Novel Disposable Microelectrode Array for Cultured Neuronal Network Recording Exhibiting Equivalent Performance to Commercially Available Arrays

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Abstract

Microelectrode arrays (MEAs) enable non-invasive recording of supra-threshold signals, i.e. action potentials or spikes, from a variety of cultured electrically active cells. While this label-free technology has been shown to be useful for pharmacological and toxicological studies, a major limitation has been the reliance on expensive recording substrates that have been manufactured with the intent of re-use. Prior work by our group has demonstrated an approach for fabricating MEAs using conventional liquid crystal display manufacturing techniques. Here, we describe and characterize the UT Dallas planar MEA which is fabricated with low cost materials and processes. We compare the performance of the UT Dallas MEAs, which consist of exposed gold microelectrodes with patterned parylene insulation over traces, with well-established commercially available MEAs using cultured murine cortical networks. Detailed electrophysiological comparisons show virtually identical performance between MEA types with respect to network metrics including recording yield across the array, network spike rate and burst rate, and virtually identical pharmacological responses to a diverse set of neuropharmacological agents.

Keywords: Microelectrode array, Disposable microelectrodes, Extracellular recording, in vitro assay, Neuronal recording

1. Introduction

Neuronal networks cultured on planar microelectrode arrays (MEAs) have utility in wide range of in vitro applications including neuropharmacology [1–4], assessing biocompatibility of novel materials [5], studying neural network plasticity [6–8], connectivity [9], and response to electrical stimulation [10,11], as well as the emergence of dynamic states [12,13]. Murine

primary cultures derived from embryonic neural tissue mature to form spontaneously active networks on the surface of MEAs. . In fact, the use of cultured cortical tissue on MEAs for pharmacological assays has been cross-validated across multiple laboratories [14]. Compared to other electrophysiological assay or imaging approaches, MEAs offer label-free, non-invasive, and long-term recording capabilities.

To create MEAs with features on the order of tens of microns, fabrication processes and materials common in the silicon microelectronics industry are used. Common conductive materials comprising MEAs include indium tin oxide (ITO), platinum, gold, or titanium nitride, whereas the insulating materials include SU-8, silicon nitride, polymimide, parylene, or polydimethylsiloxane. Advances in MEA technology have mainly focused on increasing spatial resolution and channel density through the incorporation of on-chip CMOS electronics [15–18]. Unfortunately, MEAs are typically expensive, fragile, and consist of materials that are distinctly different from conventional polystyrene culture dishes used and discarded in typical cell biology laboratories. Manufacturers expect re-use and specialized handling of these devices.

We previously reported on the application of liquid crystal display fabrication technologies to create a low-cost functional MEA [19] that made use of gold microelectrode contacts and traces insulated with parylene-C, a biocompatible polymer often used in implantable device applications [20-22].

In the present paper, we demonstrate reproducible fabrication of low-cost MEAs leveraging our fabrication process that exhibit stable electrochemical impedance profiles of gold contacts and conducting polymer modified sites under culture conditions. Developed at the University of Texas at Dallas (UT Dallas), we show that the performance of these MEAs with respect to cultured neuronal network recording is entirely consistent with that from well-

established commercially available MEAs from Multi Channel Systems (Reutlingen, Germany). Our data show that the UT Dallas MEAs offer a platform for low-cost, disposable high content assays from neuronal networks.

2. Methods

2.1 Fabrication and characterization

An array of 60 gold square microelectrodes 30 μm in length was patterned on the substrate with insulation over the leads with parylene-C. The outer dimensions of the array substrate were 3.8 cm x 3.8 cm with 2 mm x 2 mm bond pads separated by 400 μm. As shown in Fig.1, the substrate was a 500 μm thick layer of polycarbonate, a stiffer material than polyethylene naphthalate used previously [19], which improves ease of handling. The gold microelectrodes were deposited by e-beam evaporation and patterned using standard photolithography and wet etch. Parylene-C was deposited by chemical vapor deposition and then exposed to oxygen reactive ion etching for patterning.

Electrochemical characterization of the MEA was performed by electrochemical impedance spectroscopy (EIS) on individual microelectrodes from a typical array. The measurements were performed using a two-electrode setup using a potentiostat/galvanostat (CH 600D, CH Instruments, Texas, US) equipped with an electrochemical analyzer module (CHI Version 9.03, CH Instruments). Measurements were made in the presence of phosphate-buffered saline (PBS) at pH of 7.4 at room temperature by applying a sinusoidal signal with 20 mV amplitude over a range of frequencies from 10 Hz to 100 kHz to characterize the complex impedance of the working electrode and the electrolyte solution.

2.2 MEA preparation and cell culture

Commercially available MEAs from Multi Channel Systems (Reutlingen, Germany), model MCSMEA-S2-GR which consist of 60 ITO microelectrodes, were used for comparison with the novel MEAs. Microelectrodes had a diameter of 10 - 30 µm and inter-electrode spacing of 200 µm. As described in [5], MCS MEAs were first disinfected by 70% ethanol for 20 minutes under laminar flow in a biohood and then rinsed with sterile de-ionized water. To obtain better cell adhesion, the center regions of the MCS MEAs were coated with 50 µg/ml of poly-D-lysine (PDL) (Sigma-Aldrich, St. Louis, MO) overnight. After the incubation with PDL, the arrays were then washed with sterile deionized water three times to remove any excess PDL. The arrays were then coated at the center with 20 µg/mL of laminin (Sigma-Aldrich, St. Louis, MO) for at least an hour. Prior to the cell seeding the laminin was removed from the MCS MEAs.

Small, yet important modifications to the above preparation steps enabled immediate and reliable use of the UT Dallas MEAs for cell culture. First, UT Dallas MEAs were exposed to oxygen plasma treatment with the oxygen pressure of 15 psi for 1 min at 75 W (PE-50, Plasma Etch Inc, Carson City, NV). UT Dallas MEA wells were filled with PBS and allowed to soak for 24 hours at room temperature. Under a biological containment hood, UT Dallas MEA wells were treated with 70% ethanol for 20 min, washed with sterile water, filled with cell culture medium consisting of DMEM (Life Technologies), 2% B27 (Life Technologies), 5% horse serum (Atlanta Biologicals, Lawrenceville, GA), 5% fetal bovine serum (Life Technologies), and 0.2% 4mg/ml ascorbic acid (Sigma Aldrich) and incubated overnight at 37 °C in the cell culture incubator. After removal of the culture medium, ~50 µl of 50 µg/ml of PDL was applied to the center of each UT Dallas MEA recording well and allowed to incubate at 37^{o}C in a CO_2 incubator overnight, with care taken to avoid evaporation. Approximately 2-3 hr prior to cell

plating, PDL was washed from the recording wells three times with sterile water and the surface was allowed to dry. Finally, ~50 µl of 20 µg/ml of laminin was applied for at least 1 hr with the excess removed immediately prior to plating. Pilot experiments revealed that strict adherence to the above preparatory steps enabled reliable adhesion of dissociated primary embryonic cortical tissue and subsequent maturation into active networks.

The primary neuronal culture method was similar to that described in [23]. The procedure was approved by the Institutional Animal Care and Use Committee of George Mason University (Fairfax, VA). Timed pregnant, embryonic day 17, CD-1 mice (Charles River, Wilmington, MA) were euthanized with carbon dioxide followed by decapitation. Embryos were extracted in ice cold L15 (Life Technologies, Grand Island, NY). Upon isolation of the frontal cortex, the tissue was stored up to 24 hours in a hibernate media (BrainBits, Springfield, IL) supplemented with 2% B27 (Life Technologies) and 0.5 mM Glutamax (Life Technologies), Later, the hibernate media was removed, the tissue was minced by scalpels and then dissociated through incubation with DNAase and papain (Worthington Biochemical Corp., Lakewood, NJ) for 15 minutes followed by mechanical trituration using disposable graduated pipettes (Fisher Scientific, Pittsburg, PA). After centrifuging at 2500 rpm for 5 minutes, the supernatant was removed and cells were re-suspended in culture medium. The cells were counted using a hemocytometer (Life Technologies) and immediately seeded on MEAs at a density of 100,000 in a 50 µL droplet. All cultures were incubated at 37°C with 10% CO_2 and maintained in DMEM with GlutaMAX supplemented with 5% horse serum (Atlanta Biologicals), 5% FBS (Life Technologies), 2% B27, and 0.2 % of 4 mg/ml ascorbic acid (Sigma-Aldrich) for the first two days. To avoid overgrowth of glial cells, the fetal bovine serum was removed at day 3 and the cultures were thereafter maintained by a 50% media exchange twice a week for at least 21 days.

2.3 Extracellular recordings and analysis

All recordings were performed after at least 3 weeks *in vitro* to ensure that the neural networks had reached maturity and consistency in activity. The multichannel extracellular recordings were acquired with an OmniPlex data acquisition system (Plexon Inc, Dallas, TX), where each of the 60 channels of either MEA was digitized at a sampling frequency of 40 kHz. During the recording session, the culture temperature was controlled at 37 °C.

During extracellular recordings from the neuronal networks, the mean noise level was calculated for each individual channel and a threshold was set at 5 standard deviations from this mean. A spike was then detected if the signal surpassed this threshold. Channels were considered to be active if the spike rate was at least 0.1 Hz. The recorded spikes from each channel were sorted off-line into well-resolved units using Offline Sorter V.3 (Plexon Inc.). The spike sorting method was based on the 2D principle component analysis of spike waveforms followed by scanning K-Means to find and separate between clusters. Each unit presumably corresponds to the signal from an individual neuron. Consistent with [24], a burst was defined as the occurrence of a minimum of 4 spikes which were not more than 75 ms apart from each other. The minimum inter-burst interval was set to 100 ms.

2.4 Pharmacological exposure

For a subset of experiments, the pharmacological sensitivity of the cultured neuronal networks on the MCS and novel MEAs was examined. In separate comparison experiments, we applied the following compounds to the cultures on both MEA platforms: 20 µM memantine (Sigma -Aldrich), a blocker of excitatory N-methyl-D-aspartic acid (NMDA) receptors, 1 mM

pilocarpine (Sigma –Aldrich), a muscarinic cholinergic receptor agonist, 5 µM amyloid beta ($A\beta_{1-42}$) monomer (Anaspec, Fremont, CA), a biomolecule associated with Alzheimer's disease, and 5 µM bicuculline, a blocker of $GABA_A$ receptors. The comparative effects of all the compounds were examined under acute conditions.

3. Results and discussion

3.1 Microelectrode impedance stability

As shown in Fig. 2A, the measured impedance for representative electrodes paralleled previous observations for metal electrodes in saline solution [25]. To assess stability of the novel MEA microelectrodes and surrounding insulation, we compared the initial impedance and final impedance after 30 days within a cell incubator at 37 °C. The initial impedance magnitude at 1 kHz was 796.3 ± 49.6 kΩ (mean ± SEM, n=total of 16 microelectrodes from two MEAs, 8 microelectrodes per MEA) while the phase was -74.9 ± 1.1º. After 60 days, neither the impedance magnitude, 686.7 ± 98.9 kΩ, nor the phase, -73.6 ± 2.8º, were significantly altered, suggesting that novel MEA maintains electrochemical stability for at least 30 days in vitro.

To further assess MEA robustness and tolerance for handling, we examined the stability of impedance characteristics after electropolymerization of microelectrode sites with the conducting polymer poly(3,4-ethylenedioxythiophene) (PEDOT) as described for in vivo microelectrodes [26]. PEDOT deposited with the counterion tetrafluoroborate has been shown to produce a robust, durable, and stable electrochemical interface for microelectrodes [26,27]. As shown in Fig. 2B, coating with PEDOT resulted in a significant alteration in the impedance profile where the initial impedance magnitude and phase at 1 KHz were 6.1 ± 0.4 kΩ and -13.6 ± 0.4º, respectively. After 30 days, the impedance magnitude, 15.3 ± 1.4 kΩ, and the phase, -29.5 ± 1.3º were significantly different from uncoated gold electrodes.

3.2 Neuronal culture

Following surface preparation, the novel MEA supported cell body adhesion and neuronal growth (Fig 3A). With the maturation of the network, extracellular action potentials could be readily observed across multiple microelectrode recording sites (Fig. 3B); these biphasic waveforms were entirely consistent with extracellular recordings reported previously using conventional MEA platforms [28, 29]. Parallel experiments using MCS MEAs showed virtually identical extracellular potentials (Fig. 3C).

3.3 Comparative native neuronal activity across MEA platforms

To examine the suitability of the UT Dallas MEA, we compared the resulting neuronal activity with that derived from the commercially available MCS MEA. As shown in Fig. 4, cortical neuronal networks on each of the MEA platforms after approximately 3 weeks in culture displayed coordinated bursting activity. This observation for both MEA types is entirely consistent with prior work [4,14,30,31]. Multiple experiments were performed to quantitatively and systematically compare the MEA platforms. Electrophysiological parameters such as network mean spiking rate, mean bursting rate, and mean inter-spike interval showed that the activity across the UT Dallas MEAs was statistically indistinguishable from that produced on the MCS MEAs (Table 1). In addition, the activity yield, i.e., the percentage of microelectrode sites that demonstrated resolvable single unit activity, was not different between the MEA types (~70%).

3.3 Comparative neuronal activity with pharmacological compounds across MEA platforms

To further probe the consistency of the MEA platforms, we also quantitatively characterized the effects of pharmacological agents on cultured cortical networks. Excitatory reagents, pilocarpine and bicuculline, an inhibitory reagent, memantine, and a neutral biomolecule, $A\beta_{1-42}$ monomer [32], were applied to networks cultured on both substrate type. Exposure to pilocarpine and bicuculline resulted in an elevation in mean bursting and spiking rate (Fig. 5 & 6) whereas memantine produced a reduction in mean spiking rate and the $A\beta_{1-42}$ monomer failed to markedly alter spike activity. Fig. 6 summarizes the normalized changes in mean spiking rate in response to these compounds for the networks cultured on both MEAs. Linear regression fitting yielded a slope ± standard error of 0.91 ± 0.11, which is statistically indistinguishable from unity. Therefore, the degrees of activity modulation produced by all the reagents were virtually identical for both of the MEA types.

3.4 Advantages of the UT Dallas MEA

The use of MEAs for in vitro applications has been well-established since the initial demonstration over 30 years ago [30,33,34]. With more widespread use through commercial availability, a limitation has emerged with respect to the expense largely attributable to the materials, processes, and cleanroom facilities necessary for reproducible MEA fabrication. Unlike virtually all cell culture dishes and chambers, many of the available MEAs are expected to be re-used where specialized and experienced handling is required. In contrast, by leveraging flexible display fabrication approaches, the process for fabricating UT Dallas MEAs relies on materials common to biomedical applications, such as polycarbonate and parylene C, and can be readily scaled in a cost efficient manner. Based on flexible display fabrication costs, we estimate

production costs at nominal volumes to be approximately $3 per cm^2 such that the novel MEA could be fabricated for as little as $25.

4. Conclusion

We have demonstrated a new design for an inexpensive plastic MEA which is readily manufactured and disposable. Comparative analyses of electrophysiological parameters from cortical neuronal network activity from this novel MEA showed virtually identical results with a corresponding commercially available MEA of similar dimensions. We also showed, for the first time, that the primary neuronal cultures on the new disposable MEAs, when compared to networks on these commercially available MEAs, respond similarly to representative excitatory, inhibitory and neutral compounds. Although there have been prior reports on developing novel microelectrode technology, demonstrating the pharmacological consistency is often absent [15, 35]. Such a finding is not trivial considering that the substrate material could greatly impact the growth and development of primary neuronal cultures [36, 37]. The functional comparison shown in the present study demonstrates that these new disposable MEAs are entirely suitable for in vitro assay applications.

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Figure captions and table title:

Fig.1. A cross-sectional drawing of the UT Dallas MEA showing the different layers, thicknesses, and the material used in each layer (A). An optical image of the MEA with magnification showing the microelectrodes (B).

Fig. 2. Electrochemical impedance stability of the novel MEA. The impedance profile from uncoated gold microelectrodes at day 0 and day 30 with storage at 37° C in PBS (A). The impedance profile from the gold microelectrodes individually coated with PEDOT at day 0 and day 30 with storage at 37° C in PBS (B).

Fig. 3. Cultured neuronal network on a UT Dallas MEA substrate (A). Representative activity from a single microelectrode recording site from a UT Dallas MEA showing two distinct units (B). Similar activity from a sister neuronal network culture on a MCS MEA (C).

Fig. 4. Raster plots of extracellular activity from cortical neuronal networks cultured for at least 21 days in vitro on a representative UT Dallas MEA (A) and a typical MCS MEA (B).

Fig. 5. Raster plots of extracellular activity from cortical neuronal networks exposed to 1 mM pilocarpine on UT Dallas (A) and MCS (B) MEAs. Neuronal networks on both substrates responded similarly to the reagent.

Fig. 6. Normalized spiking rates for neuronal networks on UT Dallas vs. MCS MEAs in response to 1mM pilocarpine (o), 5 µM bicuculline (□), 5 µM $A\beta_{1-42}$ monomer (◊), and 20 µM memantine (x). The dashed line is the linear regression to the plotted data. The responses to the tested compounds were identical in both MEA types.

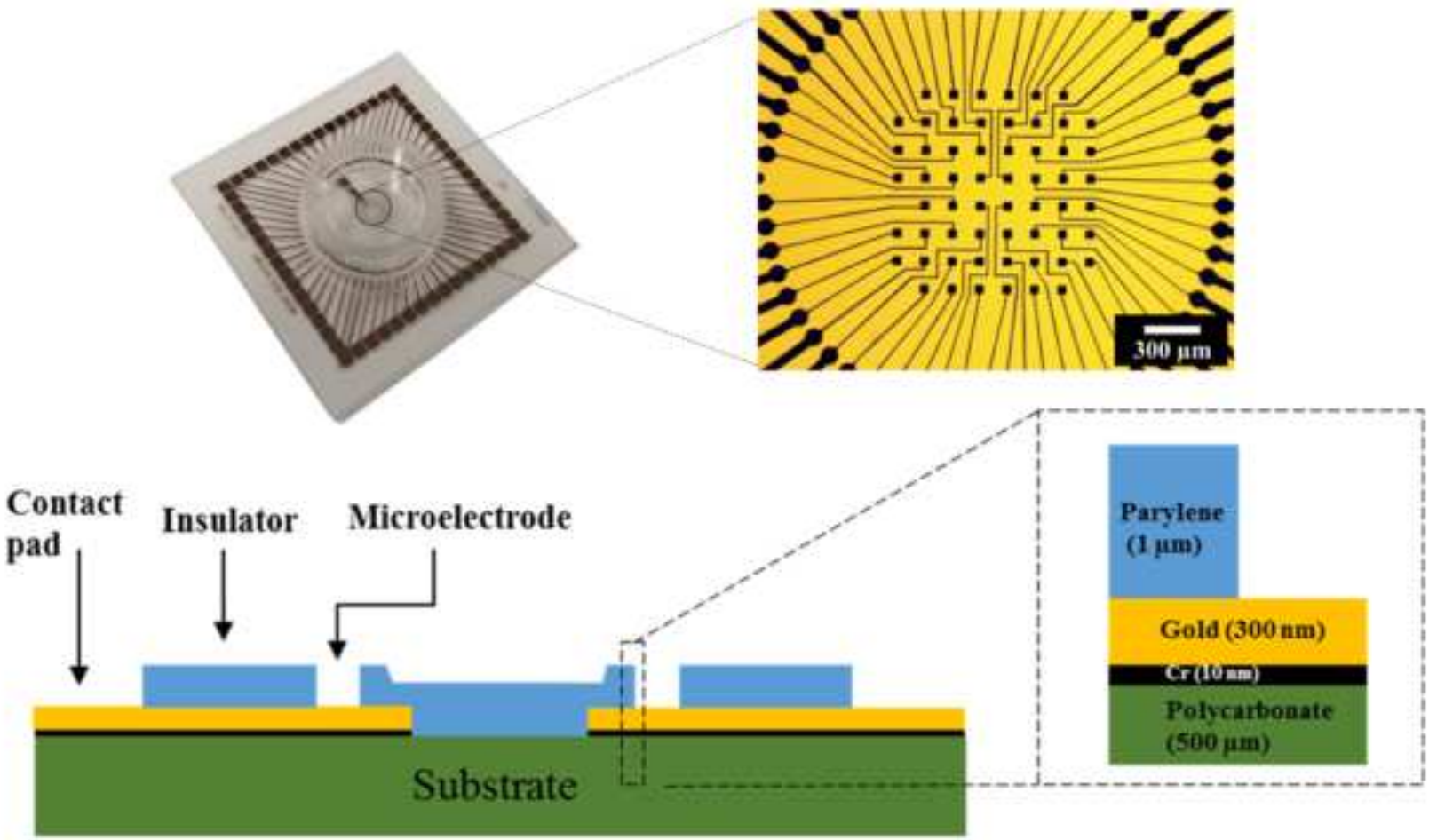
Table 1. Comparison of neuronal network parameters from sister cortical cultures seeded on novel MEAs and MCS MEAs. None of the parameters were statistically different between the MEA platforms.

Table(s)

Table 1.

	Network Number	**Spike rate (Hz)**	**Burst rate (min^{-1})**	**Inter-spike interval (s)**	**Yield (%)**
UT Dallas MEAs	11	2.8 ± 0.3	4.8 ± 1.0	1.8 ± 0.1	67 ± 6
MCS MEAs	9	2.5 ± 0.5	4.3 ± 0.8	1.7 ± 0.2	74 ± 11

Figure1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Contact pad
Insulator
Microelectrode
Substrate
Parylene (1 µm)
Gold (300 nm)
Cr (10 nm)
Polycarbonate (500 µm)

Figure2

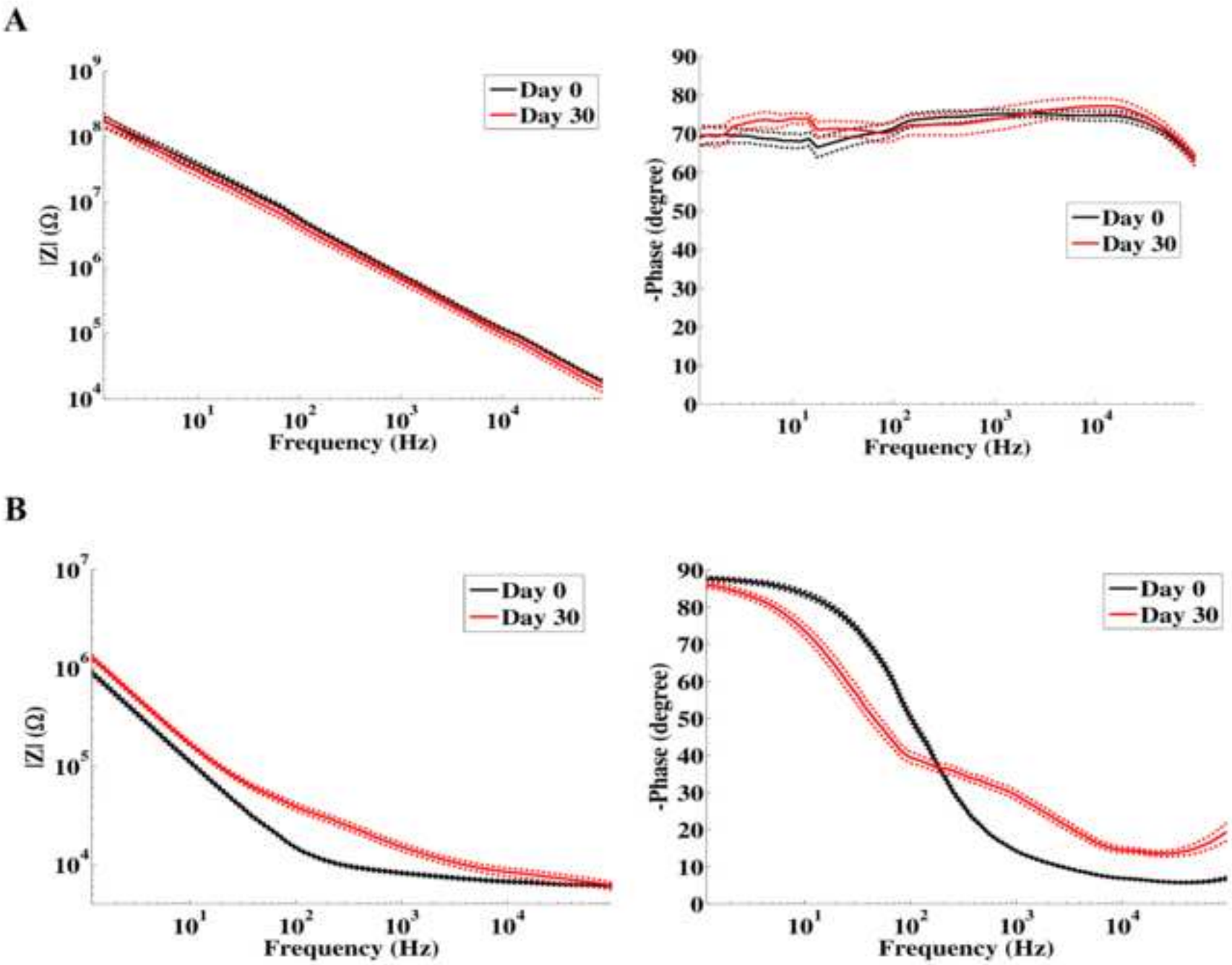
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Figure3

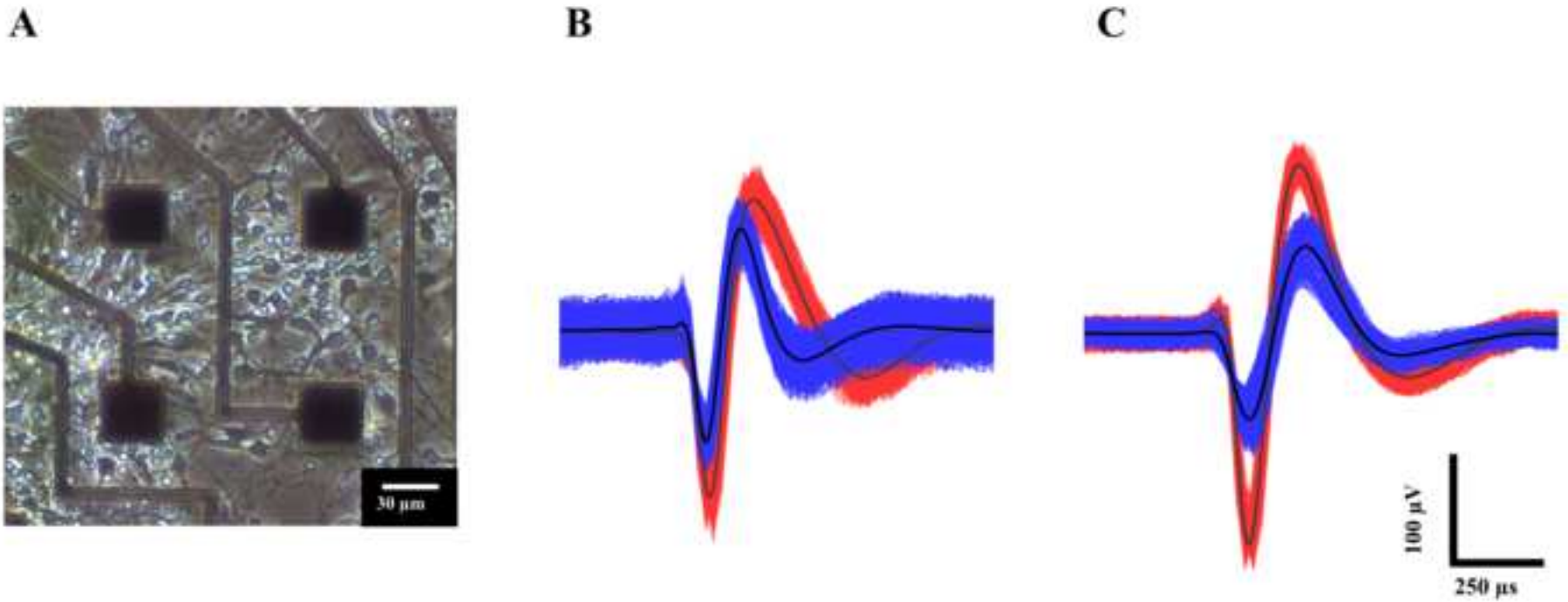


Figure4

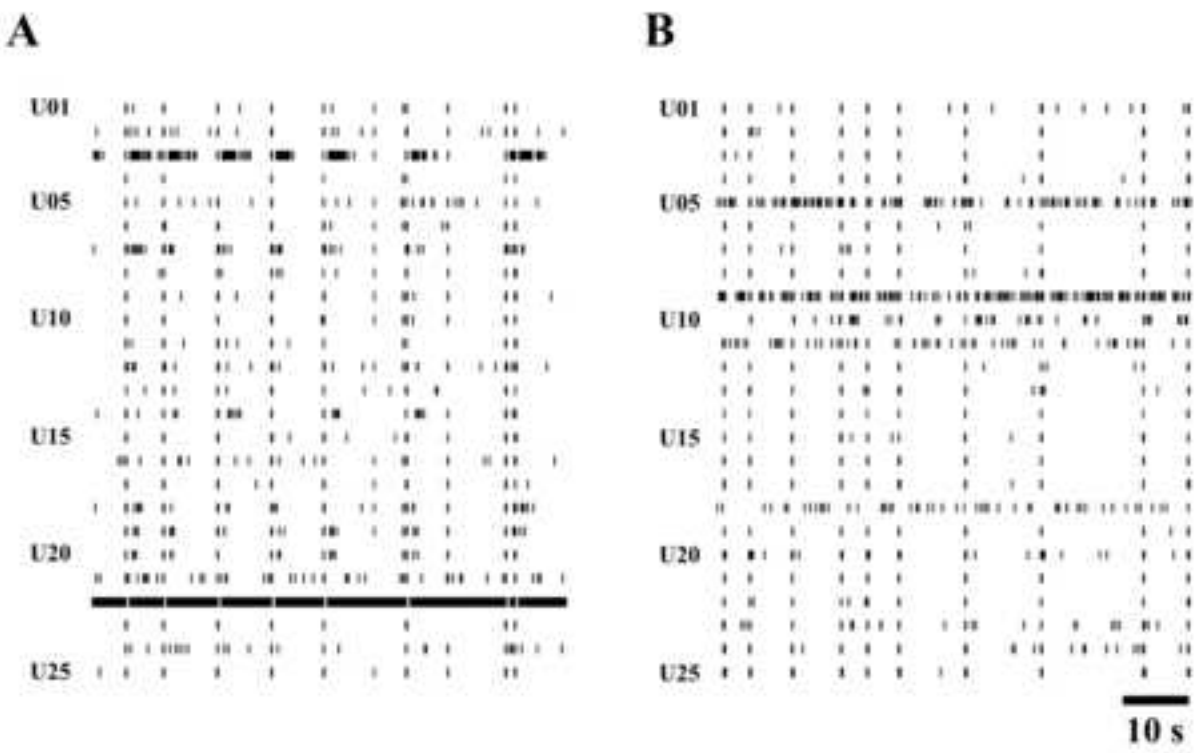


Figure5

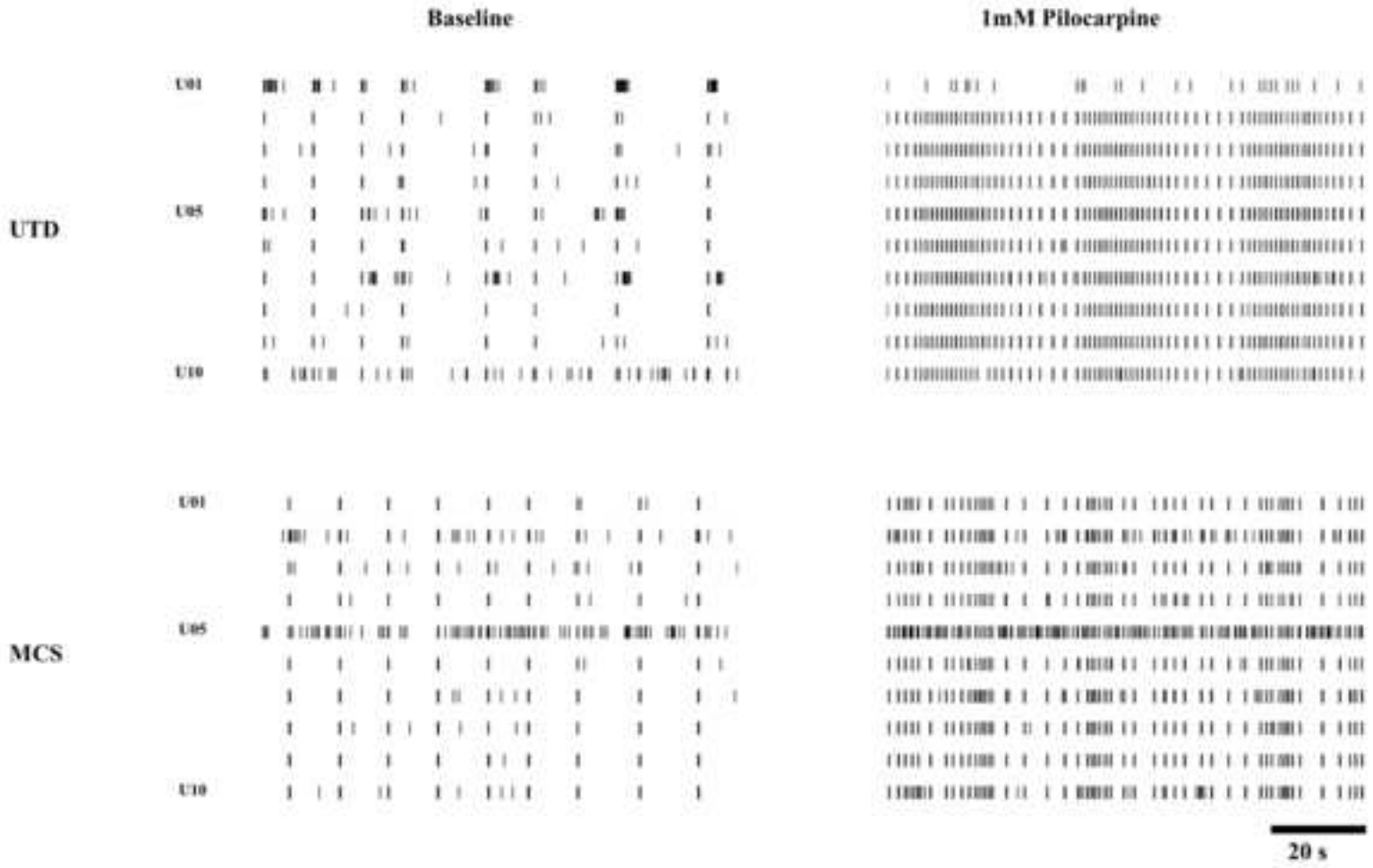
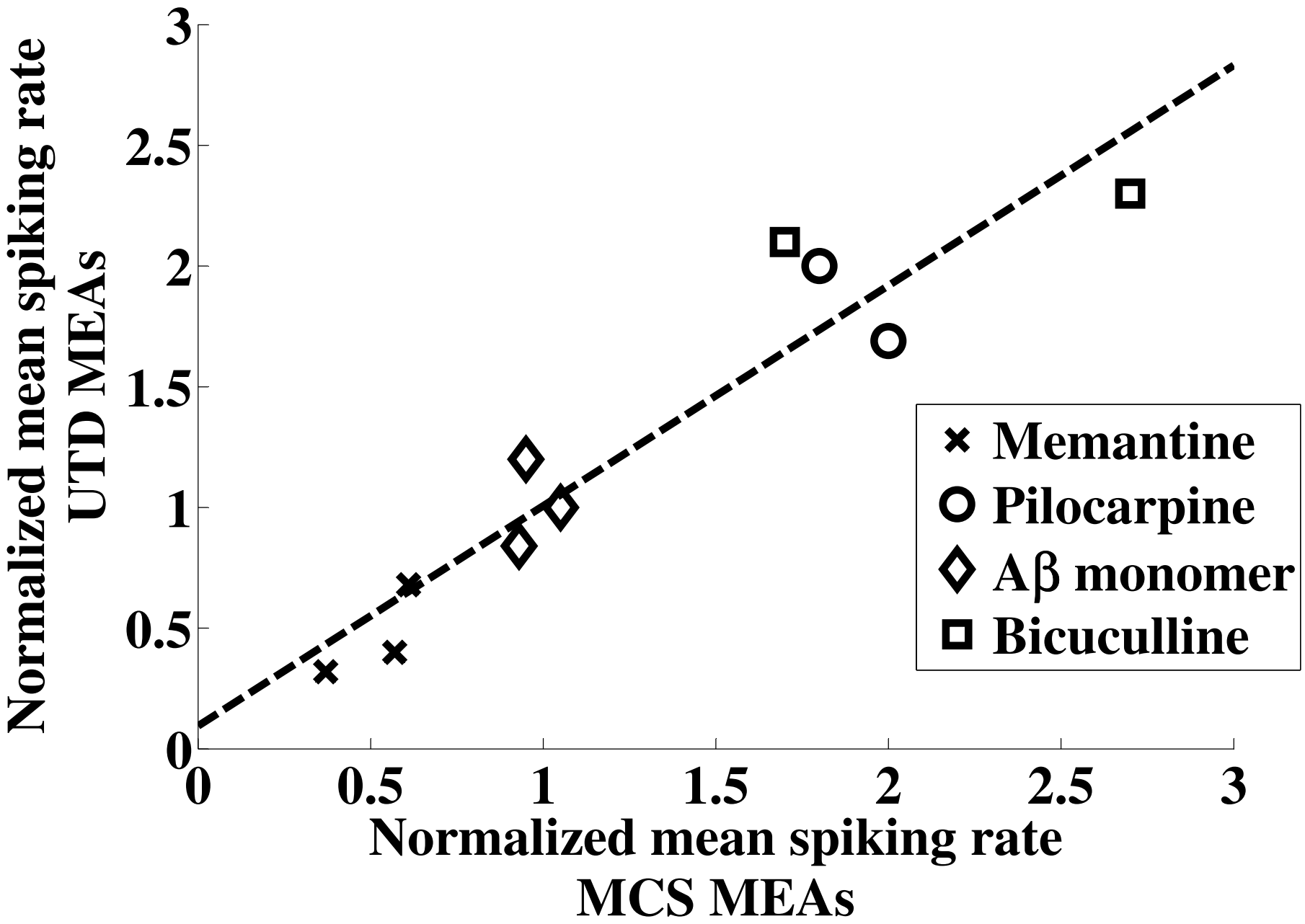


Figure 6


Normalized mean spiking rate
UTD MEAs
3
2.5
2
1.5
1
0.5
0
0
0.5
1
1.5
2
2.5
3
Normalized mean spiking rate
MCS MEAs
Memantine
Pilocarpine
Aβ monomer
Bicuculline