Demonstration of a System to Study Multisite Response to Focal Glutamate Stimulation in a Rat Retina

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THESIS
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This study was undertaken to investigate how a mammalian retina responds to focalized stimulation with a neurotransmitter as part of a larger research project seeking to establish the feasibility of a chemical-based approach to a retinal prosthesis. The specific goals of this thesis are: i) to focally stimulate a rat retina *ex vivo* with glutamate and record its response at multiple sites using a Multielectrode Array (MEA) system, and ii) to analyze the recorded signals from multiple neurons for a physiological response. For this study, retinas from dark-adapted Long Evans rats were used, and the tissue samples were prepared by carefully extracting the retina from the excised eye of the euthanized animal. The retinal tissue was placed on the MEA chip with the ganglion cell layer (GCL) contacting the electrodes while being continuously superfused with a physiological medium. The basal level of the spontaneous activity of the neurons was recorded using the MC Rack software associated with the MEA system. The GCL was stimulated by light from a custom built LED light source placed underneath the MEA system and the response of the tissue was recorded from the 60 electrodes of the MEA chip. Following confirmation of a physiological response of the tissue to the light stimulation, 0.2 µl of glutamate was ejected on the photoreceptor side of the retina through a capillary connected to a custom syringe pump. The response of the retina to this controlled ejection was recorded from 60 different sites on the tissue. The above set of stimulation experiments were repeated for three concentrations of glutamate, viz. 2 mM, 1 mM and 0.5 mM on genetically identical animals. The responses obtained from the spontaneous activity, the light-stimulated activity and the chemically-stimulated activity were analyzed using the tools available within the MC Rack software. The analyses indicate that the retina responds electrophysiologically to glutamate
stimulation and the response activity level increases with an increase in the concentration of the glutamate. The findings of this study preliminarily demonstrate the feasibility of chemically stimulating the retina in a physiological manner, which could potentially lead to a chemical based — as opposed to electrical based — retinal implant for correcting the loss of vision due to photoreceptor degenerative diseases.
CHAPTER 1 - INTRODUCTION

Retinal degenerative diseases such as Age Related Macular Degeneration (AMD) and Retinitis Pigmentosa (RP) cause blindness in people due to degeneration of the photoreceptors and afflict millions of people worldwide. Currently there are no cures or therapies available to restore the lost functionality of the lost photoreceptors. For the last several decades, researchers have been exploring various approaches, such as stem cell transplant [1], gene therapy [2] and retinal prostheses, to restore the vision in patients blinded by these diseases. While stem cell transplant and gene therapy are attractive solutions, currently the prosthetic approach remains to be the most viable solution. The widely researched retinal prosthetic approach is based on the principle of stimulating the retina by means of electrical pulses. An alternative approach based on the principle of stimulating the retina with chemicals, potentially offers some advantages over the electrical stimulation approach. However the concept is relatively new and much research is needed to fully explore the feasibility of a chemical stimulation based approach. This research was undertaken as a part of a larger research project seeking to establish the feasibility of a chemical stimulation based approach to retinal prostheses in the Microsystems and Devices Laboratory at the University of Illinois at Chicago.

1.1 Background & Significance

The retina, the sensory portion of the eye that processes visual light, is a complex structure comprising of a layered network of a variety of neurons. A schematic of the structure of the retina is shown in Figure 1.
The retina is composed of three nerve cell layers and two layers of synapses. The vision process is a complex combination of the processes of phototransduction and chemical signaling. In a normal eye, light passes through the entire thickness of the retina to reach the outer segment of the photoreceptors which contain the light sensitive pigment molecules. While the signals generated by the bipolar cells and photoreceptors comprise of graded changes in voltage across their membranes, the ganglion cells generate electrical impulses or action potentials. The photoreceptors absorb the incoming light and relay the visual information in the form of coded chemical signals through the network of cells all the way to the ganglion cells which fire action potentials that then travel to the visual cortex of the brain to process vision [4-6]. About 10-15 types of retinal ganglion cells (RGC’s) have been identified so far [7]. When afflicted by retinal
degenerative diseases, the photoreceptors degenerate and without the cells to absorb the visual signals, the information processing ceases thus causing loss of vision.

In the retinal prosthesis using electrical stimulation, researchers hope to restore the lost ability of the retina to process the visual signals by electrically stimulating the diseased retina either on the bipolar cell side or ganglion cell side. Retinal prostheses based on the electrical stimulation principle can be broadly divided into two types based on their anatomical placement within the diseased eye: epiretinal prostheses which are placed on the ganglion cell side of the retina and subretinal prostheses which are placed on the photoreceptor side of the retina. Significant progress has been made in the field electrical stimulation based prostheses over the last several decades. Clinical trials and animal testing results so far have shown immense promise at attaining successful detection of motion patterns along with an ability to identify shapes and objects within a set [8-10]. However, one main drawback of the electrical stimulation is that it stimulates all retinal neurons indiscriminately; also, this approach has issues in regards of tissue damage due to heat generation and high charge density accumulation [11, 12].

As opposed to stimulating the retina electrically, stimulating the neurons by means of neurotransmitters, offers the prospect of a more biomimetic means of restoring the lost functionality of the photoreceptors. The natural method of communication between two neurons occurs by the transfer of biochemical messages through the aid of neurotransmitters via a synaptic junction [4, 5]. Visual communication involves several types of neurotransmitters, of which glutamate is the primary excitatory neurotransmitter and GABA is the primary inhibitory neurotransmitter in the retina [6]. If one or more of the neurotransmitters can be packaged in a microfluidic dispenser and released in a modulated manner, the surviving neurons in the retina could be stimulated in a way that mimics the natural communication occurring at synapses.
1.2 Motivation & Research Objectives

To study the feasibility of stimulating the retina with neurotransmitters, a number of fundamental questions related to the retinal physiology need to be explored. Among the research issues to be studied in this regard include how the retina responds physiologically to different types, concentrations and volumes of neurotransmitters as well as the area and side on which the chemical is presented. Motivated by these interdisciplinary questions relating Bioengineering and Neuroscience which could potentially enable a novel approach to a retinal prosthesis, this thesis was undertaken to specifically explore the physiological response of the retina to focal stimulation by one type of neurotransmitter, namely glutamate, in the subretinal configuration. The specific goals of this research are:

1. To focally stimulate a rat retina ex vivo with glutamate and record its response at multiple sites using a Multielectrode Array (MEA) system.
2. To analyze the recorded signals from multiple neurons for a physiological response and assess the influence of parameters such as concentration and amounts of neurotransmitter over the time period of responses.

1.3 Literature Review of Chemical-Based Retinal Stimulation

The idea of a building retinal implant based on the concept of neurotransmitter stimulation evolved around the early 2000’s [13]. Researchers have tried to exploit the chemical stimulation idea, as discussed in the previous section, to achieve a retinal prosthesis that mimics the natural physiological vision pathways. One of the early experiments performed in the field of chemical stimulation used an inkjet printer for dispensing tiny droplets of the neurotransmitter
bradykinin, over cultured PC-12 cells for stimulating them chemically [14]. A group of researchers at Stanford University developed and microfabricated a device capable of localized chemical release through a single circular aperture of 5-10 μm in diameter where the neurotransmitter was driven by pressure through a micro channel for ejection on the surface [15]. Further advancements by the same group led to the development of a device called the Artificial Synapse Chip, a design that conceptually synthesizes the retinal function by manipulating the growth of the retinal neurites via the technique of microcontact printing and also allows for repeatable delivery of minute quantities of the neurotransmitter as intermittent chemical pulses over the surface [16, 17].

A research group at UIC has explored the concept of a retinal prosthesis wherein the glutamate packaged in a microfluidic reservoir could be ejected by means of a light powered microactuator [18]. They also developed designs for the fluid dispenser and actuator to release the chemicals packaged in the microfabricated device [19-22]. Other approaches explored in this direction involved optical activation of neurotransmitter molecules, where in ultraviolet radiation through an optical fiber was used to trigger caged glutamate molecules into their activated form over a confined neuronal area thus enabling stimulation at a single cell level [23, 24]. More recently, Finlayson and Iezzi [25] reported on the response of the retinal ganglion cells of a rat retina to localized stimulation by glutamate using micropipettes and patch clamp technique. They concluded that glutamate is an ideal candidate for a chemical-based retinal prosthesis.
1.4 Approach

For this study, retinas from dark-adapted Long Evans rats were used as the experimental model. Electrophysiological signals from the tissue were captured by means of a Multielectrode Array Recording System capable of multisite recording. Experiments were first conducted to assess the spontaneous response and light activity of the retina to ascertain the viability of the tissue sample. Following the confirmation of a physiological response, 0.2 µl of the glutamate solution was ejected on the retina through a capillary connected to a custom syringe pump. The above set of stimulation experiments were repeated for three concentrations of glutamate, viz. 2 mM, 1 mM and 0.5 mM over genetically identical animals. The responses obtained from the spontaneous activity, the light-stimulated activity and the chemically-stimulated activity were analyzed using the tools available within the MC_Rack software which forms a part of the MEA recording system.

1.5 Thesis Organization

The rest of the thesis is organized as follows: Chapter 2 deals with the experimentation work and describes in detail the model system setup and preparations required for the experiments. A detailed description of the MEA system, for capturing electrophysiological signals from the retina, is given along with the spontaneous activity experiments, light activity experiments and the chemical stimulation experiments. Chapter 3 describes the results obtained from the chemical stimulation experiments followed by an analysis of the obtained data by comparing the activity levels in light and chemical stimulation responses. The conclusions and suggestions for future research are put forward in Chapter 4.
CHAPTER 2 - EXPERIMENTATION WORK

This chapter describes the model of the system considered and the experimental work performed for achieving chemical stimulation of the retina. The model system assembled for this work comprises of four key components: 1. Retinal Sample System, 2. Chemical Delivery System, 3. MEA system and 4. Data Acquisition (DAQ) System. Figure 2 below schematically illustrates the model system.

![Figure 2. Model system comprising of different components assembled for performing the chemical stimulation experiments.](image)

The Retinal Sample System comprises of an explanted retinal tissue preserved in an artificial physiological media. The purpose of the Chemical Delivery System is to focally eject small quantities of the neurotransmitter over the retinal surface. The MEA System exists to capture the action potential activity of the neurons, which are further processed and converted into a digital format by the DAQ System. Each of these subsystems is described in detail in the following sections. For the light stimulation experiments, the same model system was used, with the exception of a custom built light source.
2.1 Chemical Preparations

This section covers a detailed description of the physiological medium preparations used to sustain the retinal tissue alive through experimental procedures and the glutamate solution preparations required for performing the chemical stimulation experiments.

2.1.1 Physiological Medium Preparation

The explanted retinal tissue from the rat eye requires preservation in a solution that most closely mimics the natural environment in which the retina remains immersed. For this purpose, AMES (A-1420 Sigma Aldrich), a medium that is used to sustain retinal tissue cultures for short term \textit{in vitro} experiments was used [26]. The AMES media is a mixture of amino acids, inorganic salts and vitamins. It needs to be buffered with sodium bicarbonate to maintain the metabolism and functionality of the extracted retinal tissue for extended periods of duration [27]. HEPES, a buffering agent that helps in maintaining the perfusion at physiological pH against changes in carbon dioxide concentration [28], was added to the AMES media. The composition of one liter (1L) of the perfusion solution comprises of AMES media (8.9 gms), sodium bicarbonate (25.3 ml) and HEPES (2.36 gms). All the above constituents were dissolved in 1L of sterile distilled water which was initially bubbled with 95\% carbon dioxide to prevent the formation of any precipitates. Immediately after the solution was made, it was sterilized by positive pressure filtration to reduce carbon dioxide loss [29]. The solution was maintained at physiological pH of 7.5 at a temperature of 32°C while being bubbled with 95\% oxygen throughout the experiment. Every batch of the perfusion was freshly prepared 2 hours prior to the experiment. The preparation methodology and ingredients used in the media are included in Appendix A1.
2.1.2 Glutamate Preparation

Glutamate used in the experiments was procured in a powdered state as L-Glutamic acid from Sigma Aldrich (G-1251). The molecular mass of glutamate is 147.13 g/mol. Considering the molecular mass of glutamate, calculations were done to prepare a 50 mM glutamate stock solution. This was achieved by dissolving 7.3565 mg of glutamate in 1 ml of sterile distilled water. Three different concentrations namely 0.5 millimolar (0.5 mM), 1 millimolar (1 mM) and 2 millimolar (2 mM) of glutamate were tested to assess the feasibility of chemical stimulation. To obtain a working solution of 2 mM glutamate, 40 µl of the stock solution was dissolved in 960 µl of freshly oxygenated AMES media whereas for a 1 mM working solution, 20 µl of the stock solution was dissolved in 980 µl of freshly oxygenated AMES media. For the 0.5 mM working solution, 10 µl of the stock solution was dissolved in 990 µl of the freshly oxygenated AMES media. The stock solution was stored in a refrigerator for up to 2 weeks. The working solution was freshly prepared 30 minutes prior to the start of an experiment and discarded at the end of each experiment.

2.2 Retinal Sample System

This section describes the dissection and tissue sample preparation of the rat retina in a detailed manner. All procedures were performed in accordance with the protocol as approved by the Animal Care Committee at University of Illinois at Chicago (a copy of this protocol can be found in Appendix A2). All experiments were performed on young male Long Evans rats weighing between 100-170 grams. The animal was dark adapted 24 hours prior to the experiment to acclimate it to the dark and prevent the influence of light in the physiological responses obtained from its retina during the experiments. The experiments were carried out in a dark room
with dim red light, which reduced the possibility of photopigment bleaching from the retina [30]. The dark adapted rat was euthanized using a carbon dioxide supply and once the animal stopped breathing, cervical dislocation was performed to ensure it was dead. This was done by pulling the neck of the animal from one end and pulling the tail from the other till a distinct crack noise was heard. This was done to dislocate the cervical vertebra which serves as a marker of safe euthanization. The eye was enucleated from the rest of the animal body and care was taken to ensure that the optic nerve did not undergo any physical damage. The excised eyes were immediately transferred to the oxygenated AMES media.

Excess tissue around the eye was removed and a small perforation was made along the ora serrata to separate the anterior portion comprising the lens and cornea. The remaining eye cup which comprised of the photo sensitive retinal portion was transferred to a Petri dish with oxygenated AMES media. The eye cup tends to curl up after the removal of the front portion of the eye. In order to flatten it out, about 4 small radial cuts were made on the eye cup causing it to evenly spread out, thus making the retina separation from the retinal pigment epithelium (RPE) much easier. The vitreous humor is present as a transparent layer lining the retina. It acts as an insulator in electrical recordings and therefore needs to be detached completely. The removal of the vitreous was achieved by tugging at the surface of the retina very gently and picking on it in with tweezers. The vitreous free retina was then gently separated from the RPE that lines the back of the eye. After it has been separated from the peripheral parts of the RPE, the retina needs to be detached from the point of entry of the optic nerve. The retina is firmly attached at this spot and needs a mild cut to be disconnected from the remainder of the eye cup.

Once the whole retina was procured, it was placed on a filter paper (Millipore) with a pore sizing of 8µm. The retinal sample was unfolded and flattened out evenly in all directions.
The retinal tissue mounted on the filter paper was then placed on the electrodes of the MEA chip (described later) with the GCL facing the electrodes. A small mesh was placed on the filter paper to ensure that the tissue was weighed down and making firm contact with the electrodes underneath. For all experimentation work, superfusion with 2 ml/min of the AMES medium was maintained in the MEA chamber to nourish the tissue for extended durations. The perfusion media was bubbled with 95% oxygen all through the experimental procedure to keep the retina alive and functional for a long period of time. The tissue was allowed to stand on the MEA for about 2 minutes which allowed it to stabilize before starting the recordings.

### 2.3 MEA and DAQ System

The MEA system comprises of several sophisticated electronic subsystems that provide a complete framework for recording, amplification and acquisition of action potentials from biological samples. The MEA system offers the possibility of recording signals from multiple sites on the tissue simultaneously, unlike the other established technique of patch clamp, which is meant for single cell recordings. As such to accomplish the first goal of this thesis, a commercially available MEA system (MEA60), which is capable of recording signals from 60 electrodes, from Multi Channel Systems was utilized [31]. The key components of the MEA system are: a) DAQ hardware (MC_Card) including amplifier, b) DAQ software (MC_Rack) for digitizing the biological signals (Figure 3).
Figure 3. Experimental setup of the MEA System including the MEA chip which houses the retinal sample, AMES media perfusion and suction arrangement.

The retinal tissue sample forms the biological signal source that comes in contact with a biosensor (MEA chip) which converts the signal to a form that can be interpreted by the amplifier (MEA 1060). The low noise amplifier filters and amplifies the biological signal through customizable gain and bandwidth settings and the final output is stored in the hardware (MC_Card) and software (MC_Rack) recording system for additional analysis, interpretation and export of data. All the above components are described in detail below. The temperature controller (TC01) aids in controlling the temperature of the MEA setup up to 105° C using a platinum sensor [31].
2.3.1. Microelectrode Array Chip

A typical MEA chip comprises of a flat glass with a circular chamber on top of the glass substrate. Within the chamber region, multiple electrodes exist that are connected to contact pads along the periphery of the MEA chip (Figure 4). Different types of MEA chips are available for recording from different types of biological samples. They come in not only different configurations of the electrode layout and the number of electrodes, but also in solid and perforated substrate types. For this thesis work, two specific types of MEA's were utilized, namely regular glass substrate MEA with electrodes in a hexagonal pattern (HexaMEA-ITO) [32] and the perforated MEA with electrodes in a grid pattern with perforations of varying diameters (pMEA200/30iR-Ti) [33]. Each of these MEA chips is described below.

![Figure 4. A Microelectrode array chip (Picture taken from ALA Science [34]).](image)

For a Hexa-MEA, the titanium nitride (TiN) electrodes are arranged in a hexagonal pattern over a base material of glass with titanium nitride (TiN) or indium tin oxide (ITO) contact pads (Figure 5). The electrode diameter varies from 10, 20, 30 μm at an interelectrode spacing of 30, 60, 90 μm respectively. The electrode impedance varies from 30 kΩ - 50 kΩ for 30 μm electrodes and 250 - 400 kΩ for 10 and 20 μm electrodes [32]. A thin silver wire with an Ag/AgCl pellet is used as a reference electrode.
Figure 5. Layout of electrodes in a hexa-MEA chip with electrode diameters varying from 10-30 µm.

For a pMEA, the titanium nitride (TiN) electrodes are arranged in a square layout over a base material of polyimide foil with perforation on a glass or ceramic carrier (Figure 6). The contact pads are made of titanium nitride (TiN) with an electrode diameter of 30 µm at an interelectrode spacing 200 µm. The electrode impedance varies from 30 kΩ - 50 kΩ with an internal reference electrode (iR). The total area of the perforations or holes is 0.8 mm with the diameter of the holes varying from 90, 75, 50, 30 to 20 µm [33].

Figure 6. Layout of electrodes in a perforated MEA chip with an electrode diameter of 30 µm and perforations of 90, 75, 50, 30 and 20 µm.
2.3.2. MEA Amplifier and Data Acquisition System

The signals from each of the electrodes of the MEA chip are amplified independently through a specially built amplifier circuitry, MEA1060. The amplifier has a very high signal to noise ratio enabling enhanced signal filtration of the raw data. The DAQ system comprises of the MC_Card and the MC_Rack software program. The MEA amplifier is connected to the DAQ computer which comes with preinstalled hardware, isolated internal power supply, MC_Card and MC_Rack software. The analog output signals of the MEA amplifier were acquired and digitized by the MC_Card which is essentially an analog to digital converter board converting real time analog signals into digital data streams. Various functions such as filtering, averaging and spike sorting can be performed on the data through the software and numerous forms of data display are available to further manipulate and extract information from the signals. The recorded data can then be plotted on graphs and analyzed for further assessment using the MC_Rack program.

2.3.3 Software Settings

A typical experiment involved setting up various components of the MC_Rack software in a ‘Virtual Rack’ configuration as described below to selectively extract the desired physiological signals from the raw data.

- **MC_Card** - It served as the basic breadboard to build an electrical circuit for capturing the retinal responses.
- **High Pass Filter** - A high pass filter at a cut off frequency of 200 Hz was used to extract the signals of interest by filtering out any low frequency field potentials.
• Spike Sorter - A spike sorter was used to provide a display of the spike count as recorded from the retina. The threshold for all experiments was set anywhere from -30 µV to -45 µV depending on the RMS value of the background noise signals.

• Input Voltage Range of Data Acquisition Board - This range was set at ±819 mV to provide an optimum data resolution on the MC_Card.

• Sampling Frequency - Data was recorded at a sampling frequency of 50 kHz/channel to avoid missing any neuronal signals.

• Temperature Control - The temperature of the MEA system was maintained at 32 °C to house the tissue in a similar physiological environment as it is used to in the eye.

2.4 Light Source

In order to perform light stimulation experiments for preliminary testing of the viability of the retinal tissue sample, a Luxeon LED (Typ Star/0, 1W, cyan) was used as the light source. The Luxeon LED setup interfaced with the MEA system has been established as an optical illumination system capable of full field retinal stimulation using the MEA60 system [35]. The LED has a wavelength of 505 nm which corresponds with the peak of the spectral sensitivity curve of the retina from Long Evans rats which is about 509 nm [36]. The LED light source is setup at a distance of approximately 1.8 cm from the bottom side of the MEA providing a variable retinal illuminance ranging from 1 to 100000 lux on the tissue sample. The light passes through the opening at the bottom of the MEA60 system. The intensity of the light from the LED, and hence the illuminance on the tissue sample, is controlled by a voltage controlled driver circuit that provides a full field light stimulation on the retina. A focusing lens helps to target the
light beam at the center of the MEA chip and hence the center of the tissue. Figure 7 illustrates the experimental setup employed for the light experiments.

![Figure 7. Experimental setup of the Luxeon LED setup integrated with the MEA60 system.](image)

2.5 Chemical Dispenser System

The chemical dispenser system comprises of a custom built syringe pump capable of releasing minute quantities of the chemicals, interfaced with a glass capillary which serves as a delivery port for dispensing the chemicals. This chemical dispenser unit was custom built in our laboratory by assembling a gas tight syringe (1801 RN 10 µL model from Hamilton Company), a nano-actuator (8301 Picomotor Actuator from New Focus Inc.) and fused silica capillaries (Polymicro Technologies). Fittings from LabSmith Inc. were used to achieve a leak proof connection between the syringe needle and the dispensing capillary. The high precision syringe pump was capable of storing 10 µl of the neurotransmitter and the tip of the capillary served as
an ejector port allowing for a single ejection of 0.2 µl of the glutamate solution in each chemical stimulation experiment [37]. Prior to the start of every chemical stimulation experiment, the syringe and the dispensing capillary were purged with the freshly prepared glutamate solution 4-5 times to ensure that the syringe and capillary were gas free with no air bubbles.

![Figure 8. Syringe pump based chemical dispenser unit integrated with the MEA system [37].](image)

### 2.6 Stimulation Experiments

To accomplish the main goal of this thesis i.e. chemical stimulation of the retina, preliminary light stimulation experiments were performed to confirm the functionality of the extracted retinal sample. In all the experiments, the spontaneous response of the tissue sample was checked to recognize the spontaneous natural bursting activity of the retinal ganglion cells. Spontaneous neuronal activity is a characteristic feature of excitable retinal cells and is often used as a representative of good health of the extracted retinal sample.

#### 2.6.1 Light Activity Experiments

A series of elementary light stimulation experiments were carried out to verify the excited electrophysiological responses of the RGC's. After a confirmation of the spontaneous
activity of the tissue sample, the LED placed underneath the MEA system, is turned on to stimulate the retina with light (Figure 7). Each light flash was one second long and the time interval between two successive light flashes was ten seconds. Four LED light flashed were made on the GCL side of the retina to ascertain the extracted tissue sample health and functionality. Figure 9 below provides a typical example of the spontaneous activity whereas Figure 10 represents the light induced response of the retinal sample. While the X-axis represents time duration in msec, the Y-axis represents the amplitude of the spikes in µV. The RMS value of the background noise is about 7.5 µV and hence the threshold for the recordings is set at -30 µV, which is 4 times the baseline RMS noise value.

![Figure 9. Example of spontaneous activity recorded from a retina sample at a single electrode.](image)
2.6.2 Chemical Stimulation Experiments

After a confirmation of the viability of the sample was established through the spontaneous activity and light stimulation experiments, the mesh was removed from the tissue sample and the superperfusion system feeding the tissue was turned off for a brief period of 45 seconds. The neurotransmitter glutamate loaded in the dispensing capillary, was connected to the end of the custom syringe pump. The ejector port of the capillary was placed on the filter paper, approximately centered over it, and a controlled focal ejection of 0.2 µl of the glutamate solution was made on the photoreceptor side of the retina using the chemical delivery system described in Section 2.5. The above experiments were repeated for three different concentrations of glutamate viz. 2 mM, 1 mM and 0.5 mM. A schematic of the capillary placement relative to the tissue sample on the MEA chip is shown in Figure 11 below.
Figure 11. A schematic of the details of the dispensing capillary interfaced with the retinal tissue sample placed in the MEA reservoir for the chemical stimulation experiments.
CHAPTER 3 - RESULTS

This chapter presents the results acquired from the chemical stimulation experiments for different concentrations of glutamate ranging from 0.5 mM to 2 mM. The raw data obtained from these experiments was represented for spontaneous activity, light activity and chemical stimulation activity. The data obtained from the chemical stimulation experiments was quantitatively analyzed on the basis of the total spike count, to determine the nature of the obtained responses. While single electrode data was analyzed for the spontaneous and light response, the data obtained from the best four electrodes, representative of increased spike activity and a sustained response, was analyzed for studying the effect of chemical stimulation.

3.1 Chemical Stimulation Results

A total of six experiments were performed with glutamate at concentrations of 2 mM, 1 mM and 0.5 mM. 3 sets of experiments were performed for 2 mM glutamate, 2 sets of experiments were performed for 1 mM glutamate and 1 experiment was performed for 0.5 mM glutamate. Four active electrodes in each of the concentration cases were monitored. The following figures show the multi unit neuronal responses as observed in single electrodes. The action potentials obtained from the four active electrodes were monitored and further analyzed for the presence of a physiological response. The figures below represent the voltage traces as observed in the actual experiment wherein the X-axis represents the time interval in msec and the Y-axis represents the amplitude of the spikes measured in µV. At the end of each experiment,
light response of the retina sample was tested again to ensure the tissue was alive during the entire course of the experiment.

3.1.1 Results from the 2 mM concentration case

Three sets of results (Set 1, Set 2, Set 3), each set containing the recordings for spontaneous and light induced activity from a single electrode and chemical triggered activity from 4 active electrodes, are presented below.

3.1.1.1 Set 1 Recordings

Figure 12 and Figure 13 show the spontaneous and light activity of the tissue sample respectively as recorded from a single electrode. Figures 15-18 show the chemically induced response at four different electrodes. Figure 14 is an electrode layout map of the MEA chip highlighting the four active electrodes that were monitored for a chemical response. The RMS value of the background noise is about 9 µV and hence the threshold for the recordings is set at -35 µV to -40 µV, which is 4 times the baseline RMS noise value.
Figure 12. A snapshot of the recording obtained from electrode no.66 for the spontaneous activity.

Figure 13. A snapshot of the recording obtained from electrode no.66 for the light stimulated response.
Figure 14. Active electrodes on the pMEA exhibiting a chemically induced response to set no.1-2 mM glutamate (red) with an approximate area of the retina covering the electrodes of the pMEA (green) and an approximate location of the dispensing capillary over the retina (black).

Figure 15. A snapshot of the recording obtained from electrode no.67 for the chemically stimulated response.
Figure 16. A snapshot of the recording obtained from electrode no.77 for the chemically stimulated response.

Figure 17. A snapshot of the recording obtained from electrode no.66 for the chemically stimulated response.
Figure 18. A snapshot of the recording obtained from electrode no.75 for the chemically stimulated response.

3.1.1.2 Set 2 Recordings

Figure 19 and Figure 20 show the spontaneous and light activity of the tissue sample respectively as recorded from a single electrode. Figures 22-25 show the chemically induced response at four different electrodes. Figure 21 is an electrode layout map of the MEA chip highlighting the four active electrodes that were monitored for a chemical response. The RMS value of the background noise is about 9 µV and hence the threshold for the recordings is set at -35 µV to -40 µV, which is 4 times the baseline RMS noise value.
Figure 19. A snapshot of the recording obtained from electrode no.61 for the spontaneous activity.

Figure 20. A snapshot of the recording obtained from electrode no.61 for the light stimulated response.
Figure 21. Active electrodes on the pMEA exhibiting a chemically induced response to set no.2-2 mM glutamate (red) with an approximate area of the retina covering the electrodes of the pMEA (green) and an approximate location of the dispensing capillary over the retina (black).

Figure 22. A snapshot of the recording obtained from electrode no.41 for the chemically stimulated response.
Figure 23. A snapshot of the recording obtained from electrode no.42 for the chemically stimulated response.

Figure 24. A snapshot of the recording obtained from electrode no.43 for the chemically stimulated response.
3.1.1.3 Set 3 Recordings

Figure 26 and Figure 27 show the spontaneous and light activity of the tissue sample respectively as recorded from a single electrode. Figures 29-32 show the chemically induced response at four different electrodes. Figure 28 is an electrode layout map of the MEA chip highlighting the four active electrodes that were monitored for a chemical response. The RMS value of the background noise is about 11 µV and hence the threshold for the recordings is set at -45 µV, which is 4 times the baseline RMS noise value.
Figure 26. A snapshot of the recording obtained from electrode no.35 for the spontaneous activity.

Figure 27. A snapshot of the recording obtained from electrode no.35 for the light stimulated response.
Figure 28. Active electrodes on the hexa-MEA exhibiting a chemically induced response to set no.3-2 mM glutamate (red) with an approximate area of the retina covering the electrodes of the hexa-MEA (green) and an approximate location of the dispensing capillary over the retina (black).

Figure 29. A snapshot of the recording obtained from electrode no.53 for the chemically stimulated response.
Figure 30. A snapshot of the recording obtained from electrode no.27 for the chemically stimulated response.

Figure 31. A snapshot of the recording obtained from electrode no.35 for the chemically stimulated response.
3.1.2 Results from the 1 mM concentration case

Two sets of results (Set 1 Set 2), each set containing the recordings for spontaneous and light induced activity from a single electrode and chemical triggered activity from 4 active electrodes, are presented below.

3.1.2.1 Set 1 Recordings

Figure 33 and Figure 34 show the spontaneous and light activity of the tissue sample respectively as recorded from a single electrode. Figures 36-39 show the chemically induced response at four different electrodes. Figure 35 is an electrode layout map of the MEA chip highlighting the four active electrodes that were monitored for a chemical response. The RMS value of the background noise is about 9 µV and hence the threshold for the recordings is set at -35 µV to -40 µV, which is 4 times the baseline RMS noise value.
Figure 33. A snapshot of the recording obtained from electrode no.38 for the spontaneous activity.

Figure 34. A snapshot of the recording obtained from electrode no.38 for the light stimulated response.
Figure 35. Active electrodes on the pMEA exhibiting a chemically induced response to set no.1-1 mM glutamate (red) with an approximate area of the retina covering the electrodes of the pMEA (green) and an approximate location of the dispensing capillary over the retina (black).

Figure 36. A snapshot of the recording obtained from electrode no.38 for the chemically stimulated response.
Figure 37. A snapshot of the recording obtained from electrode no.16 for the chemically stimulated response.

Figure 38. A snapshot of the recording obtained from electrode no.26 for the chemically stimulated response.
3.1.2. Set 2 Recordings

Figure 40 and Figure 41 show the spontaneous and light activity of the tissue sample respectively as recorded from a single electrode. Figures 43-46 show the chemically induced response at four different electrodes. Figure 42 is an electrode layout map of the MEA chip highlighting the four active electrodes that were monitored for a chemical response. The RMS value of the background noise is about 11 µV and hence the threshold for the recordings is set at -40 µV to -45 µV, which is 4 times the baseline RMS noise value.
Figure 40. A snapshot of the recording obtained from electrode no.67 for the spontaneous activity.

Figure 41. A snapshot of the recording obtained from electrode no.67 for the light stimulated response.
Figure 42. Active electrodes on the hexa-MEA exhibiting a chemically induced response to set no.2-1 mM glutamate (red) with an approximate area of the retina covering the electrodes of the hexa-MEA (green) and an approximate location of the dispensing capillary over the retina (black).

Figure 43. A snapshot of the recording obtained from electrode no.67 for the chemically stimulated response.
Figure 44. A snapshot of the recording obtained from electrode no.58 for the chemically stimulated response.

Figure 45. A snapshot of the recording obtained from electrode no.86 for the chemically stimulated response.
3.1.3 Results from the 0.5 mM concentration case

The 0.5 mM glutamate solution was ejected focally on the retinal sample. However, no significant spiking activity was observed from the tissue sample as very few spikes crossed the threshold level.

3.2 Analysis

The results obtained above are the aggregate neuronal responses of multiple retinal ganglion cells. Each electrode of the MEA chip may pick up signals from either a single ganglion cell or many ganglion cells present in the vicinity. As a result, the recordings obtained from a single electrode may be a representation of the action potentials observed in a single neuron or the combined influence of action potentials generated by numerous neurons. Figures 47-52 below, report the trend that was observed during the chemical stimulation experiments.
While the X-axis represents the time scale of the entire experiment in minutes, the Y-axis represents the total spike count as observed in each electrode. Each file recording, i.e. spontaneous, light or chemical response, is 172 seconds long and corresponds to the time span of each bar in the following graphs. These analyses were carried out using the various tools available within the MC_Rack software. No spike sorting was performed on the data.

3.2.1 Results from Set No.1 for 2 mM glutamate solution concentration

The plot in Figure 47 represents the response activity over the entire chemical stimulation experiment ranging over time period of 105 minutes. The spontaneous and light activity is shown for a single electrode (E-66) whereas the chemical stimulation activity is observed for four different electrodes (E-66, E-67, E-77, E-75).

![2mM-Set No.1](image)

Figure 47. Plot of the spike count observed before and after the ejection of 2 mM of glutamate versus the time duration of responses in set no.1. Chemical activity of four electrodes is compared with the spontaneous and light activities of a single electrode.
3.2.2 Results from Set No.2 for 2 mM glutamate solution concentration

The plot in Figure 48 represents an entire chemical stimulation experiment ranging over a time period of 136 minutes. The spontaneous and light activity is shown for a single electrode (E-61) whereas the chemical stimulation activity is observed for four different electrodes (E-41, E-42, E-43, E-61).

![2mM-Set No.2](image)

Figure 48. Plot of the spike count observed before and after the ejection of 2 mM of glutamate versus the time duration of responses in set no.2. Chemical activity of four electrodes is compared with the spontaneous and light activities of a single electrode.

3.2.3 Results from Set No.3 for 2 mM glutamate solution concentration

The plot in Figure 49 represents an entire chemical stimulation experiment ranging over a time period of 118 minutes. The spontaneous and light activity is shown for a single electrode (E-35) whereas the chemical stimulation activity is observed for four different electrodes (E-53, E-27, E-35, E-25).
Figure 49. Plot of the spike count observed before and after the ejection of 2 mM of glutamate versus the time duration of responses in set no.3. Chemical activity of four electrodes is compared with the spontaneous and light activities of a single electrode.

3.2.4 Results from Set No.1 for 1 mM glutamate solution concentration

The plot in Figure 50 represents an entire chemical stimulation experiment ranging over a time period of 117 minutes. The spontaneous and light activity is shown for a single electrode (E-38) whereas the chemical stimulation activity is observed for four different electrodes (E-38, E-16, E-26, E-36).
3.2.5 Results from Set No.2 for 1 mM glutamate solution concentration

The plot in Figure 51 represents an entire chemical stimulation experiment ranging over a time period of 87 minutes. The light activity is shown for a single electrode (E-67) whereas the chemical stimulation activity is observed for four different electrodes (E-67, E-58, E-86, E-46).
Figure 51. Plot of the spike count observed before and after the ejection of 1 mM of glutamate versus the time duration of responses in set no. 2. Chemical activity of four electrodes is compared with the spontaneous and light activities of a single electrode.

3.2.6 Results from Set No.1 for 0.5 mM glutamate solution concentration

The plot in Figure 52 represents an entire chemical stimulation experiment ranging over a time period of 87 minutes. The light activity is shown for a single electrode (E-84) whereas the chemical stimulation activity is observed for four different electrodes (E-15, E-47, E-66, E-84).
Figure 52. Plot of the spike count observed before and after the ejection of 0.5 mM of glutamate versus the time duration of responses in set no.1. Chemical activity of four electrodes is compared with the spontaneous and light activities of a single electrode.

### 3.2.7 Global plot comparing the three concentrations

The global plot serves to provide a quantitative comparison over all the experimental results obtained for the 3 glutamate concentration cases. Figure 53 represents the average spike count of chemical responses plotted as a function of time. The average spike count is calculated by taking the average of responses as observed every 30 minutes in each experiment, for all the three concentration cases of glutamate. This helps in the comparison of the chemical responses as obtained from 2 mM, 1 mM and 0.5 mM glutamate cases. The average spontaneous activity is also included which helps to draw a contrast between the pre-stimulus activity to the post-stimulus activity.
Paired t-test were performed on the global averaged data comparing the pre-stimulus mean to the post-stimulus means for the two concentrations of 2 mM and 1 mM, during the 30 minute interval. The p-value obtained for the 2 mM glutamate case was 0.0379 whereas the p-value for the 1 mM case was 0.5173. These values reveal that there was a statistically significant increase in the firing rate for 2 mM glutamate case as compared to the pre-stimulus activity i.e. spontaneous activity, and no significant increase for 1 mM concentration case. This suggests that the higher glutamate concentration of 2 mM was more effective at stimulating the retina chemically. This significance however must be interpreted with caution because the sample size is less than five i.e. N< 5 and hence these results may not be generalized for future work based on conducting similar experiments.

Figure 53. Global plot comparing the average spike count recorded for the 2 mM, 1mM, 0.5 mM glutamate concentration cases. The pre-stimulus activity (spontaneous response) is contrasted with the post-stimulus activity (chemical response) over time intervals of 30 minutes.
3.3 Discussions

The graphical results described in Section 3.2 help in providing a statistical understanding of the aggregate multi neuronal cell response to a locally applied glutamate solution. While the experimental results varied in certain aspects, general observations from the above graphs revealed that the chemically induced response in the retinal tissue is much higher than the spontaneous activity observed in same tissue sample in all experiments. An initial period of suppression of the retinal activity was observed which lasted about 7-10 minutes. With the higher concentration case of the glutamate solution i.e. 2 mM, an overall increase in the tissue activity is observed over a prolonged time period, in contrast to the lower glutamate concentration case of 1 mM and 0.5 mM. The response pattern of the chemically stimulated activity was very similar to the light induced responses in terms of spike count, i.e. the chemical responses would occur as sudden burst of high frequency spikes followed by a silent time interval with no spikes at all. For instance in one experiment, about 16 spikes were observed in a single electrode in case of the light induced response for a 5 seconds time interval which is quite close to the spike count observed for the same electrode in case of a chemical response which measured 12 spikes in 5 seconds. Based on all these observations, it may be concluded that the chemically induced responses are in fact stimulated responses of the retina.
CHAPTER 4 - CONCLUSIONS

Focalized stimulation of the rat retina by the excitatory neurotransmitter glutamate was accomplished through a model system that comprised of the MEA system, retinal sample system and the chemical dispenser unit. A quantitative analysis of the obtained data revealed that the retina responded physiologically to varying glutamate concentrations (0.5 mM, 1 mM and 2 mM) applied externally. It was observed that the higher concentration of glutamate ejected on the retina elicited an increase in the physiological neuronal activity. In conclusion, the objectives of this thesis were successfully realized.

4.1 Scope for Future Work

In regards to the work accomplished by this thesis, some key areas which could be further researched include the possibility of analyzing the multineuronal responses obtained from single electrodes into ON-OFF cell responses. The ability to specifically target excitatory-inhibitory or ON-OFF pathways provides for a fine understanding of the neural code and functional connectivity of the retina. Currently, the data obtained from one electrode represents the action potential spikes from possibly more than one cell. In order to attain a better understanding of the physiological response, commercially available spike sorting software i.e. Offline Sorter or Plexon, could be used to separate the single electrode response into multiple units which would help to identify single cell responses.
REFERENCES


APPENDIX

A.1AMES Media Preparation Instructions [29]

1. Measure out 90% of final required volume of water. Water temperature should be 15-20°C.

2. Gas the water with 100% CO2. While gently stirring the water, add 1.9 g of sodium bicarbonate or 25.3 ml of sodium bicarbonate solution [7.5% w/v] for each liter of medium.

3. Add the powdered medium to the sodium bicarbonate and water mixture. Stir until dissolved. Do NOT heat water.

4. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 3.

5. Add additional water to bring the solution to final volume.

6. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns. Positive pressure filtration will reduce CO2 loss.

7. The medium is designed to provide a physiological pH when equilibrated with 5% CO2 at 37°C. The pH can be adjusted under these conditions using 1N HCL or 1N NaOH. High CO2 is used during preparation to avoid precipitation of CaCO3.
A.2 ACC Protocol

October 21, 2010

Scott A. Shippy
Chemistry
M/C 111

Dear Dr. Shippy:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on 10/19/2010. The protocol is approved for a period of 3 years with annual continuation.

Title of Application: Developing a Retinal Implant Utilizing Chemical Stimulation

ACC Number: 10-173

Initial Approval Period: 10/19/2010 to 10/19/2011

Current Funding: Portions of this protocol are supported by the funding sources indicated in the table below.

Number of funding sources: 1

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<th>Grant Title</th>
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<td>EFRI-BSBA: Nanoactuation and Sensing of Neural Function for Engineering Future Biomimetic Retinal Implants and Therapies</td>
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This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/ea
cc: BRL, ACC File, Laxman Saggere, PAF # 2009-06949
VITA

NAME: Shushi Kabu

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POSITIONS HELD: Research Assistant, Microsystems and Devices Laboratory, University of Illinois at Chicago, Chicago, Illinois, 2010-2011

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COURSEWORK: GMP & Regulatory Requirements for Medical Products, Neural Models, Bioinstrumentation, Neuroscience, Biomaterials, Retinal Physiology, Cell & Tissue Engineering

SKILLS: Regulatory Affairs
        Electrical circuits and instrumentation
        Troubleshooting and repair of medical equipment
        Multielectrode Array Recording System
        Rat retina extraction