Optical and Electrical Stimulation of Retinal Ganglion Cells on a CMOS Microelectrode Array

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OPTICAL AND ELECTRICAL STIMULATION OF RETINAL GANGLION CELLS ON A CMOS MICROELECTRODE ARRAY

A thesis submitted to attain the degree of DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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Cover image: a topographical map indicating the signal amplitude of the spike-triggered average extracellular action potential (STA-EAP) or electrical footprint generated by a retinal ganglion cell on the HiDens microelectrode array. The scale bar is 100 μm; the blue end of the color code spectrum indicates low amplitudes, whereas the red end indicates higher amplitudes, ranging up to approximately 300 μV.

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Abstract

This thesis presents the implementation of two complementary metal–oxide–semiconductor (CMOS) high-density microelectrode array (HD-MEA) chip devices in order to interface with the ex-vivo retina for the following objectives:

1. Identification of retinal ganglion cell populations based on their light responses to projected optical stimuli.
3. Characterization of the response of the ganglion cell to voltage pulse stimuli across a cell-centered micro region, and focal electrical stimulation of targeted cells.

The retina is a part of the brain that performs complex signal processing of optical input, the result of which is a spatiotemporal pattern of electrical pulses. These pulses are generated by the neural output layer – the ganglion cell layer – and sent to higher brains centers to be interpreted as vision. The HD-MEAs, featured in this thesis, provide a direct two-way interface to the ganglion cells at high spatial and temporal resolution, which makes it possible to study the cells at a novel and higher level of detail.

There were two generations of CMOS HD-MEA systems used in this project: for the optical stimulation work, an HD-MEA featuring 11,011 electrodes, 18 μm electrode pitch (hexagonal electrode arrangement), and 126 rapidly-configurable recording channels was used; for the electrical stimulation part, the subsequent generation in this of HD-MEAs was used, which featured 26,400 electrodes (grid arrangement), 17.5 μm electrode pitch, and 1024 recording channels.

In an initial methods step, we recorded from mouse ganglion cells while stimulating the cells with bright moving bars that were projected onto the photoreceptor layer through an upright microscope. We were able to successfully identify direction selective ON-OFF ganglion cells by analyzing their responses to the moving bars.

In order to perform the classification of hamster retinal ganglion cell types, which have not previously been characterized, a series of optical stimuli were used. The optical stimuli, which had a total run time of 3.5 hours, consisted of flashing squares and moving bars of varying widths, lengths, orientations and speeds of movement. The stimuli were designed to present a range of visual features in order to elicit responses from the widest variety of ganglion cell types possible. 262 ganglion cells were recorded in seven experiments. Spike sorting and action potential template matching were performed on the data in order to obtain the spike trains of each cell. Seven normalized parameters were extracted.
from the light responses of the processed cells, five parameters of which were clustered using k-means. Ultimately, we identified seven ganglion cell types that correlate with types found in other species.

To assess electrical stimulation response characteristics of ganglion cells, voltage stimulation thresholds were first found. Biphasic pulse voltage sweeps on single electrodes (up to 300 mV in 27mV steps, pulse phase width 200 μs) were applied to the peak regions of the spike-triggered average extracellular action potential (STA-EAP) of each cell. Cell responses were read out at the axonal region, hundreds of microns from the stimulation site in order to avoid stimulus artifacts. In an attempt to reduce stimulation thresholds, we also applied monophasic pulses on single electrodes, as well as monophasic and biphasic pulses on pairs of electrodes at various electrode-to-electrode spacings; there was however no significant difference found between the stimulation configurations. We found that OFF cells had a lower threshold than ON cells using biphasic pulses.

We determined the sensitivity of ganglion cells to biphasic voltage pulse stimulation on multiple electrodes (in series) over a 105 × 105 μm² micro-region that was centered on the STA-EAP. We found that the cells were most easily stimulated in the vicinity of the STA-EAP peak or the action potential origin. The stimulation threshold was linearly correlated to the action potential distance traveled and latency, and inversely correlated to the STA-EAP amplitude. Finally, we recorded from all 24,600 electrodes using multiple electrode configuration blocks, while stimulating selected cells with biphasic pulses. We were successfully able to stimulate 4 out of 4 ganglion cells selectively.

The use of the CMOS HD-MEA as an interface to the retina provided us with a unique opportunity to examine features and characteristics of retinal ganglion cells, including extracellular subcellular-resolution activity, type-specific responses to optical stimuli, and sensitivity to extracellularly applied electrical stimuli.
Zusammenfassung

Die vorliegende Arbeit beschreibt die Anwendung zweier hochintegrierter Mikroelektroden-Array Chips (HD-MEAs) auf der Basis komplementärer Metall-Oxid-Halbleiter-Technologie (CMOS) zur elektrischen ex-vivo Ankoppelung an die Retina, zur Erreichung folgender Ziele:

1. Identifizierung retinaler Ganglionzellpopulationen anhand deren Antwortverhalten auf spezifische optische Stimulationsmuster.
2. Entwicklung einer Methode zur halbautomatischen Klassifikation von Ganglionzellen anhand deren Signale auf verschiedene Lichtstimulationen.
3. Charakterisierung der ortsabhängigen Reaktion von Ganglionzellen auf elektrische Stimulation, sowie fokussierte, elektrische Stimulation ausgewählter Zellen.


In diesem Projekt wurden zwei Generationen von CMOS HD-MEA-Systemen eingesetzt: Für die Arbeit mittels optischer Stimulation wurde ein HD-MEA mit 11'011 Elektroden, 18 μm Elektrodenraster (hexagonale Anordnung der Elektroden), sowie 126 schnell-konfigurierbaren Aufzeichnungskanälen eingesetzt; für die Anwendung elektrischer Stimulation wurde die nächste Generation dieses HD-MEAs verwendet, welches über 26'400 Elektroden (in Gitter-Anordnung), 17.5 μm Elektrodenraster und 1'024 Aufzeichnungskanäle verfügt.

In einem ersten Experiment zeichneten wir die Signale von Mausganglionzellen auf, während die Zellen mit Lichtbalken stimuliert wurden, welche über ein Mikroskop auf die Fotorezeptoren-Schicht projiziert wurden. Durch die Analyse der Zellsignale auf die sich bewegenden Lichtbalken konnten wir richtungsempfindliche ON-OFF Ganglionzellen identifizieren.

Um die Klassifikation bisher nicht charakterisierter Hamsterganglionzellen durchzuführen, wurde eine Serie verschiedener optischer Stimulationsmuster verwendet. Die optischen Stimulationsen, welche über eine Gesamtdauer von 3.5 Stunden durchgeführt wurden, umfassten blinkende Quadrate, bewegte Balken variabler Weite, Länge, Winkel und Laufgeschwindigkeit. Bei der Entwicklung der Stimulationsmuster wurde Wert darauf gelegt, Antworten aus einer grösstmöglichen Variation an Ganglionzelltypen gewinnen zu können. In sieben


Die Anwendung des CMOS HD-MEAs als Schnittstelle zur Retina ermöglichte uns die Eigenschaften und Charakteristiken der retinalen Ganglionzellen zu untersuchen, inklusive deren extrazellulären Aktivität. Die Messungen wurden mit subzellulärer Auflösung durchgeführt, die typenspezifischen Antwortsignale auf optische Stimulationen, und extrazelluläre elektrische Stimulationen wurden charakterisiert.
Chapter 1 Introduction

The field of neurophysiology has arisen out of a desire to understand the how the brain and nervous system function. Various instruments and techniques have been developed in order to measure the electrical signals produced by the building blocks of the brain and nervous system: the neurons. The most basic recording devices were single metal electrodes, which were used starting in the 1950’s (Frank and Fuortes, 1955; Fatt, 1957; Nelson and Frank, 1964). The next step was to use solution-filled glass micropipettes, which were first used for extracellular recording from muscle cells in the 1960’s (Strickholm, 1961). Intracellular recording methodologies such as patch clamp were also developed in order to record internal signals that are not detectable extracellularly; for this technique, a solution-filled micropipette punctures the membrane so as to come into direct contact with the intracellular fluid (Molleman, 2003). Optical methods have also been used in order to record electrophysiological data from neurons: for example, one of the first voltage dye recordings in neurons was made by (Tasaki et al., 1968). More recently, (Luo et al., 2012) used Ca\(^{2+}\)-sensitive fluorescent dye and intracellular Na\(^{+}\)-sensitive dye in a study of the ganglion cells in an electrically-stimulated retina; however, they found that the dyes were generally not sensitive enough to detect activity. Indeed, optical methods, while they can be used to record many cells at once, suffer from the following limitations: low signal amplitude, light scattering, pharmacological side effects, photodynamic damage, dye bleaching (Grinvald et al., 1988).

Microelectrode arrays (MEA) are devices comprised of many closely-juxtapositioned electrodes that are used to simultaneously measure voltage at the microvolt level across a planar surface. They are particularly powerful systems when it comes to recording the extracellular signals from neurons, since these cells generate extracellular action potentials as they transmit information in such processes as higher brain function, memory and vision. The MEA used and described later in this thesis is particularly suited to recording from neurons, because it features an appropriate sensitivity range for recording neuronal signals, it can record these signals at a very high temporal resolution, and it can
Chapter 1: Introduction

sample multiple neuronal signals across a selected area, so that neuronal mechanisms and connections can be studied.

The use of microelectrode arrays to record neural signals has been growing over the past four decades. One of the first MEAs, which had 30 electrodes and 100 μm electrode spacing, was used to record from chicken heart cells (Thomas et al., 1972). More recent systems, such as the commercially-available 60-electrode Multichannel Systems device (www.multichannelsystems.com), have become available and have more electrodes and higher spatial resolutions (Gross et al., 1977; Pine, 1980). MEAs have been used for recording from many neuronal preparations, including cultured cortical cells (Potter and DeMarse, 2001; Wagenaar et al., 2005; Jimbo, 2007; Bakkum et al., 2009), acute cerebellar slices (Egert et al., 2002), organotypic slices (Egert et al., 1998; Karpiak and Plenz, 2002), heart cells and retinal cells (Stett et al., 2003).

Recently, high-density (HD) complementary metal-oxide-semiconductor (CMOS)-based MEAs, such as the two sequential chip generations developed by (Frey et al., 2010a) and (Ballini et al., 2013; Muller et al., 2015), have been developed and feature up to tens of thousands of electrodes, resolutions down to 17.5 μm, and measure signals at 20 kHz; the former can record from 127 electrodes at one time, while the latter can record from 1024 electrodes. The great advantage of these systems is that their signal-to-noise ratio is much larger than that of other comparable systems (Eversmann et al., 2003a; Imfeld et al., 2008; Eickenscheidt et al., 2012), while they, however, feature a smaller number of electrodes, which can be simultaneously recorded from. Active recording and stimulating electrodes can be dynamically configured on-the-fly: large areas of cells can be scanned and probed in one experiment, and the important locations selected for further recording and analysis.

The Retina

The retina is an intricate and elegant assembly composed of layers of interconnected electrogenic cells, which together comprise a biological transducer and signal processor. Its primary purpose is to convert optical imagery and stimuli into electrical signals that can ultimately be interpreted by the higher centers of the brain. The retina is a responsive and dynamic system, which adapts to light levels and extracts the most important information from the constant stream of photons that is reaching its surface (Dowling, 2012).

The first layers of cells in the retinal signal cascade are the photoreceptors, which react when exposed to light. The two parallel systems – the cones for bright light conditions, and the rods for low-light conditions – make it possible for the retina to be fully functional across a 10 billion-fold magnitude of luminance levels (Dowling, 2012), from ambient light conditions in moonlight to those found under a mid-day sun on a ski-slope. The signals from the photoreceptors propagate through the intermediate layers of the retina, in which a complex, multi-layered system of inhibition and excitation extracts features
from the stimulus that can then be channeled to the final layer of the retina, the retinal ganglion cell layer. The ganglion cell layer is composed of several different types of ganglion cells, which respond to features of the stimulus, whether it be movement, stimulus orientation, or overall luminance levels. When a ganglion cell has been activated, it produces a burst of spikes, which are sent to the higher brain centers to be decoded (Dowling, 2012).

The retina is a complex living machine, and the extent and intricacies of its functioning have not yet been fully elucidated. It is of great interest to researchers to learn more about the workings of the retina and to unravel the mysteries which still elude us. The applications are numerous: genetic manipulation of cells to treat degenerative diseases (Tochitsky et al., 2014), visual signal encoding for implantable prosthetic devices in the blind (Chuang et al., 2014), and the development and advancement of technology that can be used to apply lessons learned from the engineering found in nature.

Use of the MEA to Investigate the Retina

The MEA features electrodes across a two-dimensional plane and is well-suited to interfacing with retinal ganglion cells, which essentially lie within a planar layer. The planar configuration of the retina is especially fortunate, as it is crucial that the electrodes be located in close proximity to the neurons of interest in order to obtain a good signal-to-noise ratio. MEAs have been used successfully to record and interface with the retina in many studies. First of all, MEAs have been used to classify ganglion cell types in the retina, which would require an immense amount of time using other methods, such as patch clamp. The MEA has also been used to learn about functionality in the retina, such as detection of movement (Borst and Euler, 2011), or how images are encoded using action potential bursts (Meister and Ii, 1999). Other groups have used the MEA to stimulate the retina with electrical pulses, in order to advance technology needed for prosthetic devices (Zrenner et al., 2011; Lorach et al., 2013a; Shepherd et al., 2013; Chuang et al., 2014).

MEA electrodes record signals from many neurons at once and are, therefore, naturally limited in the density of neurons which they can record. The HD-MEA used here features an electrode density of 3,265 electrodes per mm², bidirectional capabilities, and low noise characteristics: $2.4 \mu V_{\text{RMS}}$ in the action potential band (Ballini et al., 2013; Muller et al., 2015). There is still, however, room for improvement, as the density of ganglion cells in the human retina approaches approximately 35,000 cells in the foveal region (Curcio and Allen, 1990).

The goal of this work is to take full advantage of the capabilities of CMOS-based HD-MEAs in order to interface with retinal ganglion cells in the mouse and hamster in a bidirectional manner: (i) recording cellular activity and (ii) applying voltage to stimulate cells. Because of the routing capabilities of the device, we can select the most active regions of the retina and record the cells at those locations. The high resolution of the HD-MEA makes it possible to record subcellular details of the extracellular action potential landscape, and the large
number of recording channels enables monitoring of the activity of many cells simultaneously. We first established the use of this particular HD-MEA for retinal recording, and then incorporated it into an experimental setup, including a light projection system and perfusion system. The initial steps included to obtain light-evoked responses from ganglion cells and to apply spike sorting algorithms to read out the activity of densely-packed ganglion cells. We then proceeded with projecting a series of optimal stimuli onto the retina in order to classify ganglion cells based on their responses. Finally, we used built-in stimulation buffers to explore the response of ganglion cells to electrical stimuli and to ultimately selectively stimulate specific ganglion cells.

1.1 Structure and Scope of Thesis

The thesis is focused on the use of two HD-MEA systems for interfacing with mammalian ganglion cells in ex-vivo retina segments. The core of this work consists of the three papers listed below, which correspond to Chapters 2, 3 and 4. In Chapter 2, the basic methodology of recording from mouse ganglion cells using the HD-MEA is introduced and described. Experiments are conducted with an optical stimulus that is specifically designed to detect a particular population of ganglion cells. In Chapter 3, the optical stimuli are expanded in complexity into a stimulus series that incorporates a range of spatial, temporal and contrast features. The purpose here is to utilize the optical stimuli to classify as many hamster ganglion cell types as possible based on their cell-specific responses to the stimuli. Finally, in Chapter 3, we use the next generation MeaK HD-MEA, which has ten-fold more recording channels than the device used for previous experiments. We use the HD-MEA to characterize ganglion cell responses to electrical stimuli as well as ultimately performing selective stimulation to activate targeted cells.

1. Recording from Defined Populations of Retinal Ganglion Cells Using a High-Density CMOS-Integrated Microelectrode Array with Real-Time Switchable Electrode Selection

Michele Fiscella, Karl Farrow, Ian L. Jones, Jan Müller, Urs Frey, Douglas J. Bakkum, Peter Hantz, Botond Roska and Andreas Hierlemann

Journal of Neuroscience Methods
Volume 211, Issue 1, 15 October 2012, Pages 103–113


Ian L. Jones, Thomas L. Russell, Karl Farrow, Michele Fiscella, Felix Franke, and Andreas Hierlemann
3. **Characterization of Hamster Retinal Ganglion Cell Response to Electrical Stimulation using a High-Density 1024-channel CMOS Microelectrode Array**

Ian L Jones, Jan Müller, Thomas Russell, Felix Franke, Michele Fiscella, David Jäckel, Vijay Viswam and Andreas Hierlemann

*In preparation*

**1.2 Summary of Major Results**

**Recording, Optical Stimulation and Spike Sorting of Retinal Ganglion Cell Signals Using High Density Microelectrode Array**

Segments of mouse retina were isolated, and extracellular ganglion cell activity was recorded using a 127-channel HD-MEA. Optical stimuli were used to activate the cells, which were viable for durations of up to several hours. Principal component analysis was used to spike-sort cellular signals in order to obtain spike trains for ganglion cells. Using multiple electrode configurations, cells could be observed on up to 14±7 electrodes.

**Method for Automated Classification of Ganglion Cells using Optical Stimuli**

Retinal ganglion cells from Syrian Hamsters were recorded using a 1024-channel HD-MEA while being stimulated with a series of optical stimuli: marching squares, narrow moving bars, wide bars moving at different speeds, and wide moving bars of different widths. The stimuli probed contrast, directional, temporal, and spatial ganglion cell responses across defined ranges in order to...
activate as many cell types as possible. Data was sorted using PCA and template matching, and spike trains were extracted for each cell. Responses were transformed into a set of normalized response parameters: latency, transience, ON-OFF bias-index, direction-selective index, bar-speed response, receptive-field diameter, and bar-width response.

**Seven Cell Types Identified in the Hamster Retina**

Five parameters obtained from the automated classification method were clustered using k-means clustering. Seven ganglion cell types were found in a total of 262 recorded ganglion cells.

**Voltage Sweeps of Ganglion Cells to Find Stimulation Thresholds**

Hamster retinal ganglion cells were recorded using the HD-MEA while projecting optical stimuli onto ex-vivo retina. Cell action potential spikes were thresholded, and the spike-triggered average extracellular action potential (STA-EAPs) was obtained. Stimulation was applied at the STA-EAP peak with single-electrode voltage pulses, and the responses were read out at the distal axon. Pseudo randomized voltage sweeps were applied to obtain stimulus thresholds.
Retinal Ganglion Cells Most Easily Stimulated near STA-EAP Peak or Action Potential Origin

Voltage sweeps were applied serially at multiple electrodes in the vicinity of the STA-EAP peak to obtain stimulation threshold voltages. It was found that the cells were most easily stimulated near the STA-EAP peak or at the origination site of the action potential. The stimulus threshold was found to correlate directly with latency and distance from the STA-EAP peak; threshold correlated inversely with the STA-EAP amplitude. Latency and distance along the axon from the action potential origin were strongly positively correlated, revealing constant propagation speeds over hundreds of microns.

Focal Stimulation of Ganglion Cells Using Biphasic Voltage Pulses

We stimulated four ganglion cells at different peak-to-peak voltage amplitudes while recording extracellular activity across 26,400 electrodes (using multiple electrode configurations). We found that we were able to stimulate all cells selectively using their respective threshold voltages. At super-threshold biphasic pulse voltages of 605 mV, stimulation became nonspecific, and stimulation-induced activity of multiple cells was observed.
1.3 **Author contributions**

People involved in the project included the following:

Karl Farrow: Guidance, feedback and experimental planning with optical stimulation work.

Jan Mueller: The “go-to guy” for technical and computer-related issues and support: assistance with all MEA recording and stimulation software and hardware, including FPGA and MEA interfacing board, and the HD-MEA chip, for both the *HiDens* (Frey et al., 2010b) and *Mea1k* (Muller et al., 2015) chip generations. Extensive assistance with electrical stimulation experiments as well as planning and performance of experiments using the *Mea1k* HD-MEA. Assistance with electrical stimulation programming as well as data analysis.

Michele Fiscella: Building of retinal recording/stimulation rig, including microscope, projector, optics, vibration-isolation table, and MEA chip accessories for retina recording. Assistance with animal handling, techniques and experiments. Discussions regarding experiments and research. Planning and performance and of experiments for project in Chapter 2.

Thomas Russell: Guidance regarding project concepts, and planning. Animal handling assistance.

Felix G. Franke: Assistance with experimental planning and analysis, especially regarding mathematical and statistical aspects of data analysis and clustering. Provided support with template matching algorithm for analysis in optical stimulation project.

Alex Stettler: MEA chip wirebonding and electrode post-processing optimization. Advice regarding chip handling.
Chapter 1: Introduction

David Jäckel: Discussion of projects, ideas, suggestions, opinions and feedback.

Milos Radivojevic: Project goal discussions; provision of Matlab analysis scripts for data processing.

Vijay Viswam: Assistance with electrode characterization and support for Mea1k.

Roland Diggelmann: Programming of an excellent and invaluable graphical user interface for operating the Mea1k Chip. Provided assistance with software use and assisted with experiments.

Botond Roska: Discussed project ideas as and gave feedback regarding research results. Provided access to his group, thereby offering a unique opportunity to learn about the retina from a biological perspective.

Andreas Hierlemann: Primary supervisor for PhD. Provided feedback, advice, critique on all projects as well as extensive editing on all manuscripts, posters and abstracts.


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Chapter 2 Recording from Defined Populations of Retinal Ganglion Cells Using a High-Density CMOS-Integrated Microelectrode Array with Real-Time Switchable Electrode Selection

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2.1 Abstract  
In order to understand how retinal circuits encode visual scenes, the neural activity of defined populations of retinal ganglion cells (RGCs) has to be investigated. Here we report on a method for stimulating, detecting, and subsequently targeting defined populations of RGCs. The possibility to select a distinct population of RGCs for extracellular recording enables the design of
experiments that can increase our understanding of how these neurons extract precise spatio-temporal features from the visual scene, and how the brain interprets retinal signals. We used light stimulation to elicit a response from physiologically-distinct types of RCGs and then utilized the dynamic-configurability capabilities of a microelectronics-based high-density microelectrode array (MEA) to record their synchronous action potentials. The layout characteristics of the MEA made it possible to stimulate and record from multiple, highly overlapping RGCs simultaneously without light-induced artifacts. The high-density of electrodes and the high signal-to-noise ratio of the MEA circuitry allowed for recording of the activity of each RGC on 14±7 electrodes. The spatial features of the electrical activity of each RGC greatly facilitated spike sorting. We were thus able to localize, identify and record from defined RGCs within a region of mouse retina. In addition, we stimulated and recorded from genetically modified RGCs to demonstrate the applicability of optogenetic methods, which introduces an additional feature to target a defined cell type. The developed methodologies can likewise be applied to other neuronal preparations including brain slices or cultured neurons.

2.2 Introduction

The retina is a multilayered, light-sensitive sheet of neural tissue that encodes visual stimuli as complex spatio-temporal patterns of action potentials. The final output of the retina is encoded in the ganglion cell layer, in which densely packed neurons, called retinal ganglion cells (RGCs), generate action potentials that proceed along the optic nerve to higher brain regions (Masland, 2001a; Wässle, 2004).

Microelectrode arrays (MEA) are electrophysiological devices for simultaneously recording the extracellular activity of electrogenic cells at multiple spatial positions (Gross et al., 1995; Jimbo et al., 1998; Rutten, 2002a; Stett et al., 2003). MEA technology has been widely applied to record electrical activity in the retina (Meister et al., 1994; Segev et al., 2004; Zeck et al., 2011), to investigate retinal development (Elstrott et al., 2008; Anishchenko et al., 2010), retinal connectivity (Field et al., 2010), visual encoding (Puchalla et al., 2005; Schwartz et al., 2007; Golllisch and Meister, 2008; Pillow et al., 2008), to evaluate the efficacy of visual restoration techniques (Bi et al., 2006; Lagali et al., 2008), and for the design of artificial retinal implants (Sekirnjak et al., 2008).

Commercially available MEAs usually comprise up to 256 electrodes and feature up to 300 electrodes per mm2 (Gross et al., 1995) (www.multichannelsystems.com, www.ayanda-biosys.com, www.plexon.com). This density of electrodes is significantly lower than the density of RGCs in many mammals, including rabbits (Oyster et al., 1987), mice (Jeon et al., 1998) and monkeys (Perry and Cowey, 1985). Furthermore, when using conventional MEA technology, it can be challenging to actively target specific cell types for
recording, because the electrodes are in a fixed-configuration block, and only the activity of cells that are in the vicinity of these electrodes can be detected.

Recently, high-density MEAs, fabricated in standard microelectronics or CMOS (Complementary Metal Oxide Semiconductor) technology have emerged (Eversmann et al., 2003b; Lambacher et al., 2004; Berdondini et al., 2009) and bear the potential to perform recordings from dense populations of neurons at single-cell resolution.

In order to understand how specific features of the visual scene are encoded by the retina, a first step is to examine the synchronously-elicited action potentials of defined populations of RGCs (Ackert et al., 2006; Schwartz et al., 2007; Pillow et al., 2008; Trong and Rieke, 2008). To record the activity of a defined population of RGCs, we capitalized upon the electrode configurability capabilities of a CMOS based high-density MEA (Frey et al., 2009). In contrast to an earlier study on blind retinae (Jones et al., 2011), we here used light stimulation to evoke electrical activity from wild type retinae and selected cells according to their light response, which adds to the complexity of the setup and entails the risk of producing artifacts in the recorded signals (see below).

The accurate characterization of a population of neurons is dependent upon the extracellular recording of action potentials with a high signal-to-noise ratio that can be easily differentiated and sorted (Lewicki, 1998). However, light-induced artifacts can introduce erroneous signals and noise into electrophysiological recordings and disrupt the signal analysis process. Such artifacts may arise from the interaction of photons with the electronic components of the CMOS-based circuitry of the MEA chip. Despite the presence of light-sensitive elements in the CMOS-based circuitry, we demonstrate that it is possible to project a light stimulus directly onto the CMOS-based MEA without generating such artifacts in the recorded signals.

Consequently, the absence of light artifacts and the high signal-to-noise ratio allowed us to characterize densely packed RGCs according to their response to light stimulation.

Furthermore, the real-time switchable electrode selection of the MEA allowed the assignment of electrodes to defined physiological types of RGCs. This made it possible to stimulate and record the action potentials from a defined type of RGCs. Finally, it was possible to perform light stimulation of genetically modified RGCs that can be used as optogenetic tools directly on the CMOS-based MEA.
2.3 Methods

2.3.1 Data Acquisition System

The CMOS-based MEA features 11,011 platinum electrodes with diameters of 7 \( \mu \text{m} \) and electrode center-to-center distances of 18 \( \mu \text{m} \) over an area of 2 x 1.75 \( \text{mm}^2 \) (Frey et al., 2009). The centrally-located electrode array is surrounded by the signal amplification (0-80 dB), filtering (high pass: 0.3-100 Hz, low pass: 3.5-14 kHz) and analog-to-digital conversion (8 bit) units (Figure 2-1a).

Extracellular action potentials can be recorded at high temporal resolution (20 kHz) and with low noise levels (~7-9 \( \mu \text{V}_{\text{rms}} \), band: 100 Hz - 3 kHz, perfusion system operational but without retinal tissue). In the maximum-density recording scenario (3,161 electrodes/mm\(^2\)), each mouse RGC lies in close vicinity to multiple electrodes, which allows for recording single-cell action potentials at different spatial locations (Figure 2-1b).

A switch matrix circuitry is located under the electrode array and connects the electrodes to 126 readout channels (Frey et al., 2010b). An arbitrary subset of 126 electrodes at any location and desired inter-electrode spacing can be routed to the 126 readout channels that surround the electrode array (Figure 2-1c).

To reduce the electrode impedance and to improve the signal-to-noise ratio, a layer of Pt-black has been electrochemically deposited onto the electrodes at a current density of 0.5 nA/\( \mu \text{m}^2 \) in a solution containing 7 mM hexachloroplatinic acid, 0.3 mM lead acetate, and hydrochloric acid with an adjustment of the solution pH to 1.

For the purpose of interfacing with the semiconductor chips, custom-designed printed circuit boards have been used. The recorded data are multiplexed and sent via a single twisted-pair cable to a field-programmable gate array (FPGA) board at a rate of 16MB/s. The FPGA provides data processing features, such as error detection, digital filtering, event detection, and data reduction/compression. The preprocessed data are then sent to a personal computer for further data processing, visualization and storage.

2.3.2 Projection and Alignment of Images with the MEA

The light stimuli were designed using Psychtoolbox (http://psychtoolbox.org) within the software application MATLAB and were projected onto the electrode array by an LED projector with a refreshing rate of 60 Hz (Acer K10). The light stimulus was focused only on the electrode array area of the MEA chip by two camera lenses (Nikkor 60 mm 1:2.8 G ED, Nikon), a mirror (U-MBF3, Olympus) and a 5X objective (LMPLFLN5X Olympus) (Figure 2-1d). The light projection setup was assembled on an upright microscope (BX51WI, Olympus). The MEA chip was positioned with a joystick-controlled system (20 nm resolution along X and Y axis, Scientifica). A video camera provided a real-time
view of the electrode array. This procedure enabled the precise projection of a light stimulus exclusively on the electrode array area.

2.3.3 Preparation of Mouse Retina and Light Induced Activity Recordings

Wild-type C57BL/6J mice (P30) were obtained from Charles River Laboratories (L’Arbresle Cedex, France). All animal experiments and procedures were approved by the Swiss Veterinary Office. The retina was isolated under dim red light in Ringer’s medium (in [mM]: 110 NaCl, 2.5 KCl, 1 CaCl2, 1.6 MgCl2, 10 D-glucose, 22 NaHCO3), continuously bubbled with 5% CO2 / 95% O2. The remaining vitreous was removed to improve the contact of the retinal ganglion cells to the electrodes and, finally, a retina patch was placed ganglion-cell-side-down on the electrode array (Figure 2-1e). In order to stably secure the retina directly above the MEA, a permeable membrane (polyester, 10 μm thickness, 0.4 μm pore size) was lightly pressed against the tissue; continuous perfusion with oxygenated Ringer’s medium at a flow rate of 2.8 ml/minute and at a temperature of 35° C was provided to maintain tissue viability. Each isolated section of the retina was light-adapted to a “gray” background (blue LED, 460±15 nm, intensity ~1.0*10¹³ photons/cm²*s⁻¹; green LED, 525±23 nm, intensity ~1.67*10¹³ photons/cm²*s⁻¹) for a duration of 30 minutes prior to light stimulation and recording. We recorded from mouse retina regions between ~0.7 mm from the edge and ~0.7 mm from the center; the mouse retina features, on average, approximately 2,700 retinal ganglion cells / mm² (Jeon et al., 1998).

Different sets of light stimuli (see Light Stimulation in Supplementary Material) were focused on the photoreceptor layer and elicited action potentials from different types of retinal ganglion cells (Figure 2-1f, Figure 2-1g).
Figure 2-1. High-Density MEA Setup and Retinal Ganglion Cells. (a) Chip micrograph. The electrode array is surrounded by the first-stage amplification and filtering circuitry (1S and 2S) and the stimulation buffers (Stim). Below the array is the shift register (SR) used to program the array, and on the right-hand side are the third amplifier stages, analog-to-digital converters (3S, ADC) and the digital core. (b) Superimposed image of retinal ganglion cells (green) from PvalbCre x Thy1Stp-EYFP mouse on the electrode array. It can be seen, how each RGC is surrounded constantly by multiple electrodes. This allows the recording of extracellular action potentials simultaneously at multiple sites. (c) Examples of electrode block configurations (~126 electrodes, red squares) that can be dynamically selected from an array of 11,011 electrodes (102x108 electrodes) in order to record electrophysiological activity in different regions of the retina. Electrodes can be arbitrarily selected at a desired spacing. Electrode reconfiguration takes ~1 ms. (d) Light projection setup. A light stimulus is generated by a projector and focused only on the electrode array area of the MEA chip by two lenses, one mirror and a microscope objective. The light projection setup is assembled on an upright microscope. The intensity of the light stimulus can be decreased by neutral density filters. A video camera provides a real-time view of the retina on the electrode array. The recorded traces from the MEA chip are sent via a field-programmable gate array (FPGA) board to a computer, where they are stored for further analysis. The light stimulus is centered on the electrode array by a high-precision-
movable stage. (e) Mouse retina patch placed ganglion-cell-side-down on the electrode array. (f) Layers of the mammalian retina: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. MEA, microelectrode array. A series of computations that will occur through the different retinal layers will convert a light stimulus into action potentials, which are generated by the retinal ganglion cells (indicated by green, blue and red cells) in the GCL. These action potentials can be recorded by the MEA. For more details about cell types and retina layers see (Wässle, 2004). (g) Three different spike trains belonging to three different types of retinal ganglion cells (ON type, OFF type, ON-OFF type) in response to the same light stimulus (see Marching Square in Supplementary Material). The white bar represents a light stimulus brighter than the background light level. The gray bar represents a light stimulus darker than the background light level. Every spike train has a different color and belongs to a different retinal ganglion cell type (Fig. 1f).

2.3.4 Optogenetic Stimulation of Retinal Ganglion Cell Types

Adeno-associated viruses encoding a channel rhodopsin variant (Berndt et al., 2009) (AAV EF1α double floxed ChR2-128S-2A) were delivered by intravitreal injection into the eyes of the PvalbCre transgenic mouse (Yonehara et al., 2011). A minimum of 14 days incubation time was kept to achieve channelrhodopsin expression in the Cre-expressing RGCs. In order to identify RGCs expressing ChR2 on the MEA, we blocked photoreceptor-mediated light responses in the retina by the application of a drug cocktail containing 10 μM ABP (2-amino-4-phosphonobutyrate, agonist of the mGluR6 receptors of the ON-pathway), 10 μM CPP (3-[(±)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid, an NMDA receptor antagonist) and 10 μM NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione, a kainate AMPA antagonist). Light stimulation was performed by a flash of 40 ms, with an intensity of ~1.6*1017 photons*cm⁻²*s⁻¹ and a wavelength of 470±20 nm.

During drug application, light-induced action potentials could originate from RGCs expressing ChR2, or from intrinsically photosensitive retinal ganglion cells (ipRGCs) (Berson et al., 2002; Wong et al., 2007). The activity of RGCs expressing ChR2 and the activity ipRGCs could be separated based on the response latency to light stimulation. In control conditions with wild type retinae (without RGCs expressing ChR2), the probability to find light responses with latencies between 0-50 ms was 3.9% (Figure 2-7b). In experiments with PvalbCre transgenic retinae (with RGCs expressing ChR2), we found that 41% of the total response latencies were less than 50 ms (Figure 2-7b).

Therefore, these RGCs in PvalbCre transgenic retinae, with latencies less than 50 ms, were treated as ChR2-responding cells.
2.3.5 Data Analysis

Offline Spike Sorting was performed, on data acquired after an experiment, by a principal-component analysis (PCA) - K-means based algorithm (Lewicki, 1998; Duda et al., 2001).

Online Spike Sorting was performed in order to select a specific population of RGCs during an experiment. For this purpose, we used an independent-component-analysis (ICA) based algorithm (Brown et al., 2001; Jackel et al., 2012) (For details, see Spike Sorting in Supplementary Material).

2.3.6 Imaging of Mouse Retinal Ganglion Cells

For imaging of RGCs we used the retina from the mouse line PvalbCre xThy1Stop-EYFP. The retina was assessed with a Zeiss LSM 700 confocal microscope, 40X oil immersion lens, NA 1.2, ×0.5 digital zoom.

2.4 Results

2.4.1 Light Induced Artifacts in CMOS Technology and Light Evoked Retinal Activity

An important development was the elimination of noise introduced into the recordings by light-induced artifacts; this enabled us to record the electrogenic activity of RGCs without any interference caused by the light stimulation.

The light artifact phenomenon, which can introduce noise or offset in the electrical signal output, is due to light sensitivity characteristics exhibited by the active circuitry of CMOS-based MEAs. It is caused by photon-induced charges in the electronic components such as diodes or transistors. However, most of the short-wavelength fraction of the incident light will not pass the chip passivation layer stack, which consists of alternating layers of silicon nitride and silicon oxide of a total thickness of 1.6 μm and has been additionally applied on the chip surface to protect the chip components against penetration of liquids and associated chemical and electrochemical corrosion. The oxide-nitride layers show absorption below a wavelength between 500 and 450 nm depending on the nitride/oxide chemical composition, stoichiometry and deposition method (Cen et al., 2009). Additionally the retina patch (~250 μm thick) will absorb a part of the incident light.

Two cases have been considered for the device used here: the first includes light falling only on the sensor, i.e., the electrode array area (Figure 2-2a - top row), the second includes light falling also onto the readout circuitry, where the most light sensitive elements are located (Figure 2-2a - bottom row).
In the first case, we found that the electrode area and the underlying switch matrix are not sensitive to the light intensities and wavelengths used in our retinal experiments (Figure 2-2a, top row). In the second case, the surrounding readout circuitry was found to be light-sensitive, and was characterized by a greater sensitivity to longer wavelengths of light (Darmont, 2009) (Figure 2-2a - bottom row). The most light sensitive elements in this device are the cross-coupled diodes, used as pseudo-resistors in the feedback of the amplifiers to achieve a very low high-pass filter cut-off frequency (Frey et al., 2010b). Thus, when light hits the amplifiers, photo-induced charges cause artifacts in the recorded signal by adding offset and noise in the amplified signal (Figure 2-2a – bottom row) and may even saturate the amplifiers or bring the amplified signal out of the range of the analog-to-digital converter.

Finally, to avoid any light-induced artifacts, light was exclusively projected onto the 3.5 mm² electrode array area during the experiments. As such, it was possible to record light-evoked action potentials from RGCs in the absence of light-induced artifacts in the recorded signals (Figure 2-2b).
Figure 2.2. Light Artifact on CMOS Circuitry and Light Response of Retinal Ganglion Cells. (a) Top row: response of 126 recording channels to a light stimulus (represented by yellow-colored square) projected onto the electrode array only. Bottom row: response of 126 recording channels to light projection onto the readout circuitry and onto the electrode array. White bars represent “light on” and gray bars “light off.” Note the absence of artifacts, when light is projected exclusively on the array. The color of the traces corresponds to the following wavelengths and intensities: blue: 460±15 nm; intensity ~ 2.0•10^13 photons cm^2 s^-1; green: 525±23 nm; intensity ~ 3.3•10^13 photons cm^2 s^-1; red: 640±12 nm; intensity ~ 2.3•10^13 photons cm^2 s^-1; gray: intensity ~ 1.2•10^14 photons cm^2 s^-1 (blue, green and red sources active at the same time). (b) An example of ON and OFF light-evoked responses from mouse RGCs, as recorded by the MEA. The white bar indicates the projection of a light stimulus brighter than the background light level (“light on”). The gray bar indicates the projection of a light stimulus darker than the background light level (“light off”). The action potentials as recorded on one electrode are shown. We used the following wavelengths and intensities to elicit light-induced action potentials: blue: 460±15 nm; intensity ~ 2.0•10^13 photons cm^2 s^-1; green: 525±23 nm; intensity ~ 3.3•10^13 photons cm^2 s^-1.
2.4.2 Characterization of Mouse Extracellular Action Potentials

To determine the magnitude and spatial spread of signals produced by mouse RGCs, we analyzed approximately 60,000 extracellular light-evoked action potentials from four different retinal preparations using high-density electrode blocks (3,161 electrodes/mm$^2$).

On the CMOS-based MEA chip used here, single-cell action potentials were consistently detectable on multiple electrodes, and the propagation of action potentials along axons was observable (Figure 2-3a, Figure 2-3b).

Among the electrodes used to record the signal generated by any given cellular action potential, the central electrode (the electrode with the highest signal) recorded peak-to-peak amplitudes of 230±170 μV (Figure 2-3b, Figure 2-3c - Cornehrs left panel). The signal amplitude declined, as one moved away from the central electrode such that on average each action potential was recorded with 14±7 electrodes (Figure 2-3c - right panel). This number of electrodes covers an area equivalent to 65 x 65 μm², and all action potentials recorded within this area had a negative peak exceeding 4.5 standard deviations of the noise level. As expected, the action potential of a single RGC was distributed over a region that is larger than the cell body (Gold et al., 2006), as shown in previous retinal studies (Segev et al., 2004), or in recordings of neurons from other brain regions (Frey et al., 2009). In the mouse retina, the soma diameter of RGCs varies between 10 μm and 30 μm (Sun et al., 2002) explaining the variability observed in the spatial spread of the footprint. Furthermore, the amplitude and spatial distribution of the action potential are not only influenced by the cell morphology and distance to the recording electrodes, but also by the density of voltage sensitive ion channels and the myelination of the axon (Boiko et al., 2001; Gold et al., 2006).

Finally, the soma of mouse RGCs tends to be located within its dendritic field area (Sun et al., 2002), which approximately corresponds to the receptive fields of the cells (Brown et al., 2000). The receptive field of a RGC is the region of space, in which the presence of a light stimulus will alter or cause the firing of that RGC (Meister et al., 1994; Chichilnisky, 2001). Accordingly, we found that the center of the electrical footprint was located within each cells receptive field and the average distance between the central electrode of a footprint and its receptive field center was 120±50 μm (Figure 2-3d, Figure 2-3e).
Figure 2-3. Characterization of Extracellular Action Potentials from Mouse Retinal Ganglion Cells. (a) Superposition of 959 action potentials (gray traces) from six electrodes, indicated in Figure 2-3b. The propagation speed, evident by the staggered timing from the initial depolarization of electrode 1, was calculated to be 0.7 m/s. A biphasic somatic action potential is shown (electrode 1) as well as tri-phasic axonal action potentials (electrode 6). (b) Spatial distribution (footprint) of averaged signals of a single RGC over an area of 0.025 mm2. The thick black waveform indicates highest peak-to-peak amplitude (central electrode). Color code (right corner), yellow-red indicates the region of maximum signal amplitude (active electrodes with action potential negative peak 4.5 standard deviations above the noise level). (c) Left panel: distribution of action potential peak-to-peak amplitudes for 206 RGCs mouse at the electrode with highest signal (230±170 μV). Right panel: distribution of the number of active electrodes for 206 mouse RGCs (14±7 active electrodes). (d) ON and OFF mouse RGC footprints (black action potential waveforms) and their receptive fields. The receptive fields are determined by examining the spike-triggered average (STA) stimulus 60 ms before a spike (Chichilnisky, 2001). These two neurons were recorded on the same subset of electrodes. (e) Top panel: temporal contrast of the STA at the receptive field center. Bottom panel: Distribution of distances between the center of receptive fields and the center of 20 RGCs footprints (120±50 μm).

Chapter 2: Recording Defined RGC Populations
2.4.3 Separation of Retinal Ganglion Cells Action Potentials

In order to characterize the light responses of individual mouse RGCs, action potentials must be correctly assigned to the corresponding neurons. As described above, high-density electrode recording techniques enable the visualization of the electrical footprint of each neuron (Figure 2-3b). In order to assess, whether the additional spatial information of the action potential improves the accuracy of spike waveform assignment, we used a basic spike sorting algorithm, based on Principal Component Analysis (PCA) and K-Means clustering method (Lewicki, 1998) in an off-line procedure.

We compared the results of separating the spike waveforms, obtained from a single electrode, and those from different combinations of neighboring electrodes. (Figure 2-4a, Figure 2-4b, Figure 2-S1).

The quality of spike sorting was assessed by estimating the percentage of refractory period violations (0-2 ms) in the inter-spike interval distributions of the sorted neurons (see Spike Sorting in Supplementary Material and Figure 2-S1). The refractory period is the time needed for the membrane of a neuron to come back to its resting state before a new action potential can be fired. This time is typically between 1-2 ms and implies that the interval between two consecutive action potentials cannot be less than ~ 2 ms. Consequently, the presence of inter-spike intervals of less than ~ 2 ms, among the action potentials assigned to a single neuron, indicate an incorrect spike sorting.

In order to determine how many different neurons were in the recorded waveforms, we used the silhouette coefficients (Rousseeuw, 1987). After PCA and K-Means clustering, every action potential is represented by a score in the PCA space and it is assigned to a cluster (Figure 2-4a, Figure 2-4b, Figure 2-4c). For every action potential, assigned to a cluster in the PCA space, a silhouette coefficient can be computed, which measures how similar that point is to points within the same cluster as compared to points in the other clusters. The silhouette coefficients vary between -1 and 1, which respectively indicate “misclassified” or “well-clustered” data. The clustering solution with the highest median, computed from the distributions of the silhouette coefficients, was chosen as the correct solution (see Spike Sorting in Supplementary Material and Figure 2-S1).

In Figure 2-4a, spike waveforms, which were isolated from only a single electrode, are shown. The best clustering solution, with the highest median silhouette value, produced three groups (Figure 2-4c - top panel, Figure 2-4d - top panel). Each of the three putative RGCs had refractory period violations of 7 %, 3 % and 0 %, respectively.

In Figure 2-4b, the signals simultaneously recorded from the six surrounding electrodes as well as from the central electrode were analyzed together. In this
case, the best clustering solution, with the highest median silhouette value, yielded five clusters (Figure 2-4c - bottom panel, Figure 2-4d - bottom panel). These putative individual cells had no refractory period violations.

The median silhouette value increased from 0.5 to 0.9, when the signals of the six surrounding electrodes were included, suggesting a better grouping of the data (Figure 2-4e).

This improvement is achieved, as the added spatial information increases the separability of the waveforms produced by different neurons within the PCA feature space.
Figure 2-4. Spike Sorting with High Spatial Electrode Density. (a) 939 action potentials isolated from the central electrode (black square). The surrounding electrodes (white squares) where not used. After spike sorting, action potentials were clustered into three groups (red, green, blue clustered waveforms), representing three putative neurons. The percentages of refractory period violations (0-2 ms) in the inter-spike interval distribution were 7%, 3% and 0%, respectively. (b) 939 action potentials isolated from the central electrode (black square in center) concatenated to synchronously-recorded waveforms from six surrounding electrodes. After spike sorting, action potentials were clustered into five groups (red, green, blue, cyan, purple), and none of them had violations in the inter-spike interval distribution. The absence of violations is an indicator of correctness of sorting. (c) Principal component (PC) projection of spike waveforms from Figure 2-4a (top panel) and Figure 2-4b (bottom panel). The PC projection is used to cluster the action potential. (d) Medians of silhouette coefficient distributions as
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The ability to record from high-density blocks allows for recording from a large proportion of the densely packed RGCs of the mouse retina. Indeed, with the MEA system used here, there is more than one electrode available per RGC as the density of electrodes, 3,161 electrodes/mm², is greater than the density of RGCs in the mouse, which amounts to approximately 2,700 cells/mm² (Jeon et al., 1998). As described above, the activity of single neurons is picked up on multiple electrodes, enabling us to regularly identify on average 42±7 neurons simultaneously during light stimulation in an area of 0.025 mm² (Figure 2-5a). An example of the population of cells, from which we recorded, contained some of the expected cell types. This included RGCs that respond to increases in light intensity (ON RGCs, Figure 2-5b-c, Figure 2-5d), decreases in light intensity (OFF RGCs, Figure 2-5e, Figure 2-5f) or to both, increases and decreases in light intensity (ON-OFF RGCs, Figure 2-5g, Figure 2-5h). In addition, RGCs that were sensitive to a precise direction of motion of the light stimulus were characterized (Figure 2-5g, Figure 2-5h and Figure 2-6).

In this specific case, RGCs were characterized based on their preference to an increase or a decrease of light intensity and to the eventual preference toward a defined direction of motion of a moving bar (see Light Stimulation in Supplementary Material).

Thus, the high density of electrodes is useful not only for spike sorting, but increases the possibility to find a RGC of interest in the ganglion cell layer, where these neurons are densely packed and their extracellular action potentials mix on single electrodes. In particular, Figure 2-5a demonstrates that cells with highly overlapping electrical footprints, the receptive fields of which are overlapping to even a much larger extent (see Figure 2-3d), can be detected and assigned.
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Figure 2-5 Physiological Responses of Mouse Retinal Ganglion Cells. (a) Overlapping footprints of 39 different RGCs on a high-density electrode block (3,161 electrodes/mm²). Blue = ON RGCs, red = OFF RGCs, green = ON-OFF RGCs. This shows the possibility of separating RGCs despite physical overlapping of the neuronal extracellular action potential fields. (b) Left panel: mouse RGC footprint (blue) and light stimulus (yellow square, see Marching Square in Supplementary Material). The four small gray squares indicate four locations, spaced 100 μm apart, where the light stimulus was sequentially centered. Right panel: four raster plots showing the RGC response to light stimulation, each dot represents a single action potential. There are four different raster plots, because the light stimulus was centered at four different locations. In each raster plot the response to five repetitions of the same stimulus is shown. The firing rate of the RGC is computed by averaging these five responses.
2.4.4 Selecting a Defined Population of Retinal Ganglion Cells for Extracellular Recordings

Here we demonstrate the ability to target a specific population of RGCs by taking advantage of the high-density packing of the electrodes and high signal-to-noise ratio of the recordings as well as the rapid dynamic configurability of the MEA system.

To show the effectiveness of this procedure we chose to target ON-OFF direction-selective ganglion cells (Barlow et al., 1964; Weng et al., 2005). The main reason for choosing direction-selective cells is that they have a very characteristic response to light stimulation, making them easily identifiable (Figure 2-5g, Figure 2-5h). In fact, ON-OFF direction-selective RGCs respond most vigorously, with a transient burst of action potentials, to light stimuli moving along a preferred direction. Furthermore, they have almost no response to light stimuli moving along the null direction (opposite ~180 degrees to the preferred direction). There are four types of ON-OFF direction-selective RGCs (Barlow et al., 1964; Briggman et al., 2011). (Barlow et al., 1964; Briggman et al., 2011).

In order to target direction-selective RGCs, we first screened a region of interest by recording the activity of all RGCs from a set of highest-density electrode blocks. We scanned six adjacent regions of the ganglion cell layer, spanning an area of 0.15 mm² (Figure 2-6a). During the recording from each electrode
configuration block, a search stimulus consisting of a bar that moved in 8 different directions was presented (see Moving Bar in Supplementary Material).

In the second step, online spike sorting during the experiment was performed (Jackel et al., 2012b) see Spike Sorting in Supplementary Material), and the sorted neurons were physiologically classified according to their response to light stimulation (Figure 2-6b). During the online analysis, each isolated RGC was tested for its preference for ON vs. OFF stimuli and for its preference for a direction of motion.
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Figure 2-6. Strategy to Select and Record from a Defined Population of Retinal Ganglion Cells. (a) A region of interest within the piece of retina is stimulated with light and sequentially recorded from using high-density electrode blocks (3,161 electrodes per mm2). (b) Action potentials from densely packed RGCs are sorted and classified, based on their physiological responses to light stimulation (see Light Stimulation in Supplementary Material and Fig. 5). (c) A defined subset of electrodes
is selected at the location of a RGC of interest; these electrodes are selected so as to obtain the highest signals for each targeted RGC. (d) Polar plots of four types of selected ON-OFF direction-selective RGCs (Briggman et al., 2011). Each tuning curve has been normalized to the largest response. The black arrow indicates the direction, in which the neurons respond most vigorously, the “preferred direction.” (e) Spatial location of ON-OFF direction-selective RGCs (green, red, blue, yellow circles) across an area of six adjacent high-density electrode blocks. Gray circles indicate the remaining detected RGCs that were not classified as ON-OFF direction-selective RGCs. (f) Raster plots (10 repetitions, see Moving Bar in Supplementary Material) from four types of ON-OFF direction-selective RGCs. The gray-shaded rectangles indicate the time, during which the bar was moving across the retina. The black arrow indicates the direction of motion.

Finally, for every RGC of interest, between 5 and 7 electrodes that feature the largest-amplitude signals have been assigned to record from the respective RGCs during the rest of the experiment (Figure 2-6c).

Out of the 212 cells recorded, we found 40 ON-OFF direction selective RGCs and noted their locations (Figure 2-6d, Figure 2-6e). Finally, we targeted electrodes to a subset of ON-OFF direction-selective RGCs with the same preferred direction (Figure 2-6e, Figure 2-6f).

This allows us to simultaneously record from a group of identified cells of a defined type and to characterize not only the individual but also their collective responses to the applied light stimuli. Within 90 minutes, we were able to probe a 0.15 mm$^2$ area of the retina and to select a physiologically-defined population of cells that could then be targeted with specific light stimulation for the next ~7 hours.

2.4.5 Recording from Genetically Identified Retinal Ganglion Cells

Optogenetics is a powerful tool for investigating neuronal cell types and circuits (Yizhar et al., 2011) and an additional way to identify certain cell types. To demonstrate that optogenetics can be used in combination with our CMOS-based MEA we expressed a bi-stable channelrhodopsin (bi-ChR2) (Berndt et al., 2009) in genetically identified RGCs (see Methods). bi-ChR2-induced action potentials (Figure 2-7a) were isolated from photoreceptor-triggered action potentials by blocking glutamatergic synaptic transmission (ABP, CPP, NBQX, see Methods). In the presence of these blockers, only RGCs expressing ChR2 and the intrinsically photosensitive RGCs (ipRGCs) produced light-induced action potentials. ChR2-induced neuronal firing features shorter response latency than the latency of ipRGCs (Figure 2-7b).
2.5 Discussion

Retinal circuits encode the visual scene in parallel channels, where each RGC type conveys a different representation (Roska and Werblin, 2001a; Farrow and Masland, 2011a). The relative timing of activity among RGCs has been shown to be relevant for visual encoding (Ackert et al., 2006; Schwartz et al., 2007; Gollisch and Meister, 2008). Furthermore, the study of physiologically identified ganglion cells types demonstrates that time correlations convey significant information about the visual stimulus (Ackert et al., 2006; Pillow et al., 2008). The ability to target specific populations of RGCs is, therefore, a prerequisite for the design of experiments, aimed at understanding the population code of individual as well as combinations of visual channels.

In order to select defined populations of RGCs, we found the following process to be efficient: scanning the MEA for activity characteristic of the RGCs of interest, performing online spike sorting, and, finally, selecting a configuration
of electrodes that could most effectively be used to stimulate and record from chosen RGCs.

Elimination of the light artifact in the recorded signal greatly facilitated the quality of the data recorded from the RGCs. This is a pivotal finding, as the activity of the RGCs must be triggered by a light stimulus, while light-induced responses are simultaneously recorded by the MEA. Any artifact that would occur during recording would obscure or add noise to the extracellular action potentials. As a consequence, spike sorting could be rendered unnecessarily challenging with the possibility to completely miss action potentials. As described, the proper focusing of the light stimulus on the electrode array ensures that no artifacts occur.

In order to select a defined population of neurons, a fast and efficient online spike sorting technique is essential to classify the different cell types online during the experiment. To that end, we are currently also exploring spike sorting methods based on ICA (Harris et al., 2000; Brown et al., 2001) and template matching (Segev et al., 2004; Franke et al., 2010), which can optimally utilize the information provided by larger numbers of electrodes per neuron and concurrently reduce signal redundancy.

Furthermore, we here demonstrate that a basic and, admittedly, simple spike sorting algorithm (K-means – and use of silhouette coefficients) can then be used afterwards offline to separate action potentials from highly overlapping neurons of the retinal ensemble due to the fact that (i) the electrode with highest signal-to-noise ratio can be selected for every neuron for event detection, and (ii) the unique spatial distribution of the action potentials of every neuron, in the highly overlapping retinal ensemble, can be characterized.

This combination of spike sorting methods at different stages has proven to be very successful.

The quality of the spike sorting, finally, is critically depending on the signal quality (signal-to-noise ratio) and the electrode spacing, i.e., the average number of electrodes available to record from a neuron, with every mouse RGC being detectable here, on average, in an area of 65 x 65 μm². This MEA features an inter-electrode spacing of 18 μm, and noise levels of ~ 7 μVrms. Besides the possibility to potentially record from every neuron (electrode density larger than neuronal density), the overall surface that is covered with electrodes is an important parameter, as it is desirable to monitor many neurons of the same distinct type to investigate colony or population responses. (For more details and a comparison between different MEA devices, please see also Figure 2-S2 in the Supplementary Material).

Relative to devices previously used for retinal studies, Zeck et al. recently published a study on axonal action potentials in the rabbit retina using a CMOS-based MEA (Zeck et al., 2011). The device featured 16,384 sensor transistors at spatial resolution of 7.4 μm (16384 electrodes/mm²) on an area of 1 x 1 mm² and
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a sampling rate of 6 kHz (Lambacher et al., 2011). The work by Zeck et al. demonstrates the importance of using a MEA that features a high density of electrodes for studying the propagation speed of action potentials along axons of different RGCs types. The MEA used by Zeck et al. features noise levels from 50 to 250 μVrms, which allows the isolation of action potentials with amplitudes as low as 200 μV (Lambacher et al., 2011). Such noise levels render the characterization of small RGCs with low-amplitude action potentials difficult.

The CMOS-based MEA by Berdondini et al. has 4,096 on an area of 2.67 x 2.67 mm², a sampling rate of 7.8 kHz and noise levels of ~ 11 μVrms (Berdondini et al., 2009). The use of light stimulation with this device has not yet been reported. The density of mouse RGCs (2,700 cells/mm², (Jeon et al., 1998) is about 4.7 times higher than the MEA electrode density (567 electrodes/mm²). As a consequence, finding and targeting a defined population of mouse RGCs as well as to accurately sort their action potentials would be difficult.

The MEA system designed by Litke et al. was successfully used to record the activity of defined populations of RGCs and to study photoreceptor connectivity in the periphery of the macaque monkey retina (Litke et al., 2004; Pillow et al., 2008; Field et al., 2010). The circuitless device used by Litke et al. features 512 electrodes at a spatial resolution of 60 μm (321 electrodes/mm²) on an area of 1.7 mm² and a sampling rate of 20 kHz. In the context of the mouse retina, a spatial resolution of 60 μm decreases the number of electrodes available per neuron, which decreases the performance of the spike sorting and, therefore, reduces the possibilities to allocate the densely packed RGCs.

The circuitless MEA systems used by Segev et al. features 30 electrodes at a spatial resolution of 30 μm (1,111 electrodes/mm²) over an area of 0.12 x 0.15 mm² (Segev et al., 2004). Although this MEA device features a comparably high electrode density and low noise levels, it is limited by the low overall number of electrodes that allow the study of only small retina regions.

In summary, the developed methodology to find, locate, and to selectively record from or target a distinct type of neuronal cell that can be identified, either according to the respective signaling behavior upon specific stimuli, or by applying optogenetic methods, constitutes an important neuroscientific tool. This tool can be used to investigate population-specific signaling in different types of preparations, including retinae, brain slices, or dissociated neuronal cultures. We demonstrated that distinct neuronal populations (e.g., all 4 different types of ON-OFF direction-selective cells) can be assigned and discerned in the retina. Furthermore, we demonstrated our technique in the mouse, where genetic tools are available and genetically identified retinal ganglion cell types have been identified (Kim et al., 2008; Huberman et al., 2009; Münch et al., 2009). In future studies, this developed cell identification-and-targeting system will be used to target defined physiological types of RGCs in an effort to decode elements of the retinal code. Recording of synchronous action potentials from defined cell types will lead to a more complete understanding of how the retina
as a whole encodes a visual scene and what the code is that the brain then uses to interpret the retinal data (Pillow et al., 2008).

2.5.1.1 Acknowledgements
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2.6 Supplementary Methods

2.6.1 Light Stimulation
Three types of light stimuli were used to induce light-evoked activity and characterize RGCs:

**Marching square**: a square (200 μm x 200 μm) was flashed on a “gray” background (alternating between 2 seconds “white” and 2 seconds “black”). The square was moved along one axis in a discrete series of sequential 100 μm steps. This stimulus was used to classify ON, OFF and ON-OFF RGCs.

**Moving bar**: a bar (500 μm wide x 1000 μm long) was moved in eight directions, radially spaced at 45° or 22.5° intervals, on a “gray” background (velocity 800 μm/second). The stimulus had a positive contrast (“white” on “gray” background). This stimulus was used to classify direction-selective RGCs.

**White noise**: a random flickering checkerboard stimulus (20 pixels x 20 pixels, pixel size: 50 μm x 50 μm) was projected at 30 Hz. The stimulus was shown for 45 minutes and set to a contrast of 100%. This stimulus was used to characterize RGC receptive fields (Chichilnisky, 2001).

2.6.2 Spike Sorting
We applied two different procedures to sort extracellular action potentials: an online spike-sorting algorithm that was used during experiments and an offline spike-sorting algorithm for data analysis after the experiments. In both cases, the input data consisted of voltage traces that were recorded at a time resolution of 50 μs and band-pass filtered (500 Hz-3 KHz).
2.6.3 Offline Spike Sorting

The procedure is briefly outlined here (Jackel et al., 2012b):

(1) spikes were isolated from a single electrode trace by threshold detection at 4.5 standard deviations above the noise level;

(2) at the occurrence of every isolated spike, synchronously-recorded waveforms from 6 neighboring electrodes were concatenated;

(3) principal-component analysis (PCA) was applied to the concatenated waveforms;

(4) the extracted PCA scores of the first five principal components were clustered by the popular hard partition method K-Means (Lewicki, 1998; Duda et al., 2001);

(5) quality checks of the resulting clusters, each containing spikes from a putative RGC (unit), were performed manually:

- the percentage of refractory period violations in the inter-spike interval distribution of each unit was computed in order to determine the accuracy of the sorting (Figure 2-S1a) (Segev et al., 2004).

- the silhouette coefficients were used to estimate how many neurons were present in the clustered data (Rousseeuw, 1987) (Figure 2-S1a). The silhouette coefficients vary between -1 and 1, which respectively indicates “misclassified” and “well-clustered” data;

- to compare two sets of clustered data, we used the adjusted Rand Index (Hubert and Arabie, 1985) (Figure 2-S1b); the Rand index is used to compare partitions, and in this specific case, it was used to compute the similarity between two identical sets of action potentials that were clustered under different conditions (i.e., different number of electrodes used, or different number of clusters). If the output of the action potential clustering is identical for the two different conditions, the Rand Index will be equal to 1. Oppositely, if the output of the action potential clustering is totally different for the two different conditions, the Rand index will be equal to 0.

(6) steps 1-5 were repeated for every trace that was recorded within the high-density electrode block. Due to the detection of signals of each RGC on multiple electrodes, there was a high degree of redundancy in the data. An automatic algorithm compared the spatial distribution of extracellular action potentials to the percentage of common time stamps in order to merge the redundant units.

All data analysis steps were performed using the software application MATLAB™.
2.6.4 Online Spike Sorting:

The procedure is briefly outlined here (Jackel et al., 2012b):

1. light-induced activity was recorded over a region of interest by sequential use of high-density electrode blocks (3,161 electrodes/mm²);

2. for every electrode block, FastICA (Hyvärinen, 1999) was used to obtain the independent components (ICs) (Brown et al., 2001);

3. spike waveforms in each independent component (IC) were extracted by threshold detection at 4.5 standard deviations of the noise level;

4. spikes from different neurons were expected to have different shape in the IC signal. Thus, we classified the spikes based on their IC waveform. Principal-component analysis (PCA) was applied to the extracted waveforms;

5. the extracted PCA scores from the first five principal components were clustered using the expectation-maximization (EM) algorithm Klustakwik (Harris et al., 2000);

6. for every sorted RGC of interest, we stored the coordinates of 5-7 active electrodes with highest signal amplitude;

7. finally, only electrodes underneath the RGCs of interest were activated for simultaneous recording of the selected population.
Figure 2-S1 (related to Figure 2-4). Spike Sorting Evaluation. (a) Medians of silhouette coefficient distributions as function of the number of electrodes (electrode count: 1-7) and for different numbers of clusters (cluster count: 2-10). The highest median value of silhouette distribution (with a minimum number of violations in the interspike interval distributions) was obtained for the combination of 5 clusters and 7 electrodes (indicated by arrow and shown in Figure 2-4b). Color code (right corner): red indicates large violations in the interspike interval distributions of the clustered waveforms. (b) Rand index as function of the number of electrodes (electrode count: 1-7) and for different numbers of clusters (cluster count: 2-10). All possible combinations of clusters obtained as function of the number of electrodes (electrode count: 1-7) and for different numbers of clusters (cluster count: 2-10) were compared to the clustered data in Figure 2-4b (best clustering output). Color code (right corner): red indicates high median silhouette values.
2.6.5 Features of MEAs

Here, the different MEAs cited in the manuscript, are listed and shown. The genetically defined RGC population example, chosen for this comparison from literature (Kim et al., 2008), consists of direction-selective cells that are sensitive to upward motion. Our measurements evidence that mouse RGC action potentials are detectable, on average, over an area 65 x 65 μm². This number depends on device signal-to-noise levels and signal amplitudes: in case that signal-to-noise gets worse, the area also shrinks.

Figure 2-S2: Electrode Densities and Overall Array Area of Different MEA Systems. Left: Overall array area represented as red surface, all areas are scaled with regard to the 1mm-bar at the left bottom. Right: Electrode densities and distribution of an exemplary type of RGCs (direction-sensitive cells, sensitive to upward motion) according to Kim et al. 2008. Red dots indicate electrode locations; Black objects indicate putative RGC soma locations. The RGC type, density and spatial distribution is according to Kim et al. 2008.
Chapter 3 A Method for Electrophysiological Characterization of Hamster Retinal Ganglion Cells Using a High-Density CMOS Microelectrode Array

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Abstract

Knowledge of neuronal cell types in the mammalian retina is important for the understanding of human retinal disease and the advancement of sight-restoring technology, such as retinal prosthetic devices. A somewhat less utilized animal model for retinal research is the hamster, which has a visual system that is characterized by an area centralis and a wide visual field with a broad binocular component. The hamster retina is optimally suited for recording on the microelectrode array (MEA), because it intrinsically lies flat on the MEA surface and yields robust, large-amplitude signals. However, information in the literature about hamster retinal ganglion cell functional types is scarce. The goal of our work is to develop a method featuring a high-density (HD) complementary metal-oxide-semiconductor (CMOS) MEA technology along with a sequence of standardized visual stimuli in order to categorize ganglion cells in isolated Syrian Hamster (*Mesocricetus auratus*) retina. Since the HD-MEA is capable of recording at a higher spatial resolution than most MEA systems (17.5 μm electrode pitch), we were able to record from a large proportion of RGCs within a selected region. Secondly, we chose our stimuli so that they could be run during the experiment without intervention or computation steps. The visual stimulus set was designed to activate the receptive fields of most ganglion cells in parallel and to incorporate various visual features to which different cell types respond uniquely. Based on the ganglion cell responses, basic cell properties were determined: direction selectivity, speed tuning, width tuning, transience, and latency. These properties were clustered to identify ganglion cell types in the hamster retina. Ultimately, we recorded up to a cell density of 2780 cells/mm² at 2 mm (42°) from the optic nerve head. Using five parameters extracted from the responses to visual stimuli, we obtained seven ganglion cell types.

3.1 Introduction

Knowledge of cell types and corresponding functions in the retina provide insight into treatments for neurodegenerative diseases of the retina (Lagali et al., 2008; Bramall et al., 2010; Wright et al., 2010; Doroudchi et al., 2011). Many retinal diseases, such as age-related macular degeneration and retinitis pigmentosa, cause irreversible and progressive damage to the photoreceptors in the retina (Hartong et al., 2006); this group of cells initiates the sequence of events required for vision (Schmidt et al., 2008; Lorach et al., 2013). The photoreceptors convert visual stimuli into analog, graded-potential electrical signals that cascade through several layers of signal-processing cells to the retinal ganglion cell layer, which ultimately sends the visual signals to the brain through the optic nerve (Dowling, 2012). The ganglion cell layer has been found to contain 15–24 different types of ganglion cells (Masland, 2001; Brien et al., 2002; Rockhill et al., 2002; Dacey et al., 2003; Kong et al., 2005; Völgyi et al., 2009; Sanes and Masland, 2015). The development of methods to analyze
and assess the precise physiological properties of ganglion cell types is essential for the understanding of retinal functionality and for prosthetic device technology (Chuang et al., 2014).

One method of functionally classifying the ganglion cells in the retina is to record the neuronal responses of ganglion cells while exposing them to visual stimuli. Research in electrophysiological characterization of the retina using visual stimuli dates back to 1938, when Hartline first demonstrated the existence of two types of frog ganglion cell types that responded to either bright or dark visual stimuli (Hartline, 1938). Since 1938, the proliferation and advancement of microelectrode-array (MEA) technology has made it possible to record from groups of cells; for a review of MEA platforms, see Obien et al. (2015). The MEA is an important tool that enables the recording of extracellular signals at high spatiotemporal resolution. MEAs have been used in retinal research for recording from significant fractions of cell populations (Meister et al., 1994; Devries and Baylor, 1997; Frechette et al., 2005; Farrow and Masland, 2011). However, extracting responses of individual ganglion cells is not trivial, as each electrode of the MEA will usually detect voltage signals generated by many ganglion cells, resulting in the mixing of the signals (Lewicki, 1998); therefore, spike sorting techniques must be applied to assign action potentials (or spikes) to the ganglion cells from which they originated. To maximize the accuracy of spike sorting, it is desirable to have the greatest possible number of electrodes relative to number of ganglion cells (Lewicki, 1998; Einevoll et al., 2012; Franke et al., 2012; Jäckel et al., 2012). When the MEA electrode density is too low within a given region of the retina, ganglion cells with low-amplitude signals, or those that are located in-between and/or distant from the electrodes are likely to be undetected or discarded by the spike sorting algorithm. Standard MEA systems are therefore somewhat limited in their ability to record from most of the ganglion cells of mammalian retinæ; the ganglion cell density in the sections of hamster retina, for example, range up to approximately 3000 neurons/mm², at a 2 mm eccentricity from the optic nerve head (Tiao and Blakemore, 1976).

To maximize the number of cells recorded in the ganglion cell layer, we used a complementary metal–oxide–semiconductor (CMOS) based high-density MEA (HD-MEA). The HD-MEA is ideal for retinal recordings due to its electrode density of 3150 electrodes/mm². It has a total of 11,011 electrodes, and the selection of recording electrodes can be changed within milliseconds by means of a flexible switch matrix, which resides below the electrode array. The switch matrix capability allows for querying different regions of the retina within a 2 × 1.75 mm² area. A low recording noise level of 2.4 μV_RMS in the action potential band (100 Hz–10 kHz) enables the recording of low-amplitude neuronal signals (Frey et al., 2010).
We used visual stimuli to stimulate ganglion cells in the hamster retina for the purpose of electrophysiological characterization: flashing squares were used for determining ON and OFF responses, and various moving bars were used for direction selectivity and spatiotemporal-dependent response component measurements. The stimuli were chosen to obtain the necessary responses from as many ganglion cells as possible during the limited time that the \textit{ex-vivo} tissue was viable. Our goal was to maximize the throughput of our model by avoiding analysis and computation during the experiment that was previously necessary in other characterization studies (Carcieri et al., 2003; Zeck and Masland, 2007; Farrow and Masland, 2011).

### 3.2 Materials and Methods

#### 3.2.1 Tissue Extraction and Preparation

Eleven-week-old Syrian Hamsters/\textit{Mesocricetus auratus} (Janvier Labs, France) were anesthetized and sacrificed under protocols that were approved by the Basel-City Veterinary office, in accordance with Swiss federal laws on animal welfare. Each hamster was kept in darkness for 10 min, anesthetized (Telazol 30 mg/kg, Xylazine 10 mg/kg) and decapitated. Retinæ from both eyes were immediately removed under dim red light and immersed in Ames' Medium (8.8 g/L, supplemented with 1.9 g/L sodium bicarbonate: Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland), which was perfused with room-temperature Oxycarbon (PanGas AG, Dagmersellen, Switzerland) for at least 30 min before the optical stimuli sequence was started. To keep track of the anatomic orientation of the retina, the cornea was punctured just below the superior corneal limbus following removal of the eye from the animal, and a cut through the retinal tissue was made from the puncture location to the optic nerve head. The cornea was cut away, and the lens was extracted. The sclera was gently separated from the retinal tissue, and the remaining vitreal material was removed from the epiretinal surface; the retinal pigment epithelium was completely removed, as it would otherwise have obstructed the light path of the optical stimulus. A 1.5 $\times$ 1.5 mm$^2$ section was cut from the superior nasal or superior temporal region, near the distal edge of the retina, and the tissue section was placed on the HD-MEA (see \textbf{Figure 3-1}). The retinal section was placed such that the ganglion cell layer (epiretinal side) was in contact with the HD-MEA surface, and the optical stimuli were focused directly onto the photoreceptor layer; this anatomical orientation was maintained for each experiment.

#### 3.2.2 Physiological Apparatus

As shown in \textbf{Figure 3-1}, the HD-MEA was packaged by affixing a polycarbonate ring to it with epoxy, forming a well with a volume capacity of approximately 1 mL; the electrode array was located at the bottom of the well.
The electrodes were coated with platinum black by electrodeposition so as to maximize the signal-to-noise ratio (lower electrode impedance) and to reduce photoelectric effects caused by the visual stimuli (Novak and Wheeler, 1986; Kim and Oh, 1996; Maher et al., 1999; Chang et al., 2000; Mathieson et al., 2004; Fiscella et al., 2012). A screw-mounted meshwork could be raised or lowered manually to apply sufficient pressure to hold the retinal tissue in place on the HD-MEA (retinal tissue on the MEA is shown in Figure 3-1). To maintain viability of the tissue, a gravity-flow system provided oxygenated Ames' Medium (see previous paragraph regarding physiologic solution) at a flow rate of 2.5 mL/min. The solution was heated to 35°C with a PH01 heated perfusion cannula (Multi Channel Systems MCS GmbH, Germany) and then directed with a plastic duct (length 1 cm; inner diameter 1.0 mm) onto the meshwork, adjacent to the subretinal (photoreceptor) side of the tissue, to oxygenate the retina as well as to flush metabolites produced by it. The perfusate was continually removed throughout the experiment by a suction needle, which was located at the surface of the solution bath.

Figure 3-1: HD-MEA chip. Shown in the center of the chip is a sample of retina with a cutaway showing part of the microelectrode array (1.8 x 2 mm²) that lies underneath the retina piece; however, during an experiment, the MEA is fully covered by the retinal tissue. Around the MEA, the readout circuitry can be seen. Translucent epoxy packaging protects the periphery of the chip and the bond wires from liquid contact.
3.2.3 Light Projection

Light stimuli were programmed in a Matlab (The Mathworks, Natick, MA)-integrated program, Psychtoolbox (http://psychtoolbox.org). The stimuli were sent to a LED projector (Acer K10), which had a refresh rate of 60 Hz, the output of which was spatially reduced by two camera lenses (Nikkor 60 mm 1:2.8 G ED, Nikon), dimmed in intensity by a 10x neutral density filter, deflected by 90° with a mirror (U-MBF3, Olympus), and focused with a 5x objective lens (LMPLFLN5X Olympus). The final image on the MEA surface was 1 \times 1 \text{ mm}^2 and had the following intensity range: blue: 460 \pm 15 \text{ nm}; intensity of 2.0 \times 10^{13} \text{ photons cm}^{-2} \text{ s}^{-1}; green: 525 \pm 23 \text{ nm}; intensity of 3.3 \times 10^{13} \text{ photons cm}^{-2} \text{ s}^{-1} as in Fiscella et al. (2012). The photopic intensity of the image could be controlled in 256 discrete steps. A single pixel of the image corresponded to an optical square of approximately 1.7 \times 1.7 \mu \text{m}^2 at the retinal surface. Only blue and green channels in the projector were used because Syrian hamsters are dichromats and are not sensitive to wavelengths in the red spectrum (Jacobs, 2002).

A video camera (Leica Camera AG) was connected to the microscope so that the HD-MEA surface could be viewed and monitored in real-time for focusing and spatial adjustment of the image on the retina. The HD-MEA was mounted on a computer-controlled moveable stage platform (Scientifica), which was moved within the x-y plane by a joystick for the purpose of aligning the center of the recording electrode configuration with the center of the projected image. This adjustment ensured that the selected recording electrodes were always in the same position relative to the projected optical stimuli.

Visual stimuli frame change timestamps were sent from the stimulation computer to a field-programmable gate array (FPGA) and recorded in parallel with the electrode data stream. This information made it possible to synchronize the projected stimuli with the recorded data in the post-hoc experimental analysis steps.

3.2.4 Apparatus Control

Four computers were used to control the apparatus. One computer running Red Hat Enterprise Linux (RHEL) was used for running the software for displaying the MEA output, for sending commands to the HD-MEA chip, and for recording data from the HD-MEA. A second computer running RHEL was used to run Psychtoolbox and send the visual stimulus frames to the projector. A third computer running RHEL was used to run Matlab for simple computations.
required during the experiment. Finally, a Windows XP computer displayed the real-time feed from the video camera; this computer also ran Scientifica® software to control the moveable stage along the x-, y-, and z-axes.

### 3.2.5 Microelectrode Array Recordings

The data recorded on the HD-MEA were sampled at 20 kHz, and filtered on-chip, approximately between 0.3 Hz and 14 kHz. Prior to data post-processing, all of which was done in Matlab® (Natick, MA, USA), data were filtered with a 300 Hz high-pass filter and 8 kHz low-pass filter to reduce DC offset effects and high-frequency noise.

### 3.2.6 Experiment Preparation

Using a 1 mL pipettor tip (the end of the tip was cut off), the retinal segment was placed on the HD-MEA and aligned to the electrode array (see Figure 3-1) by removing liquid from around the retinal segment with a pipettor. The screw-mounted meshwork was lowered until it made contact with the subretinal surface. The tissue was immediately perfused with Ames' Medium, as described above, which was heated to 35°C with a TC01 temperature controller (Multichannel systems, Reutlingen, Germany). A 5-mm coverslip (Gerhard Menzel GmbH, Braunschweig, Germany) was placed between the microscope objective and the meshwork to maintain an optically aberration-free transition zone from the air to the liquid. The HD-MEA chip was plugged into the interfacing circuit board (the Neurolizer) to initiate communication with the HD-MEA. The position of the moveable stage, to which the Neurolizer was secured, was adjusted so that the center of the HD-MEA was visible in the center of the video monitoring screen. The image was then focused on the subretinal side of the retina by moving the objective lens. The retinal segment was allowed to acclimate to the MEA under a 50% contrast background for 20 min.
To find the region of the retinal segment with the greatest number of active ganglion cells, a gray scale natural movie of mice moving in a cage was projected onto the center of the retinal segment. While the movie was being projected, a sequence of 12 non-overlapping recording electrode groups (or configurations) of 126 electrodes (area of $65 \times 578 \mu m^2$; 0.0374 mm$^2$) was used to record ganglion cell activity for 30 s each at 12 different regions of the retinal segment. All spikes produced by the ganglion cells within each recorded region were extracted by spike thresholding, and peak-to-peak amplitudes of these detected spikes were computed on all electrodes (Figure 3-2). The maximum peak-to-peak amplitude on each electrode was computed, and plotted on a heat map. The plot of the electrode configuration with the greatest number of local maxima (representing putative ganglion cells) was selected as the configuration to be used for the duration of the experiment.

**Figure 3-2: Activity scan.** The location on the retina piece with the greatest number of ganglion cells was selected for each experiment. The electrodes of the MEA are shown as gray squares; the retina lies on top of these electrodes, 126 of which can be selected to simultaneously record ganglion cell activity. In this case, each rectangle shown in this image was generated by recording from a selected group, or configuration, of 126 electrodes. The colors represent the normalized peak-to-peak amplitude of the neurons found in each region.
3.2.7 Light Stimuli

The projected image was centered on the selected electrode region, and visual stimuli were run sequentially. Prior to each stimulus, a 50% contrast background was projected onto the retinal segment for a period of 5 min so that the cells could adapt to the mean projected photopic level. The stimuli were then shown sequentially, and a new data file was automatically started for each new stimulus.

**Marching Square Over Grid**

The Marching Square Over Grid stimulus was used to find receptive field centers and to characterize ON-OFF response of each ganglion cell. The stimulus featured a $100 \times 100 \, \mu m^2$ bright square that flashed at 81 non-overlapping pseudo-randomly-ordered locations over a $900 \times 900 \, \mu m^2$ area. The stimulus presentation was 2 s in duration, and five repetitions were used. Complete stimulus set duration: 27 min.

**Narrow Moving Bars**

Bright bars were used to determine the direction-selectivity properties of ganglion cells (Kanjhan and Sivyer, 2010). The bars moved along their longest axis, or width (width 1000 \, \mu m, length 500 \, \mu m), in eight radial equally-spaced directions with offsets of -250, 0, and 250 \, \mu m orthogonal to the vector of travel for each radial direction. The bars moved with a speed of 600–1200 \, \mu m/s (12.5 and 25 visual degrees/s). The purpose of the lateral offsets was to stimulate in all eight directions at every location on the selected region of retina. The bar closest to the receptive field center for each ganglion cell in each direction was then selected *post-hoc* to analyze each ganglion cell response. Complete stimulus set duration: 45 min.

**Width Test**

Bright and dark rectangular bars of a range of widths (75, 150, 300, 600, 900 \, \mu m) and maximum length were used to obtain the spatial frequency response of the ganglion cells. The bars moved along the direction of the shortest axis in four equal radially-spaced directions. The bar movement speed was 150 and 900 \, \mu m/s (3 and 19 visual degrees/s). These speeds were selected to obtain responses from both ganglion cells that responded to fast and slow stimuli. Complete stimulus set duration: 75 min.
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Speed Test

Bright and dark rectangular bars (widths: 150, 600 μm) were shown at different speeds to assess the temporal frequency response of the ganglion cells. The bars moved along the direction of their shortest axis in four equal radially-spaced directions. The bars moved at speeds of 150, 300, 600, 900, 1200, and 1800 μm/s (3, 6, 13, 19, 25, and 38 visual degrees/s). Complete stimulus set duration: 60 min.

3.2.8 Data Analysis

Spike Trains from Raw Data

Signals from many ganglion cells were present on the majority of the array electrodes in a typical recording, therefore, data processing was necessary to obtain the spike trains from each neuron. Spikes were thresholded at 3.5 times the standard deviation of the baseline signal between spikes. Groups of seven recording electrodes were selected sequentially, and above-threshold spikes were demixed using principle component analysis (PCA; Lewicki, 1998; Jäckel et al., 2012). The demixed signals were used to generate a spike-triggered average extracellular action potential (STA-EAP), which served as a spike waveform template for each neuron (Figure 3-3). Finally, a template matching function scanned the entire recording to extract timestamps for each neuron (Franke et al., 2015).

Data Quality

As a quality control check for the template matching algorithm, all multi-electrode templates generated in the previous step were compared in a pairwise fashion by computing the cross-correlation of the templates for each pair. Pairs that had a correlation coefficient of over 90% were determined to be duplicates; the multi-electrode templates of these pairs were visually inspected; and the number of spike time violations, or instances where a spike occurred within the refractory period of the ganglion cell, were computed. Spike time violations were not allowed to exceed 1% of the total number of spikes. Pairs that met the inspection and violation tolerance criteria were merged.
3.2.9 Parameter Calculation

Response Latency

The latency index describes the delay between the appearance of the stimulus and the time at which the peak firing rate occurs. This index was computed by integrating over a window of 25 ms and measuring the length of time required to reach the maximum firing rate (Farrow and Masland, 2011). Units: seconds.

Transience Index

The transience index indicates how long the ganglion cell continues to spike after the presentation of the stimulus. For each ganglion cell, the response to a flashing square was used to construct the post-stimulus time histogram (PSTH) with a duration of 1.5 s. The integral of the PSTH was normalized by dividing...
the area under the curve by 1.5 (Farrow and Masland, 2011). Range: 0 for an infinitely brief response; 1.0 for a continuous response during the 1.5 s post stimulus period.

**ON-OFF Bias Index**

The bias index (BI) describes the degree to which a ganglion cell responded to a bright or dark stimulus presentation. This index was computed using Equation (1), where \( ON \) is the spike count in response to a positive contrast stimulus, and \( OFF \) is the spike count in response to a negative contrast stimulus. Range: −1 for 100% OFF response; 0 for ON-OFF response; +1 for 100% ON response.

\[
BI = \frac{(ON - OFF)}{(ON + OFF)}
\]  

(1)

**Receptive Field Diameter**

The receptive fields for all cells were mapped using the Marching Square Over Grid stimulus. The mean number of spikes in response to the flashing square were counted per location to determine where the ganglion was most sensitive to a light stimulus. To compute the mean receptive field area, the Marching Square Over Grid stimulus response map was upsampled nine times and thresholded at 33% of the maximum value. The pixel count within this area was divided by the total pixel count and scaled to the total projected area of the Marching Square Over Grid stimulus to provide the receptive field area, \( A \). The mean diameter, \( d \) (units: \( \mu m \)), was computed as follows:

\[
d = \sqrt{\frac{4A}{\pi}}
\]  

(2)

**Direction Selectivity Index**

A direction selective ganglion cell responds maximally when the stimulus moves in a given direction across its receptive field. To compute this index, the mean firing rate during the presentation of bright bars moving in eight equally spaced radial directions was determined, and the vector average, \( D \), was calculated, as shown in Equation (3). \( v \) is defined is the firing rate vector, and \( r \) is defined as the firing rate value. Range: 0 for no directional selectivity; 1 for a unidirectional response (Taylor and Vaney, 2002).
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\[ D = \left| \frac{\sum \hat{V}_i}{\sum \hat{r}_i} \right| \]  

(3)

Preferred Speed Index

The preferred speed index indicates the preferred ganglion cell speed. During the Speed Test, moving bars were presented at different speeds. The mean peak firing response was computed at each speed, and the weighted mean of the responses was computed to obtain the peak response speed. The resulting preferred speed for each ganglion cell represented the speed at which the firing rate of the bar was greatest, across the parameter space of bar contrast and bar movement direction. Range: 0 for response to minimum speed; 1 for response to maximum speed.

Preferred Width Index

The preferred width index indicates the preferred ganglion cell width. During the Width Test, moving bars were presented at different widths. The mean peak firing response was computed at each width, and the weighted mean of the responses was computed to obtain the peak response width. The resulting preferred width for each ganglion cell represented the width at which the firing rate of the bar was greatest, across the parameter space of bar contrast and bar movement direction. Range: 0 for minimum width; 1 for response to maximum width.

3.2.10 Parameter Clustering with K-means

The following calculated indices or parameters extracted for each cell were clustered using k-means to sort the cells based on their electrophysiological responses: “Bias Index,” “Latency,” “Transience,” “DS-Index,” “Speed Index.” We ran this clustering algorithm with a preset k-value ranging from 4 to 25 and with 5000 repetitions to avoid local minima.

To assess the optimal number of clusters, we computed the Silhouette value of each member within the clusters and computed the mean Silhouette value for each group, according to Equation (4). The Silhouette value is a measure of separation between a given point and the other clusters of which it is not a member (Rousseeuw, 1987).
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The formula for the silhouette value of one point, point $i$, within the dataset is shown above. $b_i$ is the lowest mean dissimilarity (or distance) between the point $i$ and all points in another cluster, of which $i$ is not a member. $a_i$ is the mean dissimilarity, or distance, compared to all other points in the same cluster. If $b_i$ is much larger than $a_i$, point $i$ is well-separated from the other clusters and $S_i$ will approach a value of one. If, however, the opposite is true, and $b_i$ is much smaller than $a_i$, point $i$ is not well-separated from the other clusters and has probably been mis-assigned (Kaufman and Rousseeuw, 1990). We selected the number of cluster groups with the highest mean Silhouette value as the most well-separated number of clusters.

To confirm the ganglion cell type clusters obtained in the above steps, we computed the Fisher's linear discriminant for data points between all groups in a pair-wise manner. First, we computed the axis between the mean locations of each pair of clusters in 5-dimensional space (with each dimension corresponding to each parameter space for “Bias Index,” “Latency,” “Transience,” “DS-Index,” “Speed Index”). We then projected data points from each group onto this 1-dimensional axis and fitted a Gaussian function to the histogram of each group. To obtain the separation parameter, we used the following Equation (5):

$$d \over \sqrt{\sigma_1 \sigma_2}$$

where $d$ is the distance of the axis between the two group means in 5-dimensional space ($\mu_1$ and $\mu_2$, respectively), $\sigma_1$ is the standard deviation of the Gaussian fit for the first group, and $\sigma_2$ is the standard deviation of the Gaussian fit for the second group.

### 3.3 Results

Two hundred sixty two ganglion cells that responded to visual stimuli were recorded in seven separate experiments. The ganglion cells were stimulated with the following visual stimuli: Narrow Moving Bars, Width Test, Speed Test, and Marching Square Over Grid (as described in Section Materials and
Methods) in a procedure that required no spike sorting or detection of the receptive field during the experiment. Normalized descriptive parameters were extracted from the responses of each ganglion cell to the presented stimuli by quantifying the following properties: response to bright stimuli, dark stimuli or both (ON, OFF and ON-OFF responses, respectively); sensitivity to movement in a particular direction (direction selectivity (DS)); and spike train latency (brisk or sluggish) and duration (transient or sustained). The parameters were clustered to group ganglion cells into functionally-defined categories, resulting in the following ganglion cell types: ON brisk transient, OFF brisk transient, OFF DS sluggish sustained, ON-OFF brisk sustained, ON-OFF sluggish sustained, ON-OFF DS and ON DS (DS cells for multiple directions were found.
Figure 3-4: Ganglion cell distribution and signal amplitudes within selected region of retina piece. Each colored circle represents a unique spike-sorted ganglion cell that was recorded throughout one experiment and responded to at least one stimulus. The area of each circle represents each cell's peak amplitude. Locations of circles indicate the electrode where the peak amplitude of the spike-triggered average extracellular action potential (STA-EAP) were recorded. A ± 5 μm spatial jitter along the x- and y-axes was introduced into the plot to avoid circle overlaps. Overlaps occurred because the peaks of multiple ganglion cells were recorded on one electrode, which suggests that the axon initial segments of multiple neurons were closest to a given electrode. One electrode configuration was used to obtain this data: electrodes underlying cells are shown in filled gray squares. Note that not all electrodes shown in figure were used for recording.
3.3.1 Ganglion Cell Yield with HD-MEA

One of our goals was to record a large number of ganglion cells per experiment to maximize the information obtained from a given region of the retina. An average of 55.3 ganglion cells ± 28.4 (range: 29–104 ganglion cells) per experiment were spike sorted, inside a mean area of $65 \times 578 \ \mu m^2$ (0.0374 mm$^2$) containing 126 recording electrodes; we recorded and spike sorted ganglion cell data up to a maximal ganglion cell density of 2780 cells/mm$^2$ at approximately 2 mm (42°) from the optic nerve head to obtain the spike train for each detected ganglion cell (see Figure 3-4 for example of ganglion cell spatial distribution in one experiment). However, it should be noted that on average, only 68% of all cells responded to all visual stimuli.

3.3.2 Responses to Visual Stimuli

**Marching Square Over Grid**: Most ganglion cell types will respond to a marching square stimulus if it crosses the receptive field of that cell (Roska et al., 2006). Here, examples are shown of the responses of individual ganglion cells to flashing squares. In Figure 3-5A, the area over which each ganglion cell was responsive to light—the receptive field—is shown for these ganglion cells: from top to bottom, responses for ON, OFF, and ON-OFF ganglion cells are shown. In Figure 3-5B, the corresponding raster plots for ON, OFF, and ON-OFF cells in response to positive and negative contrast stimuli are shown. The uppermost gray band shows that the ganglion cell responded to the appearance of the bright square stimulus and was, therefore, determined to be an ON cell; the middle gray band shows that the ganglion cell responded to the dark square stimulus and therefore was an OFF cell; the bottom band shows a response to both bright and dark stimuli, indicating that an ON-OFF cell was found.

The **Speed Test** was used to characterize the ganglion cell response to different speeds of stimulus movement: this could also be termed the temporal response. As is shown in Figure 3-5C, some cells responded best to slower speeds (top panel), whereas others responded to intermediate (middle panel) or faster speeds (lower panel); (stimulus speed range 50–1800 $\mu m/s$). The tested range was selected based on previous experiments (Oyster, 1968; Wyatt and Daw, 1975; He and Levick, 2000; Sivyer et al., 2010), taking into account the anatomical dimensions of the hamster retina (Tiao and Blakemore, 1976).

The **Width Test** was used to characterize the spatial response: the ganglion cell response to different stimulus sizes, which is a function of the receptive field size. As is shown in Figure 3-5D, some cells responded best to narrow bars (top panel), while others responded to intermediately-sized bars (middle panel) or large sizes (lower panel); (stimulus bar width range was 75–900 $\mu m$).
Figure 3-5: Sample responses used to derive parameters. (A) Receptive fields (from top to bottom) for ON, OFF, and ON-OFF ganglion cells. (B) Raster plots (from top to bottom) for the ganglion cell responses of ON, OFF, and ON-OFF ganglion cells. (C) Speed tuning of ganglion cells; from top to bottom: low, mid and high speed response characteristics. (D) Spatial tuning of ganglion cells; from top to bottom: short, mid, and long moving bar response characteristics. (E) Polar plot for response for typical DS cell, which responds most vigorously to movements in one direction, the preferred direction. The preferred direction of this specific cell is at 225°, and the null direction is at 45°.
Classic DS ganglion cells were also identified as shown in Figure 3-5E. This cell responded maximally to a moving bar in the cell's preferred direction (in this case, 225°), and exhibited no response when the bar was moved in the opposite, or “null” direction (Barlow and Hill, 1963).

**Figure 3-6:** Responses and parameters. Histograms of responses and calculated parameters, showing the distributions for the entire dataset. The following were obtained using the responses to the Marching Square Over Grid stimulus: (A) latency values, (B) transience values, (C) bias indices, and (D) mean receptive field diameters. (E) The DS indices were computed using the Narrow Moving Bars stimulus in eight directions. (F) The bar speed response was computed from the responses to a moving bar at different speeds. (G) The bar width response was computed from moving bars with a range of widths.
3.3.3 Parameters Calculated

Descriptive parameters were extracted from the responses of the ganglion cells to the stimuli presented. The parameters were designed to represent generalized spatial, temporal and contrast sensitivity parameters for each ganglion cell across all ganglion cell types. Most parameters were normalized to a range of ±1 so as to make direct comparisons, statistical computations and parameter clustering possible. When appropriate and possible, we used the parameter computation methods similar to those used by Farrow and Masland (2011). The Marching Square Over Grid was primarily used to map out the receptive fields of the ganglion cells, but also provided temporal and contrast sensitivity response characteristics of the cells: see Figures 6A–D. To compute latency (Figure 3-6A), we measured how long a ganglion cell took to reach its maximum firing rate in response to a flashing square at the center of the receptive field: in the case of ON ganglion cells, the response to the bright stimulus was used, and in the case of OFF ganglion cells, the response to the dark stimulus was used. This latency measure peaked between 140 and 155 ms (21% of cells); 0.4% of cells exceeded 539 ms. Transience (Figure 3-6B), which measured the normalized area under the curve for this response and effectively the duration of the response, peaked between 87 and 108 ms (13% of cells), 0.4% of cells exceeded 679 ms. The ON-OFF Bias histogram (Figure 3-6C), shows a triphasic distribution at values of −1, 0, and 1, corresponding to OFF, ON-OFF, and ON cells respectively. The receptive field diameter had a monophasic distribution centered at 271.4 μm with a standard deviation of 64.3 μm (Figure 3-6D).

The Narrow Moving Bar stimulus was used to assess the direction selective (DS) component of the ganglion cells' responses, i.e., how sensitive the cell is to movement in a particular direction (Figure 3-6E). Assuming a threshold of 0.2 for a ganglion cell to be considered a DS cell (Rivlin-Etzion et al., 2012), we found that 35.1% of ganglion cells in our population were DS cells. This is consistent with the literature (Devries and Baylor, 1997; Rockhill et al., 2002).

Speed and Width Indices were computed during the Speed Test and Width Test, respectively, which involved, in the first case, varying the speed of the bar movement, and in the second case, varying the width of the bar. The Speed Index distribution appeared to be roughly trimodal: sensitivity peaks were found around the following parameter values: 0.30, 0.44, 0.60 (equivalent in speed units: 748, 1026, and 1345 μm/s; 15.7, 21.6; and 28.4 visual degrees/s; Figure 3-6F). The Width Index indicated a bimodal distribution, with parameter values at 0.34 and 0.56 (equivalent in width units: 307 and 503 μm; 6.5 and 10.6 visual degrees; Figure 3-6G).
3.3.4 Clustering of Parameters

The parameters were extracted to describe the visual response properties of the ganglion cells. After discarding neurons that failed to respond to all stimuli (125 were discarded from a total of 387 found ganglion cells), we normalized the receptive-field diameter, bar speed response and bar width response. We clustered the parameters with $k$-means from 4 to 25 and then computed the mean silhouette values for each value of $k$ to determine the number of clusters that could be obtained from this dataset, which was found to be at $k = 7$, or seven clusters. This indicated that in our dataset, there were seven distinct groups of cell types that were reasonably separable from each other using five of the parameters (using all seven of the parameters did not reveal more clusters).

3.3.5 Separability by Center-to-Center Vector Projection

The degree of cluster separability is shown graphically by axis projections between pairwise comparisons of cluster group means in the 5-dimensional space: the parameter space consisted of one dimension for each of the parameters that were used for clustering; each ganglion cell therefore had unique coordinates in 5D space, according to its parameter values. The plots were generated as follows: each two cluster groups were selected for comparison, their mean locations in 5D space were computed, data points from each group were projected on a 1D axis spanning these two locations, and Gaussian curves were fitted to the data points of each cluster group. The curves for each group show the Gaussian curves of the pair of groups being compared. To illustrate: in Figure 3-7H, the separation discriminant between cluster #2 and #6 shows a clear separation, whereas in Figure 3-7S, the clusters #3 and #1 exhibit a lower degree of separation; this is quantified the inset table in Figure 3-7, where the Separation Coefficient has a value of 11.0 for the former case, and 3.1 for the latter. The separation coefficient for each comparison was computed (see Section Materials and Methods) and plotted in the table in Figure 3-7. The separability coefficient was equal or greater than 3.1 for all groups, which, given that the data has a uniform Gaussian distribution and approximately equal standard deviations for our datasets, would indicate that the two datasets are separated by three standard deviations.
Parameter Group Characteristics

We computed the means of each group for each parameter to present the general characteristics for each group. For example, in Figure 3-8A, the ON-OFF Bias Index shows 2 OFF groups, 3 ON-OFF groups, and 2 ON groups. Furthermore, ON DS and ON-OFF DS cell types, which have been shown in the literature to exist for other mammals (Weng et al., 2005; Kanjhan and Sivyer, 2010), can be observed here. Namely, by examining Figures 8A,D, it can be seen that group 5 is an ON-OFF DS ganglion cell and group 7 is an ON ganglion cell. It can furthermore be observed, in Figure 3-8E, that group 5 responds at higher bar speeds, and group 7 to lower bar speeds, which is consistent with these two DS cell types (Wyatt and Daw, 1975). As is shown in this figure, the following cell types were found: OFF brisk transient, OFF sluggish transient, ON-OFF brisk transient, ON-OFF sluggish sustained, ON-OFF DS, ON brisk transient, ON DS.
Figure 3-8: Cluster characteristics. (A–F) The mean parameter values for each ganglion cell are plotted against one another in a pairwise fashion to show the comparative values for each group. There are seven groups representing the cell types, and the ganglion cell members of each group are color-coded.
3.3.6 Members of Clusters

In Figure 3-9, the parameters are plotted against one another, and different colors are used to distinguish the clustered groups (note the group colors also correspond to those used in Figure 3-8). The groups are most clearly apparent when each parameter is plotted against the Bias-Index. However, in Figure 3-9E through Figure 3-9O, useful parameter distributions across the other dimensions are shown. For example, in Figure 3-9D, the group labeled with red circles has a bias index near 1 and a high DS index, which means that it is an ON DS cell.
3.3.7 Confirmation Of Clustering

To visually confirm that the clustering was successful, we used a standard method of plotting the receptive fields for each of the cell types found in one experiment (Field and Chichilnisky, 2007), as shown in Figures 10A–G. We then examined the responses of two cell types that were found among our clusters: ON DS ganglion cells and ON-OFF DS ganglion cells. Figure 3-10H
shows the polar plots for ON DS ganglion cells (corresponding to cells in Figure 3-10B); responses for ON-OFF DS ganglion cells are shown in Figure 3-10I (corresponding to cells in Figure 3-10G).

3.4 Discussion

We have shown that the combination of a CMOS HD-MEA with a series of visual stimuli provided an efficient method for investigating the response characteristics of ganglion cells in ex-vivo retinal tissue. This method was designed to maximally utilize the closely-spaced electrodes on the HD-MEA to record from as many ganglion cells as possible and to obtain accurate spike trains for each cell by spike sorting subcellular-resolution recordings. Additionally, we eliminated time-intensive spike sorting and computation of receptive fields during the experiment (these steps were all performed post-hoc) to stimulate the ganglion cells in an effective parallel manner.

3.4.1 HD-MEA Platform

Similar electrophysiological ganglion cell classification studies have been conducted with both the MEA and the patch clamp. However, we believe that the HD-MEA is the platform that provides the most advantages for these types of studies. While previous studies featured MEAs with electrode pitches of 25–100 μm electrodes (Carcieri et al., 2003; Segev et al., 2006; Zeck and Masland, 2007; Farrow and Masland, 2011), an electrode pitch of 17.5 μm enabled us to record signals from each ganglion cell on tens of electrodes, providing us with subcellular features that are essential for accurate spike sorting (Lewicki, 1998; Einevoll et al., 2012; Franke et al., 2012; Jäckel et al., 2012). A fine electrode pitch also made it possible to record cellular responses and a large number of parallel visual channels at a significantly higher ganglion cell density than would be feasible with a standard MEA device, providing information about ganglion cells closer to the center of the field of view. Finally, the HD-MEA is also better suited to large-scale ganglion cell identification or classification studies than is the patch clamp (Roska and Werblin, 2001; Weng et al., 2005), because the HD-MEA provides orders-of-magnitude greater throughput. Although the patch clamp method records signals that do not require sorting, the vastly higher throughput of the HD-MEA nonetheless justifies its use, in particular in view of the straightforward spike sorting as a consequence of the high electrode density.

Our recording electrode count of 126 also made it possible to record more data than was possible with the MEA devices featuring between 30 and 61 recording electrodes that were used in the studies mentioned above. The unique dynamic configurability of the recording electrodes enabled to always record from the
most active part of the retinal sample. As mentioned in the Results Section, our maximum yield of spike-sorted ganglion cells of 2780 cells/mm$^2$ at approximately 2 mm ($42^\circ$) from the optic nerve head is approximately equal to what would be expected for cell density at that eccentricity in the hamster (Tiao and Blakemore, 1976).

3.4.2 Visual Stimuli

The visual stimuli were based on the work of Roska and Werblin (2001), Carcieri et al. (2003), and Farrow and Masland (2011): they were designed to include most basic visual characteristics (such as movement and contrast) to evoke the basic range of responses that would separate the greatest number of ganglion cell types, while being straightforward enough to analyze. However, while a common method of obtaining information about a ganglion cell's response to stimuli of varying sizes is to flash a spot of light of varying diameters (Van Wyk et al., 2006; Zeck and Masland, 2007; Farrow and Masland, 2011), this method requires locating the ganglion cell's receptive field during the experiment. To increase the efficiency of our recordings and avoid time-consuming spike sorting and signal processing, we chose to replace the flashing spots with moving bars of different widths. Moving gratings have been also been used to assess ganglion cell spatial response (Devries and Baylor, 1997; Sun et al., 2004), but we used moving bars instead to decrease the likelihood of any inhibitory effects that might suppress some ganglion cells' responses (Gollisch, 2013). Certain ganglion cell types have also been known to respond to variance in speed, most notably ON and ON-OFF DS cells (Wyatt and Daw, 1975; Grzywacz and Amthor, 2007); we therefore tested the responses of the cells to bars moving at varying speeds. Ganglion cell receptive field detection was examined by flashing a square randomly over the selected retinal region. While this method may have the disadvantage of possibly inhibiting responses in some cells (Troy, 2009), in our experience, we were able to obtain significantly more ganglion cell locations than with the random flickering checkerboard technique (Meister et al., 1994).

3.4.3 Parameter Space

The parameters that were extracted from the ganglion cell responses were meant to describe the ganglion cell behavior in a standardized manner, such that the cell type is to some degree identifiable, provided that there are sufficient parameters available (Olveczky et al., 2003; Segev et al., 2006; Zeck and Masland, 2007). As expected, the standard parameters of bias index, latency, transience and DS-index all provided response information that was useful for clustering cell types. The speed index was also found to vary amongst ganglion cell types, confirming our expectation that we would observe a cell type-
depending speed response. The width index, however, did not provide much useful information, which indicates that in our studies, this index was not a very significant factor for defining cell type.

3.4.4 Ganglion Cell Clustering

To sort the ganglion cell responses in parameter space, we chose a straightforward method for clustering: k-means. K-means is an effective data clustering method when cluster shapes are symmetrical (Kaufman and Rousseeuw, 1990); in the case of ganglion cell types, we expected that the parameters would cluster in a Gaussian distribution around a centroid, because ganglion cells of the same type would be likely to have similar parameter values across parameter space. K-means requires an a-priori cluster number, therefore, we used a range of k-values, from \( k = 4 \) to \( k = 25 \), which was selected according to the number of ganglion cells that we expected to find (Masland, 2001; Segev et al., 2006; Dowling, 2012).

The Fisher discriminant method to confirm the sorting parameters indicated that the least-well separated clusters had a discriminant value of at least 3.1 (table in Figure 3-7). As shown in Equation (4), the Fisher discriminant method is a metric of separation, based on the ratio of distance between the means (\( \mu_1 \) and \( \mu_2 \), respectively) of the Gaussian fits to the standard deviations of the Gaussian fits. Assuming a uniform Gaussian distribution and approximately equal standard deviations for our datasets, the Fisher discriminant is essentially equal to the number of standard deviations between the means of the compared datasets; the average Fisher discriminant across all pairwise comparisons in our dataset was 4.75.

It is known that most ganglion cell types tile the retina and that the receptive fields of a given type tend to have minimal overlap (Devries and Baylor, 1997; Field and Chichilnisky, 2007; Anishchenko et al., 2010). Thus, we tested our clustering for (i) coverage and (ii) overlaps. We did indeed see almost 100% coverage for all cell types: as shown in Figure 3-10, the electrode configurations under the neurons are to a large extent eclipsed by the receptive fields. However, because the number of ganglion cell types that we found was lower than the expected number for small mammals (see below for discussion), each cluster shown may have contained more than one cell type, which could explain why many receptive field overlaps are visible in these plots.

As an additional quality check of our clustering, we focused on two groups from one experiment: group 2 and group 7, which correspond to ON DS ganglion cells and ON-OFF DS ganglion cells, respectively. It has been reported that ON DS ganglion cells have a 3-lobed distribution in a polar plot, and ON-OFF DS ganglion cells have a 4-lobed distribution (Oyster and Barlow, 1967). In our
data for ON DS ganglion cells (Figure 3-10H), we observed that the majority of the responding cells formed a 3-lobed distribution; the majority of the OFF DS cells (Figure 3-10I) formed a 4-lobed distribution. We therefore concluded that our clustering successfully identified these two cell types.

Several papers in the literature have attempted to determine the number of ganglion cells by computing parameters, but the literature is still not fully in agreement as to the absolute number of ganglion cell types (Lettvin et al., 1959; Grüsser-Cornehls and Himstedt, 1973; Cleland and Levick, 1974; Hochstein and Shapley, 1976; Caldwell and Daw, 1978; Stone, 1983; Carcieri et al., 2003; Schnitzer and Meister, 2003; Segev et al., 2006). Having found seven types of cells, we are in the lower range of the spectrum for expected number of cell types; however, we did find a similar number of types as in comparable studies (Lettvin et al., 1959; Grüsser-Cornehls and Himstedt, 1973; Cleland and Levick, 1974; Hochstein and Shapley, 1976; Caldwell and Daw, 1978; Stone, 1983; Carcieri et al., 2003; Segev et al., 2006). The explanation for our lower-than-expected number of ganglion cell types is that we did not explore the entire parameter space that would be necessary to find every cell type: for example, responses to luminance changes (Farrow and Masland, 2011), or a test of rod input to discern between ON cell subtypes (Deans et al., 2002). Regarding color vision: many smaller terrestrial species have a higher density of middle-wavelength-sensitive (M) cones in the superior part of the retina, and a higher density of short-wavelength-sensitive (S) in the inferior part (Yin et al., 2009). We did not test for such color variations and we only sampled retinal tissue from the superior portion of the retina.

3.5 Outlook

This method has the potential for further development by increasing stimulus complexity to elicit responses from additional ganglion cell types, such as edge detectors, orientation-specific cells and uniformity detectors. The stimuli would need to be optimized in terms of stimulus repetitions, parameter resolution and parameter features to effectively utilize the duration of viability of the tissue. Use of an HD-MEA with more recording electrodes, such as that which has recently become available (Müller et al., 2015), would also increase the throughput of this method.

Conclusion

We have demonstrated a method for rapidly recording from populations of ganglion cells in the retina and extracting a comparatively large amount of electrophysiological data from each ganglion cell in the population. This study sought to introduce a novel combination of an HD-MEA with basic visual stimuli to introduce an efficient categorization tool for ganglion cells. The HD-MEA has the advantage of allowing one to record virtually every ganglion cell within a selected region at high spatiotemporal resolution, such that the majority
of ganglion cells are detected. The visual stimulus sequence was designed to make best use of the number of active recording channels available during the time that the retina preparation was viable and fully functional. As such, the sequence was intended to stimulate as many ganglion cells as possible (i) across as many dimensions as possible, (ii) in as short a time as possible, which was achieved by avoiding time-consuming spike sorting during the experiment so as to maximize our throughput.

### 3.6 Acknowledgements

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Chapter 4 Characterization of Hamster Retinal Ganglion Cell Response to Electrical Stimulation using a High-Density 1024-channel CMOS Microelectrode Array

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4.1 Introduction

Retinal prosthetic devices can provide a solution to certain forms of blindness, including age-related macular degeneration and retinitis pigmentosa, which afflict 4 million individuals worldwide (Lorach et al., 2013b). For such disabilities, photopically driven epiretinal devices can be used to directly stimulate the retinal ganglion cells in the retina by delivering voltage or current pulses via a microelectrode array (MEA). These electrical pulses form a substitute for the missing visual input in diseased or damaged retinae (Humayun et al., 2003, 2009; Keserü et al., 2012; Lorach et al., 2013b). It is therefore crucial that MEAs, which form the basis for prosthetic device technology, be optimized and effectively used for controlled and targeted stimulation of retinal ganglion cells.

Effective MEA systems must be capable of stimulating targeted ganglion cells with high accuracy and precision, such that only selected cells are stimulated at the appropriate time points. It is important that ganglion cells adjacent to the
targeted ganglion cell not be stimulated collaterally, as this introduces noise into
the system, thereby reducing the effective visual resolution capability of the
stimulation device. This is especially important when one considers that there
are an estimated 15 – 24 ganglion cell types, each of which responds uniquely
to optical stimuli in order to transmit specific information about that stimulus
(Roska and Werblin, 2001b; Brien et al., 2002; Rockhill et al., 2002; Dacey et
al., 2003; Kong et al., 2005; Völgyi et al., 2009; Sanes and Masland, 2015).

Therefore, the electrodes of the MEA must deliver the precise amount of charge
to evoke a response from the targeted cell, without exceeding the stimulation
threshold of adjacent ganglion cells, which are highly likely to be of another cell
type (Rockhill et al., 2000).

One of the greatest limitations of ganglion cell stimulation using MEAs has
been the low electrode density of MEA devices (60 μm electrode center-to-
center pitch), which makes it challenging to stimulate a selected target ganglion
cell (Lorach et al., 2013a; Jepson et al., 2014a). It has been shown that for
effective interaction with electrogenic neurons, such as ganglion cells, the
density of electrodes must roughly equal the density of cells (Einevoll et al.,
2012; Jackel et al., 2012a; Franke et al., 2015); in humans, the ganglion cell
density approaches values of up to approximately 35,000 cells/ mm² (Curcio and
Allen, 1990). To circumvent this electrode-to-cell ratio limitation, some
research groups have used multiple electrodes to produce a specific electrical
stimulus waveform at the location of the targeted ganglion cell (Jepson et al.,
2014a).

In response to the electrode density constraints, high density CMOS MEAs have
been developed, such as the active pixel sensor array by (Berdondini et al.,
2009), which has 4096 electrodes and a pitch of 21 μm, or the HD-MEA from
(Bertotti et al., 2014), which features 4225 electrodes with pitch of either 16 μm
or 32 μm. We propose another approach to overcome this limitation by using a
device that features more stimulation sites, a greater electrode count and lower
noise characteristics: the Complementary metal–oxide–semiconductor (CMOS)
high-density microelectrode array (HD-MEA). The HD-MEA features 26,400
electrodes of 7 μm diameter that have a pitch of 17.5 μm over an area of 3.85 ×
2.10 mm² (electrode density of 3265 electrodes/ mm²). A maximum of 1024
electrodes can be simultaneously and dynamically routed to channels for
recording and stimulation (Ballini et al., 2014; Muller et al., 2015). Using this
device, we can select region of the retina and record the extracellular activity of
ganglion cells at subcellular resolution; at this electrode density, a ganglion cell
can be recorded on up to a couple hundred electrodes if the recording electrodes
are routed such that they lie under the spike-triggered average extracellular
action potential (STA-EAP) peak and axonal regions (see Figure 4-1B, where
149 electrodes have been used to record this neuron); an amplitude map of each
cell’s action potential can be constructed from the peak-to-peak amplitudes of
the STA-EAP, as shown in Figure 4-1A. Using this information, the location of
the ganglion cell that is most sensitive to stimulation can be identified, and then electrically stimulated using one or more electrodes that feature the minimum stimulation threshold. Thereby, stimulation voltages can be minimized and very local stimulation is possible, which, in turn, entails high stimulation selectivity and precision.

We present a method for assessing the voltage stimulation response properties of ganglion cells in the hamster retina at high spatial resolution on the CMOS HD-MEA, and demonstrate the advantageous features of HD-MEA that make it possible to selectively stimulate selected ganglion cells. We characterize the stimulation threshold relative to the amplitude spatial distribution of the spike-triggered average extracellular action potential (STA-EAP) to create threshold maps, similar to those created by (Fried et al., 2009). We show that the activation of the cell can be facilitated by selecting an electrode that is near the peak of the STA-EAP, and in the vicinity of the cell’s action potential origin, both locations of which are, according to our results, in close proximity. Finally, we demonstrate selective stimulation of a ganglion cell within a densely packed region of ganglion cells.

4.2 METHODS

4.2.1 Retina Isolation

Eleven-week-old Syrian Hamsters (*Mesocricetus auratus*, Janvier Labs, France), were anesthetized and sacrificed according to protocols approved by the Basel-City Veterinary office and in accordance with Swiss federal laws on animal welfare; eyes were immediately removed, the retina dissected under dim red illumination, and segments were cut out of the retina (see Supplementary Figure 4-1C). The retina segment was cut as far distally from the optic nerve head as possible such that the ganglion cell density was relatively low (between 1,000 and 2,000 cells per mm², according to Tiao et al., 1976), and few axon bundles existed.

4.2.2 Physiological Recording Setup

The HD-MEA was packaged by attaching a polycarbonate ring to it with epoxy, which formed a well with a volume of 1 mL (see Supplementary Figure 4-1A). The MEA was located at the bottom of the well (see Supplementary Figure 4-1B), as described in (Frey et al., 2007). Electrodeposition of platinum black was conducted so as to coat the electrodes and maximize the signal-to-noise ratio, as well as to reduce photoelectric effects induced during the optical
Figure 4-1: Overview of a Retinal Ganglion Cell on the HD-MEA. The action potential generated by a ganglion cell can be measured on many electrodes. We refer to the mean of each waveform on each electrode upon spiking as a spike-triggered average extracellular action potential (STA-EAP). The spatial distribution of the action potential generated by the cell can be visualized as a contour plot of the peak-to-peak values of the STA-EAP, as shown in panel (A). Recording electrodes are shown as black dots and the amplitudes are color coded. The STA-EAP for the same ganglion cell is shown in panel (B). Zoomed-in regions show the peak region of the STA-EAP and a distal region of the axon. Note that the waveforms on each electrode in the STA-EAP peak are negative and then positive, whereas the waveforms along the axon are positive then negative.

stimuli (Novak and Wheeler, 1986; Kim and Oh, 1996; Maher et al., 1999; Chang et al., 2000; Mathieson et al., 2004; Fiscella et al., 2012). The platinum black increased the electrode surface area by a factor of 80 to 130 (Kim and Oh, 1996) thereby reducing the impedance of the electrodes and facilitating electrical stimulation. A meshwork mounted on a height-adjustable support was used to secure the retinal tissue in place on the HD-MEA while sufficient pressure was applied. To physiologically support the cell, a gravity-flow system delivered oxygenated Ames’ Medium at 2.5 mL/minute. The solution was
heated to 35° C with a PH01 heated perfusion cannula (Multi Channel Systems MCS GmbH, Germany) and then diverted with a plastic duct (length 1cm; inner diameter 1.0 mm) to the subretinal side of the tissue, in order to provide oxygen and to remove metabolic by-products.

4.2.3 Optical Stimuli

Optical stimulus sequence scripts were written in a Matlab (The Mathworks, Natick, MA)-embedded program called Psychtoolbox (http://psychtoolbox.org). The stimuli were sent to an Acer K10 LED projector (frame rate 60 Hz). Along the projected optical path from the projector were two camera lenses (Nikkor 60 mm 1:2.8 G ED, Nikon) that reduced the image, a 10x neutral density filter to reduce optical intensity, a mirror (U-MBF3, Olympus), and finally a 5x objective lens (LMPLFLN5X Olympus) that focused the image onto the retina. The image projected onto the MEA surface had a total area of 1 × 1 mm² and was characterized by the following intensity range: blue: 460±15 nm; intensity of 2.0 × 10^{13} photons cm⁻¹ s⁻¹; green: 525 ± 23 nm; intensity of 3.3 × 10^{13} photons cm⁻¹ s⁻¹ as in (Fiscella et al., 2012). The brightness of the image could be adjusted in 256 increments. One LED pixel of the projector corresponded to a projected square of approximately 1.7 × 1.7 μm² at the subretinal plane, where the image was focused. Exclusively blue and green LEDs were used, since Syrian hamsters are dichromats and, therefore, not sensitive to wavelengths within the red spectrum (Jacobs, 2002).

4.2.4 Ganglion Cell Scanning, Selection and STA-EAP Extraction

Ganglion cells were located using a sparse electrode configuration of up to 1024 electrodes, where every other electrode was routed as a recording electrode (approximate total area of 0.32 mm²): ganglion cell signals were recorded while projecting a natural movie onto the photoreceptors at the subretinal surface. The ganglion cell spikes were plotted and detected using custom-written MEA interface software. Electrodes within the recording region that showed a high rate of activity were manually selected, and the spikes were thresholded by an adaptive filter at 3.5 times of the root mean square value (RMS). 100 spikes were averaged to create a STA-EAP for each detected ganglion cell; the STA-EAP was plotted over the recording electrodes. Ganglion cells were then selected as target cells only if the STA-EAP peak and approximately 500 μm of the axon were visible.

4.2.5 Finding Initial Ganglion Cell Threshold

Once a target ganglion cell was selected, it was tested for a stimulus response. Its STA-EAP was plotted using the MEA interface software, and the electrode on which the amplitude of the STA-EAP was greatest was selected as the main
stimulating electrode. A positive-then-negative biphasic pulse (pulse width of 200 μs, interpulse delay of 50 ms) was then delivered in pseudo-random order across a voltage range of 10:100 bits using 5 repetitions while the data were recorded. A recording electrode that laid under the axon was selected as a readout electrode. The data from the readout electrode was subsequently loaded into Matlab and plotted as a function of voltage. The threshold – or voltage at which the ganglion cell responded to 50% of the voltage pulses – of the ganglion cell was then computed.

4.2.6 Creating Electrode Configuration for Selected Ganglion Cells

A maximum-density configuration was then manually routed by selecting electrodes in the MEA interface software: this configuration consisted of electrodes underneath the region of the STA-EAP peak and approximately 500 μm of the distal axonal region. The configuration was sent to the MEA chip for all subsequent recordings. In order to make efficient use of the limited preparation viability time, two ganglion cells were simultaneously selected, and configurations of electrodes underlying both were created and routed for the procedure.

4.2.7 Optical Stimulation

The basic ganglion cell electrophysiological light response properties were determined using an optical stimulus, which was used to test for ON/OFF characteristics. To measure ON and OFF responses as well as ganglion cell receptive-field size, flashing bright and dark spots on a middle-gray background were centered on the STA-EAP peak location and shown in sequence. The spots consisted of different diameters displayed in pseudorandom order: 75, 150, 300, 600, 900 μm; two second stimulus presentation duration; fifty-percent contrast against a gray background; two second inter-stimulus delay; five repetitions. The mean firing rate across all repetitions for the presentation of each spot size and contrast was computed by dividing the number of spikes over the stimulus duration by the duration. The size and contrast resulting in the maximal mean firing rate was selected as the optimal spot stimulus for that cell.

4.2.8 Voltage Stimuli

Voltage pulses were controlled by a Python script, which was used to do the following: create the stimulus pulse phase number and polarity; send electrode configurations to the chips; connect electrodes to the stimulation buffers; send the stimulation pulse trains; write meta-data files for post-hoc analysis steps. Following each electrode configuration change, the potential of each electrode was centered within the recording range so that the recorded signals would not saturate during recording (since sending a configuration induces a voltage offset.
in each channel). Pulse waveforms could be specified at a temporal resolution of 50 μs, and in pulse amplitude increments of 2.75 mV (up to a voltage step of 2.5 V, while still maintaining an acceptable rise time of 50 μs (Muller et al., 2015)). In our experiments, we used the following pulse shapes: positive monophasic, negative monophasic, positive-then-negative biphasic and negative-then-positive biphasic (see Figure 4-8).

4.2.9 Electrical Stimulation Sequence

The electrical stimulation protocol consisted of several stages. First, the threshold voltage was determined for a group of ganglion cells. Second, a variation of stimulus pulse phase number and polarity, including monopolar and bipolar pulses, and the simultaneous use of 2 stimulation electrodes with opposing voltages were explored to determine whether any of these was more efficacious than the others in reducing the ganglion cell threshold. Third, ganglion cells were stimulated with several electrodes across the spatial plane (in increments of 17.5 μm) in order to determine the location at which the cell was most easily stimulated. Finally, we demonstrated selective stimulation of ganglion cells by stimulating with one stimulation electrode while recording multiple configurations, such that the activity seen on the entire MEA (an area of 3.85 × 2.10 mm²) in response to this stimulation could be reconstructed.

4.2.10 Single Electrode Voltage Stimuli

Voltage sweeps consisting of stimulus pulses across a discrete voltage range were sent to the main stimulation electrode to obtain threshold values using single electrodes. The first sweep consisted of a series of positive-then-negative biphasic pulses: 28 to 165 mV peak-to-peak amplitudes in 14 mV increments in pseudorandom order; 15 repetitions; 200 μs pulse width for each phase; stimulus frequency 20 Hz. The voltage sweep was then repeated for positive monophasic and negative monophasic pulses on the main stimulation electrode: 28 to 165 mV peak to peak amplitudes in 14-mV increments in pseudorandom order; 15 repetitions; 200 μs pulse width; stimulus frequency 20 Hz.

4.2.11 Dual Electrode Voltage Stimuli

Voltage sweeps were sent simultaneously to electrode pairs, where the polarity of each pulse phase on one electrode was always the opposite of the pulse phase on the other electrode. To determine the effect of orientation relative to the axon, electrode pairs were chosen along four orthogonal vectors. A positive-then-negative voltage pulse was delivered to the main stimulation electrode, while the inverse was delivered to the neighboring electrodes. The reverse was then applied: a negative- positive voltage pulse was delivered to the main stimulation electrode and the inverse was delivered to the neighboring electrodes. The paired electrode sequence was conducted in the same manner for electrodes that were spaced by one electrode (35 μm horizontally or
vertically and 45 \( \mu \text{m} \) diagonally, on a regular electrode grid arrangement with 17.5 \( \mu \text{m} \) pitch), all of which were electrodes neighboring the central electrode.

4.2.12 Threshold Map

Threshold maps were generated to determine each ganglion cell’s stimulus sensitivity with respect to the location of the stimulation. Essentially, electrodes in the vicinity of the peak of the ganglion cell’s STA-EAP were stimulated across a range of voltages, and the resulting response thresholds were measured. First, we located the electrode on which the STA-EAP for each ganglion cell was highest; the eight electrodes closest to this electrode were selected, which comprised an initial stimulus electrode configuration. Each of the electrodes within this configuration was stimulated in turn (single electrode, biphasic positive-then-negative waveform, 50 \( \mu \text{s} \) pulse width, 20 repetitions, inter-stimulus delay 100 ms, inter-series delay 200 ms). The threshold voltages at each of these electrodes were computed during the experiment. An annulus configuration of 16 electrodes beyond the periphery of the 9 central electrodes was then stimulated using a higher voltage range (since a higher voltage was usually required to elicit a response farther from the STA-EAP center, and using excessive voltage amplitudes in the center caused the cell to stop responding). Finally, the last annulus of 24 electrodes was stimulated, also with an adjusted voltage range, based on the responses at the previous electrodes.

4.2.13 Amplitude and Frequency Map of Retina

An optical stimulus scan of all activity on the chip was performed to determine the general features of the population activity of the entire retina segment. 26 stored electrode configurations were created for this purpose, each of which had groups of electrodes that were randomly distributed; the union of all configurations included the set of 26,400 electrodes of the HD-MEA. These electrode configurations were sent in sequence to the chip, and each was used to record for 120 seconds while stimulating the retina with a natural movie optical stimulus.

4.2.14 Focal Stimulation

We stimulated selected ganglion cells at different voltages while recording the activity on the entire HD-MEA to determine to what degree the stimulus was specific and selective. To maximize the efficiency of the experiments, two ganglion cells were stimulated separately in an alternating fashion. The electrode nearest to the STA-EAP peak for each ganglion cell was first selected as the stimulating electrode. 26 groups of electrodes, or electrode configurations, were generated with the following requirements: the stimulation electrodes for both ganglion cells were included; all recording electrodes on the HD-MEA were distributed throughout the configurations in a random manner, such that the configurations together included all 26,400 electrodes. Each
electrode configuration was sent to the chip sequentially, and a voltage sweep was performed at the stimulation electrode for the ganglion cell while recording from the selected configuration. Stimulation parameters: 55 mV to 605 mV bit peak to peak amplitudes in 55 mV increments in pseudorandom order; 15 repetitions; 50 μs pulse width; stimulus frequency 20 Hz.

4.2.15 Analysis

Determination of Stimulation Thresholds

Stimulation thresholds obtained from voltage sweeps were computed by determining the point at which the cell responded 50% of the time to a voltage stimulus. The timestamp of the axonal response at the maximum voltage was manually detected on the selected readout channel, located under the axon, approximately 500 μm distant from the stimulation site. A temporal window of 10 samples (0.5 ms) was selected around the timestamp on the readout channel data. The data in the window were thresholded at 3.5 standard deviations from the mean of noise. Thresholding was computed for all repetitions and all voltages, and a sigmoidal curve was fitted (with a generalized linear regression using a binomial distribution) to the means of the percent response at each voltage. The inflection point of the curve was selected as the threshold voltage. All response traces and sigmoidal fits were plotted and manually inspected during the analysis phase for accuracy.

Stimulation Pulse Voltages

Stimulation voltages were defined as the peak-to-peak voltage for either monophasic or biphasic pulse waveforms: in the former case, the voltage difference between the baseline and maximum voltage deviation; in the latter case, the difference between the peak maximum and peak minimum stimulus pulse voltage. Voltage pulse waveform data for one stimulation buffer was recorded in parallel to the electrode channel data at 20 kHz to verify voltage pulse values.

Threshold Maps

Threshold maps were created using stimulus thresholds found at each routed electrode located under the respective ganglion cell. X- and y- coordinates for each threshold were determined based on the electrode locations. The resulting three-dimensional data were plotted using a color-coded scatter plot; “hotter” colors correlated to a higher stimulus threshold, and “cooler” colors correlated to a lower stimulus threshold. When a neuron did not respond to a stimulus, the location was left blank.
Amplitude and Frequency Map of the Retina

To create an amplitude map of the recorded activity from the 26 optical stimulation scan configurations, the maximum peak-to-peak values on each electrode were computed: the minimum value of the signal recorded on each electrode for each configuration was computed and subtracted from the maximum found on that electrode. The peak-to-peak values for each electrode were then assigned a pixel based on the electrode’s position on the chip, and a heat map was created. To create a frequency map, the activity on each electrode was thresholded at 3.5 times the standard deviation of the signal noise, and spikes were detected. The number of spikes per unit time was computed (spikes per second), and this value was assigned to a heat map based on the electrode position. For the amplitude map, brighter colors correlated to higher peak-to-peak voltages, and darker colors correlated to lower peak-to-peak voltages. For the activity map, brighter colors correlated to a higher mean firing rate, and darker colors correlated to a lower mean firing rate.

Focal Stimulation Movie

In order to assess the response of ganglion cells to voltage stimulation at each stimulus voltage value of the voltage sweep across time, movies were generated. To create each movie frame, the median peak-to-peak amplitude on every electrode over all repetitions was determined for each electrode configuration. Within each frame, a pixel, determined by the electrode’s coordinates, was assigned the amplitude value for the corresponding electrode. The pixels were assembled across all configurations to reveal the activity recorded on the entire MEA at every time sample. This pixel assembly process was repeated at all sample time points from 1 frame (0.05 ms) prior to stimulation to 159 frames (7.95 ms) following the stimulus time to create a movie.

Focal Stimulation Still Frames

To portray focal stimulation in two dimensions, we computed the maximal peak-to-peak voltages recorded on all electrodes over the movie duration from focal stimulation movies at sub-threshold, threshold, and super-threshold voltage stimulation values. We performed image enhancement of the resulting images in Adobe Photoshop to increase contrast and visibility.

Spike sorting

All spike sorting was done manually using the Matlab-based software, UltraMegaSort2000 (Hill et al., 2011). In order to spike-sort the data and obtain timestamps for a given ganglion cell, we used groups of 9 neighboring electrodes near the location of the real-time STA-EAP peak that was found during the experiment in the MEA interface software.
STA-EAP Generation

The STA-EAP shows the mean waveform shape that is produced by a ganglion cell every time it fires. To compute the STA-EAP, we spike-sorted the data acquired from the cell during optical stimulation and obtained the spike times. We then cut out 20 samples (1 ms) before and 25 samples (1.25 ms) after each spike time (maximally 300 spike times per cell) on the trace recorded at each electrode. The baseline offset was found by taking the median value of the first 10 samples at each electrode, and the cutout waveform at each electrode was offset to zero by subtracting its baseline offset value. The waveforms on each electrode were then averaged to obtain the STA-EAP.

Ganglion Cell Response Metrics

By combining information from the optically and electrically stimulated ganglion cells, we obtained data about each cell’s electrophysiological characteristics. First, we investigated whether the latency over distance (or propagation speed) was constant. We computed the latency on each electrode during optical stimulation. To do so, we upsampled the STA-EAP eight times and then located the time of the maximum negative peak on each electrode; the electrode, at which the peak occurred earliest, was designated as the origin of the action potential. The time difference between the negative peak occurrence on each electrode and the occurrence of the peak at the origin was taken to yield the latency. Latency was then plotted as a function of distance from the electrode at the action potential origin. To investigate the dependency of stimulation threshold on physiological characteristics, we plotted the stimulation threshold at each electrode (obtained in the Threshold Maps analysis step) as a function of distance from the action potential origin, STA-EAP amplitude, and latency.

Equivalent Current Values

The experiments described in this paper all used voltage-controlled stimuli. However, since many publications use units of charge, approximately equivalent charge value for our stimulation values can be calculated as follows.

\[ q = VC \]  \hspace{1cm} (3)

where \( q \) is the charge in picocoulombs, \( V \) is the voltage in millivolts, and \( C \) is the capacitance in nanofarads, the latter of which was approximately 1.57 nF for the MEA electrodes (see (Muller et al., 2015) for normalized electrode impedance measurements).
4.3 Results

4.3.1 Localization of Ganglion Cell and Extracellular Signal Features

The electrical activity of every ganglion cell was recorded on multiple electrodes simultaneously, such that electrophysiological features of the cell could be observed at subcellular resolution. In Figure 4-1A, the amplitude distribution of the spike-triggered average extracellular action potential (STA-EAP) landscape on 170 electrodes is depicted as a contour map, which shows the interpolated spatial distribution of the signal amplitude relative to the recording electrodes (shown as black dots). The peak-to-peak amplitude values range from 26 to 476 μV for this sample ganglion cell. In Figure 4-1B, the corresponding STA-EAP has been plotted, such that the ganglion cell’s action potential waveform on each electrode can be viewed. The zoomed-in circled areas show more details near the negative peak value of the STA-EAP as well as a distal axonal portion of the STA-EAP approximately at 500 μm distance. At the negative peak, the waveform was negative-then-positive and larger in amplitude, while in the distal direction along the axon, the signal became attenuated and inverted, such that it was first positive-then-negative. We observed these action potential polarity inversions and distal amplitude attenuations for all recorded cells; the polarity inversion occurred 90.7 ± 26.5 μm (n=9) away from the STA-EAP peak, distally along the axon. The contour plots for three ganglion cells recorded within a 400 by 400 μm² area (23 by 23 electrodes) in one retina segment are shown in Figure 4-2. The distal axonal regions are labeled for each cell; note that the axons of all neurons are aligned in parallel with one another in the southeast direction.

4.3.2 First Steps in Stimulation

Action Potential Propagation for Optical and Electrical Stimulation.

During optical stimulation or when spontaneously firing, a ganglion cell will generate an action potential that is thought to originate at or near the axon initial segment, and then propagates distally along the axon (Sekirnjak et al., 2008; Fried et al., 2009), as shown in Figure 4-3A. As is indicated by the color coded dots, the action potential propagates along the distal axon (in the northeasterly direction on the contour plot); this propagation can clearly be observed as a temporal shift to the right on the traces at each consecutive electrode in the traces plot. Likewise, when this cell is stimulated with a voltage pulse (Figure 4-3B), an action potential is induced and propagates along the axon in the same manner as in the optically stimulated or spontaneous case, which can is clearly
similar to the case of optical stimulation or spontaneous firing. The stimulus pulse, however, causes a transitory voltage offset (to the height of the pulse amplitude), which introduces noise into the readout channels and interferes with the shape of the action potential waveform on the first few electrodes along the propagation path.

Figure 4-2: Ganglion Cells with Typical Parallel Axon Orientation. Contours of STA-EAPs from 3 spike-sorted neurons are shown here. The axons, which are oriented parallel to each other in the southeasterly direction and lead to the optic nerve head in the intact retina, are marked with grey shading. Electrodes are shown as gray squares. Contour lines represent 50 μV increases in STA-EAP amplitude.
Figure 4-3: Action Potential Propagation.

Shown here are the responses of the same ganglion cell to optical stimulation and to electrical stimulation, (A) and (B), as recorded simultaneously on multiple electrodes along the axonal region. The action potential waveform, as it propagates distally (in this figure, upwards), from the vicinity of the STA-EAP peak along the axon, can be seen on each electrode. Each trace is shown in gray and the mean is shown in black. The point at which the EAP signal reaches its maximum negative peak value is at the bottom of the plot, and is measured on an electrode near the STA-EAP peak. At each consecutively more distant electrode, the maximum negative peak value is offset to the right, meaning that the signal is propagating away (distally from the soma) from the initial starting point. The color-coded circles at the left next to the traces indicate the latency of the respective action potential; grey and black lines represent the traces and mean voltage that were recorded at that electrode. The colored circles also correspond to the locations shown on the ganglion cell contour to the left. (A)
Examples of Responses to Voltage Sweeps

Ganglion cells were stimulated at the STA-EAP peak using a range of voltages at regular increments between a low and high value (voltage sweeps) in order to find their stimulus thresholds. In Figure 4-4, examples are shown of such voltage sweeps on three ganglion cells using positive-then-negative biphasic pulses on single electrodes (the stimulus phase and polarity can be observed in the stimulus artifacts, highlighted with a blue background, which deviate in the positive direction and then in the negative direction). The electrode configuration consisted of electrodes under the STA-EAP and distal axon region, such that recorded data from several electrodes were available for the post-processing analysis. The stimulus response traces, plotted in this figure, were read out at a selected electrode that was between 300 and 500 μm from the stimulating electrode for each cell. In Figure 4-4A, the cell was stimulated between 28 and 303 mV in steps of 55 mV. The first 3 steps did not result in any response, whereas the stimulus pulse at 193 mV resulted in a response 80% of the time. Following the two consecutively higher voltage pulses, the cell responded to the stimulus with 100% response probability. The second cell, as shown in Figure 4-4B, is another example of a voltage sweep response; in this case, however, no intermediate response stage was found: the cell did not respond to a voltage pulse of 154 mV, but did respond to 187 mV, and the threshold value lays between these two values, at 175 mV.

The third sample cell shows what occurs when the cell is in the vicinity of other cells or of an axon bundle. As can be seen in Figure 4-4C, the threshold for this cell was around 165 mV. However, as the voltage of the stimulus pulse was further increased, the response amplitude increased and the waveform shape broadened, indicating that more than one cellular or axonal region was most likely responding at these higher voltages (please see Discussion section on this topic).
Chapter 4: Electrical Stimulation Response

Finding Thresholds: Sigmoidal Fit of Responses

Voltage thresholds were computed by fitting a sigmoidal curve to the response probabilities. At the bottom of Figure 4-4A and Figure 4-4B, the data and fitted curves are shown for the responses of these two cells to voltage sweeps: the stimulation threshold is marked with a grey dotted line at the 50% response probability value.
4.3.3 Spatially distributed stimulation

**Sample cell with latency, amplitude, threshold map**

We investigated the spatial distribution of the action potential latency, the signal amplitude, and the stimulation threshold distribution for the recorded ganglion cells. As is shown in the sample cell in Figure 4-5, the action potential from an optically stimulated ganglion cell originated at the STA-EAP peak value. The action potential subsequently propagated distally from the STA-EAP peak in a symmetrical fashion, and continued distally along the length of the axon for 700 μs (to the right in this figure). The speed in this case was $8.1 \times 10^4$ m/s. Once the STA-EAP had been extracted from the optically stimulated ganglion cell, we proceeded with electrical stimulation on multiple electrodes. As is shown in the zoomed-in square panel, we conducted a voltage sweep on each member of a 7 by 7 square of electrodes (excluding the corner electrodes) that was centered on the peak of the STA. This voltage sweep made it possible to determine the threshold required to evoke a response from the cell using each electrode. The threshold values ranged from 77 to 505 mV within a radius of 49 μm from the STA-EAP peak.

![Figure 4-5: Example of Latency, STA-EAP Amplitude and Stimulation Threshold Map for one Cell.](image)

The colored dots in the uppermost part of the figure show the locations of electrodes that were used to record from a selected ganglion cell. The area of each dot is scaled to the amplitude of the ganglion cell’s action potential on the recording electrode. The jet-color code shows the latency of the signal along the axon from the point of origin of the action potential; the origin is the location of the electrode at which the action potential first reaches its maximum negative peak. Within the zoomed-in region, a threshold map is shown. The origin mentioned above is marked with a green dot. The STA-EAP, where the peak-to-peak
amplitude is greatest, is marked with a light blue star. This map was generated by determining what voltage elicits a response from the cell 50% of the time at each electrode. Within each square, the STA-EAP as measured on that electrode is shown. On blank squares, in this case, at the 5th row and 5th column, it was not possible to elicit a response within the voltage range that was used.

**Threshold Distribution**

Threshold maps from six ganglion cells were generated by voltage stimulation scanning to obtain the spatial distribution of stimulation thresholds shown in Figure 4-5 and Figure 4-6. Each threshold map consists of the stimulation threshold obtained on each electrode within a selected region centered on the STA-EAP peak for that cell. If no response was elicited from the cell, the square left blank. In Figure 4-6, the grey balls and sticks indicate the

![Threshold Map Examples](image)

**Figure 4-6: Threshold Map Examples.** Threshold maps, which show the spatial distribution of the stimulation threshold, are shown for 5 ganglion cells. Each square is centered on a single electrode and the color of the square represents the threshold for this cell at that electrode. Within each square is shown the STA-EAP as seen on that electrode. At locations for which there is no square, it was not possible to elicit a response from the cell. The STA-EAP peak and action potential origin are marked. The direction of the axon is shown below each threshold map (the ball represents the peak of the STA-EAP, and the line is oriented in the direction of the distal axon). The origin (the location at which the action potential first reaches its maximum negative peak) is marked with a with a green dot. The STA-EAP, where the peak-to-peak amplitude is greatest, is marked with a light blue star. On blank squares with waveforms, for example the 3rd row and 2nd column in (A), it was not possible to elicit a response within the voltage range that was used.
orientation of the distal axon relative to the STA-EAP peak.

**STA-EAP Peak and Action Potential Origin**

In all cases, the STA-EAP peak and the action potential origin were found in close proximity to one another: the mean distance between them was $24.5 \pm 22.5 \, \mu m$. From the data shown in **Figure 4-5** and **Figure 4-6**, it was found that the minimum threshold voltage location was $17.2 \pm 19.5 \, \mu m$ from the negative signal peak, and $27.6 \pm 17.5$ from the action potential origin location.

### 4.3.4 Analysis of Threshold Maps: Physiological Response Characteristics of Ganglion Cells

**Latency vs. Distance from AP Origin**

The propagation speed of the action potential from the initiation point (AIS or soma) along the subsequent RGC axonal segments was observed to be fairly constant at $0.778 \pm 0.103 \, \text{m/s}$. As shown in terms of latency per distance along the axon from the origin, in the examples in **Figure 4-7A**, the two variables were strongly correlated, as shown in the lower table for row A in **Figure 4-7**. However, propagation in the opposite direction (away from the distal direction), represented by the negative distance and latency values, was faster than in the distance direction, at $1.500 \pm 1.218 \, \text{m/s}$ over a maximum distance of $106 \, \mu m$, although not significantly different from the speed in the distal direction ($t$-test value of 0.177).

**Threshold vs. Distance from AP Origin, STA-EAP Amplitude and Latency**

As shown in **Figure 4-7B**, stimulation thresholds were found also to be correlated to the distance along the axon distal from the peak of the STA-EAP (**Figure 4-7B**) with high correlation coefficients for all cells with the exception of cell #3, as shown in the lower table under Row B. Stimulation thresholds were also correlated to the latency of the action potential propagation along the axon (**Figure 4-7D**) for all cells, but also with the exception of cell #3. As shown in **Figure 4-7C**, the threshold had an inverse correlation to the STA-EAP peak-to-peak amplitude. At the location on the ganglion cell, for example, where the signal amplitude was half that of the maximum, the stimulus threshold increased by fifty percent. Finally, the squares solution to a linear system of equations, which computed the combined correlation of distance, STA-EAP amplitude and latency to the threshold, showed that the threshold was strongly correlated to these factors (**Figure 4-7E**).
Figure 4-7. Physiological Response Characteristics of Ganglion Cells. We investigated several metrics that we extracted from the stimulation threshold maps of 6 ganglion cells to determine what factors are important for stimulating ganglion cells efficiently. Each consists of pair-wise plotted parameters for six cells, which are labeled in red-highlighted numbers; the cell positions are the same in each panel. (A): Latency versus distance. Latency was determined by measuring the time point at which the negative peak value on each electrode was reached referenced to the location where the action potential originated. Latency in the distal direction (away from the soma) was strongly correlated with the distance along the axon from the origin; root mean square and correlation coefficients were very high. In two cases, the propagation speed in the proximal direction (antidromically) was faster. (B): Threshold voltage versus distance. A correlation of voltage to distance from the
Threshold vs. Pulse Phase Number, Pulse Phase Polarity and Stimulation Electrode Number

Thresholds for ganglion cells were assessed by using biphasic and monophasic pulses on single electrodes, as well as biphasic and monophasic pulses on pairs of electrodes (each electrode in the pair had a polarity opposite to that of the other electrode); the stimulus pulse phase, polarity and numbers of simultaneously stimulating electrodes are shown in the upper panel of Figure 4-8. For example, in the first row, the was a single-electrode positive then negative biphasic pulse; in the fourth row, a single-spaced (spacing of 17.5 μm or 25.7 μm, depending on pair orientation with respect to MEA grid) pair of electrodes with opposing monophasic pulses was used. The results of thresholds using pairs of stimulating electrodes are also shown: electrodes were either neighboring electrodes, spaced by 17.5 μm (or 25.7 μm if diagonally oriented, see inset box at bottom of Figure 4-8), or electrodes with an additional electrode between them at a distance of 35 μm (or 49.5 μm, if diagonally oriented). The first rows (Figure 4-8, rows 1 – 3) show the results for a single-electrode stimulation. The biphasic stimulus had a large range, from 75 to 275 mV. The ranges for both positive and negative monophasic pulses were narrower, but these stimuli only elicited responses from cells in 33% of the cases for monophasic positive and in 50% of the cases for monophasic negative. Rows 4 – 7 in Figure 4-8 show the monophasic pulses on pairs of electrodes. When the electrodes are spaced by one electrode (rows 4 and 6), the range is narrower, but for spacing of 2 electrodes (rows 5 and 7), the range and standard deviations are greater. Finally, rows 8 – 11 show results for biphasic pulses on electrode pairs, which all had comparable ranges. A t-test was run across all possible pairs of the described stimulation configuration combinations described above, but there was no significant difference in threshold values between any of them.

<table>
<thead>
<tr>
<th>Panel</th>
<th>Electrode Configuration</th>
<th>Threshold Range (mV)</th>
<th>Standard Deviation</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel 1</td>
<td>Single electrode, positive then negative biphasic pulse</td>
<td>Large</td>
<td>25.5 mV</td>
<td>0.89</td>
</tr>
<tr>
<td>Panel 2</td>
<td>Pair of electrodes, opposite monophasic pulses, spaced by 17.5 μm</td>
<td>Narrow</td>
<td>3.4 mV</td>
<td>0.78</td>
</tr>
<tr>
<td>Panel 3</td>
<td>Pair of electrodes, opposite monophasic pulses, spaced by 25.7 μm</td>
<td>Narrow</td>
<td>4.0 mV</td>
<td>0.82</td>
</tr>
<tr>
<td>Panel 4</td>
<td>Pair of electrodes, opposite monophasic pulses, spaced by 35 μm</td>
<td>Narrow</td>
<td>5.1 mV</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Inset Tables. The column header cell# refers to the cell in each number in each panel (please see (A) for corresponding cell positions, highlighted in red, which are the same for every panel); the columns correspond to the panel numbers. The upper table shows the root mean square value for each cell in each panel; the lower panel shows the correlation coefficient for each cell in each panel.
Figure 4-8: Stimulus Thresholds for Different Stimulus Waveforms, Polarities, and Electrode Configurations (Single Electrodes or Pairs of Electrodes). This box plot shows stimulus thresholds acquired from cells by using a variety of stimulus waveforms and polarities on one electrode, and on pairs of electrodes. In cases where more than one electrode was used (where multiple orientations relative to the axon direction were tried, the minimum threshold was used). Columns 1 – 3, in the light gray region show ganglion cell stimulus thresholds using a single electrode. The remaining columns (4 – 11) display pairs of electrodes with either monophasic or biphasic stimuli and with pair spacings of either 1 electrode (17.5 μm or 25.7 μm, depending on electrode pair orientation, as shown in the inset box at the bottom of the figure) or 2 electrodes (35.0 μm or 49.5 μm, depending again on electrode pair orientation). Voltage pulse shapes and spacings between the stimulating electrodes are indicated at the top of the plot. Note in the gray inset box that the amplitudes, A, for both biphasic and monophasic pulses are measured peak to peak; phase width is marked with d, and was 200 μs in all cases. The box at the bottom of the figure shows the electrode pitch measurements for pairs of electrodes either aligned with the MEA grid, or diagonal to it. Dark gray electrodes are the active stimulating electrodes, and the light gray electrodes depict the non-stimulating electrodes.
Cell Type

We calculated the thresholds for 14 cells, 6 of which were ON, 8 of which were off for single-electrode biphasic (positive then negative) stimulus pulses (200 μs phase width). The mean stimulation threshold value for ON cells was 241.2 ± 68.6 mV and the mean threshold value for the OFF cells was 151.6 +/- 75.3 mV. A t-test resulted in a p-value of 0.041, which indicated that OFF cells had a significantly lower threshold than ON cells (Supplementary Figure 4-2).

Focal Stimulation

For focal stimulation of selected ganglion cells we used biphasic pulses (which we found to be the most reliable single-electrode stimulus pulse waveform) on single electrodes while recording from all other HD-MEA electrodes by using multiple electrode configurations. The activity of the segment of retina that was used for focal stimulation can be seen in Supplementary Figure 4-3; the tissue is homogeneously active, both in terms of firing frequency, and signal amplitude. A more detailed view of one of the focally stimulated ganglion cells is shown in Figure 4-9: the contoured outline of the stimulated cell (in red) is shown to have been in close proximity to many other cells that have been detected upon optical stimulation with a natural movie. The mean standard deviation of the signal (noise) for regions without platinum black was 4.078 ± 1.007 μV and the noise for regions with platinum black was 2.590 ± 1.141 μV. These noise levels were significantly different (a t-test resulted in a p-value of 1.5289×10^{-10}). Noise measurements were made in regions without tissue.
Figure 4-10 shows an example of focal stimulation of the cell in Figure 4-9: at sub-threshold (165 mV) only a stimulation artifact was produced within 8 ms of the stimulation, and no cellular activity was observed in the surrounding region (Figure 4-10A). However, as the stimulus voltage was increased, the threshold voltage for this cell was reached. At the closest voltage sweep to threshold voltage (220 mV, Figure 4-10B), the cell was selectively stimulated: the stimulation artifact can be observed as in the sub-threshold stimulation, as can be the axonal activity, which is indicated by the red arrow. Finally, at voltages exceeding the threshold (605 mV), other cells in the vicinity were stimulated.

Selective stimulation is shown for this cell and three other cells in the Supplementary Data Movies, which contains movies for each focally stimulated ganglion cell at sub-threshold, threshold and super-threshold stimulus voltage values. At the threshold for all cells, the stimulus artifact is visible, followed by the propagation of one action potential in the southerly direction; in the case of rgc1, the action potential can be seen rebounding after it reached the edge of the array, which was also where the edge of the tissue was located for this experiment. In all super-threshold movies, multiple action potentials on multiple axons can be observed, and the rebounding effect occurred in all cases.
4.4 DISCUSSION

4.4.1 Recording Ganglion Cells in the Ex-Vivo Retina

Using the HD-MEA and custom-written interface software, we were able to locate ganglion cells during our experiments while stimulating with optical stimuli. Thresholding at the peak of the STA-EAP was usually sufficient to completely isolate the activity of a specific cell from other cellular activity, and the axonal signal was clearly measurable hundreds of microns from the stimulation site, as shown in Figure 4-1 and Figure 4-2. STA-EAP peak amplitudes were on the order of hundreds of microvolts, whereas axonal signals were an order of magnitude smaller (5 - 20 µV). Furthermore, as shown in Figure 4-1B, phases of the action potential became inverted in polarity from negative then positive to positive then negative as the action potential propagated. We observed this polarity inversion on essentially all ganglion cells and assign it to the transition zone between the axon initial segment and the distal axon. The inversion was found to occur at 90.7 ± 26.5 µm (n=9).

Since ganglion cell axons all run towards the optic disc in the intact retina (Radius and Anderson, 1979) subsets of them are aligned in a parallel fashion, as shown in a very typical example in Figure 4-2. The axons tend to form bundles (Kanamori et al., 2010), which can cause complications when trying to selectively stimulate a targeted cell, since a stimulation pulse next to an axon bundle may cause many ganglion cells to be activated; this is even more of a problem nearer to the optic disc, where the axon density becomes larger.
and Anderson, 1979). However, the axonal alignment provides the advantage of allowing us to determine whether we stimulated cells in orthodromic or antidromic directions.

**Propagation Speed**

We noted a very strong correlation between latency and distance from the origin of the action potential along the distal axon (0.986 ± 0.011), which means that conduction velocity was fairly homogeneous along the axons of different cells at 0.77 ± 0.10 m/s over a maximum distance of 600 μm (see Figure 4-7A), which is slower than that found in other studies of unmyelinated axonal propagation, such as that in the rabbit retina (Zeck et al., 2011), which was measured at 1.3 ± 0.3 m/s; this may have been due to an inter-species difference, a ganglion-cell type difference, or a measurement technique difference. At 1.50 ± 1.22 m/s, the propagation speed in the proximal direction (away from the axon) was somewhat higher (p=0.22; n = 6). However, (Muller et al., 2015) measured mean propagation velocities of 0.74 m/s in unmyelinated axons of cultured cortical neurons using the same HD-MEA that we used.

4.4.2 Electrically Evoking a Cellular Response

As shown in Figure 4-3, the action potential propagation that resulted from an optical or electrical activation of the cell was the same, in terms of waveform shape, amplitude and latency along the distal axon. The only difference, in terms of the STA-EAP, was that the 0.5 ms-long stimulus artifact disrupted the waveform shape for the first few tens of microseconds (marked with a blue background and reduced in magnitude by a factor of fifty in Figure 4-3). The large similarity means that one could effectively replace the optical input that naturally occurs under in-vivo conditions through targeted electrical pulses; this finding is important in view of potential prosthetic devices, since such device must mimic the natural input as closely as possible in order to provide precise neural input to the brain. Note that the action potential attenuated from hundreds to tens of microvolts along the axon during the first 100 μm; however, due to the low-noise characteristics of the HD-MEA (2.4 μVrms), the signal was visible on single traces (in gray) along a large part of the distal axonal region.

It has been shown that the action potential in the ganglion cell originates in the axon initial segment, a few tens of microns from the soma, and that this region is most excitable (Carras et al., 1992; Jensen et al., 2003; Kole et al., 2008; Fried et al., 2009). Since the negative peak value of the STA-EAP in microelectrode array recordings is found near the soma (Bakkum et al., 2013; Muller et al., 2015), i.e., in the vicinity of the axon initial segment, we decided to use the location of this peak as the main stimulation location in our experiments. We used a biphasic (positive-then-negative) pulse shape as our primary stimulus, because it has been shown to be an effective stimulus.
(Wagenaar et al., 2004), and since we found biphasic pulses to be more reliable (biphasic pulses elicited responses from 100% of the stimulated cells, while monophasic negative pulses elicited responses from 33% of the stimulated cells). We found that peak-to-peak voltage pulse amplitudes on the order of 150 mV (equivalent to a charge of 235 pC or current of 1.175 μA) were sufficient to evoke a response from ganglion cells (see Figure 4-8). The stimulation values are comparable to the stimulation thresholds found for rat and monkey retina (Sekirnjak et al., 2006; Jepson et al., 2013).

4.4.3 Finding Thresholds

We obtained the stimulus thresholds by stimulating cells at the electrode featuring their highest STA-EAP amplitude, and reading out distally, hundreds of micrometers away, along the axon. Due, again, to the fact that we could observe the action potential on single traces along the axon, obtaining the threshold was a matter of counting the number of times that there was a response at the axon at each voltage and fitting the result. It could happen, presumably due to the proximity of an axon bundle or simply other cells, that more than one cell was stimulated or recruited, as shown in Figure 4-4C. In such a case, the action potentials from the other cells summated, resulting in a response waveform that was larger in magnitude and wider in duration than the response of the targeted cell. In this instance, the response waveform near the threshold value was either manually compared with the light-stimulated STA-EAP and confirmed as the response of the targeted cell, or the data was discarded.

4.4.4 Threshold vs. Ganglion Cell Type

Ganglion cells of different types contain a unique distribution of sodium channel types (Fried et al., 2009), have dendritic stratification at different levels in the inner plexiform layer (Rockhill et al., 2002; Azeredo da Silveira and Roska, 2011), and, additionally, have different connectivity based on cell type (Field and Chichilnisky, 2007); it would therefore be within the realm of likelihood that cell threshold may vary according to cell type. Indeed, it was found in a patch clamp study that the electrical stimulation thresholds varies according to cell type in the retina (Fried et al., 2009). However, a recent paper using MEAs showed no significant difference in stimulation threshold for different cell types (Jepson et al., 2013). Our hamster data showed a difference between OFF and ON cells: OFF cells had a significantly lower mean threshold (Supplementary Figure 4-2). This finding is in contrast to (Sekirnjak et al., 2006), who found that OFF parasol cells in monkey had a higher threshold than ON cells. This difference may be due to the fact that we compared the entire class of ON cells to OFF cells and did not differentiate between subclasses.
4.4.5 Stimulus Threshold Maps

Threshold maps are a method for probing a cell’s response to stimulation at different locations and of determining the optimal location to stimulate (Fried et al., 2009). As mentioned in the Results section, we found that the ganglion cells were most easily stimulated near the peak of the STA-EAP and the action potential origin. Therefore, in order to sample an area where the lowest threshold was expected, we stimulated at a radius of 3 electrodes (52.5 μm along the horizontal and vertical axes, and 74.3 μm along the 45 and 135 degree axes) from the electrode next to the STA-EAP peak. As shown in Figure 4-5 and Figure 4-6, threshold maps evidence indeed lowest stimulation thresholds near the STA-EAP peak or action potential origin (the location of the first negative peak occurring upon cell firing). However, the minimal threshold values within the maps did not exhibit a smooth or symmetrical increase away from the center, as can be seen in Figure 4-5 and Figure 4-6. This may partially be due to contact variations between the different electrodes and the surface of the cell, which could affect the charge transmission and distribution, thereby changing the measured threshold. Electrode impedance variations are too low to account for such threshold distribution inhomogeneities (Muller et al., 2015).

4.4.6 Threshold Factors

We analyzed the response of the threshold maps combined with the information obtained from the STA-EAP to determine what factors the stimulation threshold was dependent upon. We then analyzed the dependency of the threshold on action potential distance travelled, STE-EAP amplitude, and latency. We found that the threshold was directly correlated to distance travelled along the axon and to latency, as shown in Figure 4-7B and Figure 4-7C. Since the stimulus threshold has been found to be lowest at the maximum channel density on the axon, (Fohlmeister and Miller, 1997; Boiko et al., 2003; Sekirnjak et al., 2006; Fried et al., 2009), it is obvious that the threshold increases farther away. Latency, which we found to be strongly correlated with distance (Figure 4-7B), was therefore also correlated with the threshold. Finally, we found an inverse correlation between threshold and STA-EAP amplitude, as shown in Figure 4-7C; this is also logical, since the STA-EAP decreases as one moves away from the cell, and therefore the cell will be more difficult to stimulate at a greater distance, a phenomenon that was also observed by (Jensen et al., 2003; Sekirnjak et al., 2008; Habib et al., 2013); the former two authors reported a nonlinear correlation (monkey), while we and the latter author observed linear correlations (hamster and rabbit). The root mean square values for two of the cells (#2 – 3) in Figure 4-7B were quite low, suggesting a poor fit for the fitted line. We also checked the correlation coefficients for these cells, which showed a strong correlation for cell #2, but not for #3. However, to show that the threshold could be more accurately explained by the combined values of
distance travelled, STE-EAP amplitude, and latency, we performed an ordinary least squares solution to a linear system of equations (Strang, 1986). This resulted in a correlation coefficient of $0.608 \pm 0.209$ for all cells, indicating that the stimulation threshold is dependent on these three factors combined.

### 4.4.7 Stimulation Electrode Number and Pulse Phase Count and Polarity

The most obvious and widely-implemented manner of stimulating ganglion cells is to use a single electrode with a distant counter-electrode in order to inject a negative current or to create a transitory localized negative voltage gradient (Rutten, 2002b; Wagenaar et al., 2004; Merrill et al., 2005; Cogan, 2008). This current changes the ionic concentration in the vicinity of a given ganglion cell, bringing its membrane to its threshold voltage and causing it to depolarize (Cogan, 2008). This method works well enough, but the electrical field extends over a comparably large distance to the reference electrode (which serves as a counter-electrode) and is thereby diffuse (Merrill et al., 2005). There is also the danger that the stimulating current will be high enough to inadvertently stimulate adjacent ganglion cells, especially as the axons of ganglion cells in the retina can become very dense and may be in the vicinity of the ganglion cell STA-EAP peak location that is being stimulated (Rizzo et al., 2003; Wilms and Eckhorn, 2005; Behrend et al., 2011; Jepson et al., 2013).

An alternative to single electrode stimulation is to use multiple electrodes; in fact, this technique was applied by (Jepson et al., 2014b) using 3 electrodes simultaneously, albeit with a lower resolution MEA (60 μm spacing). In the case of electrode pairs, when one electrode is injecting a current, the other electrode is injecting current of the opposite sign. When opposing currents are injected, the electric field generated is theoretically confined to the immediate region close to the electrode pair (which behave as a source and sink) and, more importantly, close to the ganglion cell being stimulated. This electric field confinement should produce a targeted stimulatory impulse.

We tested our electric field confinement by stimulating with not only single electrodes with biphasic and monophasic pulse waveforms, but with two electrodes that were stimulated at the same time with opposing pulse waveform polarities (see pulse waveforms in Figure 4-8, Columns 4 – 11). As noted in the Results section, we did not see any significant differences in stimulation threshold when changing the number of phases of the stimulus, or using pairs of electrodes to deliver the stimulus pulse. The fact that monophasic negative pulses were not significantly better than the biphasic pulses could be due to the fact that in effect, the effective negative current injected was not actually different: indeed, the negative current injected, which has been found to be the factor that triggers the action potential, is the first derivative of the voltage (Wagenaar et al., 2004). The negative voltage transient in both cases was essentially the same for the same peak-to-peak voltage of a mono- or biphasic
pulse, since their peak-to-peak voltages were equivalent. One explanation as to why difference was found between single electrodes and pairs could be that in the case of electrode pairs, the electric field between the two electrodes is confined to such a degree, that the cell is at the periphery of this electric field and is therefore not as efficiently stimulated as would be expected for focal stimulation.

It should be noted that one advantage of using the electrode pair stimulation paradigm was that the vector orthogonal to the vector containing the two electrodes had minimal artifact near the stimulation site. If the orientation was correctly selected with regard to the axonal orientation, the response to the stimulus could be read very close to the stimulation site (see Supplementary Figure 4-4B).

4.4.8 Chip-Wide Activity and Amplitude

The ganglion cell activity was homogeneously distributed across the 2 x 2 mm² retina segment on the HD-MEA, which was to be expected, since the segment was taken from the periphery of the retina, where the density does not vary appreciably over these dimensions (Tiao and Blakemore, 1976). As shown in Supplementary Figure 4-3A, the mean firing rate of the ganglion cells did not vary noticeably across the region, and the peaks indicate the locations of many active cells. Furthermore, locations with peak firing rates may have been locations where two or more cells were firing, since thresholding does not differentiate between spikes from one cell and spikes from another. On the flanking regions of the HD-MEA (shown in light gray and labeled as “Pt Bright” in Supplementary Figure 4-3), the cellular activity can also be observed: note that the dotted line, which delineates the profile of the retina piece, extends over the platinum bright region. Although the noise is higher in the platinum bright regions, the cells are still be detected. In Supplementary Figure 4-3B, an amplitude map is shown. The amplitudes are homogeneous; mostly yellow (-150 μV) signal amplitude are visible where the retina is located, with blue peaks of large negative amplitudes, up to -400 μV. Note that to the far left in the the platinum bright region, the amplitude is approximately at -100 μV, while in platinum black region, the amplitudes are closer to zero. The amplitude difference between the platinum bright and platinum black regions is due the fact that platinum bright electrodes have a higher impedance (measured as capacitance: 0.04 nF) than the platinum black electrodes (2 nF) (Muller et al., 2015).

4.4.9 Focal Stimulation

As shown in the close-up in Figure 4-9, where the spike-sorted ganglion cells are shown, the cells are quite densely packed in the retina near to a targeted cell. Nonetheless, at a low enough stimulation voltage, no activity of neighboring
cells was observed (Figure 4-10A, B). At very small voltages (at and below 165 mV), the targeted cell also did not respond. Once the threshold (at 220 mV) had been reached, it was possible to stimulate this targeted cell exclusively (Figure 4-10B), since the electrode density was high and provided access to the most stimulable region of the cell. Higher voltages (at and above 275 mV) appeared to activate more than one cell, as shown in Figure 4-10C, which was to be expected, since the cell count was high in this region.

While these three images are two-dimensional representations of sub-threshold, threshold and super-threshold stimulation, the movies in the Supplementary data show this much more convincingly. Indeed, in the case of four out of four ganglion cells, selective stimulation could be achieved. Below the threshold (55 - 220 mV) there was no response, and only a stimulation artifact was measurable. However, in the movies where the cell was stimulated at threshold (165 - 275 mV), the axon potential can be seen propagating in a southerly direction (at approximately 270°) following stimulation. Significantly above threshold (at the maximum voltages that we tried for these cells, which was 605 mV), there was a complex propagation that involved the activation of multiple cells, as well as antidromic propagation (see super-threshold Supplementary Movies #3, #6, #9 and #12), which presumably occurred from collateral stimulation of axons of other ganglion cells. In all cases of super-threshold stimulation (especially in Supplementary Movie #6), the axonal activity was along the same vector (north to south), which can be explained by the alignment and bundling of the axons. Moreover, we observed a rebound effect following stimulation of one ganglion cell at threshold voltage. Since the rebound occurred at the dissected edge of the tissue (the edge of the tissue was aligned with the southern edge of the HD-MEA), the phenomenon could have been due to an impedance mismatch at the severed distal end of the axon (Goldstein and Rall, 1974; Debanne et al., 2011), which was also observed in cortical cultures using a similar HD-MEA (Bakkum et al., 2013).

Selective stimulation of ganglion cells has been the focus of other studies, most notably amongst them (Jepson et al., 2013). In this work, the authors used a MEA to apply a current stimulus by using up to three electrodes simultaneously to focally stimulate cells in the macaque monkey. Our equivalent (using Equation (1)) threshold pulse charge of 235 pC is larger than their range of 50 to 100 pC per electrode. This may however, be due to ganglion cell differences between the species or to the fact that we are using voltage-controlled pulses and they are using current-controlled pulses. Another difference is that we used a pulse phase width of 200 μs, while they used up to 300 μs. Although we can make a theoretical conversion from from voltage to charge units, we are not entirely certain what the currents are and where they flow. Nonetheless, we believe that the smaller electrode pitch (17.5 μm electrode spacing versus 60 μm) provides a significant advantage in focal stimulation, since a larger electrode density makes it possible to selectively access cells in a region of higher ganglion cell density and to focus on their most stimulable regions.
Indeed, other HD-MEA systems do feature high densities of electrodes, but suffer from much higher noise, fewer and larger stimulation spots, and less fortunate electrode charge transfer characteristics as a consequence of the use of dielectric electrode materials (Eversmann et al., 2003a; Bertotti et al., 2014). The low electrode noise enables us to record from each cell at subcellular resolution, including extended regions of the axons; the high overall electrode number and density makes it possible to stimulate the cell at its lowest threshold region, to observe its action potential propagation, and to observe activity from neighboring cells.

4.5 Conclusion

We demonstrated here that we were able to locate the STA-EAPs of ganglion cells in a segment of retina and record optically induced extracellular activity at subcellular resolution. We then stimulated the cells with voltage pulses and applied voltage sweeps so as to determine the stimulation threshold for each cell. Voltage maps were generated to explore the threshold variation as a function of location relative to the STA-EAP. We showed that voltage pulses applied on two electrodes simultaneously did not reduce the threshold, which may have been due to excessive confinement of the electric field generated between the two electrodes. Finally, we applied the threshold voltage for targeted ganglion cells while recording from the entire HD-MEA area, clearly showing that we selectively stimulated the targeted cell, despite the close proximity of other cells in that region. Our results have shown the significance of the electrode density of the MEA in order to achieve accurate selective stimulation in the retina, both in terms of spatially sampling the cell’s extracellular signal, as well as locating the optimal location to stimulate.

4.6 Outlook

The foveal region of the human retina, which is essential for high-acuity vision and reading, contains a high density of ganglion cells (Dowling, 2012). In order to stimulate ganglion cells independently, and avoid inadvertent stimulation of other ganglion cells (which causes collateral cell activation, it is clearly essential that the stimulation device be of sufficient resolution to interface with the ganglion cells. Indeed, at the present time, prosthetic MEAs must be implanted in the peripheral regions of the retina, since the ganglion cell density nearer to the fovea is too high for the latest technology available (Shepherd et al., 2013). The HD-MEA presented here provides the opportunity to advance a step further towards high-accuracy and high-precision stimulation. While this may still not be sufficient for future high-end prosthetic devices, it shows the benefits of increased resolution, and how the currently available electrode resolution can most effectively be harnessed.
Chapter 4: Electrical Stimulation Response

4.7 ACKNOWLEDGEMENTS

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4.8 SUPPLEMENTARY DATA

Supplementary Figure 4-1: HD-MEA Showing MEA and Retina Piece on MEA. (A) shows an “en face” view of the packaged Mea1k Chip, with the HD-MEA in the center (outlined in white). (B) A zoomed-in region of the chip showing a more detailed view of the HD-MEA, part of which is black due to the platinum black electrodeposition. (C) Here, the same zoomed-in region is shown with a segment of retina on top of the platinum black region of the MEA (white arrow points to the retina segment).
Supplementary Figure 4-2: Cell Types. The threshold values of ON and OFF cells, stimulated with a biphasic positive-then-negative voltage pulse, are plotted with a random jitter along the x-axis to aid visibility of data points. ON thresholds are indicated by red spots; OFF thresholds are indicated by black spots.
Supplementary Figure 4-3: Activity and Amplitude Map across Entire HD-MEA. In Panel (A), the firing frequency as measured on the entire chip during optical stimulation is shown. This image was created by thresholding the action potentials of all cells measured on all the 26,400 electrodes (using multiple recording electrode configurations). On the regions to the sides, where the firing rate is zero, there was no retinal tissue. White dotted line shows location of retina segment. (B) shows the action potential amplitudes for the same tissue. Platinum black was only deposited in the central region of the HD-MEA (marked in dark gray and labeled Pt Black). The mean standard deviation of the signal (noise) for regions without platinum black was 4.078 ± 1.007 and the noise for regions with platinum black was 2.590 ± 1.141. These noise levels were significantly different (a t-test resulted in a p-value of 1.5289×10⁻¹⁰). Noise measurements were made in regions without tissue. Black dotted line shows location of retina segment.
Chapter 4: Electrical Stimulation Response

Electrical Stimulation Movies

Four electrical stimulation movies show voltage stimulation and responses of four separate ganglion cells, numbered 0 through 3, upon “subthreshold”, “threshold” and superthreshold stimulus. Each movie file contains the median frames recorded on all HD-MEA electrodes during stimulation at one voltage. For sub-threshold recordings, only the stimulus artifact can be seen. The movie files at the stimulus threshold show axonal propagation of the ganglion cell being stimulated. Super-threshold movie files show the activation of multiple ganglion cells being stimulated in the vicinity of the targeted cell. Files are named as follows:

[file number]_movie_cell[cell number]_08_[sub/thresh/super]_[stimulation pulse amplitude]mV.avi

Below is a table of the stimulated cells that should serve as a guide to the files: “Threshold” indicates the computed threshold for this cell, derived by fitting a sigmoidal curve to the responses to a voltage sweep; “Sub-Threshold” is the voltage used to stimulate the cell below threshold; “At Threshold” is the voltage used to stimulate the cell just above threshold; “Super-Threshold” is the maximum voltage used to stimulate the cell.

<table>
<thead>
<tr>
<th>Cell Number</th>
<th>Threshold (mV)</th>
<th>Sub-Threshold</th>
<th>At Threshold</th>
<th>Super-Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>193</td>
<td>165</td>
<td>220</td>
<td>605</td>
</tr>
<tr>
<td>1</td>
<td>171</td>
<td>165</td>
<td>220</td>
<td>605</td>
</tr>
<tr>
<td>2</td>
<td>160</td>
<td>55</td>
<td>165</td>
<td>605</td>
</tr>
<tr>
<td>3</td>
<td>256</td>
<td>220</td>
<td>275</td>
<td>605</td>
</tr>
</tbody>
</table>
Chapter 5 Conclusion

The use of low-noise and high-density MEA systems to study the mammalian retina provides a unique and powerful tool to investigate the functionality of the retinal circuitry at the cellular level. Not only can the extracellular action potential propagation of cells be measured at high spatial and temporal resolution, but the high electrode density also makes it possible to record signal levels down to tens of microvolts from subcellular features on up to a couple hundred electrodes simultaneously. These planar HD-MEAs – the 127-channel HiDens (Frey et al., 2010a) and the 1,024-channel Mea1k (Ballini et al., 2014; Muller et al., 2015) – are especially well-suited to interfacing with the cells of the neural output layer of the retina: the ganglion cells. The ganglion cells lie along a planar surface, which puts them in close proximity to the electrodes of the HD-MEA. Thus, the HD-MEA can both detect voltage fluctuations generated by the cells as well as deliver electrical impulses to them, serving as a bidirectional electrical neuro-interface to the retina.

Ganglion cells have been studied with microelectrodes and patch clamp for decades (Granit and Svaetichin, 1939; Kuffler, 1953; Rodieck and Stone, 1965; Lukasiewicz and Werblin, 1988), with MEAs for the past quarter century (Meister et al., 1994; Zrenner et al., 2011). The HD-MEAs featured in this work (Frey et al., 2009), make it possible for us to observe details and features of the extracellular signals of ganglion cells that would not necessarily be detected using other modalities or systems. For selected neurons, we were able to record regions of the ganglion cells that we believe to be the initial segment of the axon, as well as regions of the axon that extended up to distances of hundreds of microns. We observed phenomena, such as the inversion of the action potential waveform as it propagated from its origin to the axonal regions, and measured amplitudes, latencies, and action potential propagation speeds on multiple electrodes.

Our optical stimulus ganglion cell classification system enabled us to sort out cell types in a semi-automated fashion. Indeed, compared to other studies using MEAs, where a dozen cells are recorded in one experiment, our throughput on the order of fifty cells per experiment was an improvement (Farrow and Masland, 2011b). The increase in cell yield was due primarily to the high
electrode density, as well as to the fact that cells did not have to be spike-sorted during the experiment. However given a commonly-estimated 20 - 24 ganglion cell types in the mammalian retina (Ammermuller and Kolb, 1995; Linberg et al., 1996; Masland, 2001a; Cohen et al., 2002; Kong et al., 2005), our identification of seven functional cell types did fall short of our expectations. A categorization of all cell types in a hamster retina was an ambitious undertaking, and there are certainly aspects to be improved. Firstly, if the optical classification system were modified such that it would run on the Mea1k, the throughput would be increased by an order of magnitude. Naturally, with the generation of massive amounts of recorded data over extended periods, data storage considerations must be taken into account. Also, while spike sorting and STA-EAP template matching worked reasonably well and is being continually improved (Franke et al., 2014), spike sorting this quantity of data from in excess of a thousand channels would require a considerable amount of computational power and algorithm optimization. Finally, the complexity of the optical stimulation sequence would most certainly need to be increased and incorporate, for example, natural movie stimuli. Although natural movies are challenging to analyze, they would stimulate a larger variety of cell types (Farrow and Masland, 2011b).

The electrical stimulation capabilities of the Mea1k made it possible to deliver precisely-timed voltage pulses to the cells. We therefore had the opportunity of not only recording at subcellular resolution, but of stimulating at subcellular resolution: in effect, we had a true bidirectional interface with the ganglion cells. Since ganglion cells respond to voltage pulses differently depending on where they are stimulated – most likely due to different trans-membrane sodium channel distributions along the axon (Fried et al., 2009) – we were interested in mapping out the response of the cell to electrical stimuli at different spatial locations. We found that we could most easily stimulate near the STA-EAP peak. We also used different combinations of pulse phases and polarities on both single and pairs of electrodes to stimulate at the STA-EAP peak. Surprisingly, we did not observe the expected trends in the stimulation threshold of the cells, as the stimulation pulses all performed more or less equally well. In future, additional waveform variations would need to be tested, possibly using stimulation electrode configurations with more than two electrodes, along the lines of (Jepson et al., 2013); in addition, once fully characterized and implemented in this MEA, current controlled stimulus pulses should give greater control over stimulation accuracy: current-controlled stimulation is not susceptible to impedance variations due to platinum black electro-deposition irregularities; however, forcing a defined current through an electrode may require excessively high voltages that then harm the cells; voltage-controlled pulses, on the other hand, ultimately produce current pulses, the magnitude of which varies according to electrode impedance (Wagenaar et al., 2004); their advantage is that the preset maximum voltage will not be exceeded. Nonetheless, we found that biphasic or monophasic voltage pulses on single electrodes were adequate for stimulating ganglion cells selectively.
Indeed, by applying voltage at the stimulus threshold for each cell, the stimulus artifact and subsequent single axonal propagation could clearly be observed.
Chapter 6 Outlook

It would be of great interest to be able to relate the extracellular activity of a cell to the anatomy of the cell, since then it would be known precisely what regions are being recorded and what is being stimulated; the next step in HD-MEA electrical stimulation would be to attempt correlations between extracellular electrical signal features and cell morphology. Experimental evidence indicates it to be likely, for example, that the STA-EAP peak marks the location of the axon initial segment or axon hillock (Sekirnjak et al., 2008; Bakkum et al., 2013); this hypothesis is supported by the existence of high-density sodium channel bands in this region (Fried et al., 2009). In order to do prove this correlation, the anatomical parts must be identified with techniques such as immunostaining (Boiko et al., 2003). This would also reveal the precise location of the soma, which we have, as of yet, been unable to identify using the STA-EAP alone, and ultimately contribute towards validating HD-MEA recording as a complementary method with the gold standard of traditional patch clamp recording.

There are still common technological hurdles to be overcome in the interfacing of ganglion cells and HD-MEAs. While we recorded in a relatively low density peripheral area of the hamster retina (in the low thousands of cells/mm²), there are other regions in the retina that have much higher ganglion cell densities, such as the visual streak in the rabbit, which has 5,000 cells/mm² (Oyster et al., 1981), and the human fovea, which has up to 35,000 cells/mm² (Curcio and Allen, 1990). The majority of visual tasks performed by humans, such as high-acuity visual discrimination and reading, involve the fovea; but to interface with such a great density of neurons, the electrode size and pitch must be further decreased, although not to the extent that the signal quality decreases (Cogan, 2008). Furthermore, it must be possible to deliver sufficient current to activate the targeted cells.

The applications for methods involving optical and electrical stimulation of ganglion cells are numerous, especially with respect to the development of visual restoration solutions in patients. Firstly, an understanding of the
particular ganglion cell types and associated functionality is essential in this endeavor: since each cell type responds differently to optical stimuli, all cells should be electrophysiologically identified, and the typical spike train response of each cell type should be known. With this information, the relevant cells can be artificially stimulated using a prosthetic device in response to an optical stimulus, and the brain will be able to interpret the information correctly. For example, when a bright bar is moving in a particular direction, the prosthetic device should stimulate ON-OFF direction selective cells. Secondly, once each cell is identified, it must be stimulated precisely at the right point in time such that it conveys the correct information about the optical stimulus. This requires a high level of accuracy, since other ganglion cells should not be stimulated collaterally. Moreover, minimal use of power is always desirable in such devices, as the power available in implanted devices is limited, as is device heat dissipation (Margalit et al., 2002). It is for these reasons that knowledge about how to stimulate ganglion cells efficiently and, above all, selectively is important in this field.

In conclusion we see great potential for the HD-MEA interface to retina platform. The research presented here has highlighted the many advantages that the device offers in learning about retinal ganglion cells; we look forward to the increased implementation in the laboratory and further advancement of this technology, which we anticipate will result in expanding our scientific knowledge of the circuitry of the retina and lead to the development of increasingly sophisticated medical applications.
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Chapter 8 Publications

8.1 Journal Articles


8.2 Conference Contributions


Chapter 8: Publications

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