloxane (PDMS) to form a 15 mm diameter culture well.

C. Chip Surface Preparation and Cell Culture

Prior to cell culture, the chip surface is sequentially coated with adhesion proteins, 100 μg/ml poly-L-lysine and 10 μg/ml laminin. The hippocampal neuronal cultures used in the extracellular stimulation experiments reported here are seeded and cultured from E18 rats according to well-established methods [5]. For each culture, half of the media is exchanged with fresh media every three days before imaging (Fig. 3).

D. Microsystem Setup

A custom-made PCB provides sockets for the assembled stimulation chip and interfaces with two PCI data acquisition

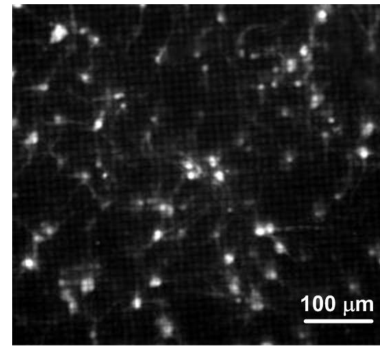

Fig. 3. Fluo-4 fluorescence of disperse culture growth on MEA surface at day-14.

cards (NIDAQ, National Instrument) to a host PC. A user interface program generates stimulation patterns, allowing the control of pixels to be activated, duty cycles, and numbers of consecutive stimuli. The patterns are bit streamed into a custom made LabView program clocked by internal counters on the NIDAQ cards. Chips containing neuronal cultures are positioned under a 10X objective mounted on a upright fluorescence microscope. Images are recording using a cooled CCD camera at 100-msec-per-frame with 490 nm excitation and 520 nm emission filters. External triggers sent to both the camera control box and the LabView program synchronize shutter opening 154 msec prior to electrode activation. Experiments are performed with cell cultures immersed in perfused DMEM-HEPES solution at 75 ml/hr perfusion rate. Continuous perfusion with solution warmed at 37°C maintains a constant temperature at array surface, and mitigates heating from the MEA due to power dissipation. An Ag/AgCl reference electrode is immersed into the solution tapered to the chamber wall.

E. Dye Loading and Imaging Analysis

The high-affinity calcium indicator, Fluo-4-AM, is used to measure intracellular calcium concentration changes as an indirect measure of action potential response [6]. Prior to each experiment, Fluo-4-AM dyes are bulk loaded into matured cell cultures at 1 μM in filtered phenol-red-free HEPES-buffered DMEM. Imaging analysis is performed by creating a differential fluorescence change (DFF) movie from the original recording. Maximum change in fluorescence in DFF is measured as $\Delta F/F_0 = [(F_{pk} - B_{pk}) - (F_0 - B_0)]/(F_0 - B_0)$, where F_0 and F_{pk} is the baseline fluorescence and peak of the fluorescence transient respectively, and B_0 and B_{pk} are the associated background fluorescence intensities. Look-up tables are adjusted so that pixels associated with increasing calcium fluorescence appear dark versus a white background in DFF.

III. Results

A. Artifact characterization

Prior to MEA stimulation, we calibrate calcium transients with loose-patch electrophysiology recording of spontaneous

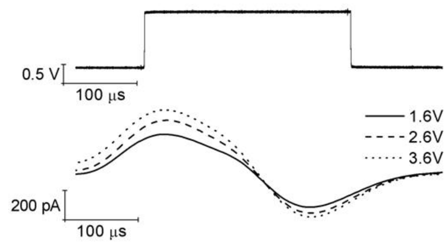

Fig. 4. Square voltage pulse stimulus produced by individual electrodes in the MEA (top) and the triggered stimulus artifact in 1X DMEM-HEPES solution (bottom).

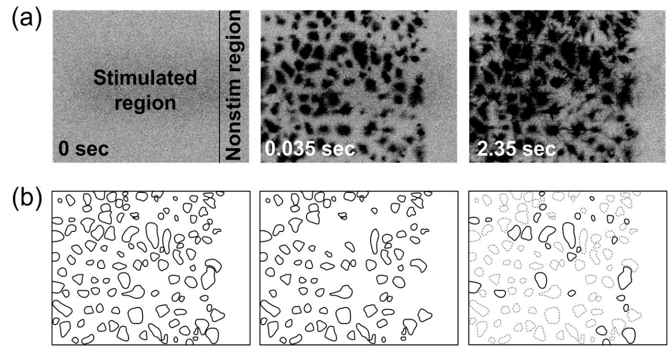

Fig. 5. Regional stimulation on dispersed hippocampal culture. (a) Times series montage of stimulated response. (b) Cells activated in the initial stimulation (left), cell activated in the repeated trial (middle), and non-overlapping cells from both stimulation trials (right).

activities in day-14 dissociated hippocampal cultures. Spike trains with firing rates exceeding 6.5 Hz are compared to discrete Ca^{2+} signals summed consecutively in the soma. A DFF of 1.27% is the calibrated response of a single AP.

Because of the capacitive coupling of the electrode to the media, square voltage pulses applied to the electrode produces a biphasic current stimulation. Each electrode with a capacitance of 0.9 pF delivers 20 pC of charge at 1.6 V. Peak biphasic current is approximately 85 pA/V of amplitude for the rising edge of the pulse and 35 pA/V of amplitude for the trailing edge (Fig. 4).

B. Proof of Principle

In initial experiments,a large population of hippocampal cultures are imaged to discern cell activities to pre-programmed electrical stimuli from the MEA electrodes. Objective lens is positioned above a crossover line so that cell response in both stimulated and non-stimulated region can be observed within the same field of view. Fig. 5a shows the DFF time series of induced cell activities after stimulation with fourteen 200 μsec voltage pulses at 2.7 V. Significant fluorescent change is induced in almost all neurons in the stimulated region while neurons in the non-stimulated region remain idle. Stimulation in a repeated trials shows a 7.7%±1.9 peak fluorescence change versus 13.5%±1.4 in the initial trial. Spatial wise, a high degree of overlap of activated cells is observed between both stimulation trials. Fig. 5b shows activated neuron contours in each trial. It is observed that approximately 20% of the cells did not activate in the second stimulation trial, and post-stimulation neurons does not exhibit fluorescent decay. Both indicate probable undesired electrophoresis. This leads to the analysis of safe and effective stimulation, addressed in the next section.

C. Single Neuron Stimulation

To study the efficacy of localized stimulation for activating single cells, a train of fourteen voltage pulses at 10 msec apart is applied to four adjoining electrodes directly below targeting cells in multiple experiments. Fig. 6 shows a representative cell response. The DFF on the stimulated cell shows a 1.4% peak fluorescence change above the baseline at 1.6V stimulation amplitude in response to the elicited action potential. This

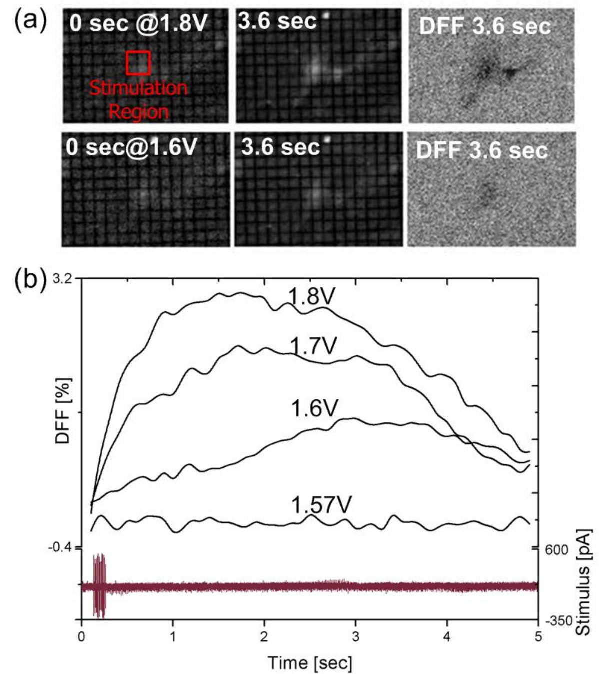

Fig. 6. Stimulation threshold detection with calcium imaging. (a) An isolated neuron is stimulated through electrodes located directly underneath the cell (boxed in red). (b) Temporal DFF profiles with bleach correction shows fluorescence percent change for each applied stimulation amplitude. Captured electrode stimuli is plotted on the same time scale.

contrasts with a DFF that remains at baseline in the absence of a stimulus. Using 1.27% DFF as for the calibrated response of a single AP, an average stimulation amplitude of 1.5 V determines the activation threshold.

D. Simultaneous stimulation of multiple neurons

Simultaneous stimulation of multiple neurons using MEA electrodes are also tested. In the cultures shown in Fig. 7, initial stimulation applied to cell N_1 by activating a group of 4x4 electrodes at suprathreshold results in a 3.5% fluorescence

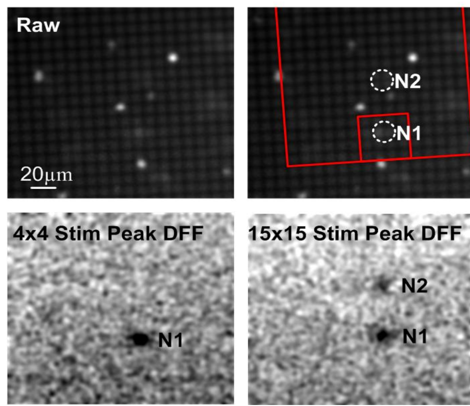

Fig. 7. Simultaneous stimulation of multiple cells with two different stimulation regions (boxed in red, top row). Peak DFF fluorescence in cell N_1 and N_2 in response to the two stimulation trials (bottom row).

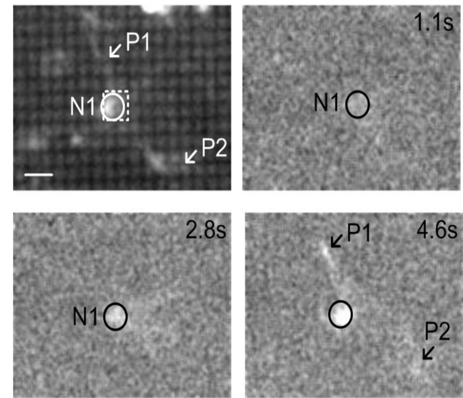

Fig. 8. Time series of $\Delta F/F_0$ on calcium signals in a hippocampal network with stimulated response progressive spread into neighboring regions. Raw Fluo-4 fluorescence is shown in the top left figure with stimulation region is boxed with dash lines. Scale bar equals to 25 μm.

change. The same stimulus is subsequently applied to an expanded region of 15x15 electrodes to simultaneously stimulate both N_1 and N_2. Cell N_1 exhibits fluorescence increase (2.4%) with similar kinetics. Cell N_2 also exhibited a fast onset fluorescence change at 1.8% in response to the stimulation, contrasting with DFF that remains at baseline in the initial stimulation trial. Overlaying DFFs of both N_1 and N_2 shows a ΔT of 600 msec at peak fluorescence, suggesting variations in membrane capacitance and channel conductances.

E. Stimulation induced network response

Neurons in hippocampal networks exhibit strong excitatory synaptic connections [7]. An important application of the MEA is to evoke spatiotemporal activity patterns as a result of localized stimulation of a neuron within a network. With the network shown in Fig. 8, stimulus at threshold amplitude is applied under cell N_1. The stimulation results in a 3.3% fluorescence change in N_1 from the baseline that progressively spreads outwards. Surrounding cells P_1 and P_2 also exhibited smaller fluorescence increase at 1.49% and 1.51% respectively with approximately 2 sec time lag. Immunohistochemical staining for neural and synaptic markers is performed on the network. The fixation indicates a probable connection from N_1 to P_1 and P_2.

IV. Conclusion

In conclusion, we described here a high density active MEA and demonstrated selective extracellular stimulation of neural activity in cultured cells through non-Faradaic MEA electrodes. Combined with optical sensing technique, network activation following multiple targeted cell stimulation can be effectively studied using this modality.

Acknowledgment

This work was supported in part by the New York State Foundation for Science, Technology, and Innovation (NYSTAR), and the Gatsby Initiative in Brain Circuitry.

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