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Use of cortical neuronal networks for *in vitro* material biocompatibility testing

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ABSTRACT

Neural interfaces aim to restore neurological function lost during disease or injury. Novel implantable neural interfaces increasingly capitalize on novel materials to achieve microscale coupling with the nervous system. Like any biomedical device, neural interfaces should consist of materials that exhibit biocompatibility in accordance with the international standard ISO10993-5, which describes *in vitro* testing involving fibroblasts where cytotoxicity serves as the main endpoint. In the present study, we examine the utility of living neuronal networks as functional assays for *in vitro* material biocompatibility, particularly for materials that comprise implantable neural interfaces. Embryonic mouse cortical tissue was cultured to form functional networks where spontaneous action potentials, or spikes, can be monitored non-invasively using a substrate-integrated microelectrode array. Taking advantage of such a platform, we exposed established positive and negative control materials to the neuronal networks in a consistent method with ISO 10993-5 guidance. Exposure to the negative controls, gold and polyethylene, did not significantly change the neuronal activity whereas the positive controls, copper and polyvinyl chloride (PVC), resulted in reduction of network spike rate. We also compared the functional assay with an established cytotoxicity measure using L929 fibroblast cells. Our findings indicate that neuronal networks exhibit enhanced sensitivity to positive control materials. In addition, we assessed functional neurotoxicity of tungsten, a common microelectrode material, and two conducting polymer formulations that have been used to modify microelectrode properties for *in vivo* recording and stimulation. These data suggest that cultured neuronal networks are a useful platform for evaluating the functional toxicity of materials intended for implantation in the nervous system.

1. Introduction

The international standard ISO10993 provides regulatory guidance aiming to ensure that new biomedical devices consist of materials that are biocompatible. As described in ISO10993-5, consideration of new medical device materials typically begins with *in vitro* testing where cytotoxicity serves as the main endpoint. In general, cytotoxicity testing is considered to be a standardized and sensitive approach to assess whether or not a novel material, or residual reagent used in the fabrication process, can induce deleterious biological effects. While a negative cytotoxicity test result does not necessarily imply suitability of the material for *in vivo* use, these tests can be useful to identify reactive materials. The standard describes systematic methods of exposing materials or material extractions to cells for multiple cell viability assays.

In clinical settings, implantable devices are being increasingly used to provide therapeutic neuromodulation to individuals suffering from neurological disease and injury. In addition, emergent devices such as implantable microelectrode arrays (MEAs) for brain machine interface applications leverage advances in fabrication and material science to achieve microscale systems (Kozai et al., 2012; Lewitus et al., 2011; Ware et al., 2013). Clearly, materials which are enabling for these novel implantable devices should neither induce cytotoxicity nor negatively influence the function of neuronal tissue.

In the present study, we examine the utility of living neuronal networks as functional assays for *in vitro* material biocompatibility. Neural tissue derived from primary murine dissection can be

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cultured on substrate-integrated MEAs to form functional networks. Such networks are spontaneously active and action potentials or neuronal firings can be monitored via the MEA. Previous work has demonstrated that neuronal networks cultured on MEAs provide a valuable platform for neurotoxicology (Defranchi et al., 2011; Johnstone et al., 2010; Martinoia et al., 2005; O'Shaughnessy et al., 2004; Pancrazio et al., 2003, 2001) and neuropharmacology (Hascup et al., 2010; Kulagina et al., 2004; Novellino et al., 2011; Xiang et al., 2007). Spike firing rate, amplitude, bursting, and synchronization of firing among neurons, or units, can be readily observed and quantified (Gross et al., 1995; Selinger et al., 2004; Wagenaar et al., 2005). Systematic analysis of a large set of chemical compounds across several laboratories demonstrated the reproducibility and reliability of the MEA method (Novellino et al., 2011).

Given the well documented use of frontal cortex derived neuronal networks for neuropharmacological applications (Hascup et al., 2010; Novellino et al., 2011; Piet et al., 2011; Xiang et al., 2007), we examined the utility of frontal cortex networks for functional testing of biomaterials. We describe a method for exposing networks which provides (1) stable neuronal activity under control conditions, and (2) consistency with ISO10993-5 guidance. Using materials considered either conductive or insulating, exposure to established negative controls failed to significantly alter neuronal network activity while positive controls elicited marked reductions in spike firing rate. We validated our method against an established cytotoxicity measure using L929 fibroblast cells and show that neuronal networks exhibit enhanced sensitivity to positive control materials. Lastly, we report the functional neurotoxicity of tungsten, a common microelectrode material, and two conducting polymer formulations that have been used to modify microelectrode properties for *in vivo* recording and stimulation.

2. Material and methods

2.1. Materials and sample preparation

The testing materials were divided into two categories: conductor and insulator. For each category a positive and negative control material was selected. For conductors, gold (Au) and copper (Cu) and for insulators, polyethylene (PE) and polyvinyl chloride (PVC) were chosen as negative and positive controls, respectively. The choice of control materials was in line with the ISO 10993-12 standard as well as prior work on the cytotoxicity of implantable devices (Hooper and Cameron, 2007). According to the same standard, a positive material is one that, when tested by a specific procedure, will cause a reactive response while a negative control will induce a non-reactive or minimal response under the same test procedure. In addition to positive and negative controls, the functional assay was used to test three other materials including tungsten (W), poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) (PEDOT-PSS) and poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate)-carbon nanotubes (PEDOT-PSS-CNTs).

For Au, W, and Cu, thin films were deposited onto 8 mm × 10 mm rectangular silicon (Si) coupons using the following process. 100 mm diameter wafers of (100) Si (Wafer World, West Palm Beach, FL) were cleaned using a standard RCA clean process (Kern and Puotinen, 1970) and then placed in a 4-pocket E-beam evaporator and loaded with ceramic crucibles containing titanium (Ti) and either Au, W, or Cu, respectively. 500 Å of Ti was evaporated onto the surface of the Si as it allows the target metals to attach to the surface of the Si. 5000 Å of the target metal was then evaporated onto the Ti film surface. The wafers were removed from the evaporator and diced into 8 mm × 10 mm rectangular coupons using a dicing saw equipped with a diamond blade. The samples were then solvent cleaned ultrasonically in acetone and isopropanol followed by a de-ionized water rinse.

A bulk high density polyethylene (PE) sheet was purchased from Ridout Plastics Co. Inc. (San Diego, CA) and cut into the 8 mm × 10 mm rectangular coupons using a dicing saw. Tygon F-4040-A tubing designed for use with fuels and industrial lubricants, which consists of polyvinyl chloride (PVC) with a plasticizer, was used as a positive control material (Hooper and Cameron, 2007).

PEDOT-PSS and PEDOT-PSS-CNTs were electrochemically deposited on gold coated 8 mm × 10 mm Si coupons using both galavnostatic and potentiostatic techniques (Cui and Zhou, 2007; Kim et al., 2010). Prior to electrochemical deposition, Au coated coupons were first rinsed in de-ionized water and then cleaned by voltammetric cycling in 0.1 M H_2SO_4 from 0 to 1.2 V. All the samples were sterilized by ethylene oxide exposure for 12 h.

2.2. Microelectrode array preparation

Planar microelectrode arrays (MEAs) were purchased from ALA Scientific Instruments (Farmingdale, NY). Each MEA had 60 electrodes with an electrode diameter of 10 μm and inter-electrode spacing of 200 μm. MEAs were disinfected by 70% ethanol for 20 min under laminar flow in a biohood and then rinsed with sterile de-ionized water. To obtain better cell adhesion, the center of the 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 DI water three times to remove any excess PDL which can be toxic to neuronal cells (Hollenbeck and Bamburg, 2003). The arrays were then coated at the center with 20 μg/mL of laminin (Sigma-Aldrich, St. Louis, MO) for at least an hour. Upon removal of the laminin, the MEAs were allowed to dry by air flow under laminar flow in biohood.

2.3. Cell culture

2.3.1. Fibroblast culture

Mice fibroblast cells, NCTC clone 929 (strain L) which are commonly known as L929 mouse fibroblasts were obtained from ATCC (Manassa, VA) and then cultured in Dulbecco's Minimum essential medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 2 mM GlutaMAX™-I CTS™ (Life Technologies), and 1% antibiotic/ antimycotic solution (Sigma-Aldrich, St. Louis, MO). The L929 cells were cultured in 75 cm^2 tissue culture flasks (CytoOne T75 filter cap; USA Scientific, Ocala, FL) in a water jacketed incubator at 37 °C, 95% relative humidity, and 5% CO_2. The L929 cultures were maintained within the flask, receiving a 50% media change every two days until they were required for a cytotoxicity assay or the culture confluence level exceeded 90%.

2.3.2. Primary neuronal culture

The primary neuronal culture method was similar to that described in Knaack et al.(2013). 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,). Upon isolation of the frontal cortex, the tissue was minced by scalpels and then dissociated through incubation with DNAase and papain (Worthington Biochemial Corp., Lakewood, NJ) for 15 min followed by mechanical trituration using disposable graduated pipettes (Fisher Scientific, Pittsburg, PA). After centrifuging at 2500 rpm for 5 min, 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