Doctoral Thesis

Combining CMOS-based microelectrode arrays with genetic labeling to study visual processing in the retina

Author(s):
Fiscella, Michele

Publication Date:
2014

Permanent Link:
https://doi.org/10.3929/ethz-a-010271475

Rights / License:
In Copyright - Non-Commercial Use Permitted
Combining CMOS-Based Microelectrode Arrays with Genetic Labeling to Study Visual Processing in the Retina

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

presented by

MICHELE FISCHELLA
Laurea Specialistica in Biotecnologie Molecolari e Industriali, Università di Bologna

Born on 8th April 1983
Citizen of Italy

Accepted on the recommendation of

Prof. Dr. Andreas Hierlemann
Dr. Botond Roska
Dr. Felix Franke

2014
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thesis Abstract</td>
<td>6</td>
</tr>
<tr>
<td>Sommario Tesi</td>
<td>9</td>
</tr>
<tr>
<td>Major Findings</td>
<td>13</td>
</tr>
<tr>
<td>Author’s Contributions</td>
<td>16</td>
</tr>
</tbody>
</table>

### Chapter 1: Microelectrode Array Technology for Recording Retinal Activity

1.1 Neurons and Action Potentials                                      | 19   |
1.2 Basic Techniques for Recording Neural Electrical Activity          | 20   |
1.3 Microelectrode Arrays                                              | 22   |
1.4 Retina and Microelectrode Arrays                                   | 26   |
1.5 Sorting Extracellular Action Potentials                            | 28   |
1.6 Retinal Ganglion Cell Types                                       | 32   |
1.7 Summary                                                            | 33   |
References                                                             | 34   |

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

2.1 Abstract                                                           | 41   |
2.2 Introduction                                                       | 41   |
2.3 Methods                                                            | 43   |
2.3.1 Data Acquisition System                                          | 43   |
2.3.2 Projection and Alignment of Images with the MEA                  | 44   |
2.3.3 Preparation of Mouse Retina and Light Induced Activity Recordings| 45   |
2.3.4 Optogenetic Stimulation of Retinal Ganglion Cell Types          | 45   |
2.3.5 Data Analysis                                                    | 46   |
2.3.6 Imaging of Mouse Retinal Ganglion Cells                         | 46   |
2.4 Results                                                            | 48   |
2.4.1 Light Induced Artifacts in CMOS Technology and Light Evoked Retinal Activity | 48   |
2.4.2 Characterization of Mouse Extracellular Action Potentials        | 51   |
2.4.3 Separation of Retinal Ganglion Cells Action Potentials          | 53   |
2.4.4 Physiological Characterization of Retinal Ganglion Cells        | 56   |
2.4.5 Selecting a Defined Population of Retinal Ganglion Cells for Extracellular Recordings | 58   |
2.4.6 Recording from Genetically Identified Retinal Ganglion Cells    | 59   |
2.5 Discussion                                                         | 62   |
Acknowledgements                                                       | 65   |
References                                                             | 65   |
Supplementary Methods                                                  | 69   |
Chapter 5: Conclusions and Outlook

5.1 Conclusions: The Importance of Microelectronics-Based MEAs for Accessing Sub-Cellular Neural Features and Defined Neural Population Activity

5.2 Outlook: Limits of the HiDens MEA and Development of MEA1k

References
Thesis Abstract

This thesis presents the application of a microelectronics-based microelectrode array (MEA) system for studying the concerted activity of defined neurons in the retina. The principal motivation behind this work is that, in the past ten years, neurosciences were significantly influenced by genetic characterization of cell types. As a consequence, it became important to develop reliable methodologies to study information encoding/decoding by defined neural populations. We focused our investigation on retinal neurons specialized in extracting certain features of the visual scene, namely the movement of objects (including, e.g., direction, velocity). The analysis of electrical signals from tens of retinal neurons required sophisticated data processing techniques, which included modeling and sorting of neural activity.

The retina is a layered neural tissue lining the back of the eye that converts visual inputs into electrical signals. The retina contains ~60 cell types forming circuits, which extract precise features from the visual scene. Retinal ganglion cells (RGCs) are the output retinal neurons and are organized in a two-dimensional layer, which is easy to access by microelectrode array technology. Importantly, ~20 RGCs send electrical signals to the brain through parallel information channels. The retina can be isolated and subsequently stimulated by light for 8-10 hours without disrupting the original cell connectivity, and it is possible to stably record RGC electrical activity during those 8-10 hours under perfusion conditions. The retina is an ideal model system for neuroscience research for the following reasons: (1) it can be isolated preserving original circuitry; (2) light, the primary retina input, can be conveniently modulated in space and time; (3) microelectrode array (MEA) technology can be used to stably record RGC population activity during hours.

MEA technology is routinely used for recording RGC activity through multiple electrodes, located at different spatial positions. Commercial MEAs feature ~60 electrodes spaced more than 100 µm. In this thesis, we used a high-density MEA system (HD-MEA), developed at
ETH Zürich, which features low noise levels (7 - 9 $\mu$V$_{rms}$ during tissue recording) and more than 11,000 recording sites with a center-to-center electrode distance of 18 $\mu$m. HD-MEAs are a fundamental research tool in neuroscience mainly for three reasons: (1) HD-MEAs enable to potentially detect every neuron sitting on the array; (2) HD-MEAs can resolve electrical signal features at sub-cellular resolution; (3) HD-MEAs allow for isolating single-cell activity more accurately than commercial MEAs, and, thereby, massively ease spike sorting, the assignment of the electrical signals to the individual neurons. Since the HD-MEA system used in our experiments was built by complementary metal oxide semiconductor (CMOS) technology, we had to address the problem of light-induced artifacts in the semiconductor chips during retinal recording.

We first designed and built a microscope setup for precise light stimulation of retinal tissue on the HD-MEA; we successfully stimulated retina with light patterns and were able to record retinal ganglion activity without light-induced artifacts in the acquired electrical signals. Next, we developed a method for finding and recording defined population of RGCs. Spike sorting was performed online during, and electrodes were assigned to RGCs of interest (e.g., direction-selective ganglion cells). We implemented a spike sorting method based on independent-component analysis (ICA) for accurate online identification of RGCs. Finally, we demonstrated that (i) densely packed RGCs could be recorded and identified and that (ii) recording of genetically identified RGCs by optogenetic stimulation was possible on HD-MEAs.

The concerted activity of RGCs provides vision. Therefore, the ability to record from a defined population of RGCs enables to address the following computational-neuroscience-related questions:
(1) How does a population of RGCs encode the visual scene?
(2) How does the brain decode retinal signals for reconstructing the visual scene?
(3) What is the combination of RGCs that carries most information about the visual scene?
In order to address the questions listed above, we studied direction-
selective ganglion cells, a population of retinal neurons that responds only to defined directions of a moving object. We reconstructed directions of moving objects by using the concerted activity of the direction-selective ganglion cells in combination with computational techniques, such as linear or Bayesian decoders. Finally, based on measurement data, we simulated the activity of direction-selective ganglion cells to investigate whether their response characteristics are optimally combined for the task of inferring directions of moving objects.

The data sets acquired within this thesis serve as starting point for developing new data analysis techniques, which will be used for the next generation of HD-MEA (26.000 electrodes). Finally, understanding the physiological activity of complete RGCs populations is fundamental for testing medical therapies, which aim at restoring vision in patients with eye diseases.
Sommario Tesi

Questa tesi presenta l’applicazione di un array di microelettrodi, fabbricato con una tecnologia di microelettronica, al fine di studiare l’attività di definite popolazioni di neuroni nella retina. La motivazione principale dietro questo lavoro sta nel fatto che, negli ultimi dieci anni, le neuroscienze sono state significativamente influenzate dalla caratterizzazione genetica di molteplici tipi di cellule. Di conseguenza, è diventato fondamentale sviluppare delle metodologie affidabili, per studiare come le informazioni vengono codificate e decodificate da definite popolazioni di neuroni. Questa tesi è focalizzata su dei neuroni della retina, specializzati nell’estrazione di determinate caratteristiche di oggetti in movimento (esempio: direzione, velocità). Infine, per studiare i segnali elettrici di decine di neuroni della retina, è stato necessario utilizzare sofisticate tecniche di analisi dei dati, che includono la generazione di modelli dell’attività dei neuroni e la separazione dei segnali di singoli neuroni (quest’ultima tecnica è conosciuta col nome di “spike sorting”).

La retina è un tessuto di neuroni localizzato nella parte interna dell’occhio che trasforma gli input visivi in segnali elettrici. La retina contiene circa 60 diversi tipi di cellule che formano elaborati circuiti al fine di estrarre precise informazioni dell’input visivo. Le cellule gangliari della retina (RGCs) sono i neuroni responsabili dell’output della retina e sono organizzate in una struttura bidimensionale, che è facilmente accessibile dalla tecnologia di array di microelettrodi. In totale, sono stati caratterizzati circa 20 tipi diversi di cellule gangliari della retina che mandano al cervello informazioni attraverso canali elettrici paralleli di informazione. La retina può essere isolata e stimolata per mezzo della luce per circa otto-dieci ore senza danneggiare i circuiti originali e di conseguenza è possibile registrare in modo stabile l’attività elettrica delle cellule gangliari. In conclusione, la retina è un modello ideale per la ricerca nel campo delle neuroscienze per i seguenti motivi: (1) può essere isolata preservando le connessioni fra i neuroni; (2) la luce, l’input principale, può essere modulata nello spazio e nel tempo; (3) array di microelettrodi possono essere utilizzati per registrare in modo
stabile l’attività elettrica di popolazioni di cellule gangliari.

Gli array di microelettrodi sono spesso utilizzati nelle neuoscienze per registrare l’attività delle cellule gangliari per mezzo di molteplici elettrodi localizzati in differenti posizioni. Gli array di microelettrodi commerciali (MEA) solitamente contengono circa 60 elettrodi e la distanza fra due elettrodi è circa 60 µm. In questa tesi, è stato utilizzato un sistema ad alta densità di elettrodi (HD-MEA), sviluppato al politecnico di Zurigo, con bassi livelli di rumore (7 – 9 µV_{rms}, con material biologico sul biosensore) e più di 11.000 elettrodi con una distanza fra due elettrodi di 18 µm. Sistemi di microelettrodi ad alta densità (HD-MEA) sono uno strumento essenziale per la ricerca in neuroscienze principalmente per tre ragioni: (1) gli HD-MEA permettono di registrare potenzialmente ogni neurone sul biosensore; (2) gli HD-MEA possono registrare segnali elettrici a una risoluzione subcellulare; (3) gli HD-MEA possono isolare i segnali di singole cellule più accuratamente rispetto ai sistemi commerciali e di conseguenza il processo denominato “spike sorting” viene significativamente semplificato (spike sorting è il metodo mediante il quale i segnali elettrici di diversi neuroni vengono separati). Il sistema HD-MEA utilizzato nei nostri esperimenti è stato realizzato attraverso tecniche di microelettronica CMOS (complementary metal oxide semiconductor), di conseguenza è stato necessario affrontare il problema di artefatti indotti dalla luce per la stimolazione della retina.

Per cominciare, è stato necessario progettare e assemblare un microscopio per la precisa stimolazione della retina sull’array di microelettrodi. Dopo la realizzazione del microscopio è stato possibile stimolare la retina per mezzo della luce e registrare l’attività elettrica delle cellule gangliari senza nessun artefatto nei segnali elettrici registrati. Successivamente, è stato sviluppare un metodo per localizzare e registrare l’attività di definite cellule gangliari durante l’esperimento. La separazione dei segnali elettrici di differenti cellule (spike sorting) è stata effettuata online durante gli esperimenti. Una volta localizzate e identificate le cellule di interesse (esempio: cellule gangliari sensitive alla direzione del movimento),
gruppi di 5-7 elettrodi sono stati assegnati esclusivamente alle cellule di interesse. Per la separazione dei segnali elettrici è stato implementato un metodo basato su “independent component analysis (ICA)”. In conclusione, siamo stati in grado di dimostrare i seguenti punti: (i) l’attività elettrica di cellule gangliari della retina, densamente distribuite, può essere registrata e l’attività elettrica di singole cellule caratterizzate; (ii) l’identificazione di cellule gangliari geneticamente identificate è possibile sul biosensore ad alta densità di elettrodi attraverso metodi di stimolazione optogenetica (per optogenetica si intende l’introduzione di canali ionici esogeni in cellule; l’attivazione di questi canali ionici esogeni avviene attraverso la luce e modifica l’attività fisiologica dei neuroni).

L’attività elettrica simultanea delle cellule gangliari è fondamentalmente ciò che rende possibile l’interpretazione da parte del cervello dell’input visivo, dunque il senso della vista. Di conseguenza, la possibilità di registrare popolazioni di cellule gangliari della retina permette di esplorare le seguenti tematiche nel campo delle neuroscienze computazionali:

(1) In che modo una popolazione di neuroni codifica l’input visivo per mezzo di segnali elettrici?

(2) In che modo il cervello decodifica l’input visivo dai segnali elettrici delle cellule gangliari della retina?

(3) Quale combinazione di neuroni contiene maggiori informazioni sull’input visivo?

Per rispondere alle domande elencate in precedenza, abbiamo deciso di studiare le cellule gangliari sensitive alla direzione del movimento, una popolazione di neuroni che nella retina codifica la direzione del movimento degli oggetti. È stato possibile decodificare le direzioni di oggetti in movimento utilizzando l’attività elettrica delle cellule gangliari in combinazione con tecniche computazionali come ad esempio modelli con decodificazione lineare e Bayesiana. Infine, utilizzando i segnali elettrici delle cellule gangliari della retina, ne abbiamo simulato l’attività per capire se le loro caratteristiche di risposta alla stimolazione visiva sono combinate ottimamente per decodificare la direzione degli oggetti in movimento.
I dati acquisiti in questa tesi sono stati utilizzati come punto di partenza per sviluppare nuove tecniche di analisi dei dati, che saranno utilizzati direttamente nella nuova generazione di HD-MEA (26.000 elettrodi). Infine, lo studio dell’attività elettrica fisiologica di popolazioni di cellule gangliari della retina è fondamentale per implementare terapie mediche che hanno lo scopo di reintrodurre la capacità visiva in pazienti con malattie agli occhi.
Summary of Major Findings

Recording Retinal Activity without Light-Induced Signal Artifacts

Light stimuli with different wavelength and power were tested on the HD-MEA. By centering light stimuli on the array, it was possible to successfully record retinal activity. Importantly, we found that the amplifiers that surround the electrode array are the main light-sensitive components of the HD-MEA. Light-induced retinal activity was recorded at low noise levels by means of hundreds of densely packed electrodes (Chapter 2).

Characterizing Electrical Activity of Retinal Neurons at Subcellular Resolution

High spatial electrode density enabled to record electrical activity from densely packed mouse RGCs from 14+7 electrodes per RGC. Defined populations of retinal neurons could be recorded from for long time periods (>8 hours) at subcellular resolution (Chapter 2 and Chapter 3).

Optogenetic Stimulation of Transgenic Retinas on HD-MEA

Channelrhodopsin (ChR2-128S-2A) was delivered by Adeno-Associated Viruses (AAV) to activate genetically identified neurons in the retinae of the Pvalb<sup>Cre</sup> transgenic mouse (Chapter 2).
Recording from Populations of ON-OFF Direction-Selective Retinal Ganglion Cells

For the first time, all four known types of ON-OFF direction-selective ganglion cells were simultaneously recorded from. Spike trains of multiple cells were used to decode information about directions of moving objects (Chapter 2 and Chapter 3).

Decoding and Modeling of the Activity of ON-OFF Direction-Selective Retinal Ganglion Cells

The combined activity of ON-OFF direction-selective ganglion cells was decoded by linear or Bayesian decoders. We found that Bayesian methods decoded motion direction more accurately than linear methods. In addition, the retinal direction-selective system was robust with respect to parameters changes, such as changes in velocity. In contrast, decoding accuracy was negatively influenced by the size of the moving object and motion directions close to the cell’s preferred directions. Finally, we simulated ON-OFF direction-selective ganglion cell activity and found that: (1) ON-OFF direction-selective ganglion-cell tuning curves are sufficiently wide so as to avoid large decoding errors for given stimulus directions; (2) the retinal direction-selective system would not perform much better with an arrangement of cells that cover more than four preferred directions (Chapter 3).
HD-MEA Enables Accurate Spike Sorting

High-density electrode recording of neural activity significantly improved spike sorting of densely packed neural populations. For offline analysis, we implemented a simple sorting algorithm based on principal-component analysis (PCA) and K-means. A simple, although slow, PCA K-means spike sorter was already sufficient to accurately sort the electrical signals. Finally, for fast online analysis during experiments, we implemented an independent-component-analysis (ICA)-based spike sorter (Chapter 2 and Chapter 4).
Author’s Contributions

The core team in this project included:

- **Michele Fiscella**: building the microscope setup for electrophysiology experiments, design of experiments, animal experiments, packaging of HD-MEA, immunohistochemistry, *in vivo* injections of viruses, fluorescent and confocal microscopy, data analysis, modeling of neural activity, and writing scientific manuscripts.

- **Felix Franke**: design of experiments, data analysis, modeling of neural activity, and writing scientific manuscripts.

- **Jan Müller**: Hardware and software support for HD-MEA.

- **Karl Farrow**: design of experiments, animal experiments and data analysis.

- **Urs Frey**: design of hardware and software for HD-MEA recording system.

- **David Jäckel**: data analysis.

- **Ian L. Jones**: technical support with animals and HD-MEA recording setup.

- **Douglas J. Bakkum**: technical support with software.

- **Peter Hantz**: biological experiments with optogenetics.

- **Rava A. da Silveira**: modeling of neural activity, and writing scientific manuscript.
Chapter 1

Introduction: Microelectrode Array Technology for Recording Retinal Activity
1.1 Neurons and Action Potentials

The neuron is the functional unit of the nervous system. Neurons form complex networks that compute and store information. Neurons communicate by electrical signals that are exchanged through specialized contacts called synapses. The electrical activity of multiple neurons provides the basis for the perception of the world and influences the behavior of the organism.

A neuronal cell has three main morphological and functional compartments: dendrites, soma and axon (Fig. 1a). The dendrites are a tree-like structure that receive and integrate electrical inputs. The soma is the cell compartment, where electrical signals are generated (more precisely in a region called “axon hillock”). The axon is a thin cable-like structure (diameter <1 µm) that conveys electrical signals to other neurons.

When an electrical signal reaches the terminal part of an axon, chemical molecules, known as neurotransmitters, are released to change the electrical activity of synaptically connected neurons. Changes in ion concentrations inside or outside neurons cause electrical signals by changing the voltage across the membrane. In particular, ions flow through proteins that form pores in the cell membrane and are called ion channels (Fig. 1b).

One type of electrical signal that is used by neurons, for computing and exchanging information, is the action potential. An action potential is a transitory change (~ 1 ms) of the cellular membrane voltage.

At rest, the intracellular membrane voltage of a neuron is between -90 mV and -40 mV. When intracellular membrane voltage becomes higher than approximately -40 mV (threshold for generating action potentials), Na\(^+\) and K\(^+\) voltage-dependent channels will open. First, voltage-dependent inward Na\(^+\) currents depolarize the cell membrane (i.e., the voltage of the intracellular compartment becomes more positive than the voltage of the extracellular compartment) (Fig. 1b). Second, voltage-dependent outward K\(^+\) currents hyperpolarize cell membrane (i.e., the voltage of intracellular compartment becomes more negative than the voltage of the extracellular compartment) (Fig. 1b). The combination of
Chapter 1

voltage-dependent Na\(^+\) and K\(^+\) currents yield an action potential (Fig. 1c). Neural action potentials can be recorded and studied by intracellular or extracellular electrodes and by optical methods.

**FIGURE 1**

(a) Basic morphology of a neuron. Waveforms below the axon compartment represent the action potential at different times. Action potential starts at the axon hillock (t1). Action potential travels along the axon (t2). Action potential reaches the synapse and causes neurotransmitter release (t3). Red dots indicate neurotransmitter molecules.

(b) Basic action potential mechanism: 1) At rest, the cell is negatively polarized, i.e., the intracellular compartment is more negatively charged with respect to the extracellular space. Na\(^+\) ions feature a larger concentration outside the cell. K\(^+\) ions feature a larger concentration in the cell cytosol. Blue and green rectangles indicate ion channels. 2) When a neuron starts to depolarize and reaches a membrane voltage threshold at -40 mV, Na\(^+\) voltage-sensitive channels open and cause an inward Na\(^+\) current that depolarizes the cell membrane (cytosol becomes more positive than extracellular compartment). 3) K\(^+\) voltage-sensitive channels open and cause an outward K\(^+\) current that hyperpolarizes cell membrane. (c) Intracellular action potential waveform. Numbers refer to the different steps in Fig. 1b.

**1.2 Basic Techniques for Recording Neural Electrical Activity**

The patch-clamp technique uses a glass micropipette as electrode for recording **intracellular** action potentials (Hamill et al., 1981) (Fig. 2a) and allows for recording sub-threshold events, such as excitatory post-synaptic potentials (EPSPs) and inhibitory post-synaptic potentials (IPSPs). Furthermore, the patch-clamp technique enables recording of inhibitory and excitatory neural currents. Since a single
electrode records from a single neuron, electrical signals can be easily correlated and assigned to the respective cell. Although the patch-clamp method can record a large variety of electrical signals, it is an invasive and low-throughput technique. In contrast to patch-clamp, action potentials can be recorded non-invasively and at high-throughput by metal microelectrode arrays (MEAs) (Thomas et al., 1972). In fact, microelectrode arrays can record extracellular action potentials in vivo or in vitro at multiple spatial locations (Fig. 2b). Recent advances in microelectrode array fabrication (“in-cell” recordings by mushrooms electrodes) (Hai et al., 2010) enabled to record sub-threshold signals. Since a single electrode of a microelectrode array records extracellularly from multiple neurons at the same time, electrical signals have to be sorted and assigned to the respective cells.

Action potentials can also be optically recorded by calcium indicators (Fig. 2c) (Miyawaki et al., 1997). Although optical recordings are non-invasive and high-throughput, the time resolution of this technique is not sufficiently high for the detection of single-cell action potentials (Briggman and Euler, 2011). Optical methods can also not record sub-threshold neuronal signals (EPSP, IPSP).

The work described in this thesis uses high-density MEA (HD-MEA) technology for recording action potentials of neural populations.

**FIGURE 2**

(a) Basic scheme of the Patch-clamp technique. Action potential amplitude is ~100 mV peak-to-peak. (b) In vitro microelectrode array technique. Gray squares represent
metal electrodes. Circles represent cells. Action potential amplitude is ~100 µV peak-to-peak. Top right small inset shows in vivo recording with multiple electrodes (gray triangles) inserted in the brain. (c) Optical recordings. Action potential causes Ca\(^{2+}\) influx into the cells. Intracellular Ca\(^{2+}\) increases cell fluorescence by binding exogenous proteins, known as calcium indicators.

1.3 Microelectrode Arrays
For understanding the connectivity and function of neural circuits, it is crucial to record the synchronous electrical activity of neural populations. For recording neural populations, microelectrode array technology can be used either in vivo or in vitro.

In vivo microelectrode arrays are inserted in the brain for recording and isolating extracellular activity of multiple neurons (Maynard et al., 1997; Blanche et al., 2005). In vivo microelectrode arrays have been used to study neural computations in the visual cortex of primates (Graf et al., 2011) or for understanding memory processes in the hippocampus (Buzsaki et al., 1992). In vivo microelectrode arrays were also used as a form of prosthetic devices for paralyzed human patients (Hochberg et al., 2006).

In vitro planar microelectrode arrays are widely used in electrophysiology research for drug discovery (Natarajan et al., 2011), for studying dissociated brain cells (Shahaf and Marom, 2001), brain slices (Egert et al., 1998), retinas (Meister et al., 1994) and heart cells (Sanchez-Bustamante et al., 2008). In addition, recently developed MEA technology allows for recording neural electrical activity even at sub-cellular resolution (Frey et al., 2009; Zeck et al., 2011; Bakkum et al., 2013).

One of the first in vitro planar MEAs was used by Thomas et al. to record action potentials from chicken embryonic heart cells (Fig. 3a). Gold electrodes, on a glass substrate, were covered with platinum black through electrochemical deposition. The array contained 30 electrodes arranged in 2 rows of 15 electrodes. The 2 rows of electrodes were 50 µm apart, and the distance between 2 electrodes in a row was 100 µm. Single electrode size was 7 x 7 µm².

Few years after Thomas et al., a similar planar MEA with 36 metal electrodes on a glass substrate was developed (Gross et al., 1977), and action potentials from snail neurons were successfully recorded.
Finally, a planar MEA with 2 parallel rows of 16 gold electrodes on a glass substrate was used to record, for the first time, from dissociated cell cultures of rat cervical ganglia (Pine, 1980). Electrodes measured 8 x 10 µm², and were spaced 250 µm apart. An alternative to metal electrodes for recording neural electrical activity is the use of open-gate field-effect transistors (FETs). In particular, the ion-sensitive field-effect transistor (ISFET) measures ionic concentrations in a solution and was used to record electrical activity from nerve cells (Bergveld, 1972) (Fig. 3b). The exposed open gate of the transistor behaves like a sensor to record electrical activity of neurons (polarization of the transistor gate region through moving ionic charges).

Commercially available MEAs for multisite electrophysiology consist of 64 titanium-nitride-coated (TiN) passive metal or indium-tin-oxide electrodes on glass or silicon substrates (e.g., Multi Channel Systems GmbH, Germany; Panasonic Inc., Japan; Plexon Inc., USA). In such devices, electrodes are typically hundreds of micrometers distant, and signal conditioning (amplification, filtering, digitalization) is carried out off-chip.

**FIGURE 3**

*Figure 3. Microelectrode Arrays and Field-Effect Transistor.*
(a) Microelectrode array scheme adapted from Thomas et al., 1972. (b) Schematic representation of an ion-sensitive field-effect transistor, adapted from Bergveld, 2003.
As an alternative to commercial systems, microelectronics-based MEAs were realized in recent years (Eversmann et al., 2003; Berdondini et al., 2009; Frey et al., 2010). Such devices rely on using standard integrated circuit or complementary metal oxide semiconductor (CMOS) technology. Differently from commercial systems, CMOS-based MEAs feature high-density electrodes (electrode distance below 40 µm), and signal conditioning is carried out on-chip.

The CMOS-based MEA developed by Eversmann et al. contains an array of 128 x 128 open-gate field-effect transistors in an area of 1 x 1 mm² (Fig. 4a). The center-to-center electrode distance is 7.8 µm. Cell electrical activity can be recorded with a time resolution of 2 kHz from 16,384 sensors simultaneously. Readout amplifiers are located under each pixel. Recorded output currents are transferred off-chip to current-to-voltage converters and digitalized by analog-to-digital (ADC) converters. The high-density of sensors in combination with small space for readout circuitry resulted in quite high noise levels (70-250 µV rms) (Hutzler et al., 2006).

Berdondini et al. developed an active pixel sensor (APS) CMOS-based MEA with metal electrodes (Fig. 4b). The APS-MEA contains an array of 64 x 64 metal electrodes in an area of 2.67 x 2.67 mm². The center-to-center electrode distance is 40 µm. Full recordings of cell activity by 4,096 electrodes can be done with a time resolution of 7.7 kHz at noise levels of 10-20 µV rms.

The high-density (HiDens) CMOS-based MEA developed by Frey et al. has been fabricated in 0.6-µm CMOS technology and features 11,011 metal electrodes in an area of 2 x 1.75 mm² (Fig. 4c). The center-to-center electrode distance is 18 µm, and it is possible to simultaneously read out 126 channels with a sampling frequency of 20 KHz. Flexibility in the electrode selection (126 electrodes at arbitrary positions can be selected at the same time) is attained through an analog switch matrix integrated underneath the electrode array, which consists of 13,000 SRAM cells and analog switches to define the routing from the electrodes to the amplifiers of the channel units. Neural activity can be recorded at comparably low noise levels (7-9 µV rms) (Frey et al., 2009; Fiscella et al., 2012). Moreover, the
HiDens MEA can be used for stimulating neuron electrically with sub-millisecond time precision (Muller et al., 2012; Bakkum et al., 2013).

In summary, CMOS-based MEAs allow to record neural population activity at high spatio-temporal resolution and high signal-to-noise ratio. Such features simplify sorting of extracellular action potentials and facilitate the identification of defined neural types (see section 1.5). We used the MEA developed by Frey et al. within this thesis to study visual coding in the retina, a neural tissue in the eye that converts the visual scene into electrical signals for the brain.

**FIGURE 4**

(a) CMOS-based MEA chip of Eversmann et al. 2003. (b) CMOS-based MEA chip of Berdondini et al. 2009. (c) CMOS-based MEA chip of Frey et al. 2010.

*Figure 4. CMOS-Based Microelectrode Arrays.*
Chapter 1

1.4 Retina and Microelectrode Arrays

The retina is a layered neural tissue lining the back of the eye (Fig. 5a). The retina converts the visual scene into electrical signals by circuits composed of 5 principal neuron classes: Photoreceptors, Horizontal cells, Bipolar cells, Amacrine cells and Retinal ganglion cells (Wassle, 2004).

Light is converted in graded membrane potentials by the photoreceptors. There are 2 types of photoreceptors: cones for day vision and rods for night vision. Photoreceptors convey electrical signals to another cell type, the bipolar cell. There are ~ 10 types of bipolar cells (Helmstaedter et al., 2013) divided in 2 general classes, ON bipolar cells (response to increases in light intensity) and OFF bipolar cells (response to decreases in light intensity). Bipolar cell electrical signals converge to retinal ganglion cells (RGCs), the output layer of the retina. Importantly, horizontal cells and amacrine cells are localized at the synapses between photoreceptors and bipolar cells and at the synapses between bipolar cells and retinal ganglion cells, respectively. Horizontal and amacrine cells are inhibitory neurons that have a crucial role in retinal computations (Masland, 2012a; Thoreson and Mangel, 2012).

A retina layout scheme is shown in Fig. 5b.

Retinal ganglion cells’ axons project to the brain and send information about the visual scene through action potentials. There are ~ 20 RGCs types that convey information about the visual scene into 20 separated channels (Azeredo da Silveira and Roska, 2011; Masland, 2012b). Retinal ganglion cells are organized in a 2-dimensional layer. Extracellular action potentials of multiple retinal ganglion cells can be recorded by planar MEAs (Meister et al., 1994). MEA technology enabled to study the simultaneous activity of RGC populations revealing distinct types of spatio-temporal correlation in the spike trains (Meister et al., 1995). Such correlated activity of retinal ganglion cells is caused by common inputs from chemical synapses or by direct coupling due to electrical synapses (Brivanlou et al., 1998). It has been suggested how the brain could use correlated activity among retinal ganglion cells for extracting precise features of the visual scene, for example, the sudden reverse of...
motion of a moving object (Schwartz et al., 2007) or the spatial structure of an image (Gollisch and Meister, 2008). In addition, spatio-temporal correlations in retinal responses allow for a more accurate modeling of ganglion cell responses to visual stimulation and for a more accurate decoding of the visual scene (Pillow et al., 2008). Consequently, it is crucial to simultaneously record the concerted activity of RGCs for understanding how vision is encoded in the retina and decoded by the brain.

**FIGURE 5**

(a) Basic scheme of the eye. The retina is shown in red. Retinal signals are transmitted to the brain by the optic nerve. (b) Basic retinal cell types and circuitry. The visual scene is converted into action potentials by computations that happen through different retinal layers. Retinal ganglion cell action potentials can be recorded by MEA technology. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. MEA, microelectrode array.
MEA technology is also used for more medically oriented studies with the goal to restore vision in patients affected by blindness or poor vision. In most cases, photoreceptor degeneration is the cause of vision impairment (Rattner et al., 1999). Two main approaches are currently pursued to try to restore vision: (1) electrical stimulation (2) genetic techniques that introduce light-sensitive molecules. In the case of the electrical stimulation approach, MEA technology is directly used to electrically stimulate retinal ganglion cells and to characterize the evoked responses (Sekirnjak et al., 2008). The main goal of electrical stimulation is to elicit a ganglion cell population response as similar as possible to the light-induced physiological response caused by upstream retinal neurons. In the case of genetic techniques, light-sensitive ion channels are introduced and expressed either in damaged photoreceptors (Busskamp et al., 2010) or to convert non-photosensitive cells, such as bipolar cells, into light-sensitive cells (Lagali et al., 2008). MEA technology is subsequently used to test if the genetics-based visual restoration causes light-induced responses in retinal ganglion cells. Furthermore, MEA technology has been used for retinal developmental studies (Elstrott et al., 2008) and for understanding how photoreceptors are connected to defined types of retinal ganglion cells (Field et al., 2010).

All studies mentioned above show the potential of in vitro planar MEAs for retinal electrophysiology. Importantly, in each of these studies recorded action potentials have to be assigned to the proper neuron through “spike sorting”, an essential step for obtaining significant results in MEA-based neural recordings.

1.5 Sorting Extracellular Action Potentials
A single electrode on a planar MEA records extracellular action potentials from multiple neurons. Therefore, mixed action potentials have to be sorted in order to obtain single-unit spike trains. The data analysis technique for separating mixed action potentials is known as spike sorting (Lewicki, 1998; Einevoll et al., 2012). The first step in spike sorting is the extraction of action potentials from filtered voltage traces (e.g., high-pass filter cutoff: 500 Hz, low-pass filter cutoff: 3000 Hz) (Fig. 6a). For spike waveform isolation, a
threshold must be computed. The threshold could be a fixed voltage value (Segev et al., 2004), the standard deviation of the voltage trace (Hill et al., 2011), or a measure that involves the median of the voltage trace (Quiroga et al., 2004).

Afterwards, isolated spike waveforms are aligned using the minimum negative peak (Fig. 6b). The negative peak is typically less influenced by noise and is, therefore, used for alignment. A more precise alignment can be obtained by up-sampling spike waveforms. Signal up-sampling is especially important for recordings at low temporal resolution.

**FIGURE 6**

(a) Spike detection: voltage trace containing action potentials (black) and detection threshold (red line). (b) Spike alignment: extracted action potentials are aligned with respect to the negative peak. (c) Feature extraction and clustering: spike waveforms are projected on principal components (PC1 = first principal component, PC2 = second principal component). Projected spike waveforms are clustered by using a k-means algorithm. (d) Spike trains: every vertical line represents a single action potential. Action potentials from different neurons are indicated by different colors (blue, green). Spike train quality checks are performed, i.e., the presence of action potentials within time intervals smaller than 2 ms (refractory period violations).
The next step in the spike sorting procedure is the feature extraction from aligned spike waveforms. Basic features, such as spike amplitude and spike width, have been used for spike sorting (Meister et al., 1994). Alternatively, principal component analysis (PCA) techniques can be used for feature extraction (Wallisch et al., 2009). PCA extracts features by using all voltage samples that are contained in each spike waveform (Fig. 6c). PCA will reduce the dimensionality of the data so that all data variability will be compressed in few dimensions (orthogonal data projection method).

After feature extraction, data have to be clustered in order to assign each spike waveform to a single unit that represents the activity of a single neuron (Fig. 6c). For example, the algorithm k-means assigns a spike to the cluster with the closest mean by measuring Euclidian distance. Alternatively, parametric algorithms model each spike cluster by a multivariate Gaussian distribution (Pouzat et al., 2002; Bar-Hillel et al., 2006). Spikes are assigned to the cluster for which their probability is the highest. Finally, a single-neuron cluster of spikes cannot always be represented by a defined probability distribution due to, for example, bursting activity. Therefore, non-parametric methods, such as hierarchical clustering, have been implemented for spike sorting (Fee et al., 1996).

Finally, after clustering, spike waveforms are divided into hypothetical units. Thus, for confirming that sorted spikes belong to single neurons, quality checks have to be performed on the resulting spike trains (Fig. 6d). Neurons cannot fire two consecutive action potentials within a time interval smaller than 1-2 ms (refractory period). A common quality check consists of checking if single units contain consecutive spikes within the refractory period (Segev et al., 2004). If a sorted unit contains consecutive spikes within the refractory period, the spike sorting process obviously assigned spikes of more than one neuron to the same unit. An additional quality check consists of checking how many spikes are missing due to threshold for spike extraction (Hill et al., 2011).

The spike sorting process can become very complicated if multiple neurons are recorded by a single electrode (Fig. 7a). The main reason is that the waveforms of two or more neurons can have
similar shapes and amplitudes (Fig. 7b) (Fiscella et al., 2012). Therefore, spike sorting can become problematic if not impossible. Microelectronics-based MEAs, however, offer the potential to simultaneously record single-neuron action potentials on multiple electrodes at several spatial locations (Fig. 7c). Therefore, the action potentials of multiple neurons can be sorted more easily due to the spatial signal redundancy. There are two main reasons why high-density MEAs simplify spike sorting: (1) electrodes with the largest spike amplitude can be chosen for spike extraction; (2) each neuron will have unique action potential spatial profile or distribution (Fig. 7d). Spike sorting techniques that make use of the detectability of action potentials through multiple electrodes have been implemented. Such algorithms use independent-component analysis (ICA) (Brown et al., 2001; Jackel et al., 2012) or filter-based approaches, known as template matching methods (Franke et al., 2010).

**FIGURE 7**

(a) Recording neural activity by a low-density MEA. Circles represent 2 different cells, neuron A (green) and neuron B (blue). Squares represent electrodes; red indicates electrodes that can record the neurons’ action potentials, gray indicates electrodes that cannot record the neurons’ action potentials. (b) Extracellular action potentials from neurons A and B as recorded by electrodes numbered in Fig. 7a on a low-density MEA. (c) Same as Fig. 7a but for a high-density MEA. (d) Same as Fig. 7b but for a high-density MEA.

Figure 7. Spike Sorting of High-Density MEA data.

1. Recording neural activity by a low-density MEA. Circles represent 2 different cells, neuron A (green) and neuron B (blue). Squares represent electrodes; red indicates electrodes that can record the neurons’ action potentials, gray indicates electrodes that cannot record the neurons’ action potentials. (b) Extracellular action potentials from neurons A and B as recorded by electrodes numbered in Fig. 7a on a low-density MEA. (c) Same as Fig. 7a but for a high-density MEA. (d) Same as Fig. 7b but for a high-density MEA.
1.6 Retinal Ganglion Cell Types

A reliable spike sorting process is crucial for identifying retinal ganglion cell types and for evaluating the recordings of their activity by microelectrode arrays. Retinal ganglion cells can be classified by analyzing the morphology of the cell body and the dendrites (Sun et al., 2002). In addition to morphology, stratification of ganglion cell dendrites in the inner plexiform layer in combination with electrophysiology was used for characterizing ganglion cell types (Roska and Werblin, 2001).

In recent years, mouse lines expressing endogenous genes by cell-type specific promoters have been characterized (Siegert et al., 2009). Such transgenic mouse lines allow for classifying retinal ganglion cells by their unique gene expression. Here is a list of the most recently characterized retinal ganglion cells:

- A new type of OFF ganglion cell that detects upward motion (Kim et al., 2008);
- OFF ganglion cells that detects approaching motion (Munch et al., 2009);
- ON direction-selective ganglion cells (Yonehara et al., 2009);
- ON-OFF direction-selective ganglion cells (Huberman et al., 2009; Trenholm et al., 2011);
- ON-OFF ganglion cell known as local edge detector (Zhang et al., 2012);

Alternative approaches for characterizing ganglion cell types included studies by using microelectrode arrays. Ganglion cells were characterized by spike train temporal patterns (Zeck and Masland, 2007) or by light responses to a set of light stimuli (Farrow and Masland, 2011). It was found that it is possible to record the activity of defined ganglion cell types by using MEAs. For example, ON-OFF direction-selective ganglion cells (Barlow and Hill, 1963) can be easily identified on MEAs. In fact, ON-OFF direction-selective ganglion cells receive ON and OFF excitatory inputs by bipolar cells (Fig. 8a); therefore they respond to light onset and light offset (Fig 8b). In addition, ON-OFF direction-selective ganglion cells receive asymmetric inhibition inputs by starburst amacrine cells (Fried et al., 2002)(Fig. 8a); therefore they respond
asymmetrically to motion direction (Fig 8c). Such combination of light responses is unique for ON-OFF direction-selective cells enabling their characterization by specific light stimulation patterns.

**FIGURE 8**

(a) ON-OFF direction-selective ganglion cell circuitry; ON bipolar cells and OFF bipolar cells provide excitatory inputs (black cells). Starburst amacrine cells provide asymmetric inhibitory inputs (red cells are connected, faint red cells are not connected). (b) Light responses to positive-contrast flash (white rectangle, ON) and negative-contrast flash (gray rectangle, OFF). Each line indicates an action potential. (c) Light responses to a moving bar along 2 opposite directions indicated by arrows below the raster plots.

**Figure 8. ON-OFF Direction-selective Cell Circuitry and Light-Induced Response.**

1.7 Summary

Microelectronics-based high-density MEAs (HD-MEA) offer the potential to record and identify neural populations. *In vitro* planar HD-
MEA technology is particularly suited for recording retinal output due to full accessibility of all neurons in the ganglion cell layer. The retina is an ideal model for studying neural computations, because its input parameters, such as light intensity and spectral composition, can be manipulated in space and time. In addition, the retina can be studied ex vivo without disrupting circuitry connections along different layers of neurons. Furthermore, it is possible to stably record retinal activity from any of its cell types during light stimulation for more than 8 hours after isolation.

The neural datasets recorded by HD-MEAs can be used to develop new spike sorting strategies. The ultimate goal would be to fully automatize spike sorting and to develop new algorithms for online characterization of neural activity. The automatization of spike sorting is especially important considering that new microelectronics-based HD-MEAs are capable of recording from thousands of electrodes. In such a scenario, manual supervision becomes problematic and time consuming.

In the following chapters, the main work described of this thesis is intended to explore the potential of microelectronics-based HD-MEAs for retinal electrophysiology. Extracellular recordings will be performed in the mouse, a model organism where sophisticated genetic manipulation techniques have become available in the recent years. In addition, the population responses of specific retinal neurons that detect the direction of a moving object will be analyzed in the rabbit retina. Finally, the related datasets containing hundreds of recorded neurons will be used for developing and testing of spike sorting techniques.

References


Chapter 1


Chapter 1


information about upward and downward image motion. PloS one 4:e4320.
Chapter 2

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

Michele Fiscella\textsuperscript{1}, Karl Farrow\textsuperscript{2}, Ian L. Jones\textsuperscript{1}, David Jäckel\textsuperscript{1}, Jan Müller\textsuperscript{1}, Urs Frey\textsuperscript{3}, Douglas J. Bakkum\textsuperscript{1}, Péter Hantz\textsuperscript{2}, Botond Roska\textsuperscript{2} and Andreas Hierlemann\textsuperscript{1}

\textsuperscript{1} Bio Engineering Laboratory, ETH Zurich, Basel, Switzerland.  
\textsuperscript{2} Neural Circuits Laboratory, Friedrich Miescher Institute, Basel, Switzerland.  
\textsuperscript{3} Riken Quantitative Biology Center, Kobe, Japan.

Published in:  
Journal of Neuroscience Methods  
Volume 211, Issue 1, 15 October 2012, Pages 103–113
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 RGCs 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, 2001; Wassle, 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, 2002; 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; Gollisch 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 (Pine, 1980; 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., 2003a; 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 retinas (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 µm and electrode center-to-center distances of 18 µm over an area of 2 x 1.75 mm² (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 (Fig. 1a).

Extracellular action potentials can be recorded at high temporal resolution (20 kHz) and with low noise levels (~7-9 µV rms, band: 100 Hz - 3 kHz, perfusion system operational but without retinal tissue). In the maximum-density recording scenario (3,161 electrodes/mm²), each mouse RGC lies in close vicinity to multiple electrodes, which
allows for recording single-cell action potentials at different spatial locations (Fig. 1b).
A switch matrix circuitry is located under the electrode array and connects the electrodes to 126 readout channels (Frey et al., 2010). 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 (Fig. 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/µm² 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) (Fig. 1d). The light projection setup was assembled on an upright microscope (BX5IWI, 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 CaCl$_2$, 1.6 MgCl$_2$, 10 D-glucose, 22 NaHCO$_3$), continuously bubbled with 5% CO$_2$ / 95% O$_2$. 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 (Fig. 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*10$^{13}$ photons*cm$^{-2}$*s$^{-1}$; green LED, 525±23 nm, intensity ~1.67*10$^{13}$ photons*cm$^{-2}$*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 / mm$^2$ (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 (Fig. 1f, Fig. 1g).

2.3.4 Optogenetic Stimulation of Retinal Ganglion Cell Types

Adeno-associated viruses encoding a channelrhodopsin variant (Berndt et al., 2009) (AAV EF1a double floxed ChR2-128S-2A) were delivered by intravitreal injection into the eyes of the $P_{valb}^{Cre}$ 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-sulfamoylbenzo[f]quinoxaline-2,3-dione, a kainate AMPA antagonist). Light stimulation was performed by a flash of 40 ms, with an intensity of \( \sim 1.6 \times 10^{17} \text{photons} \cdot \text{cm}^{-2} \cdot \text{s}^{-1} \) 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 retinæ (without RGCs expressing ChR2), the probability to find light responses with latencies between 0-50 ms was 3.9% (Fig. 7b). In experiments with Pvalb\(^{\text{Cre}}\) transgenic retinæ (with RGCs expressing ChR2), we found that 41% of the total response latencies were less than 50 ms (Fig. 7b). Therefore, these RGCs in Pvalb\(^{\text{Cre}}\) transgenic retinæ, 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 Pvalb\(^{\text{Cre}}\) \( \times \) Thy1\(^{\text{Stp-EYFP}} \). The retina was assessed with a Zeiss LSM 700 confocal microscope, 40X oil immersion lens, NA 1.2, \( \times 0.5 \) digital zoom.
**Figure 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 Pvalb\textsuperscript{Cre} x Thy1\textsubscript{Sp-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.
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, microwelectrode 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 (Wassle, 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).

2.4 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 (Ding 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
Chapter 2

(Fig. 2a - top row), the second includes light falling also onto the readout circuitry, where the most light sensitive elements are located (Fig. 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 (Fig. 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) (Fig. 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., 2010). Thus, when light hits the amplifiers, photo-induced charges cause artifacts in the recorded signal by adding offset and noise in the amplified signal (Fig. 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 (Fig. 2b).
Figure 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/mm²).

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 (Fig. 3a, Fig. 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 (Fig 3b, Fig. 3c - 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 (Fig. 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 (Fig. 3d, Fig. 3e).

**FIGURE 3**

(a) Superposition of 959 action potentials (gray traces) from six electrodes, indicated in Fig 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).

**Figure 3. Characterization of Extracellular Action Potentials from Mouse Retinal Ganglion Cells.**

(a) Superposition of 959 action potentials (gray traces) from six electrodes, indicated in Fig 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).

### 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 (Fig. 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. (Fig. 4a, Fig. 4b, Fig. 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 Fig. 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 (Fig. 4a, Fig. 4b, Fig. 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 Fig. S1).

In Fig. 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 (Fig. 4c - top panel, Fig. 4d - top panel). Each of the three putative RGCs had refractory period violations of 7%, 3% and 0%, respectively.

In Fig. 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 (Fig. 4c - bottom panel, Fig. 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 (Fig. 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 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 Fig. 4a (top panel) and Fig. 4b (bottom panel). The PC projection is used to cluster the action potential.

(d) Medians of silhouette coefficient distributions as function of the number of clusters for waveforms from Fig. 4a (top panel) and Fig. 4b (bottom panel). The solid black circles indicate the optimal clustering solution with the highest median value (see also Fig. S1).

(e) Distribution of silhouette coefficients for clustered waveforms from Fig. 4a (top panel) and Fig. 4a (bottom panel). The median values correspond to the solid black circles in Fig. 4d.

2.4.4 Physiological Characterization of Retinal Ganglion Cells

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 (Fig. 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, Fig. 5b–c, Fig. 5d), decreases in light intensity (OFF RGCs, Fig. 5e, Fig. 5f) or to both, increases and decreases in light intensity (ON-OFF RGCs, Fig. 5g, Fig. 5h). In addition, RGCs that were sensitive to a precise direction of motion of the light stimulus were characterized (Fig. 5g, Fig. 5h and Fig. 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, Fig. 5a demonstrates that cells with highly overlapping electrical footprints, the receptive fields of which are overlapping to
even a much larger extent (see Fig. 3d), can be detected and assigned.

**FIGURE 5**

Figure 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 (long horizontal line at the top of each raster plot). The white bar at the bottom of the raster plots represents “light on” and indicates an increase in light intensity of the stimulus. The gray bar at the bottom of the raster plots represents “light off” and indicates a decrease in light intensity of the stimulus. This cell is classified as ON-RGC, because it responds exclusively to increases in light intensity. According to the response to this light stimulation, it is possible to classify RGCs as: ON (if they respond to an increase of light intensity of the stimulus, Fig. 5b-d); OFF (if they respond to a decrease of light intensity of the stimulus, Fig. 5e-f); ON-OFF (if they respond to both, an increase and a decrease of light intensity of the stimulus, Fig. 5g-h).

(c) **Top panel**: polar plot showing the responses of the RGC in Fig. 5b to motion of a bar in 8 directions at 45° radial intervals (see Moving Bar in Supplementary Material). The response is quantified by counting the number of action potentials that were fired by the RGC in response to the bar moving along the different directions. The tuning curve is normalized to the largest response. The arrow indicates the vector sum response corresponding to the preferred direction. The length of the arrow indicates the extent of direction-selectivity (the arrow is visible in Fig. 5g-h, because these two RGCs are direction-selective, whereas RGCs in Fig. 5b-f are not direction-selective and, therefore, the arrow is too short to be visualized). **Bottom panel**: distribution of time intervals between consecutive spikes (inter-spike interval distribution).

(d-h): Same as in Fig. 5b-c and showing the characterization of five neighboring RGCs.

### 2.4.5 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 (Fig. 5g, Fig. 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). 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 mm\(^2\) (Fig. 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., 2012) see Spike Sorting in Supplementary Material), and the sorted neurons were physiologically classified according to their response to light stimulation (Fig. 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.

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

2.4.6 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
Chapter 2

genetically identified RGCs (see Methods). bi-ChR2-induced action potentials (Fig. 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 (Fig. 7b).

**FIGURE 6**

Figure 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.

**FIGURE 7**

(a) Trace showing action potentials from a *Pvalb* RGC expressing ChR2-128s light-sensitive cation channels. The inset shows 500 ms raster plot after a 40 ms light pulse. The red line indicates the light pulse.

(b) Top panel: distribution of times between light stimulation offset and the first action potential in experiments with wild type retinae (control). In this experiments there are not RGCs expressing ChR2.

Bottom panel: distribution of times between light stimulation offset and the first action potential in experiments with *Pvalb* RGCs expressing ChR2.

Note the presence of the early peak in the histogram of the *Pvalb* Cre-ChR2-128S retinae (Fig. 7b) and the lack of short latencies in the control experiment with wild type retinae (Fig. 7b). Photoreceptor-mediated light response was blocked in all experiments by using synaptic blockers (see Methods).
2.5 Discussion
Retinal circuits encode the visual scene in parallel channels, where each RGC type conveys a different representation (Roska and Werblin, 2001; Farrow and Masland, 2011). 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 µm$^2$. This MEA features an inter-electrode spacing of 18 µm, and noise levels of ~ 7 µV$_{rms}$. 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 Fig. 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/mm$^2$) on an area of 1 x 1 mm$^2$ and 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 µV$_{rms}$, 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 A.M, 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 retinas, 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.
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).

Acknowledgements
This work was financially supported by the FP7 of the European Community through the ERC Advanced Grant 267351 “NeuroCMOS”. Michele Fiscella acknowledges individual support through a Swiss SystemsX interdisciplinary PhD grant 2009_031. Karl Farrow acknowledges individual support through Marie Curie and EMBO long-term fellowships. We acknowledge Miguel H. Teixeira for imaging of mouse retinal ganglion cells.

References
Bi A, Cui J, Ma YP, Olshevskaya E, Pu M, Dizhoor AM, Pan ZH (2006) Ectopic expression of a microbial-type rhodopsin restores visual


Chapter 2


Supplementary Methods

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).

**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).

**Offline Spike Sorting:**

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

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 (Fig S1 a) (Segev et al., 2004).
   - the silhouette coefficients were used to estimate how many neurons were present in the clustered data (Rousseeuw, 1987) (Fig S1 a). 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 L., 1985) (Fig. S1 b); 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™.

**Online Spike Sorting:**

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

(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 (Hyvarinen, 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 S1**

(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 Fig. 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 Fig. 4b (best clustering output).
Color code (right corner): red indicates high median silhouette values.

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 S2**

Figure 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)
accord ing 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.

Supplementary References


Chapter 3

Visual Coding with a Population of Direction-Selective Neurons

Michele Fiscella¹, Felix Franke¹, Karl Farrow², Jan Müller¹, Botond Roska³, Rava Azeredo da Silveira⁴,⁵, and Andreas Hierlemann¹

1 Bio Engineering Laboratory, ETH Zurich, Basel, Switzerland
2 Neuro-Electronics Research Flanders Imec, Leuven, Belgium
3 Neural Circuits Laboratory, Friedrich Miescher Institute, Basel, Switzerland
4 Department of Physics, Ecole Normale Supérieure, Paris, France
5 Laboratoire de Physique Statistique, Centre National de la Recherche Scientifique, Université Pierre et Marie Curie, Université Denis Diderot, Paris, France

Submitted to Journal of Neurophysiology
3.1 Abstract
The brain decodes visual-scene information from the action potentials of ~ 20 retinal ganglion cell types. Among the retinal ganglion cells, direction-selective ganglion cells (DSGCs) encode motion direction. Several studies focused on encoding or decoding of motion direction, by recording multiunit activity, mainly in the visual cortex. However, such analyses have not been carried out at the level of the retina. In this study, we simultaneously recorded, from all four types of ON-OFF DSGCs of the rabbit retina by using a microelectronics-based high-density microelectrode array (HDMEA), and decoded their concerted activity by using probabilistic and linear decoders. Furthermore, we investigated how the modification of stimulus parameters (velocity, size, angle of moving object) and the use of different tuning curve fits influenced decoding precision. Finally, we simulated ON-OFF DSGCs cell activity, based on real data, in order to understand how tuning curve widths and the angular distribution of the cells' preferred directions influence decoding accuracy.

We found that probabilistic decoding strategies outperformed linear methods and that decoding accuracy was robust towards changes in stimulus parameters, such as velocity. Removing of noise correlations among cells by random shuffling trials caused a drop in decoding accuracy. Moreover, we found that tuning curves are broad in order to minimize large errors at the expense of a higher average error, and that the retinal direction Selective system would not substantially benefit, on average, from having more than four types of ON-OFF DSGCs or from a perfect alignment of the cells' preferred directions.

3.2 Introduction
Detecting motion is a fundamental task in vision, and a large part of the visual system is devoted to processing motion information. Aside from photoreceptors, possibly the most studied visual neurons are the retinal direction-selective ganglion cells (DSGCs) (Barlow and Hill, 1963; Weng et al., 2005; Sun et al., 2006; Kim et al., 2008). These have been investigated in fish (Nikolaou et al., 2012),
amphibians (Kühn and Gollisch, 2013), mammals (Huberman et al., 2009; Yonehara et al., 2009; Trenholm et al., 2011), and similar cells exist in insects (Maisak et al., 2013). DSGCs encode the direction of a moving object by responding vigorously to motion along a so-called ‘preferred direction’ and weakly along the opposite direction, the so-called ‘null direction’. Several studies have focused on motion coding in the retina (Berry et al., 1999; Frechette et al., 2005; Schwartz et al., 2007), and a great deal of work has been devoted to understanding the circuit mechanisms responsible for direction selectivity (Yoshida et al., 2001; Euler et al., 2002; Fried et al., 2002; Yonehara et al., 2013), but less attention has been given to examining the coding properties of DSGCs.

Four types of ON-OFF DSGCs are present in the mammalian retina: each comes with a preferred direction along one of the four cardinal body axes (temporal, dorsal, nasal and ventral) (Oyster and Barlow, 1967). The axons of retinal ON-OFF DSGCs project to the superior colliculus and the thalamus (Kay et al., 2011) and, additionally, as recently discovered, there is a circuit linking retinal ON-OFF DSGCs to neurons in the visual cortex (Cruz-Martin et al., 2014). Neurons that respond asymmetrically to motion have also been identified in the thalamus (Marshel et al., 2012; Piscopo et al., 2013) and in the visual cortex (Ohki et al., 2005; Hubel and Wiesel, 2009) of various species, and, recently, a number of studies have quantified the coding of directional information by populations of cortical and thalamic neurons (Graf et al., 2011; Stanley et al., 2012). However, similar quantifications in population coding have not been carried out in the context of the retina, in part because populations of specific DSGCs from given types had not been recorded from simultaneously.

In the present paper, we address the coding of directional information by populations of ON-OFF DSGCs; its content is two-fold. We present population recordings of ON-OFF DSGC outputs. We quantify the coding performance of the population by using various theoretical methods. We then first derive the precision of directional coding using an optimal decoder, and examine the dependence of this precision upon stimulus parameters (velocity,
object size) and decoder properties (time scale, complexity of decoders, noise assumption of responses). In a second step, we investigate the way in which the coding performance depends upon single-cell properties (shape of the tuning curves) and population properties (relative arrangements of tuning preferences, precision of tuning preferences), as all ON-OFF DSGCs were recorded from simultaneously.

Since the spiking outputs of retinal ON-OFF DSGCs were recorded simultaneously, we have access to the statistics of noise correlations, which may be relevant to coding. In the present work, we assessed the effect of such noise correlations on the decoding performance by randomly shuffling the trial order among multiple cells. A more detailed analysis of noise correlation is, however, due to the required comprehensive treatment, subject of a separate article (Franke et. al, submitted).

3.3 Material and Methods

3.3.1 Retina Preparation

All animal experiments and procedures were approved by the Swiss Veterinary Office. New Zealand female albino rabbits (females, 2.5 - 3 kg) were obtained from Charles River Laboratories (L’Arbresle Cedex, France). The eyes were dissected under dim red light conditions in Ames solution (Sigma, A1420) continuously equilibrated with 5% CO₂ - 95% O₂. The vitreous was removed, and a retina patch (~ 2 x 2 mm²) was isolated between 1 mm eccentricity and 4 mm eccentricity, along the ventral direction (Vaney, 1994). The retina patch was placed ganglion-cell-side-down on the electrode array and perfused by Ames solution (pH 7.4, 36° C) equilibrated with 5% CO₂ - 95% O₂. Retinal ganglion cell extracellular activity was recorded for 7-8 hours.

3.3.2 Light Stimulation

For retinal light stimulation, we used the light projector Acer K10 (60 Hz refreshing rate) in a previously developed setup, in which extracellular electrophysiological measurements under light projections can be performed on a microscope stage. More details
about the microscope and the optics for focusing the light stimulus on the retina can be found in Fiscella et al., 2012. We simultaneously used blue (460 ± 15 nm) and green (523 ± 23 nm) projector LEDs for stimulating retinal photoreceptors and we applied the following light stimuli in our experiments:

- Static flashing square: this stimulus was shown to test ganglion cell ON and OFF responses. A positive-contrast square stimulus (0.2 x 0.2 mm²) was turned ON for two seconds and turned OFF for two seconds with a background irradiance of 0.2 µW/cm² and a square stimulus irradiance of 2.2 µW/cm². After each presentation of the stimulus, its center was moved by 0.1 mm to cover a larger area of the retina.

- Moving bar: this stimulus was used to characterize directional tuning properties of the ganglion cells. The bar length (dimension parallel to the movement direction of the bar) was always 1 mm in order to separate ON and OFF responses. Background irradiance was 0.2 µW/cm² and bar stimulus irradiance was 2.2 µW/cm². In experiments aimed at decoding motion direction, the bar moved at a constant velocity of 1.6 mm/s along 36 equidistant angular directions radially spaced at 10 degrees. Two different bar widths (dimension orthogonal to the movement direction of the bar) were tested: 0.5 and 1 mm.

In addition, for experiments aimed at decoding motion direction across different stimulus velocities (0.4, 0.6, 0.8, 1.2, 1.6 mm/s), due to experimental time limit, the bar moved along 18 equidistant angular directions radially spaced at 20 degrees intervals. Bar width was 0.5 mm.

Finally, for measuring cell responses to different stimulus parameters, such as irradiance (n = 8), velocity (n = 8) and size (n = 5), due to experimental time limit, the bar moved along 8 equidistant angular directions radially spaced at 45 degree intervals.

- Moving flashing square: this stimulus was used to map ganglion cell receptive fields. A positive-contrast square stimulus (0.08 x 0.08 mm²) was turned on for one second and turned off for one second (5 repetitions). The stimulus moved sequentially in discrete non-overlapping steps over an area of ~ 1 mm² with a background
irradiance of 0.2 µW/cm² and a square stimulus irradiance of 2.2 µW/cm².

3.3.3 Microelectrode Array Recordings

For extracellular recordings we used a microelectronics-based high-density microelectrode array (HDMEA) (Frey et al., 2009; Fiscella et al., 2012). The microelectrode array features 11,011 platinum electrodes with diameters of 7 µm and electrode center-to-center distances of 18 µm over an area of 2 x 1.75 mm². 126 electrodes can be arbitrarily selected for simultaneous recording. Extracellular action potentials were recorded at a sampling frequency of 20 kHz.

In Fig. 1, we show the strategy for recording from a defined population of retinal ganglion cells by the microelectronics-based HDMEA:

1) We let the retina patch settle down on the HDMEA for 30 minutes before starting light stimulation (Fig. 1a, step 1).

2) We recorded light-induced ganglion cell activity by high-density electrode blocks (3,161 electrode/mm²) that were placed below the area where the stimulus was shown. Importantly, we sorted single-cell activity online during the experiment to find a set of electrodes that recorded ON-OFF DSGC activity (1-2 hours, Fig. 1a, step 2).

3) Once we knew the location of ON-OFF DSGCs, we assigned 5-7 electrodes per cell and recorded the light-induced concerted activity of a population of ON-OFF DSGCs (4-5 hours, Fig. 1a, step 3).

Spike sorting was carried out manually by using the software UltraMegaSort2000 (Hill et al., 2011) on non-overlapping groups of 5-7 electrodes. All recorded traces were band-pass filtered (500 Hz-3 KHz), and all resulting multi-channel spike clusters were manually inspected (Fig. 1b). To verify that each cluster came from a single retinal ganglion cell, the percentage of refractory period violations was estimated by using the number of inter-spike intervals lower than 1.5 ms (Fig. 1c). Only clusters with refractory period violations of less than 2% of all spikes were used for subsequent analysis. Duplicate units were removed by comparing the spatial distributions.
of extracellular action potentials, receptive field location, and by temporal cross correlation of spike trains. All data analysis steps were performed using the software application MATLAB™.

FIGURE 1

(a) Step 1: rabbit retina patch, ganglion cell side down on the electrode array (sensor area is shown by dashed red rectangle). Step 2: scanning of the ganglion cell layer by high-density electrode blocks in order to find locations of defined cell types (indicated by different colors). Step 3: assignment of 5-7 electrodes per ganglion cell for simultaneous recording of light-induced activity from identified and defined populations of retinal neurons.

(b) Average multichannel spike waveforms of 9 spiking units, isolated from 5 neighboring electrodes (continuous line = mean voltage, dashed line = s.d. voltage). Electrodes were chosen so as to feature the largest-amplitude signals of the neurons of interest (here, e.g., all 5 electrodes recorded large signals for unit 9.

(c) Interspike interval (ISI) distribution for unit 9 shown in panel b. The red region indicates the ISI violation time (0-1.5 ms).

3.3.4 Selection of Retinal ON-OFF Direction-Selective Ganglion Cells

To characterize the ganglion cell’s responses to motion, we used the number of spikes that each neuron fired during the presentation of a
moving bar light stimulus. This procedure was repeated 100 times for each of the 36 movement directions. To estimate the cell’s directional tuning curve, the responses to all trials were then averaged (Fig. 2c).

To compute a direction selectivity index for each cell, we normalized the tuning curves by their integral. For each ganglion cell, the vector sum of the normalized responses to motion in all stimulus directions was computed, which resulted in a vector pointing in the cell’s preferred direction. The magnitude of this vector is the direction selectivity index (DSI) (Fig. 2d) (Taylor and Vaney, 2002). Only ON-OFF ganglion cells with a DSI>0.2 were selected as direction-selective neurons (Rivlin-Etzion et al., 2012).

3.3.5 Tuning Curve Parameterization

Several parameterizations of direction-selective tuning functions have been suggested (Dayan and Abbott, 2001; Nowak et al., 2011). We tested the most common ones in terms of fit quality and decoding performance (Swindale, 1998). We analyzed the following different forms of model tuning functions, \( F_i(\theta) \), of neuron \( i \) with preferred direction \( \theta_i \) and stimulus \( \theta \).

1. Gaussian:

\[
F_i(\theta) = b_i + \frac{c_i}{\sigma_i \sqrt{2\pi}} e^{-\frac{D(\theta, \theta_i)^2}{2\sigma_i^2}},
\]

where \( b_i \) is a constant reflecting the baseline firing rate of the neuron, \( c_i \) controls the peak firing rate, \( \sigma_i \) controls the width of the tuning function, and \( D(x, y) \) is the angle between \( x \) and \( y \).

2. von Mises function (Oesch et al., 2005; Elstrott et al., 2008):

\[
F_i(\theta) = c_i e^{\frac{1}{\alpha_i^2} \cos(\theta - \theta_i)},
\]
where $c_i$ and $\sigma_i$ control the peak height and width of the tuning function.

3. Flat-topped von Mises function (Swindale, 1998):

$$F_i(\theta) = c_i e^{\kappa_i \cos(\eta_i \sin(\theta - \theta_i) - 1)},$$

where $c_i$, $\kappa_i$, and $\eta_i$ control the peak firing rate, the width, and the ‘flatness’ of the tuning function, respectively. We fitted the parameters to the mean firing rates of the recorded neurons iteratively, one parameter at a time, by using a one-dimensional minimization procedure, until either convergence or a predefined maximum number of iterations was reached.

4. Half-wave rectified cosine function (Theunissen and Miller, 1991):

$$F_i(\theta) = \left[ \frac{c_i \cos(\theta - \theta_i) - b_i}{1 - b_i} \right],$$

where $\left[ \cdot \right]$ is the rectification at 0, and $c_i$ and $b_i$ control the peak firing rate and width of the tuning function.

### 3.3.6 Noise Characteristics of ON-OFF Direction-Selective Ganglion Cells

We analyzed the Fano factors of the cells (i.e., the variance of cell’s response to a stimulus divided by its mean) and found that they were strongly stimulus-dependent and ranged from ~0.5 (for motion directions close to the preferred direction of the ON-OFF DSGC) to ~1.5 (for motion directions close to the null direction of the ON-OFF DSGC). We found that the variance-to-mean (see Fig. 3b) relation
was well characterized by a power law: $\sigma_N^2 = \sigma_N^2(\mu) = a\mu^b$ with $a = 4.03$ and $b = 0.51$ (fitted with Matlab’s curve-fitting toolbox, green line, Fig. 3b), where $\sigma_N^2$ is the cell’s variance, and $\mu$ its mean response. We therefore modeled the noise characteristics of a population of $N$ ON-OFF DSGCs with an independent multivariate Gaussian distribution with angle-dependent means, 

$$F(\theta) = \begin{bmatrix} F_1(\theta), \ldots, F_N(\theta) \end{bmatrix}^T$$

and variances 

$$C(\theta) = \begin{bmatrix} \sigma_N^2(F_1(\theta)), \ldots, \sigma_N^2(F_N(\theta)) \end{bmatrix}^T.$$ 

### 3.3.7 Decoders

We decoded the stimulus direction from the simultaneous responses (spike counts), $r = [r_1, \ldots, r_N]$, of $N$ neurons. In defining the spike counts, we used different widths of the time bins in order to examine the influence of the time binning on decoding. We used time bin widths ranging from 0.01 s to 0.3 s, as well as the full duration of a stimulus presentation. The decoding error was defined as the error between the direction of the true stimulus, $\theta$, and the estimated direction, $\hat{\theta}$, over all directions. The maximum error is equal to 180 degrees. For every direction of motion we computed the root mean squared error (RMSE):

$$RMSE = \sqrt{E\left[\left(\theta - \hat{\theta}\right)^2\right]}.$$ 

Finally, we averaged the RMSE across all motion directions in order to get the average RMSE for every configuration of decoding cells (Fig. 3c).

We tested several decoders, described hereafter.

85
1. Population vector decoder (Georgopoulos et al., 1986):

\[
\hat{V} = \frac{1}{N} \sum_{k=1}^{N} r_k V_k,
\]

where \(\hat{V}\) and \(V_k\) are the vectors pointing in the directions \(\hat{\theta}\) and \(\theta_k\) respectively, where \(\theta_k\) is the preferred direction of neuron \(k\).

2. Optimal linear estimator (Salinas and Abbott, 1994):

For symmetric tuning functions, the optimal linear decoding is given by:

\[
\hat{V} = \frac{1}{N} \sum_{k=1}^{N} r_k D_k,
\]

with:

\[
D_k = \sum_j Q^{-1}_{kj} V_k.
\]

The matrix \(Q\) can be computed from the tuning functions and captures the overlap among tuning functions (Salinas and Abbott, 1994). For equidistant preferred directions and identical tuning functions, this decoding method is equivalent to the population vector; in general, this decoder can take into account variations among the different tuning curves.

3. Maximum likelihood decoder:

If we assume that each stimulus appears with equal probability (flat prior probability), the maximum likelihood (ML) decoder is given by:

\[
\hat{\theta} = \arg\max_{\theta} \left(L(\theta, r)\right),
\]
where \( L(\theta, r) \) is the likelihood of stimulus \( \theta \) for a population response \( r \). Since we assume in this work that neurons are independent, \( L(\theta, r) \) can be written as:

\[
L(\theta, r) = \prod_k p(r_k | \theta),
\]

where \( p(r_k | \theta) \) is the probability of observing \( r_k \) spikes from neuron \( k \) when a stimulus \( \theta \) is presented. \( p(r_k | \theta) \) is the distribution of spike counts for neuron \( k \). We modeled the spike distribution in two ways. In some of the analyses, we used a Poisson distribution:

\[
p_{\text{Poiss}}(r_k | \theta) = \frac{F_i(\theta)^{r_k} e^{-F_i(\theta)}}{r_k!}.
\]

In most of the analyses, however, we used a distribution, which satisfied the observed sub-Poisson variance, \( \sigma^2_{N,k}(\theta) = aF_k(\theta)^b \) (see the section “Noise characteristics of ON-OFF direction-selective ganglion cells”); we modeled the ON-OFF DSGC response by a Gaussian distribution with the corresponding variance:

\[
p_{\text{Gauss}}(r_k | \theta) = \frac{1}{\sigma_{N,k}(\theta) \sqrt{2\pi}} e^{\frac{-D(\theta, \theta_k)^2}{2\sigma_{N,k}(\theta)^2}}.
\]

### 3.3.8 Decoding Performance Bound

In parallel with decoders, we used the Fisher information in order to obtain a bound on the coding performance of a population of ON-OFF DSGCs. The Fisher information is a stimulus-dependent quantity, calculated as:
\[ I_F(\theta) = \left\langle \left( \frac{\partial \log P(r|\theta)}{\partial \theta} \right)^2 \right\rangle, \]

where the brackets denote an average over the distribution of population responses. For the case of Poisson variability in the spike count, this expression simplifies to:

\[ I_F(\theta) = \sum_k \frac{F'_k(\theta)^2}{F_k(\theta)}. \]

The Fisher information yields a lower bound on the standard deviation of an unbiased deterministic decoder, and thus also the RMSE, via the Cramér-Rao bound, \( \text{std}(\hat{\theta}) \geq \frac{1}{\sqrt{I_F(\theta)}}. \)

We express our results in terms of the average over all stimulus directions, \( S \):

\[ \varepsilon_{\text{mean}} = \frac{1}{S} \sum_{i=1}^{S} \frac{1}{\sqrt{I_F(\theta_i)}}, \]

and the maximum Cramér-Rao bound over all stimulus directions:

\[ \varepsilon_{\text{max}} = \max_{\theta} \sqrt{\frac{1}{I_F(\theta)}}. \]

### 3.4 Results

#### 3.4.1 Spiking Output of Retinal ON-OFF Direction-Selective Ganglion Cells

We used a HDMEA (Fiscella et al., 2012) to record action potentials from given types of retinal DSGCs. Action potentials of a single cell were recorded over multiple electrodes (Fig. 2a), so that we could
use the 5-7 electrodes with highest signal amplitudes for a given cell (Fig. 2a, yellow-red region) for multi-channel spike sorting (Hill et al., 2011).

**FIGURE 2**

**Figure 2. Extracellular Recording from Retinal ON-OFF DSGCs.**
(a) Spatial profile of an action potential of a single ganglion cell. Red-yellow area: high amplitude signals. Black waveform: average single-cell action potential on a single electrode (204 electrodes). The amplitude color scale refers to the initial minimum of the waveform (Fig. 1b).
(b) Raster plot of ganglion cell responses to 5 ON and 5 OFF stimuli.
(c) Tuning curve of ganglion cell responses to motion of a bar. The red arrow points in the preferred direction of the ganglion cell. Black solid line: mean response; dashed gray lines: standard deviation.
(d) Preferred directions of 103 ON-OFF DSGCs recorded from 8 retinas. The length of each vector indicates the magnitude of the direction selectivity index (DSI); 0 = not direction selective, symmetric response; 1 = direction selective, asymmetric response (see Methods). T = Temporal, D = Dorsal, V = Ventral, N = Nasal.
(e) Histogram of ON-OFF DSGC vector angles from Fig. 1d. Nasal = -0.1±13.7 degrees, Dorsal = 85.8±10.2 degrees, Temporal = 181.8±8.4 degrees, Ventral = 266.8±10.5 degrees (mean±s.d.).
(f) Raster plot of ganglion cell responses to motion; simultaneous responses of 12 ON-OFF DSGCs. Responses are colored according to the ganglion cell’s preferred directions (Red = Temporal, Green = Dorsal, Blue = Nasal, Yellow = Ventral). Each row shows the activity of a single ganglion cell for different directions of motion. Bottom arrows indicate directions of motion.
(g) Receptive field locations of the ON-OFF DSGCs in panel f.
We then distinguished the ON-OFF DSGCs from the other ~20 types of retinal ganglion cells by using two characteristic response properties of ON-OFF DSGCs: (1) ON-OFF DSGCs respond to both positive and negative contrast stimuli (Fig. 2b); (2) ON-OFF DSGCs respond asymmetrically to a moving object (Fig. 2c) (Barlow and Hill, 1963). We recorded from 103 retinal ganglion cells, which obeyed these criteria, from a total of 8 rabbit retinas (Fig. 2d). As expected, we found four groups of ON-OFF DSGCs, with preferred directions aligned with the temporal, dorsal, nasal, and ventral body axes (Fig. 2e, Fig. 2f) (Oyster and Barlow, 1967).

All our analyses were carried out on the ganglion population responses to stimuli in which a bright bar moves on a dark background (See Methods). ON-OFF DSGCs responded to the leading edge and the trailing edge of the bar (Fig. 2f). As expected from minimal overlap among the dendritic fields of ON-OFF DSGCs of same types (Vaney, 1994), we observed that cells with the same preferred direction tiled the visual field (Fig. 2g).

3.4.2 Coding Precision of Retinal ON-OFF Direction-Selective Ganglion Cells

In order to characterize the coding performance of a population of ON-OFF DSGCs, we asked with what precision a near-optimal decoder can retrieve directional information. We initially used groupings of four ON-OFF DSGCs with the four cardinal preferred directions. In principle, if one would know the mean response of each cell as a function of motion direction (direction-tuning curves) (Fig. 3a) as well as the noise characteristics of the response (Fig. 3b), one could construct an optimal maximum-likelihood (ML) decoder (see Methods for precise definitions and details) (Pouget et al., 2000; Dayan and Abbott, 2001).

We call our decoder ‘near-optimal’ as it applies maximum-likelihood decoding but uses fits of direction-tuning curves and an approximated noise distribution (Fig. 3b). In figure 3c (top panels), we show decoding results by using a maximum-likelihood decoder for a single cell, and for a group of four ON-OFF DSGCs. For every configuration of cells, we quantified the accuracy of decoding as the
average root mean squared error (RMSE) across all motion directions (Fig. 3c, bottom panels). In calculating this quantity, we used von Mises fits of the direction-tuning curves and a Poisson or a sub-Poisson power law for the dependence of the variance on the mean. For the discussion in this section here, we first fitted the ON-OFF DSGC mean tuning curves by von Mises functions, which are commonly used for fitting direction-selective cell responses (Swindale, 1998; Oesch et al., 2005; Elstrott et al., 2008; Graf et al., 2011). However for the discussion in the section “Influence of tuning curve shape, response variability and noise correlations on the coding performance” below, we analyzed also how other tuning function models influence decoding performance. By using von Mises functions, we obtained an average RMSE respectively for Poisson and sub-Poisson noise of 10.5±2.4 degrees and 11.0±2.5 degrees over all possible groupings of four ON-OFF DSGCs with the four cardinal preferred directions (Fig. 3d-e, n = 600, 5 retinas).

As the brain may use simpler decoders than an optimal maximum-likelihood decoder, we investigated how the decoding precision degrades with reduced decoder complexity. In Fig. 3f-g, we present decoding analyses carried out with different decoders, namely the population vector (PV) (Georgopoulos et al., 1986) and the optimal linear estimator (OLE) (Salinas and Abbott, 1994). Simpler decoders, such as PV and OLE, performed significantly worse than the ML decoder and yielded an average RMSE of 19.7±6.2 degrees and 17.4±7.4 degrees, respectively (n = 600, 5 retinas) (p<0.05, Mann-Whitney U-test, Kolmogorov-Smirnov test).

The above results refer to the coding performance of groups of four cells with different preferred directions (we refer to these as ‘quadruplets’, Fig. 3c right panels). The performance is obviously improved by having more than a single quadruplet. However, increasing the number of quadruplets to 6 (i.e., the number of cells to 24) reduced the average RMSE by no more than 50% (Fig. 3h) as compared to a single quadruplet.
**FIGURE 3**

(a) Response of a single ON-OFF DSGC to a bar moving along 36 directions, angularly spaced by 10 degrees, n = 100 sweeps per direction.

(b) Characterization of the response variability of ON-OFF DSGCs (n = 103). Fit: power law, $\sigma^2 = a \mu^b$, where $\sigma^2$ = variance and $\mu$ = mean, black line. Poisson variability of responses with mean equal to variance is indicated by the blue line.

(c) Top left panel, decoding results by using the responses of a single ON-OFF DSGC. Blue circles indicate decoding errors in single trials (von Mises fit, Poisson noise, maximum-likelihood decoder): the farther a blue circle lies from the center, the higher the decoding error in the corresponding trial (max. error = 180 degrees). Bottom-left panel, RMSE along 36 different motion directions spaced 10 degrees, using a single cell. The average RMSE in this example with a single cell was 58.5 degrees. Top right and bottom panels, same as left panels, but decoding was performed using the signals of a quadruplet of ON-OFF DSGCs with different (approx. orthogonal) preferred directions; here, the average RMSE was equal to 6.4 degrees.

(d), (e), (f), (g), Average RMSE distributions for 600 quadruplets of ON-OFF DSGCs from 5 retinas, using four different decoding methods (For more details, see section “Decoders” in Material and Methods). The size of the moving bar is 0.5 x 1 mm$^2$ and the velocity 1.6 mm/s.

(h) Average RMSE as a function of the number of quadruplets used for decoding (error bars, s.d.). The dashed line indicates the average RMSE for quadruplets shown in panel D. For every quadruplet configuration, n = 100.

**Figure 3. Coding Precision of Retinal ON-OFF DSGC Populations.**

- (a) Response of a single ON-OFF DSGC to a bar moving along 36 directions, angularly spaced by 10 degrees, n = 100 sweeps per direction.
- (b) Characterization of the response variability of ON-OFF DSGCs (n = 103). Fit: power law, $\sigma^2 = a \mu^b$, where $\sigma^2$ = variance and $\mu$ = mean, black line. Poisson variability of responses with mean equal to variance is indicated by the blue line.
- (c) Top left panel, decoding results by using the responses of a single ON-OFF DSGC. Blue circles indicate decoding errors in single trials (von Mises fit, Poisson noise, maximum-likelihood decoder): the farther a blue circle lies from the center, the higher the decoding error in the corresponding trial (max. error = 180 degrees). Bottom-left panel, RMSE along 36 different motion directions spaced 10 degrees, using a single cell. The average RMSE in this example with a single cell was 58.5 degrees. Top right and bottom panels, same as left panels, but decoding was performed using the signals of a quadruplet of ON-OFF DSGCs with different (approx. orthogonal) preferred directions; here, the average RMSE was equal to 6.4 degrees.
- (d), (e), (f), (g), Average RMSE distributions for 600 quadruplets of ON-OFF DSGCs from 5 retinas, using four different decoding methods (For more details, see section “Decoders” in Material and Methods). The size of the moving bar is 0.5 x 1 mm$^2$ and the velocity 1.6 mm/s.
- (h) Average RMSE as a function of the number of quadruplets used for decoding (error bars, s.d.). The dashed line indicates the average RMSE for quadruplets shown in panel D. For every quadruplet configuration, n = 100.
3.4.3 Variability of Coding Precision with Respect to Stimulus Parameters

As the response characteristics of direction-selective neurons vary with stimulus properties, such as bar velocity and bar size, we investigated to what extent these variations influence the coding of directional information (Fig. 4a and 4e) (Oyster et al., 1972; Wyatt and Daw, 1975; Nowak et al., 2011). While the peak of the tuning curve depends strongly on the bar velocity (Fig. 4b) (decrease of 50.4% when the bar velocity is varied from 0.4 mm/s to 1.6 mm/s), the width of the tuning function remains virtually unchanged (Fig. 4c) (2.9% decrease for the same variation in bar velocity).

Changing the width of the moving bar (see inset in Fig. 4e) influenced tuning curve peak and width as well. Specifically, doubling the width of the moving bar from 0.5 mm to 1 mm caused an average 14.6% decrease in the directional peak magnitude (Fig. 4f) and an average 12.7% decrease in the tuning curve width (Fig. 4g). The decreases in peak response and tuning curve width are due to the fact that ON-OFF DSGCs have a large inhibitory receptive field surround (Chiao and Masland, 2003).

Varying the velocity of the moving stimulus modulated the average RMSE by less than 1 degree (Fig. 4d ML decoder with von Mises fit and Poisson noise): it dropped from 9.8±1.4 degrees for a stimulus velocity of 1.6 mm/s to 9.5±1.8 degrees for a stimulus velocity of 0.4 mm/s (Fig. 4d). In contrast, the width of the moving bar influenced the decoding results more substantially. Doubling the width of the moving bar from 0.5 mm to 1 mm caused an increase of the average RMSE equal to 8.7 degrees, i.e., a 80% significative increase (p<0.05, Mann-Whitney U-test, Kolmogorov-Smirnov test) (Fig. 4h).

Although the velocity of the stimulus did not influence decoding accuracy, the increasing size of the bar significantly worsened decoding. Therefore, a single quadruplet is less accurate when decoding the movement direction of objects larger than its receptive fields.

Owing to the shape of the tuning curves, the decoding performance depended on the stimulus direction. Motion directions ~45 degrees away from the preferred directions were decoded with lower average
RMSE (70% significative decrease, for 0.5 mm bar widths) as compared to motion directions close to the preferred directions (p<0.05, Mann-Whitney U-test, Kolmogorov-Smirnov test) (Fig. 4i, right panel, black line). On the other hand, decoding accuracy for bar widths of 1 mm had a uniform error across all stimulus directions (Fig. 4i, right panel, red line).

**FIGURE 4**

*Figure 4. Influence of Stimulus Velocity and Size on the Decoding Performance.*

(a) Tuning curves for stimulus velocities of 0.4 mm/s (blue line) and 1.6 mm/s (black line). Stimulus size was 1 mm x 0.5 mm (length x width). The bar moved perpendicular to its shorter edge.
(b) Tuning curve peak as a function of stimulus velocity. For every cell, each tuning curve peak value, across all stimulus velocities, was normalized with respect to the highest tuning curve peak value (100% indicates highest tuning curve peak, $n = 10$).

(c) Tuning curve width (computed from von Mises fit) as a function of stimulus velocity. For every cell, each tuning curve width, across all stimulus velocities, was normalized with respect to the highest tuning curve width (100% indicates highest tuning curve width, $n = 10$).

(d) Average RMSE for 5 stimulus velocities ($n = 108$ quadruplets of ON-OFF DSGCs, error bars, s.d.). Decoding performed by using von Mises fit, Poisson noise, and a maximum-likelihood decoder. Stimulus size was 1 mm x 0.5 mm (length x width).

(e) Tuning curves for bar widths of 0.5 mm (black line) and 1 mm (red line). Stimulus velocity was 1.6 mm/s.

(f) Tuning curve peak as a function of bar width (normalized as in panel B, $n = 10$).

(g) Tuning curve width (computed from von Mises fit) as a function of bar width (normalized as in panel C, $n = 10$).

(h) Average RMSE for stimulus bars of different widths, black indicates 0.5 mm and red indicates 1 mm ($n = 600$ and $n = 464$ quadruplets of ON-OFF DSGCs, error bars, s.d.). Decoding performed by using von Mises fit, Poisson noise, and a maximum-likelihood decoder. Stimulus velocity was 1.6 mm/s.

(i) Left panel, tuning curves for stimulus bars of different widths. Right panel, average RMSE as a function of stimulus direction relative to ON-OFF DSGCs preferred directions. Error bars indicate s.d. in all panels.

3.4.4 Influence of Tuning Curve Shape, Response Variability and Noise Correlations on the Coding Performance

The tuning curve of ON-OFF DSGC has been commonly fitted by using von Mises functions (Swindale, 1998; Oesch et al., 2005; Elstrott et al., 2008; Graf et al., 2011). We examined to what extent the functional shape of the tuning curve affected the coding performance, by comparing the latter in the case of von Mises fits (4 free parameters) to four other cases: Gaussian fit (4 free parameters); half-wave rectified cosine (4 free parameters); bimodal von Mises fit (6 free parameters); flat-topped von Mises fit (5 free parameters) (Fig. 5a, see Methods). In each case, we evaluated the goodness of the fit by computing the coefficient of determination, $R^2$, for 103 ON-OFF DSGCs. The highest average $R^2$ resulted for the flat-topped von Mises fit (0.96±0.04), the bimodal von Mises fit (0.95±0.06) and the half-wave rectified cosine fit (0.95±0.05) (Fig. 5b). The average $R^2$ for von Mises and Gaussian functions were significantly lower: 0.92±0.08 and 0.92±0.10 (Fig. 5b), respectively, than that obtained with the flat-topped von Mises function ($p<0.05$, Mann-Whitney U-test, Kolmogorov-Smirnov test).
FIGURE 5

Figure 5. Influence of Tuning Curve Fit on Decoding Performance.
(a) Various tuning curve fits. The different fit types are indicated in the legend by different colors and numbers.
(b) Mean coefficient of determination, $R^2$, for 103 ON-OFF DSGCs (all four preferred directions, 8 retinas) fitted by four different functions. Colors as in panel A.
(c) Average RMSE using six different functional fits of the tuning curves. Colors as in panel A (5 retinas, 600 quadruplets, dimensions of the moving bar: 0.5x1 mm$^2$ and velocity of 1.6 mm/s). Decoding results were obtained by using a maximum-likelihood decoder. Decoding was performed by Poisson variability of responses. Error bars indicate s.d. in all panels.
(d) Relative decoding accuracy ratio using Poisson and sub-Poisson fits to fit the variability in cell responses (5 retinas, 600 quadruplets, size of the moving bar equal to 0.5 x 1 mm$^2$ and velocity equal 1.6 mm/s).
(e) Relative decoding accuracy ratio of unshuffled and shuffled trials used for decoding directions of motion (5 retinas, 600 quadruplets, dimensions of the moving bar: 0.5 x 1 mm$^2$ and velocity 1.6 mm/s).

The flat-topped von Mises functions, which represented the best fit to the tuning curves of ON-OFF DSGCs, also yielded the smallest average RMSE of 9.4±2.7 degrees ($p<0.05$, Mann-Whitney U-test, Kolmogorov-Smirnov test), compared to 10.5±2.4 degrees for the von Mises fit, 10.6±3.0 degrees for the Gaussian fit, 11.1±3.5 for the half-wave rectified cosine and 10.1±2.6 degrees for the bimodal von Mises fit (Fig. 5c). For the sake of comparison, we also report the average RMSE obtained from spline interpolation of the tuning curves, which amounts to 8.6±2.5 degrees (black bar, Fig. 5c). The decoding results computed above were obtained by using a
maximum-likelihood decoder and Poisson variability of the responses. Using sub-Poisson noise (Fig. 3b) influenced decoding performance and introduced relative changes between 1-7% among the tuning curve fits (Fig. 5c-d).

Finally, we measured the effect of noise correlations on decoding accuracy by randomly shuffling cell response trials between multiple cells (Averbeck et al., 2006; Cohen and Kohn, 2011). For example, decoding of unshuffled responses, from four cells, means that we used the simultaneously recorded activity of these four cells during trial #1. Oppositely, decoding of shuffled responses, from four cells, means that we used the activity that is not recorded synchronously, and therefore cell responses are obtained by different trials (e.g. trial #1, trial #5, trial #7 and trial #25 respectively for cell #1, cell #2, cell #3 and cell #4). Random shuffling of the trials before decoding significantly lowered relative decoding performance by approximately 5-7% for all tuning curve fit types (p<0.05, Mann-Whitney U-test, Kolmogorov-Smirnov test) (Fig. 5e-f), despite the fact that none of the decoders is based on the assumption of correlated noise in the probability density function. Surprisingly, the performance of the decoders was not improved in any instance by shuffling the trials, which is in contrast to the fact that noise correlations would be harmful for encoding stimulus information.

3.4.5 Influence of Time Bin size on Decoding Performance

The results reported in the previous sections were obtained from spike counts within a time bin that covered the total duration of the stimulus presentation. In order to quantify how the bin size influences decoding performance, we repeated the analysis with spike counts derived from overlapping time bins with sizes ranging from 10 s to 300 ms (Fig. 6). For a meaningful comparison, we always carried out the analysis on selected ON-OFF DSGCs with overlapping receptive fields, and, therefore, with synchronous light-induced responses (Fig. 6a-b). We found that decoding performance saturated for bin sizes larger than 100 ms (average RMSE smaller than 16.2 degrees, Fig. 6c-d). In contrast, for bin sizes smaller than 100 ms, decoding performance started to decrease with an average
RMSE of 23.9 degrees for bins sizes of 50 ms and 67.1 degrees for bin sizes of 10 ms (Fig. 6c-d). In conclusion, the brain could decode the combined activity of multiple ON-OFF DSGCs in time bins between 100-200 ms, a time span that is behaviorally relevant in order to process visual inputs (Thorpe et al., 1996).

**FIGURE 6**

(a) Overlapping receptive fields of four simultaneously recorded ON-OFF direction-selective cells (four different preferred directions).
(b) Simultaneous responses for a bar moving in the direction indicated by black arrows at the top of the panels. Each panel shows 100 responses of a single cell. Preferred directions of the respective cells are indicated by colored arrows within the panels.
(c) Decoding performance at consecutive time points of cell responses. Each curve represents decoding results obtained with a different temporal bin size to count cell spikes. For decoding we used a von Mises fit for the tuning curves, Poisson variability of responses and a maximum-likelihood decoder.
(d) Decoding accuracy as a function of bin size (ms) for 5 quadruplets of ON-OFF DSGCs.

### 3.4.6 Influence of Tuning Curve Width on Decoding Performance

If any direction is to be encoded with ON-OFF DSGCs, organized along the four cardinal directions, one expects ON-OFF DSGCs’ tuning curves to be broad. Here, we quantify the dependence of the
coding performance on this width (see tuning curve parameterization in Methods, $\sigma_i$ parameter for von Mises fit); we do so by assuming a von Mises form for the shape of the tuning curve and by establishing bounds, $\epsilon_{\text{mean}}$ and $\epsilon_{\text{max}}$, on the decoding accuracy over a range of tuning curve widths (see “Decoding performance bound,” in Methods). As we varied tuning curve width, we fixed the integral of the tuning curve, so that we compare cases, which share a given total number of spikes (and, hence, a given metabolic cost), and in which each stimulus direction appears with equal probability. Quantifications on coding performance as function of tuning curve widths have been already performed in the context of visual cortical neurons (Seung and Sompolinsky, 1993).

**FIGURE 7**

![Graphs showing influence of tuning curve width on decoding precision.](image)

**Figure 7. Influence of Tuning Curve Width on Decoding Precision.**

(a-b) Two examples (solid and dashed lines, von Mises function) of four simulated tuning curves with preferred directions separated by 90 degrees for two different widths of 40 and 20 degrees. The integral of the tuning curves was fixed.

(c) $\epsilon_{\text{mean}}$, computed with model tuning curves, as a function of the tuning curve width (continuous line = mean, dashed line = s.d.). Red cross: lowest $\epsilon_{\text{mean}}$ value. Green dots: $\epsilon_{\text{mean}}$ values for measured tuning curves ($n = 600$ quadruplets).

(d) $\epsilon_{\text{max}}$, computed with model tuning curves, as a function of tuning curve width (continuous line = mean, dashed line = s.d.). Red cross: lowest $\epsilon_{\text{max}}$ value. Green dots: $\epsilon_{\text{max}}$ values for measured tuning curves ($n = 600$, quadruplets).
We assigned tuning curves to four ON-OFF DSGCs with homogeneously distributed preferred directions (Fig. 7a-b). The value of the integral of each model tuning curve was drawn from the distribution of the corresponding quantity in the data. We modeled 200 ON-OFF DSGCs quadruplets in which the tuning curve widths ranged from 10 to 70 degrees. For model tuning curves, the lowest obtained $\epsilon_{\text{mean}}$ value was 3.7 degrees and corresponded to a narrow tuning curve with a width of 18 degrees (Fig. 7c, black solid curve – red cross). We also derived the minimum value of the maximum estimation error over stimulus directions, $\epsilon_{\text{max}}$, and compared these figures with real data. The lowest $\epsilon_{\text{max}}$ value was 7.2 degrees and corresponded to broader tuning curves with a width of 36 degrees (Fig. 7d, black solid curve – red cross). We found that the data (green dots, Fig. 7c-d) clustered near the optimal $\epsilon_{\text{max}}$ value, i.e., the tuning curves were wider (41.1±7.3 degrees) than predicted from optimizing the average error, but comparable to the prediction obtained by minimizing the maximum error over stimuli. This suggests that tuning curves are sufficiently wide so as to avoid large decoding errors for given stimulus directions.

### 3.4.7 Influence of the Arrangement of Preferred Directions on Population Coding

As previously mentioned, roughly speaking, the preferred directions of ON-OFF DSGCs are aligned with the four cardinal directions. But, more precisely, the preferred direction in each ON-OFF DSGC is slightly jittered away from ‘its’ cardinal direction (Fig. 2d-e). These considerations naturally beg for quantitative analyses in response to two questions:

I. How much more accurate is coding for preferred directions arranged along the four cardinal directions as opposed to those randomly arranged on the circle?

II. To what extent does the jitter in the cardinal direction affect the coding performance?
In order to answer these questions, we compared the coding performance for several arrangements of cells:

1) **ON-OFF DSGCs** with equidistant preferred angular directions (Fig. 8a). We refer to this model as “Equidistant PD”.

2) **ON-OFF DSGCs** with preferred directions along the cardinal directions (Fig. 8b). We refer to this model as “Cardinal PD”.

3) **ON-OFF DSGCs** with mean preferred directions along the cardinal directions, but where individual preferred directions are jittered by a small increment, the distribution of which was derived from the data (Fig. 8c). We refer to this model as “Cardinal Jittered PD.”

4) **ON-OFF DSGCs** with preferred directions (PD) randomly distributed over the circle (Fig. 8d). We refer to this configuration as “Random PD”.

Arranging only 4 cells in a “Random PD” configuration was obviously disadvantageous and resulted in higher $\varepsilon_{\text{mean}}$ and higher $\varepsilon_{\text{max}}$ compared to “Cardinal PD” and “Equidistant PD” configurations (Fig. 8e-f). However, for a larger number of cells, $\varepsilon_{\text{mean}}$ converged to similar values for all possible angular arrangements of the preferred directions (Fig. 8e). Similarly, $\varepsilon_{\text{max}}$ decreased when the number of cells increased, although the “Equidistant PD” configuration resulted in a $\varepsilon_{\text{max}}$ approximately 20% lower than that of “Cardinal PD” and “Random PD” configurations (Fig. 8f). Direction selectivity is established by spatial asymmetric inhibition of ON-OFF DSGCs through starburst amacrine cells (Fried et al., 2002; Briggman et al., 2011; Yonehara et al., 2013; Park et al., 2014). Establishing more than four direction-selective cells would require a rearrangement of the inhibitory circuits, and in particular a more precise spatial distribution of starburst amacrine cells dendrites (Euler et al., 2002; Briggman et al., 2011). Importantly, here we show that the retinal direction-selective system would not substantially benefit on average, in terms of decoding accuracy, from implementing a more complex cellular/molecular machinery featuring a direction-selective system with more than four preferred directions. Having more cells with more than four preferred directions would decrease $\varepsilon_{\text{max}}$ by
about 20%, which corresponds to ~ 1 degree improvement in decoding accuracy (Fig. 8f). Such improvement in decoding performance could be not enough behaviorally relevant in order to implement a more complex coordinate system, which features more than four preferred directions.

FIGURE 8

![Diagram showing different coordinate systems and error bars for different conditions.](image-url)
Next, we asked to what extent jitter in the preferred directions of the DSGCs influences coding performance. We compared $\varepsilon_{\text{mean}}$ and $\varepsilon_{\text{max}}$ of ON-OFF DSGCs with preferred directions angularly spaced at 90 degrees without jitter and with jitter (Fig. 8b-c and Fig. 8g-h). We found that the direction-selective systems did not benefit on average ($\varepsilon_{\text{mean}}$, Fig. 8g) from perfectly aligning cells along the four cardinal directions. Only in the case of one quadruplet or two quadruplets of ON-OFF DSGCs (one quadruplet contains 4 ON-OFF DSGCs with 4 different preferred directions), a perfect alignment of the preferred directions along the four cardinal directions is slightly advantageous and reduces $\varepsilon_{\text{max}}$ by 5-15%. Again, these results indicate that the cellular or molecular burden needed for perfect alignment with the four cardinal directions may not be worth the marginal improvement in coding performance.
3.5 Discussion

We recorded spiking activity from populations of ON-OFF DSGCs and analyzed their concerted activity for decoding the direction of motion of moving objects in the visual scene. Furthermore, we tested different decoding strategies (PV, OLE, ML, see Methods) and reported, how a change in stimulus parameters (velocity, object size, direction of motion) influenced the decoding of visual stimuli. In addition, based on real data and a theoretical framework, we analyzed, how the observed tuning curve widths and the distribution of the preferred directions along the four cardinal directions influenced the coding performance.

Populations of functionally identified retinal ON- and OFF-cells have been recorded from the macaque retina, and it was shown that recording of their concerted activity allows for extracting more information from the visual scene (Pillow et al., 2008). Furthermore, several studies used physiologically identified orientation- and direction-selective neurons in the primary visual cortex for decoding directions of moving objects (Britten et al., 1992; Graf et al., 2011; Montijn et al., 2014). Quantifications of direction coding, however, has not yet been carried out in the case of retinal direction-selective populations.

In the context of data on primate primary visual cortex, a linear decoder and a maximum-likelihood decoder yielded comparable accuracy in angular motion estimation (Graf et al., 2011). We found that in the case of the retina, however, the coding accuracy was ~2 times higher for a Bayesian decoder as compared to a linear decoder. In a recent study in the mouse (Montijn et al., 2014), it was shown that a maximum-likelihood decoder significantly outperformed linear methods, such as population vectors, when decoding motion directions by visual cortical neurons. This is consistent with our result (Fig. 3d-g) and indicates that the neural code in front-end sensory areas may not be optimized for a simple linear readout scheme. In addition, in the work of Montijn et al. 2014, it was shown that increasing the population size (1 to 80 cells) for the maximum-likelihood decoder quickly saturated the decoding performance after ~35 neurons with a ~60-70% improvement in decoding precision.
Similarly, we found that increasing the population size (from 4 to 24 cells) for the maximum-likelihood decoder improved the decoding accuracy by ~ 50% (Fig. 3h), and that adding more cells (e.g., total of 80 cells) further improved decoding performance, but it did not improve decoding accuracy by more than a total of ~70% (data not shown).

The most informative region of a bell-shaped direction-tuning curve depends on the respective noise levels (Butts and Goldman, 2006). In the presence of small noise, high-slope regions are the most informative, whereas, in the presence of large noise, high-firing-rate regions (tuning curve peaks) are the most informative (Butts and Goldman, 2006). For groups of four direction-tuning curves with preferred directions that are angularly spaced at 90 degrees, it was shown that the most informative regions change as a function of noise levels (Butts and Goldman, 2006). In the work of Butts and Goldman, for noise levels reported as “3x” with Fano factor values similar to ours, the most informative tuning curve regions were located at ~ 45 degrees from the cells’ preferred directions (Butts and Goldman, 2006). Similarly, we report that, in the retinal direction-selective system, the lowest decoding errors occurred at ~ 45 degrees from the cells’ preferred directions (Fig. 4i). However, the result from Butts and Goldman was derived for the cricket cercal sensory system (Miller et al., 1991), where the Fano factor increased with increasing cell activity, whereas we found an inverse relationship in the rabbit retina: the Fano factor was lowest at the peak of the tuning functions (Fig. 3b).

In other studies it was shown that heterogeneity in neural responses could be advantageous for decoding (Chelaru and Dragoi, 2008; Tripathy et al., 2013). For example, neural population diversity reduced the negative effects of noise correlations on the decoding performance (Shamir, 2014). The preferred directions of ON-OFF DSGCs are jittered and, therefore, are heterogeneous (Fig. 2d-e) (Oyster and Barlow, 1967). In our simulations with different angular arrangements of the ON-OFF DSGCs’ preferred directions (Fig. 8), we could not find a substantial positive effect by preferred direction
heterogeneity across ON-OFF DSGCs pointing to the same cardinal direction.

In mammals, approximately 20 retinal ganglion cell types convey information about the visual scene to the brain (Masland, 2001; Roska and Werblin, 2001; Azeredo da Silveira and Roska, 2011; Dhande and Huberman, 2014). In the last decade, genetically identified retinal ganglion cells have been molecularly characterized and with them, their target areas in the brain (Huberman et al., 2008; Kim et al., 2008; Huberman et al., 2009; Kay et al., 2011; Dhande et al., 2013). Recently, it has been shown that retinal direction-selective neurons in the mouse transmit, through lateral geniculate neurons, information about motion directions to cortical neurons in the primary visual cortex (Cruz-Martin et al., 2014). Moreover, it has been demonstrated that dendritic spines of individual neurons in the mouse visual cortex can be tuned to diverse preferred orientations, and that the tuning of a given neuron (i.e., its preferred tuning direction as defined by its spiking output) can be predicted by the prevalent tuning of its dendrites when all the dendritic-spine signals are averaged (Chen et al., 2013). Thus, it is possible that the inputs of retinal ON-OFF DSGCs are combined in the visual cortex to decode the direction of a moving object. In future investigations of sensory input coding, it will be advantageous to record the simultaneous activity of genetically defined sensory neurons, as reported in this work, and to trace their brain targets in order to possibly understand how the activity of multiple cells is combined to perform the respective computations.

Acknowledgements

The authors acknowledge Marcel Stimberg (École Normale Supérieure Paris, France) for the code to do fits with Von Mises functions. The Circular Toolbox was provided by Philipp Behrens (Bernstein Center for Computational Neuroscience, Tübingen, Germany). We acknowledge Thomas Lee Russell and Ian Lloyd Jones at ETH Zurich for technical support with animals. This work was supported by the Advanced ERC Grant “NeuroCMOS” under contract number AdG 267351, the SNSF Sinergia Project
CRSI13_141801, and the CNRS through UMR 8550. Michele Fiscella acknowledges individual support through a Swiss SystemsX interdisciplinary PhD grant No. 2009_031.

References


108


Chapter 4

Applicability of Independent Component Analysis on High-Density Microelectrode Array Recordings

David Jäckel¹, Urs Frey², Michele Fiscella¹, Felix Franke¹, Andreas Hierlemann¹

¹ Bio Engineering Laboratory, ETH Zurich, Basel, Switzerland.
² Riken Quantitative Biology Center, Kobe, Japan.

Published in:

Journal of Neurophysiology
Volume 108, Issue 1, 1 July 2012, Pages 334–348
4.1 Abstract
Emerging complementary metal oxide semiconductor (CMOS)-based, high-density microelectrode array (HD-MEA) devices provide high spatial resolution at subcellular level and a large number of readout channels. These devices allow for simultaneous recording of extracellular activity of a large number of neurons with every neuron being detected by multiple electrodes. To analyze the recorded signals, spiking events have to be assigned to individual neurons, a process referred to as “spike sorting.” For a set of observed signals, which constitute a linear mixture of a set of source signals, independent component (IC) analysis (ICA) can be used to demix blindly the data and extract the individual source signals. This technique offers great potential to alleviate the problem of spike sorting in HD-MEA recordings, as it represents an unsupervised method to separate the neuronal sources. The separated sources or ICs then constitute estimates of single-neuron signals, and threshold detection on the ICs yields the sorted spike times. However, it is unknown to what extent extracellular neuronal recordings meet the requirements of ICA. In this paper, we evaluate the applicability of ICA to spike sorting of HD-MEA recordings. The analysis of extracellular neuronal signals, recorded at high spatiotemporal resolution, reveals that the recorded data cannot be modeled as a purely linear mixture. As a consequence, ICA fails to separate completely the neuronal signals and cannot be used as a stand-alone method for spike sorting in HD-MEA recordings. We assessed the demixing performance of ICA using simulated data sets and found that the performance strongly depends on neuronal density and spike amplitude. Furthermore, we show how postprocessing techniques can be used to overcome the most severe limitations of ICA. In combination with these postprocessing techniques, ICA represents a viable method to facilitate rapid spike sorting of multidimensional neuronal recordings.

4.2 Introduction
In the field of neurophysiology research, extracellular recordings of neural activity have become an important means to study intercell interaction and firing patterns to understand better the physiology and
the information processing of neuronal networks. In multiunit recordings, the electrodes monitor the simultaneous activity of a large number of individual neurons. For the analysis, the spike trains of the individual neurons then have to be extracted from the recorded data, a process usually referred to as “spike sorting” (Lewicki, 1998). Generally, the spike-sorting tasks consists of two fundamental steps: to 1) detect action potential (AP) events in the data; and 2) classify them into groups. Whereas a main problem for the spike detection task is to deal with data recorded under low signal-to-noise-ratio (SNR) conditions, the most severe challenge to the classification problem is the presence of overlapping spikes from different neurons. The sorting performance can be drastically increased by using multielectrode devices such as tetrodes (Gray et al., 1995). In these systems, which feature several closely spaced electrodes, an AP is simultaneously measured on more than one electrode. In addition to the temporal cues of the waveform, these multielectrode devices reveal information about the spatial cues of the spike shape distribution. This additional information can be efficiently used to separate units.

Planar microelectrode arrays (MEAs) are arrangements of electrodes for extracellular measurements of multiple cells on a chip surface. They are widely used to study the dynamics of the neuronal networks, as they enable simultaneous access to a large number of neurons. Traditional MEA systems incorporate 60–200 passive metal electrodes on a silicon or glass surface, which are connected to external circuitry and typically feature interelectrode distances of 100–200 µm (Stett et al., 2003). Since the signal of a neuron is detected by at most one electrode on such MEAs, the spike-sorting problem is the same as for single-electrode recordings (Shoham et al., 2003; Zhang et al., 2003).

Recently, “active” MEAs based on complementary metal oxide semiconductor (CMOS) technology have been developed (Eversmann et al., 2003; Berdondini et al., 2005; Hutzler et al., 2006; Frey et al., 2010). These devices feature signal-conditioning circuitry on-chip and provide much larger electrode densities and, thereby, enable to conduct electrophysiological experiments at cellular or subcellular level. The MEA used for our experiments (Frey et al., 2010) features 11,011 electrodes (3,161 electrodes/mm²) as well as 126 read-out channels.
The possibility to select arbitrarily a subset of electrodes for recording or stimulation entails the possibility to use different electrode configurations, such as high-density (HD) or sparse arrangements. The HD of the electrodes on the array enables recordings at subcellular resolution, with the activity of every neuron being measured by multiple electrodes. Although this feature improves sorting capabilities, the large number of channels and the highly redundant nature of HD-MEA data pose challenges to the strategies for event detection and classification. Particularly, two issues arise when applying standard analysis techniques to HD-MEA data.

- **How to Perform Event Detection in Redundant Data?**
  A neuronal AP will produce spikes on several electrodes, leading to threshold crossing events (TCEs) on these electrodes. Ideally, these events should be grouped and considered as one single spiking event, since their origin is the same AP of the same cell. This could be performed by merging TCEs, which are spatially and temporally closely aligned. However, this task gets challenging for large numbers of spatially highly overlapping neurons.

- **Which Features Should be Used for Unit Separation?**
  For recordings with hundreds to thousands of electrodes, the feature space needs to be reduced in a way that only the electrodes are used that prominently contribute to unit separation. Only a few methods have been published that specifically target spike sorting of HD, redundant recordings. A sequential approach that targets one electrode at a time is proposed in (Litke et al., 2004). This simple and robust method lacks efficiency by repeating the clustering of the same cells many times and does not handle the overlap problem. Another approach based on template matching has been presented in (Segev et al., 2004) using data from MEA recordings with up to 30 electrodes. The overlap problem is addressed by this approach; this method, however, is formulated for a limited number of electrodes and templates and requires prior knowledge of the neurons and their waveforms. The challenges of efficient, automatic spike-sorting and validation techniques for multielectrode systems have been discussed in (Einevoll et al., 2012).
Independent component (IC) analysis (ICA) (Hoyer and Hyvarinen, 2000; Stone, 2002) is a blind source separation technique that can be used to demix a set of independent source signals that were linearly mixed across a number of observed signals. The high potential of ICA to be applied to spike-sorting problems has been discussed in (Brown et al., 2001). Neurons have been regarded as independent signal sources, which are linearly mixed across the recording electrodes. ICA has the following requirements. 1) The source signals are non-Gaussian and statistically independent. 2) A linear, instantaneous mixture of the source signals is assumed to produce the observed signals. 3) The number of observed signals needs to be equal to or larger than the number of source signals. Although neurons are not independent from each other, as they can be synaptically connected or may receive common input, the individual spike trains can be regarded as statistically independent, since dependence in this context refers to instantaneous overlaps rather than time-delayed dependence (Brown et al., 2001). Therefore, concerning the first requirement, ICA will only fail to separate two neurons if they fire always precisely at the same time.

Recently, some studies attempted to combine closely spaced electrode recording techniques with computationally efficient ICA algorithms such as FastICA (Hyvarinen, 1999). In (Hermle et al., 2004; Snellings et al., 2006), ICA is applied as a preprocessing step on recorded data to reduce cross talk and increase data quality. The major obstacle for an efficient use of ICA has been, however, requirement 3. In standard in vivo experiments with tetrodes, the number of neurons is likely to be larger than the number of recording sites. One attempt to overcome this restriction for ICA included to perform k-means clustering of detected waveforms in a preprocessing step and to decompose the waveforms of each cluster individually with ICA (Takahashi et al., 2003), since the number of neurons in the clusters is expected to be lower than the number of recording sites. A second approach included to increase the number of recording sites to 12 (Takahashi and Sakurai, 2005). The large number of electrodes of HD-MEAs is suited to meet requirement 3, which renders ICA a good candidate to separate HD-MEA data. Ideally, the independent source signals, found by ICA,
correspond to individual activity signals of neurons. Applying threshold detection on each source signal then yields the sorted spike times of the neurons that have been recorded on the array. Making use of the redundancy in the data, ICA should also provide an increase in the SNR of the demixed signal compared with the recorded signals and, therefore, improve the spike detection performance. By separating the neuronal sources, it also holds promise to solve the problem of overlapping spikes.

In this study, we explore the applicability of ICA for blind and rapid spike sorting of HD-MEA recordings.

4.3 Methods

4.3.1 Data Acquisition System

Recordings were made with the HD-MEA recording system described by (Frey et al., 2009; Frey et al., 2010) The array is integrated into a microsystem chip, fabricated in a 0.6-µm CMOS process. It accommodates a total of 11,011 electrodes of 7-µm diameter on an area of 2.00 × 1.75 mm² (18-µm hexagonal center-to-center pitch, density of 3,161 electrodes/mm²). One hundred twenty-six bidirectional channels are implemented on-chip, featuring recording and stimulation electronics. The channels are connected to the electrodes via a flexible switch matrix lying underneath the array. This system provides routing flexibility to select almost arbitrary electrode configurations, which can be changed within milliseconds.

The programmable gain amplifiers (0–80 dB) allow for recording neuronal signals throughout a wide range of amplitudes, which depend on the respective cell type. Offset and fluctuations resulting from the electrode-saline interface are removed by first-order high-pass filtering the analog signals (tunable cutoff frequency 0.3–100 Hz). The frequency range is limited toward the high-frequency end by means of a tunable second-order low-pass filter (3.5–14 kHz). The signals are multiplexed and digitalized with 8-bit analog-to-digital converters with a sampling rate of 20 kHz.

The data were stored on a standard PC, and the analysis was conducted using MATLAB. Before postprocessing, all the data were digitally band-pass filtered (500–3,000 Hz).
FIGURE 1

(a) 2 s of recorded data from 6 selected electrodes, labeled in a.
(b) Data segment indicated by a gray rectangle in a. The spikes of 3 retinal ganglion cells (RGCs) that were identified by supervised spike sorting were colored (red, green, and violet).
(c) Superposition of all detected spike waveforms within 50 s of data (green neuron: 187 spikes; red neuron: 191 spikes; violet neuron: 1,046 spikes). The colored line shows the averaged waveform [spike-triggered averaging (STA)].
(d) Cell-specific templates (only green and red neuron shown for visualization purposes). Black dots indicate electrode positions on the microelectrode array (MEA). Colored waveforms correspond to the STA of the neuron on the respective electrode. The numbered electrodes refer to those in a-c. Scale bar: 100 µV/1.8 ms.

4.3.2 Extracellular Recordings from Retinal Ganglion Cells
We used the rd1 mouse retina to record spontaneous activity (Stasheff, 2008) from retinal ganglion cells (RGCs). All animal experiments and procedures were approved by the Swiss Federal Veterinary Office. The retinæ were isolated at ambient light from the C3H/HeNCrl (rd1) mouse strain at P80 in Ringer medium (in mM: 110 NaCl, 2.5 KCl, 1 CaCl₂, 1.6 MgCl₂, 10 D-glucose, 22 NaHCO₃, bubbled with 5% CO₂-95% O₂). Once a piece of the retina was isolated, it was placed with the RGC layer adjacent to the MEA. The retina was fixed on the array by a permeable membrane (polyester, 10-µm thickness, 0.4-µm pore size) and superfused with Ringer medium at 36°C.
All recordings shown in this study were done with electrode configurations of blocks at highest possible spatial resolution. The largest HD block that can be simultaneously read in the configurable
array is $6 \times 17$ electrodes and covers an area of approximately $80 \times 320 \ \mu m^2$.

An example of recorded RGC activity is shown in Fig. 1. Three neurons were identified in an HD block, using a manually supervised custom-designed spike-sorting method based on principal component analysis (PCA) and expectation-maximization (EM) clustering (KlustaKwik; (Harris et al., 2000)). The characteristic multichannel signature of the neurons is obtained by spike-triggered averaging (STA) of the individual aligned traces (Fig. 1c). This cell-specific footprint will be referred to as template, namely the distribution of the average spike shape across the electrodes. Figure 1d shows the templates of two cells that have significant energy on a large number of electrodes.

Templates and firing characteristics of recorded neurons were found to be very similar across several recording experiments ($n=10$).

### 4.3.3 Assumption of Linear Dependence Cells

ICA requires a linear and instantaneous mixture of the source signals across the electrodes. This requirement implies that the signals of the sources (neurons) on the different electrodes are linearly dependent and do not contain phase shifts. The assumption of linear dependence can be validated by testing the degree of linearity within the waveforms of the neuron template.

We measure the linearity between two vectors, $a$ and $b$, using the normalized cross-correlation coefficient (CC):

\[
CC_{ab} = \frac{\langle a, b \rangle}{\|a\|\|b\|}
\]

The CC can range from $-1$ to $1$, where two vectors with a CC of $1$ are perfectly linearly dependent and two vectors with a CC of $-1$ are inversely linearly dependent. A CC of $0$ indicates that the vectors are orthogonal. Therefore, the linearity assumption is met if the absolute CC values of the spike waveforms of the individual neuron templates are close to $1$. 

120
4.3.4 ICA

Let the recorded time-series signals on \( M \) electrodes be \( X = [x_1(t), ..., x_M(t)]^T \) and \( S = [s\sim_1(t), ..., s\sim_N(t)]^T \) be the intrinsic signals generated by \( N \) single neurons. Given the assumption of linearity, stated in the previous section, we can model the recordings as a linear mixture of the neuronal signals:

\[
X = A \cdot \mathbf{\hat{S}}
\]

Under the additional assumptions that the individual signals are non-Gaussian and statistically independent and that there are more recording sites than neurons, the mixing matrix \( A \) can be estimated blindly by applying ICA (FastICA; (Hyvarinen, 1999)) directly to the recorded data. The ICs, which ideally represent individual-neuron signals, are obtained by:

\[
S = W \cdot X
\]

The ICs are the rows in \( S = [s_1(t), ..., s_M(t)]^T \), and \( W = A^{-1} \) is the demixing matrix. In the following, we will refer to the columns of \( A \) as the mixing coefficient vectors (MCVs), whereas the rows of \( W \) will be called demixing coefficient vectors (DCVs). Note, that the estimated mixing matrix has a dimension of \( M \times M \).

If the individual templates are known, we can evaluate the separation by directly demixing the templates with \( W \). For a given neuron, \( i \), consider the template matrix \( F^i = [f_{i1}, ..., f_{iM}]^T \), for which the \( j \)th row is the STA waveform \( f_{ji} \) at the electrode \( j \). Then the demixed template is:

\[
H^i = W \cdot F^i
\]
In the case of perfect separation, $H^i$ contains the intrinsic neuron waveform in the $i$th row and zeros in all the other rows. ICA offers three main features if all the assumptions are met. 1) The redundancy is reduced so that only ICs 1, ..., $N$ contain significant signals (spikes), whereas ICs $N + 1$, ..., $M$ contain only noise. This allows for extracting the number of neurons from the number of ICs containing spikes. 2) The recordings are demixed in a way that every IC only contains the spikes of one corresponding neuronal source. Consequently, applying threshold detection to the ICs yields the sorted spike times. 3) ICA achieves an increase in SNR compared with single-channel signals by accumulating signals of several electrodes in the ICs.

The use of ICA, however, typically entails two major problems if the linearity assumption is not totally fulfilled. 1) One IC can contain signals from more than one neuron. In that case, demixing does not achieve perfect separation. 2) A neuron can contribute signals to two or more ICs. In that case, more than $N$ ICs contain neuronal signals, and ICA does not completely reduce the redundancy.

### 4.3.5 Evaluation Criteria

In the following, we formulate several evaluation metrics to characterize the performance of ICA with regard to the points elaborated in the previous section.

- **SNR**

We define the SNR of neuron $i$ in the recorded signal $X$ as

\[
SNR_{EL}^i = \frac{\max(\text{abs}(f_j^i))}{\sigma_j}
\]

where $j$ is the electrode on which the template $F^i$ has its highest peak value, and where $\sigma_j$ is the standard deviation of the noise signal on that electrode. The $SNR_{EL}$ thus denotes the peak value in the template divided by the noise standard deviation.
Chapter 4

Next, we define the SNR of the neurons in the ICs. FastICA normalizes the DCVs so that every IC signal has unit variance. Instead, we want the noise on the ICs to have unit variance. Therefore, we first normalize all the DCVs:

\[ \hat{w}_k = \frac{w_k}{\sqrt{(w_k)^T C w_k}} ; \ k = 1, \ldots, M \]

Here, \( w_k \) is the \( k \)th row of \( W \), and \( C \) is the instantaneous noise covariance matrix between the electrodes. \( \hat{W} \) is the new demixing matrix with normalized DCVs, having \( \hat{w}_k \) on its \( k \)th row. This normalization ensures equivalent noise levels on all the ICs and allows for comparison of their signals. The SNR of neuron \( i \) in the ICs is the peak value of the demixed template \( \hat{H}_i = \hat{W} \cdot F_i \):

\[ SNR_{IC}^i = \max(|\hat{H}_i|) \]

- **Redundancy**
  Another important ICA performance measure is how well redundancy in the ICs is reduced compared with the recorded signals. Therefore, the redundancies \( RED_{EL}^i \) and \( RED_{IC}^i \) are defined, where the first denotes the number of electrodes and the latter denotes the number of ICs, on which the signal of neuron \( i \) exceeds the threshold value of five times the noise standard deviation.

- **Separability**
  We also introduce a measure for the separability of the neurons on the ICs. A neuron \( i \) with high separability must have a high peak signal on an IC \( k \), on which all other neurons have only low peak signals. Thus its separability is the difference between its peak on IC \( k \) and the highest peak of any other neuron on IC \( k \). We determine \( k \) by maximizing the
separability, taking into account that the relevant peak can feature either positive- or negative-sign amplitude.

\[(8)\]

\[
SEP_{IC}^i = \max_{k=1,\ldots,D} (f_{pos}^k, f_{neg}^k);
\]

\[
f_{pos}^k = \max(\hat{w}^k F^i) - \max_{q \neq i}(\hat{w}^k F^q);
\]

\[
f_{neg}^k = \min_{q \neq i}(\hat{w}^k F^q) - \min(\hat{w}^k F^i);
\]

Analogously, we define the separability of a neuron in the recorded signals as the difference between the peak of its template and the highest peak of any other neuron template on the same electrode, divided by the noise standard deviation on that electrode.

\[(9)\]

\[
SEP_{EL}^i = \max_{j=1,\ldots,M} \left( \frac{g_{pos}^j}{\sigma_j}, \frac{g_{neg}^j}{\sigma_j} \right);
\]

\[
g_{pos}^j = \max(f_j^i) - \max_{q \neq i}(f_j^q);
\]

\[
g_{neg}^j = \min_{q \neq i}(f_j^q) - \min(f_j^i);
\]

### 4.3.6 Simulation of Recorded Neuronal Activity

For evaluation purposes, simulated data were generated. In this study, we used two types of simulated data sets. Data set A contained simulated activity of three neurons that had spatially overlapping templates that were extracted from digitally unfiltered, recorded data. This data set is mainly used for visualization purposes. Spike sorting to extract the templates for data simulation was performed using manually supervised PCA and EM clustering.

For a systematic analysis, we simulated RGC activity at different cell densities in data set B. Therefore, we used eight well-isolated,
manually selected neuronal templates extracted from recorded unfiltered data as model templates. Higher spatial resolution was obtained by interpolating the model templates on a grid (5-µm pitch). By modifying position, orientation, amplitude, and spatial extension of the model templates, individual neuron templates were simulated. The modified templates were positioned on a gridlike structure with equidistant points. Peak-to-peak amplitudes were set randomly (uniformly distributed between 50 and 300 µV). The grid of neurons covered an HD block of 90 electrodes on an area of 130 × 185 µm². We simulated 5 different configurations with average neuron distances (D) between 40 and 20 µm (average distance to the 6 neighboring neurons in a hexagonal arrangement). Out of 20 simulations of 30 s each, which were generated for each configuration, a subset of 358 neurons per configuration was considered for the analysis. Surrounding neurons outside the electrode block were simulated but not included in the analysis. An overview of the different configurations represented in data set B is given in Table 1 and illustrated in Fig. 2.

<table>
<thead>
<tr>
<th>Configuration</th>
<th>Average Neuron Distance, µm</th>
<th>Cell Density, Neurons/mm²</th>
<th>Approximate Number of Neurons per Simulation (Included in Analysis/Simulated)</th>
<th>Number of Simulations Used</th>
<th>Neurons Considered in Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>722</td>
<td>18/32</td>
<td>20</td>
<td>358</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>943</td>
<td>24/40</td>
<td>15</td>
<td>358</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>1,283</td>
<td>30/50</td>
<td>12</td>
<td>358</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>1,848</td>
<td>46/77</td>
<td>8</td>
<td>358</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>2,887</td>
<td>71/111</td>
<td>5</td>
<td>358</td>
</tr>
</tbody>
</table>

Table 1: Simulation Overview For Configurations in Data Set B.
Different numbers of simulations were considered for different configurations to have equal sample sizes in the analysis. For comparison of these values, please keep in mind that the average electrode distance is 18 µm, the electrode density is 3,161 mm², and the number of electrodes used in the simulations is 90 (high-density arrangement of 9 × 10 electrodes).
Individual, uncorrelated spike trains were simulated for both data sets using sets of \( \gamma \)-distributed interspike intervals (ISI), which proved to be a good model for the spiking behavior of RGCs (Levine, 1991). The mean firing rates ranged between 30 and 50 Hz for data set A and between 5 and 50 Hz for data set B. A refractory period of 2 ms was introduced. We upsampled the template waveforms to 160 kHz and then randomly downsampled them to the respective sampling frequency for every simulated AP, this way imitating recording conditions, under which the spikes are not always digitalized at the exact same position (Pouzat et al., 2002; Quiroga et al., 2004). Spike shape variability (Fee et al., 1996a) was induced by multiplying the template waveforms of every spiking event on all electrodes with a random factor (normally distributed \( \mu = 1, \sigma = 0.1 \)). A digitally unfiltered noise signal, which was recorded under experimental conditions with a retina preparation on the array that had no visible spiking activity, was added to the simulated spike data. The resulting signal was quantified to a least significant bit, similar to the one used in the measurements (5 \( \mu \text{V} \)). This way, the simulated data had similar characteristics as recorded data, and the same data handling and processing steps could be used.

**FIGURE 2**

Figure 2. Sample arrangements of neurons (circles) and electrodes (black dots) for the 5 configurations in data set B. Neurons lying outside the electrode block (gray) were also simulated, but only neurons inside the electrode block (black circles) were considered for the evaluation. \( D \), average neuron distance.

## 4.4 Results

### 4.4.1 Spatiotemporal Template Analysis

A main requirement for ICA is that the independent sources (i.e., the neuron templates) are linearly mixed over all recording electrodes. This
requirement implies that the waveforms of a neuron on the different recording electrodes are linearly dependent. Therefore, the degree of linear dependence between the waveforms of a RGC template was analyzed.

A single neuron template was reconstructed from two overlapping blocks, recorded at highest spatial resolution (Fig. 3a). Because of the chip architecture, the electrodes are not sampled at the same point in time. We corrected for this by upsampling (160 kHz) and resampling the recorded data at defined time points (20 kHz). After spike detection, the multichannel spike traces of the identified neuron were again upscaled by a factor of 4 to allow a more precise spike alignment and averaging (upsampled resolution: 12.5 µs).

Figure 3b shows the superimposed waveforms from 40 electrodes, illustrating that they are not exactly in phase but shifted by up to 3 samples (150 µs). The electrode position where the AP wave appears 1st (dark blue wave in Fig. 3b) will be referred to as AP reference (white marker in Fig. 3c). The color of each waveform indicates the distance between the corresponding electrode and the AP reference. An increase in the phase shift is observed for increasing distance. This phase shift is due to the AP propagation delay.

The spatial extent of the propagation delay is visualized in Fig. 3c. For every electrode, the precise position of the negative peak in the upscaled averaged waveform was used to determine the temporal delay compared with the AP reference. After emergence close to this reference point, the AP spreads into all directions. The timing delays vs. the travelling distances for the individual waveforms, shown in Fig. 3d, give an estimate of the propagation speed. The slope of the linear fit corresponds to a velocity of 0.55 m/s.

For better visibility, we focus in the following on the waveforms recorded from the 12 electrodes providing the highest signals (peak-to-peak voltages > 60 µV, marked with a red dot in Fig. 3a, a and c. In any case, signals with low amplitude only moderately influence ICA compared with high amplitude signals.

The CC matrix (Fig. 3e, left) contains the CC values (see Eq. 1 in Methods) for the waveforms of all electrode pair combinations of the selected electrodes. The electrodes were arranged in the matrix with
respect to ascending time delay, which is displayed in the inset above the matrix plot. Electrodes 1–7 (electrodes in purple, bluish area in Fig. 3c) as well as 8–12 (electrodes in green, yellowish area in Fig. 3c) form electrode groups with high CC values for electrode pairs within each group but relatively small CC values for electrode pairs between both groups. The decrease in linearity is caused by a relatively large phase shift between electrodes 7 and 8. Similar abrupt phase shifts were observed for many RGCs and are presumably a characteristic physiological feature of these neurons. For electrode pairs

![Figure 3](image)

**Figure 3. Ganglion Cell Template Analysis at High Spatiotemporal Resolution.**
(a) RGC template; the blue point indicates the center of gravity, and the blue line shows a Gaussian-fit equipotential line; scale bar: 100 µV/1.6 ms.
(b) Superimposed averaged waveforms with the color code indicating the distance to the reference point marked in c. The phase shift grows with increasing distance.
(c) For every averaged waveform, the action potential (AP) timing was determined based on the occurrence of the respective negative peak value. The position of the earliest AP occurrence (AP reference) is indicated by the white dot, and the background color shows AP delay with respect to this reference point.
(d) Distance to reference point vs. AP delay for all recorded waveforms (blue points) of the RGC. The red line shows a linear fit with a slope of 1.8, corresponding to a velocity of 0.55 m/s.
(e) left: cross-correlation coefficient (CC) matrix for waveforms on 12 electrodes with highest amplitude (red dots in a and c), arranged according to AP delay in ascending order. The increasing delay (shown at the top) causes a decrease in the CC, with values
as low as 0.61. Two main groups with high intragroup but low intergroup CCs appear, particularly electrodes 1–7 and 8–12. Right: CC matrix for the same 12 waveforms, however, with the phase shift being corrected by alignment according to the occurrence of the negative peak value. The resulting CCs range between 0.89 and 1. Without significant phase shift, such as for electrodes 5 and 6 or for electrodes 10–12, the resulting CCs are very close to 1.

This example shows that somatic AP signals, recorded at high spatiotemporal resolution, contain significant propagation delays and thus cannot be regarded as an instantaneous mixture. However, even if there was no propagation delay, we could not assume a perfect linear mixture for a second reason. The complex physiological structure of the neurons has effects on the spike waveforms. To analyze this, the waveforms were temporally aligned according to the occurrence of their negative peak value, and the resulting CC matrix was determined (Fig. 3e, right). For this case, the interelectrode CC values have a mean of 0.97 and a smallest value of 0.89, compared with a mean of 0.89 and smallest CC value of 0.61 in the nonaligned case. These characteristics of limited linear dependence between the waveforms of a neuronal unit on different recording electrodes, mainly caused by the phase shift due to the AP propagation delay, imply that the linearity assumption of ICA is not fulfilled.

4.4.2 Nonlinearity Effects on Linear Demixing and ICA

Next, we investigated how nonlinearity in the neuronal templates affects the possibilities of separating neuronal signals by means of linear demixing and ICA. For this, we used data set A, which contained simulated activity of three spatially overlapping neuronal templates (Fig. 4, a and b) extracted from a retinal recording.

In a first trial, the template of every neuron was manipulated to achieve linearity across the electrodes. This was realized by replacing the template waveform in each channel by scaled versions of the waveform observed on the electrode with maximum signal so that the peak-to-peak amplitude value at each electrode was preserved. The DCVs, directly derived from the manipulated templates (see Eqs. 10–11 in Appendix) led to perfect separation in the demixed templates (Fig. 4c, top), which means that there is only one high peak signal in each
component (row). This shows that neuronal templates can be completely separated by means of linear demixing, given the assumption of perfect linear mixtures under the boundary condition that there are more electrodes than neurons.

The DCVs were extracted in the same way from the realistic templates, and the demixed templates were computed (Fig. 4c, bottom). The signals along the diagonal of both plots have similar magnitudes, suggesting that the accumulation of the template energies in the components leads to a similar SNR increase for the linearized and the real case. However, compared with the linearized ideal case, there is significant cross talk. As a result, e.g., neuron 3, having the smallest spike amplitudes and being therefore the most challenging to demix, fails to be separated.

In a next step, ICA was applied to the simulated data set A, which contained spikes from these three neurons. The demixed templates and corresponding DCVs maps (Fig. 4d) show that some of the responses (i.e., for ICs 1, 3, 5, and 6) feature a reversed sign, which is due to the fact that ICA cannot derive the correct sign of the source signals. Besides this, ICA leads to comparable SNR and separability on the first three ICs as the demixing using directly derived DCVs from the templates.

If the linearity criterion of ICA would be met, we would not only achieve perfect separation, but also redundancy reduction so that the number of ICs with significant signals would be equal to the number of neurons. This would imply that we find only one high peak signal per column of the demixed templates. Since there are still signals in the demixed templates for ICs 4–6, ICA does not completely reveal the right number of sources here.

We observe that ICs 2 and 4 have significant DCV weightings in the area of neuron 3 (black arrows in Fig. 4d, right) while having nearly orthogonal MCVs (CC = 0.11). However, the positive weightings (red) in the DCV of IC 4 are centered around a subset of three electrodes, whereas one electrode has large negative weight (blue). The CC values of spike waveforms of the three electrodes with positive weights are >0.995, however, significantly lower for combinations with the waveform of the electrode with negative weight (CC values 0.79, 0.81,
and 0.83). The effect of splitting the source between different ICs was also consistently found on simulated data sets containing spikes from only single neurons (data not shown). This suggests that ICA splits the source due to the phase shift between the waveforms across the electrodes described in the previous section. As a consequence, the similarity of MCVs, as used in (Takahashi and Sakurai, 2005), might be a poor indicator for determining if the sources underlying two ICs are coming from a single or two separate neurons.

Interestingly, there are cases where a neuron does not exhibit the best separation performance on the first IC on which its signal is visible. Whereas neuron 3 has a strong signal but no separability on IC 2, better separability is achieved on IC 4 ($SEP_{IC2}^3 = 0$ vs. $SEP_{IC4}^3 = 4.8$). At the same time, the DCV for IC 4 has large weights on a smaller area (Fig. 4d, right), and therefore it features less template energy and a smaller SNR increase compared with IC 2 ($SNR_{IC2}^3 = 9.1$ vs. $SNR_{IC4}^3 = 5.6$).

**FIGURE 4**
Figure 4. Nonlinearity Effects on Linear Demixing and ICA.
(a) STA templates of 3 spatially overlapping neurons identified and extracted from retinal recordings. Scale bar: 100 µV/1.8 ms.
(b) Individual templates and respective demixing coefficient vectors (DCVs), directly derived from the templates (see appendix).
(c) Demixed templates using the DCVs, which were directly derived from the templates. In this representation, the rows refer to the demixing components, and the columns refer to the neuron templates. Top: the manipulated, idealized case where linearity across each template is given. Bottom: the realistic case where linearity is not given. The DCVs used here are shown in b.
(d) Left: template responses for the 1st 6 independent components (ICs) obtained by applying IC analysis (ICA) on the simulated data set. Right: corresponding DCV maps. The arrows indicate an example where significant weightings for demixing 1 source (neuron 3) are found on 2 ICs (ICs 2 and 4).

4.4.3 Advantages of ICA for Resolving Overlapping Spikes
We have shown in the previous section that the violated linearity criterion complicates clean separation of the neuronal sources using ICA-based linear demixing. However, in the following, we show that ICA has substantial advantages compared with traditional PCA methods for dealing with overlapping spikes in the case of sufficient separability on the ICs.

Figure 5a shows individual spike traces and average waveforms of the neurons in Fig. 4 on nine selected electrodes. The high firing rates of the simulated neurons caused many overlapping spikes, which evidently challenge alignment and classification of the spikes. To demonstrate this challenge, multichannel PCA (i.e., PCA performed on the concatenated single-channel waveform traces; see Litke et al. 2004) was applied to the spike traces of the three neurons on the selected electrodes. Figure 5b shows the resulting PCA scores of the first two principal components for each individual neuron; the black dots indicate spikes of the neuron of interest, and the gray dots mark spikes of other neurons. Violet dots indicate spikes that overlapped with another neuron within a time frame of 10 samples (0.5 ms). The nonoverlapping events are located in different, defined regions of the PCA space (ellipses) and can thus be separated using the representation of the PCA scores. However, the overlapping events are distributed all over the space and cannot be correctly identified using standard clustering methods.
In the following, ICs 1, 3, and 4 (from Fig. 4d) were considered for separating neurons 1–3 (Fig. 4, a and b). The spike traces projected on the corresponding ICs (traces along the diagonal in Fig. 5c) exhibit clean waveforms, which indicate that the overlaps shown in Fig. 5a have been resolved. Therefore, the ICA separation for this example is sufficient to enable proper spike assignment based only on threshold detection on the ICs.

The capability to resolve overlaps is also demonstrated by applying PCA to the IC spike traces of the three neurons for each IC independently. The representation of the scores (Fig. 5d) shows clear separation of the spikes of the respective neuron of interest (black dots) from the spikes of other neurons (gray dots). The overlapping spikes (violet dots) also lie within the cluster, which enables a correct assignment.

An important advantage of ICA usage for dealing with overlapping spikes appears here. Since the ICs can be treated independently, event detection is applied to the individual ICs. Thus two neurons that fire simultaneously evoke signals on two ICs and can also be detected as two independent spike sources.

It is important to mention in this context that, as shown in the previous section, ICA yields different separabilities on different ICs. However, it is not known which of the IC achieves the best separability for every neuron. Therefore, the best IC cannot be selected blindly.

4.4.4 ICA Performance Evaluation

To use ICA, followed by simple threshold detection for spike sorting, the neuronal sources need to be well-separated, and each neuron needs to be prominent and detectable on exactly one IC. To evaluate the applicability of such an ICA-based spike-sorting method, we analyzed the performance of ICA with regard to achieving three goals: 1) SNR increase; 2) separation of the neuronal sources; and 3) redundancy reduction. For this purpose, we used the basic evaluation metrics, which were defined in Evaluation Criteria.
FIGURE 5

Figure 5. Using ICA to Resolve Overlaps.
(a) Template waveforms (colored) and individual spike traces (gray, only 80 traces are shown per neuron for better visualization) on 9 selected electrodes for the neurons shown in Fig. 4. Many overlapping spikes are visible in the gray traces.
(b) Principal component analysis (PCA) applied to the multichannel spike traces on the 9 selected electrodes in A. Black dots indicate spikes from the neuron of interest, and gray dots denote spikes from other neurons. Spikes, which temporally overlapped with a spike from another neuron (timing difference of 10 samples or less), are colored violet (the respective overlapping spikes from the other neurons are not shown). Note that overlaps mostly lie outside of the main cluster.
(c) Spike traces on ICs 1, 3, and 4 after applying ICA. The clean waveforms along the diagonal indicate that separability is given and that overlaps have been resolved.
(d) PCA applied individually to the IC spike traces on the 3 selected ICs.

ICA was applied to simulated RGC activity at different cell densities (data set B; see METHODS). For calculation of the evaluation criteria, the noise signals and the templates that were used in the simulations have been considered.

First, the SNR of the neurons on the raw data (SNR_{EL}) was compared with that on the ICs (SNR_{IC}; Fig. 6a), whereat an overall increase in the SNR_{IC} was observed. The SNR ratio r_{SNR} = SNR_{IC}/SNR_{EL} was found to be particularly large for neurons with high SNR_{EL}, which suggests that
ICA is more effective in demixing signals of high-SNR neurons. However, the SNR increase drops for configurations with higher cell densities. Whereas for $D = 40 \ \mu m$, 82% of the neurons have $r_{SNR} > 1$, for $D = 20 \ \mu m$ this is only the case for 44% of the neurons (Fig. 6b).

Next, we addressed the question of how well the neuronal sources are separated in the representation of ICs. Separability $SEP_{IC}$ denotes the difference between the peak of a neuron on an IC and the next highest peak of another neuron. If a neuron has a large enough $SEP_{IC}$, the sorted spikes can be obtained by simply applying threshold detection to the respective IC. We define the condition for a neuron to be separable if it has an $SEP_{IC}$ above a threshold of 5. Note that the $SEP_{IC}$ is given in units of standard deviations of the noise in the IC, like the $SNR_{IC}$, and, therefore, every neuron with an $SEP_{IC} > 5$ also has an $SNR_{IC} > 5$.

Figure 6c shows the separability and SNR values of the neurons for different configurations; the color code indicates the template energy. The dashed lines confine the thresholds with regard to detectability, and therefore all neurons positioned in the upper right area of the plot are detectable and separable according to the defined criteria. For largely spaced neurons, separability increases approximately linearly with the SNR. The overall separability drops for larger neuron densities, and several large-SNR neurons feature low separability for the most tightly spaced configuration $D = 20 \ \mu m$. ICA yields an increase in separability compared with the raw data (Fig. 6d). However, a substantial decrease in separability is observed for tightly spaced neuron configurations, reflected by the low percentage of neurons featuring $SEP_{IC} > 5$ or $SEP_{EL} > 5$.

The third performance criterion is the reduction of redundancy in the IC space. The redundancies in the IC and electrode space were computed for a total of 1,460 simulated neurons in all configurations. Figure 6e shows the histogram counts for $RED_{EL}$ and $RED_{IC}$. A majority of the neurons yields $RED_{IC}$ values between 1 and 2, and thus ICA performs well in reducing the dimensionality of the data.

Note that the zero value in the y-axis is not shown, and thus neurons with $RED_{IC} = 0$ were excluded in this graphic. Whereas low $RED_{IC}$ values are desired, as they mean that the individual neurons are
not detected many times, RED$_{IC} = 0$ means that the neuron cannot be detected at any of the ICs.

Finally, the effect of an increased SNR (Fig. 6, a and b) on the detection of the neurons was evaluated. We found that for the configurations with $D \geq 30 \, \mu m$, more neurons are detectable on the ICs (Fig. 6f) due to the increase in SNR obtained by using ICA. One has to note, however, that the SNR increase, as shown in Fig. 6a, is relatively small for low-SNR neurons. Therefore, ICA only slightly increases the percentage of detectable neurons. For configurations with $D < 30 \, \mu m$, the percentage of detectable neurons based on the ICs drops below the percentage of detectable neurons based on the raw signals, which is approximately constant across all configurations. We conclude that ICA fails to improve signal quality for very dense neuron populations but is beneficial for lower density populations.

The presented analyses revealed that ICA, applied to HD-MEA recordings, yields only limited separation performance and is, therefore, not suited to be used as a stand-alone spike-sorting tool in combination with threshold detection.

### 4.4.5 ICA Applied to Recorded Data

An example of ICA, applied to block recordings with spontaneous RGC activity is illustrated in Fig. 7. The DCVs, depicted in Fig. 7a, yield spatially localized high weightings. Note that the input to ICA is the multielectrode signal without any information on the electrode positions and that the neuronal templates are localized in space. Therefore, spatially localized high weighting values in the DCVs are a good indicator that the signals, underlying the ICs, originate from neuronal units.

In looking at the spike waveforms, some ICs (e.g., ICs 1 and 7 in Fig. 7b) feature high separability and, practically, represent single-unit spike trains of neurons. However, the problems of limited separability and dimensionality reduction that were discussed in the previous sections are also visible in the IC signals. Several ICs (e.g., ICs 3–6 and 8) presumably contain spikes from multiple neurons as indicated by spike waveforms of different amplitudes on the IC. On the other hand, in
some cases, spikes from a single neuron were observed on multiple components (red arrows in Fig. 7b).

**FIGURE 6**

<table>
<thead>
<tr>
<th>a</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

*Figure 6. ICA Performance Evaluation.*
(a) Signal-to-noise-ratio (SNR) conditions for electrodes (SNR<sub>EL</sub>) vs. ICs (SNR<sub>IC</sub>) for simulated neurons (red crosses) of 3 different configurations. The dashed line shows SNR<sub>EL</sub> = SNR<sub>IC</sub>.
(b) Percentage of neurons, for which SNR<sub>IC</sub> > SNR<sub>EL</sub> for the 5 configurations.
(c) SNR vs. separability for neurons of 3 different configurations. The dashed lines indicate threshold values for separability of neurons (SEP<sub>IC</sub> = 5 and SNR<sub>IC</sub> = 5). The color code indicates the template energies.
(d) Percentage of neurons with SEP<sub>IC</sub> > 5 (blue) and SEP<sub>EL</sub> > 5 (red).
(e) Histogram showing the counts of redundancies RED<sub>EL</sub> and RED<sub>IC</sub> for a total of 1,130 neurons (330 neurons having RED<sub>IC</sub> = 0 were excluded from the graphic representation). The dashed line indicates RED<sub>EL</sub> = RED<sub>IC</sub>.
(f) Percentage of neurons that can be detected according to the criteria SNR<sub>IC</sub> > 5 (blue) and SNR<sub>EL</sub> > 5 (red) for the 5 configurations.

4.4.6 Approaches for ICA-Based Spike Sorting
Since most ICs do not represent single-unit spike trains (Fig. 7b), the spikes cannot be sorted by just applying event detection to the ICs. The reduced redundancy in the data as well as the increased SNR and...
separability are, however, still valuable features of the IC representation. In this section, we propose to use postprocessing techniques to overcome the most severe limitations arising from the nonlinearity of the templates.

For the case that an IC contains spikes of multiple units, the spikes can be separated by means of PCA-based clustering of the IC spike waveforms. This is exemplarily shown for IC 4 in Fig. 7, c-e. The green cluster, which exhibits large spike signals in this IC, corresponds most likely to the neuron that can be associated with this component. The smaller spikes, grouped into the red cluster, can be discarded and may be detected on other ICs. Note that the peak-to-peak amplitudes in the red cluster (Fig. 7e) show large variability, suggesting that these spikes arise from multiple neurons.

The problem of multiple detection of neurons can be addressed by using an aggregation method, which, e.g., compares the identified spike times of the questionable neurons and merges them if they have a specific number of spike times in common (Litke et al. 2004). Additionally, waveform similarity and ISI statistics can be included as aggregation criteria (Fee et al., 1996b).

Furthermore, ICA-based spike sorting can be performed in an iterative procedure: ICA is applied to the data, and spikes are identified by applying threshold detection to the ICs. Following clustering and merging, the STA waveforms of identified neurons are subtracted from the raw data. In a next iteration, ICA is applied to the residual signals. Similar, subtractive methods were proposed for spike sorting using optimal filters (Gozani and Miller, 1994) and template matching (Vargas-Irwin and Donoghue, 2007).

This iterative scheme, which adds a nonlinear feature to the linear ICA approach, is motivated by two reasons. On the one hand, neurons featuring large signal amplitudes render the separation of neighboring, spatially overlapping neurons with smaller amplitudes difficult. Therefore, the identification and subtraction of dominant neuronal sources allows for identifying less dominant signals in the subsequent ICA iteration. On the other hand, ICA facilitates the detection and classification of a spike A even though it temporally overlaps with another spike B. The proper subtraction of spike A will improve
detection and classification of spike $B$ in the proximate iteration in case that it cannot be identified on another IC.

**FIGURE 7**

(a) Spatial map of DCVs for the 1st 8 components. The coefficients are normalized with regard to the resulting IC signal having a standard deviation of 1; therefore, no absolute values are shown here. The white line in the corresponding color bars represents the 0 value.

(b) Corresponding IC signals (left) and close-up (right, data segment is indicated by a gray rectangle on left plot). Except for the large spikes in ICs 4 and 6, which originate from the same neuron (red arrows), the spikes on different components belong to different neurons, as they are not correlated in their timing.

(c) Threshold detection and clustering for IC 4. Top: dashed line representing the threshold level; detected events are colored. Bottom left: superimposed IC traces for all detected events above threshold within 20 s. Bottom right: 1st and 2nd PCA scores for detected events, which can be clustered (colors).

(d) Spatial spike distributions for both clusters over selected electrodes marked by the white rectangle in a, showing the average spike shape (colored) and the individual traces (gray). Scale bar: 100 µV/1 ms.

(e) Peak-to-peak amplitude values of the 2 clusters on pairs of electrodes, EL1–EL2 and EL1–EL3; the electrodes are indicated in d.
An algorithm based on the described approaches was implemented. ICA was applied to the band-pass filtered recordings, decomposing the data into ICs. AP events, identified by applying threshold detection to the IC signals, were clustered (KlustaKwik), based on the principal components of the IC spike traces. Clusters with high standard deviation on the multichannel spike traces were believed to be erroneous and discarded. After an intermediate merging step, during which clusters of multiple-detected neurons were aggregated, the STA spikes were subtracted from the raw data. In the next iteration, ICA was applied to the residual signal. This iterative scheme was repeated for a defined number of iterations. A detailed description of the individual algorithm steps is given in ICA-Based Spike-Sorting Algorithm in Appendix.

Figure 8 shows the templates (black) of 18 sorted cells from recorded RGC activity on an HD block. The DCV weightings (background colors) largely overlap with the active electrodes of the neuron templates. The individual traces are depicted in gray. Concurrent high-amplitude spiking activity of neighboring neurons results in visible gray traces near the neuron template, which can be observed for several neurons (e.g., for templates 1, 2, 4, 5, and 6). These traces, which lie mostly outside the DCV active area, indicate that ICA allowed to classify correctly the spikes despite overlaps.

Finally, an unsupervised version of the spike-sorting algorithm was applied to the simulated data set B, and the sorting output was matched with the simulated data. A sorted neuron was assigned to a simulated neuron, if the number of matching spikes exceeded 10% of the total number of spikes of the simulated neuron. We classified the simulated neurons as identified (if detected as 1 neuron), identified multiple (if detected as 2 or more separate neurons), falsely merged (if detected but merged with 1 or more simulated neurons), or as not found (if not detected at all). The classification percentages of the simulated neurons are depicted in Fig. 9a. As for ICA alone, the sorting performance was found to depend on the cell density. Additionally, the neuronal signal amplitude played a dominant role. As shown in Fig.
9c for the different configurations, most detected neurons featured a certain amplitude range, whereas neurons with lower amplitudes passed constantly undetected. This correlates to the observation that the separability of a neuron on the IC strongly depends on its SNR. To quantify the sorting quality, each sorted neuron was assigned to a simulated neuron (based on the number of matching spikes), and the numbers of true-positive (TP), false-positive (FP), and false-negative (FN) events were computed. We used the performance measures “sensitivity” \([\text{TP}/(\text{TP}+\text{FN})]\) and “precision” \([\text{TP}/(\text{TP}+\text{FP})]\); a sensitivity value of 1 means that all spikes were detected (no FNs), and a precision value of 1 signifies that only correct spikes were detected (no FPs). Figure 9b shows box plots of the performance results. The blue boxes indicate the interquartile ranges (IQR), and the black whiskers the highest and lowest data values that are within 1.5 times the IQR. Therefore, the lower performance bounds are given by the lower border of the blue boxes (for 75% of the neurons) and by the lower whiskers (for 87.5% of the neurons). Although there were numerous neurons with poor performance (outliers: red circles), the majority of the sorted neurons (indicated by the medians: red lines) yielded sensitivity and precision values >0.95 throughout all configurations. The performance was again found to depend on the cell density. For configurations \(D \geq 35 \mu m\), 87.5% of the neurons yield in performance values >0.93.

The iterative approach allowed to increase the number of detected neurons (i.e., substantial increases for iterations 2 and 3). This is evident from the number of detected neurons after each ICA iteration, shown in Fig. 9d.

We also compared the detection performance for overlapping and nonoverlapping spikes (Table 2). This analysis revealed that the ICA approach performs well in classifying spikes despite the fact that they are temporally overlapping with spikes from nearby neurons. The error probability associated with overlaps (right column in Table 2) was determined using Eq. 16 in Appendix. These error probabilities varied between 1.4 and 6% for the different configurations and are on the same order as the probabilities for nonoverlap errors. Accordingly, even for the highest-density case, only 1 out of 16 overlapping spikes was
missed due to spatiotemporal interference with a spike from another neuron.

**FIGURE 8**

![Image of STA templates and individual traces](image)

*Figure 8.* STA templates (black) and individual traces (gray) of neurons as identified using the iterative ICA-based approach. The DCVs are indicated through the color code in the background. Scale bars: 100 µV/1 ms. For visualization purposes, the templates were individually scaled, which results in variable-size scale bars.

**Table 2:** Classification Performance (false-negative events) of Nonoverlapping vs. Overlapping Spikes.

For this particular analysis, all sorted neurons with sensitivities >0.6 were considered. Overlaps were defined as spikes featuring a time difference of 10 samples or less to spikes of other neurons that were closer than 50 µm. The observed error probabilities ($p_O$) for nonoverlapping spikes ($p_E$) and for overlapping spikes ($p_{OE}$) were used to compute the probability associated with overlapping spikes $p_O$ shown in the last column, using Eq. 16 in Appendix. $D$, distance.
Chapter 4

FIGURE 9

(a) Percentage of simulated neurons classified as identified, identified multiple, falsely merged, and not identified for the different configurations.
(b) Box plots of the sensitivity and precision values for all sorted neurons, showing the median values (red horizontal line), the interquartile ranges (IQR; blue boxes), highest and lowest data values that are within 1.5 times the IQR (black whiskers), and outliers outside 1.5 times the IQR (red circles). (c) Histogram counts of neuron classification vs. neuronal-signal peak-to-peak amplitude for 3 configurations.
(d) Cumulative sum of detected neurons vs. ICA iterations.

Figure 9. Evaluation of Spike Sorting as Applied to Simulated Data Sets.

4.5 Discussion

Recently, ICA has received increasing attention as a tool to analyze biomedical signals, such as EEG or functional MRI, as well as for spike sorting of optical brain recordings (Stone, 2002; Reidl et al., 2007; Mukamel et al., 2009; Hill et al., 2011). Being an automatic tool for source separation of redundant data sets, ICA represents a promising candidate to facilitate rapid spike sorting of HD-MEA data.

In this paper, the suitability of ICA for demixing HD-MEA recordings was evaluated for the first time. We analyzed neuronal activity, recorded at high spatiotemporal resolution, and found that the fundamental requirement for ICA, a linear mixture of the source signals, is not fully satisfied by the characteristics of the data. Instead, the
linearity between the waveforms of a neuron on different electrodes is decreased, which is mainly due to AP propagation delays.\textsuperscript{1} The compromised linearity was found to be a limiting factor already in sparse neuron arrangements, which impeded perfect source separation. As a consequence, ICA, followed by threshold detection, cannot be used as a stand-alone method for spike sorting of HD-MEA data. These findings presumably also hold for other devices and planar microelectrode systems (Csicsvari et al., 2003; Takahashi and Sakurai, 2005; Du et al., 2009). The limitations of the applicability of ICA as a consequence of the nonlinear characteristic of the neuronal signals, as shown for tetrode recordings in (Shiraishi et al., 2009), cannot be completely compensated by spatial oversampling using high electrode density.

The analysis of ICA applicability using simulated data sets of RGC activity as presented here revealed that the ICA performance strongly depends on the neuronal density in the preparation. Particularly, when the neuronal density approached the electrode density (3,161 electrodes/mm\textsuperscript{2}) and the number of simulated neurons (111) exceeded the number of electrodes (90), the separation performance clearly decreased. Neurons that produced high-SNR signals entailed superior separation performance. Except for very dense neuronal populations, ICA led to a significant overall SNR increase, which allowed for detecting more neurons. Moreover, the redundancy was clearly reduced in the ICs, which helps to overcome the problem of detecting the same APs multiple times on several electrode signals. For a limited number of neuronal sources, ICA automatically provided separation, which could serve to resolve efficiently overlapping spikes from these sources.

We showed that limitations arising from the nonlinearity of the sources could be addressed by combining the ICA output with postprocessing techniques. In particular, we proposed an algorithm, based on applying PCA and clustering, to the detected IC traces. For densities up to 1,300 neurons/mm\textsuperscript{2}, >80% of the neurons were detected (>70% correctly identified as single neurons) using the unsupervised, ICA-based algorithm. The detection of the majority of the neurons for these densities was highly accurate (87.5% of the neurons had sensitivity and
precision values above 0.86 and 0.91). Additionally, the algorithm performed well in resolving overlapping spikes. The percentage of misclassified overlaps (FNs), compared with nonoverlaps, was increased by factors of up to 2, and the particular error probability associated with the overlaps was between 1.4 and 6.3%.

The method of combining ICA with the proposed postprocessing techniques was not efficient for sorting the complete neuronal population but yielded good results in sorting large fractions of the cells with high accuracy. By iteratively subtracting identified spike waveforms from the data and applying ICA, the number of detected neurons could be increased by 15–68% for the different simulated neuron densities.

In conclusion, our results suggest that ICA applied to HD-MEA data does not yield complete separation of the neuronal signals. However, the IC-representation of the data has some valuable features, e.g., the reduced redundancy, which entails that spikes from a neuron are only prominent on one or a few ICs. This allows for treating the individual ICs as separate signals, which facilitates spike detection in redundant MEA data. In addition, the increased separability contributes to resolving overlaps. These features make ICA a valuable tool to serve as a preprocessing step to spike sorting.

**Acknowledgments**

We thank Jan Müller, Ian Jones, Ralph Streichan, Branca Roscic, Douglas Bakkum, and Alexander Stettler for help with the measurements and the experimental setup. U. Wahlen is acknowledged for contributions to data analysis. Three anonymous reviewers are expressly thanked for providing very helpful comments on an earlier version of this paper.

**References**


Appendix

- Estimation of the Demixing Matrix Based on the Templates

The linearity assumption implies that the template waveforms can be viewed as scaled versions of an intrinsic neuronal signal $s^j$ with coefficients:

$$F^i = [f^i_1, ..., f^i_M]^T$$
$$= [a^i_1 \cdot s^i, ..., a^i_M \cdot s^i]^T$$

(10)

The mixing coefficients $a^i_1, ..., a^i_M$ describe how $s^j$ is mixed across the electrodes. If the neuron templates are known, the demixing coefficients in $A$ can be directly derived by:

(11)
\[ a_{ji} = \arg\min_a \| f_j^i - a_{ji} \cdot s_j^i \| ; \quad j = 1, \ldots, M, i = 1, \ldots, N \]

The average of the template waveforms is used for the intrinsic signal:

\[
\mathbf{s}_i = \frac{1}{M} \sum_{j=1}^{M} f_j^i
\]

- **ICA-Based Spike-Sorting Algorithm**

This section describes the individual steps of the spike-sorting algorithm and the parameters that were used for spike sorting of the simulated data sets. 1) All data are band-pass filtered between 500 and 3,000 Hz. 2) FastICA is applied to the full-length data, and the number of estimated ICs equals the electrode number. The following steps 3–5 are successively performed for every IC signal. 3) Spikes are detected on the IC by threshold detection. The noise level is estimated based on the median (Donoho and Johnstone, 1994)

\[
\sigma_n = \text{median} \left\{ \frac{|x|}{0.6745} \right\}
\]

which has been shown to be robust for variable firing rates (Quiroga et al., 2004). A threshold level of \(5 \cdot \sigma_n\) is used. 4) PCA is applied to the aligned IC spike waveforms. The scores from the first three resulting principal components are clustered using KlustaKwik, which automatically estimates the number of clusters. The cluster with the largest average IC spike signal is selected for further processing, and other clusters are discarded. 5) As spike traces from a well-isolated cluster are expected to have low variation, the standard deviation of the traces is used as a measure of cluster quality. The traces on the three electrodes with highest spike signals are normalized by the peak-to-peak amplitude of the cluster template, and the standard deviation of
the normalized traces is computed (relative standard deviation, RSTD). The RSTD has been experienced to be a robust quality measure, as it compensates for the effect that the degree of spike trace variation also depends on spike amplitude. The resulting cluster from step 4 is discarded if the RSTD exceeds a threshold of 0.12. 6) After repeatedly conducting steps 3–5 for all ICs, pairwise comparisons between the obtained clusters are performed, and two clusters, A and B, are merged if they appear to belong to the same neuronal unit according to the following criteria: i) if the number of spikes shared by both clusters exceeds 30% of the number of spikes in cluster A or B; and ii) the similarity between the aligned average waveforms of clusters A and B is measured by means of their normalized Euclidian distance:

\[
D = \sqrt{\frac{1}{M} \cdot \frac{1}{L} \cdot \sum_{j} \sum_{\tau} (f_{j\tau}^{A} - f_{j\tau}^{B})^2}
\]

where \( f_{j\tau}^{A} \) is the \( \tau \)th sample of the cluster template for cluster A at the \( j \)th electrode, \( M \) is the number of electrodes considered, and \( L \) is the waveform length. For this measure, only electrodes on which the clusters had significant energy were considered. The threshold value for merging was set empirically (merge if \( ED < 4.3 \)). The merging is organized in the following way. In a first step, the clusters are compared for the criterion of common spike times and accordingly merged. In a second step, the distances for all cluster pairs are calculated, and the cluster pair with the smallest distance is merged if the condition is fulfilled. After merging, the cluster-pair distances are recalculated, and the merging condition is checked again for the pair with smallest distance. 7) The STA waveforms of the identified neuronal clusters are subtracted from the raw data, and steps 2–6 are subsequently applied to the residual data. This iterative scheme is repeated for a total of five iterations. After each iteration, newly identified clusters are aggregated with previously obtained clusters using the merging method described in step 6.
- Computing the Overlap-Specific Error Probability

From the spike-sorting results, the observed probabilities of an FN error for nonoverlapping \( (p_E) \) and overlapping \( (p_{OE}) \) spikes can be extracted. The observed probability of missing an overlapping spike can also be formulated as:

\[
p_{OE} = p_E + (1 - p_E) \cdot p_o
\]

\( p_o \) is the specific error probability for overlapping spikes, i.e., the probability to miss a spike participating in an overlap, although it would have been detected if the other spike was not there:

\[
p_o = \frac{p_{OE} - p_E}{(1 - p_E)}
\]

**Supplementary References**


153
Chapter 5

Conclusions and Outlook
5.1 Conclusions: The Importance of Microelectronics-Based MEAs for Accessing Sub-Cellular Neural Features and Defined Neural Population Activity

Microelectronics-based MEAs allow for recording of extracellular neural activity at sub-cellular resolution. The ability to record neural action potentials by multiple electrodes, at closely neighbored spatial locations, is highly relevant in neuroscience studies for the following reasons:

1) Axonal signals are difficult to access by traditional intracellular techniques, i.e., patch-clamp, due to the small axon size (< 1 um in diameter). Sub-cellular axonal activity can, however, be recorded extracellularly by high-density electrode arrays (Zeck et al., 2011; Bakkum et al., 2013). Investigating axonal signal dynamics is highly relevant for understanding (I) how neural populations exchange information and (II) if physiological changes along axons are involved in signal processing and learning mechanisms (Alle and Geiger, 2006; Grubb and Burrone, 2010).

2) Recording from neurons with a defined genetic profile (Huberman et al., 2009; Fiscella et al., 2012). The possibility to record synchronous activity from defined neural populations by MEA technology, is highly relevant, because genetically identified cells are routinely used in neuroscience research (Siegert et al., 2009; Huang and Zeng, 2013). In addition, precise and efficient tools for in vivo gene manipulation in adult animals are already available (Ran et al., 2013; Wang et al., 2013).

3) Recording of concerted activity of defined neural populations for studying sensory information encoding/decoding (Meister et al., 1995; Pillow et al., 2008; Tanabe, 2013). For understanding encoding/decoding mechanisms in neural circuits, it is crucial to know wiring between neurons. Recently, viral tracing of neural circuitries and electron microscopy reconstruction, together with knowledge of genetically identified cell types, is increasing our understanding on how
multiple neurons wire together in order to perform computations (Wickersham et al., 2007; Kay et al., 2011; Helmstaedter et al., 2013; Maisak et al., 2013; Yonehara et al., 2013). Consequently, it is important to precisely select and record from defined neurons for understanding how sensory information is processed. Finally, recording simultaneously from multiple neurons gives the opportunity to measure noise correlations among neurons (Averbeck et al., 2006). Since it is currently controversial, whether noise correlations are relevant for decoding sensory information, targeted recordings from defined neurons could shed light on this debated issue (Oram et al., 1998; Series et al., 2004; Graf et al., 2011; Eyherabide and Samengo, 2013).

4) Screening for evaluating efficacy of medically relevant treatments, e.g., visual restoration. Visual restoration in mouse models with impaired vision has been recently demonstrated (Lagali et al., 2008; Busskamp et al., 2010). In such studies, commercial MEAs with low electrodes density have been used to test light-induced activity in retinal ganglion cells. Although light-induced activity was successfully demonstrated in such restored retinas, it would be important to study if similar signal features - at the level of defined neuronal population - are present in animals with restored vision in comparison to wild type animals. For example, fine time correlation structures have been reported among retinal ganglion cells (Brivanlou et al., 1998; Berry et al., 1999; Schwartz et al., 2007) in wild type animals: the MEA system, used for retinal studies in this thesis, would be capable to investigate if such time correlations are present in defined ganglion cells of visually restored retinas.

Finally, genetically modified mice with unknown phenotype could be screened to quantify how much and if specific types of retinal ganglion cells are affected by gene mutations.

5) Precise Electrical Stimulation of Neurons. Electrical stimulation of retinal ganglion cell is an alternative strategy, to molecular methods for restoring vision (Sekirnjak et al., 2008). High-density electrode arrays with electrical stimulation capabilities offer the potential to selectively
stimulate a single retinal ganglion cell or a group of defined retinal ganglion cells. Since the retina splits the visual information in ~20 separated channel (Wassle, 2004), it is crucial that a retinal prosthesis is capable to stimulate precisely defined ganglion cell types without interfering with neighboring cells or neighboring axons from distant cells (Jepson et al., 2013).

5.2 Outlook: Limits of the HiDens MEA and Development of MEA1k

The device used in this thesis, named “HiDens MEA”, features a recording area of 2.0 x 1.75 mm², including 11,011 electrodes at a pitch of 18 µm. Electrode configuration, at highest possible density, covers an area approximately 0.1 x 0.3 mm². Therefore, for sampling an 1 x 1 mm² area of tissue with high-density electrode blocks, it is necessary to scan approximately 33 sequential electrode blocks (see Chapter 2, Fig. 6). The time needed for sequentially scanning electrode blocks is disadvantageous in the context of acute electrophysiological recordings as in the retina. For example, long scanning times are necessary to find defined ganglion cell types that respond to slow moving objects, i.e., ON direction-selective cells (Oyster et al., 1972; Sun et al., 2006). The same scanning process will be similarly slow for finding sub-cellular structures as axons in dissociated neural cultures. Therefore, a MEA with more than 126 simultaneously recordable channels would significantly speed up the time needed for tissue scanning. As a consequence, it would be easier to find neurons or sub-cellular features of interest. Importantly, a new MEA should feature comparably low noise levels and electrode densities close to that of the HiDens version in order to facilitate neuron spike sorting.

The HiDens MEA can be used to distinguish activity from overlapping neurons (see chapter 2, Fig. 5). Nevertheless, due to a sampling area size of 0.1 x 0.3 mm² (at highest possible electrode density), activity from neurons located at the edges of a recording electrode block will be only partially sampled. For data analysis, it is crucial: (1) to select the electrode with highest signal-to-noise ratio in order to properly distinguish action potentials from noise; (2) to choose 5 to 7 electrodes
to accurately separate mixed action potentials from different cells (see chapter 2, Fig. S1). A larger overall sampling area would reduce sampling time and sampling bias of neurons at the edges of the recording blocks.

In order to quickly and efficiently access neural networks information and overcome limitations of the HiDens MEA, we developed a new MEA system, named MEA1k, that features a recording area of 3.85 x 2.10 mm², including 26,400 electrodes at a pitch of 17.5 µm. Importantly, the new MEA1k system allows for recording simultaneously from 1024 electrodes (~ 8 times more than HiDens MEA) (Ballini et al., 2013). In particular, electrode blocks at maximum density cover an area 0.4 x 0.4 mm² (~ 5 times more than with the HiDens MEA) (Muller et al., 2013).

MEA1k allows for disconnecting recording electrodes during electrical stimulations, therefore stimulation signals will not saturate the recording electronics, and the stimulation artifact will be lower (Shadmani et al., 2014). Furthermore, MEA1k features a 10-bit analog-to-digital-conversion unit (ADC) in contrast to the 8-bit ADC in HiDens MEA. A 10-bit ADC unit allows for using lower amplification for recording small-amplitude electrical signals.

MEA1k, however, poses an important challenge for data analysis, in particular for spike sorting. In fact, with an ~8 fold increase of simultaneously recorded electrodes, human supervision for spike sorting will become very time consuming, if not impossible (Hill et al., 2011) (see Chapter 3, Fig. 1). For these reasons, automatized procedures for spike sorting based on template matching (Franke et al., 2010) are currently tested on data acquired by HiDens MEA.

In summary, technological advances will help to further push the limits of extracellular electrical recordings and open new avenues for retinal and neuronal investigations.
References
Chapter 5


CURRICULUM VITAE

MICHELE FISCELLA

Personal Information

Date of birth: 8th April 1983
Citizenship: Italian
Civil status: Married
Residence permit: Swiss C permit (EU/EFTA, 5 years)
Address: Isteinerstrasse 70, 4058 Basel, Switzerland
E-mail: michele.fiscella@bsse.ethz.ch
Mobile: +41 (0)786029267
Website: http://www.bsse.ethz.ch/bel/

Education

Sep 2008 – Jul 2014: SystemsX Interdisciplinary PhD student at ETH Zurich (Bio-Engineering Laboratory, Prof. A. Hierlemann) and Friedrich Miescher Institute (Neural Circuits Laboratory, Dr. B. Roska), Basel, Switzerland.
Thesis project: Combining CMOS-Based Microelectrode Arrays with Genetic Labeling to Study Visual Processing in the Retina.

Dec 2005 – Mar 2008: MSc in Molecular and Industrial Biotechnology at University of Bologna, Italy.
Thesis lab: Karolinska Institute, Dr. A. Simon, Stockholm, Sweden.
Overall mark: 110/110 cum laude.

Oct 2002 – Dec 2005: BSc in Biotechnology at University of Bologna, Italy.
Thesis project: Apoptosis and IAP, Nuclear Shuttling and Cellular Localization.
Thesis lab: VU University Medical Center, Prof. G. Giaccone, Amsterdam, The Netherlands.
Overall mark: 108/110.

Overall mark: 100/100.

Research Experience

Sep 2008 – Now:
Research Assistant (PhD), ETH Zurich and Friedrich Miescher Institute, Basel, Switzerland.
Activities: Design of a light-projection microscope for retina stimulation. Use of a microelectronics-based biosensor (HD-MEA) for recording activity from genetically-identified neurons. Electrophysiology experiments. Data analysis and modeling of neural network activity. Participation in international meetings with poster and oral presentations, supervision of students, animal handling, writing of grants and scientific manuscripts, publication of original scientific articles.
Methods: Electrophysiological recordings by microelectronics-based microelectrode arrays, spike sorting and modelling of multiple spike trains by MATLAB, tissue and primary cells isolation from mouse and rabbit, cell culture, immunofluorescence assay, confocal and fluorescent microscopy, in vivo injections of Adeno-associated viruses (AAVs).
Intern, Karolinska Institute, Department of Cell and Molecular Biology, Stockholm, Sweden.

Activities: Developmental biology research. Study of molecular features of regeneration processes in the amphibian *Notophthalmus viridescens* (animal model for tissue regeneration). Research on the de-differentiation process, which involves mature muscle cells during limb regeneration.

Methods: Cell culture, cloning, PCR, DNA and protein gel electrophoresis, microinjections of DNA vectors into cells, immunofluorescence assay, confocal and fluorescent microscopy.

Intern, VU University Medical Center, Department of Medical Oncology, Amsterdam, The Netherlands.

Activities: Cancer biology research. Analysis of protein sequences that are involved in the nuclear shuttling of cIAP1 and cIAP2, which have an anti-apoptotic role.

Methods: Cell culture, DNA cell transfection, cloning, PCR, DNA and protein gel electrophoresis, spectrophotometry, immunofluorescence assay, fluorescent microscopy.

Publications


Selected Conference Contributions

- Bernstein Conference, Tübingen, Germany, Poster Presentation (2013).
- 7th Federation of European Neuroscience Societies (FENS), Amsterdam, The Netherlands. Poster presentation (2010).

CV Fiscella
Additional Courses

- Clarity workshop at center for microscopy and image analysis (University of Zurich, Switzerland, 2014).
- Summer school “Advanced Course in Computational Neuroscience” (Będlewo, Poland, 2013).
- Scientific writing workshop (Friedrich Miescher Institute, Switzerland, 2013).
- Summer school “Advanced Course in Computational Neuroscience” (Będlewo, Poland, 2013).
- Summer school “Advanced Course in Computational Neuroscience” (Będlewo, Poland, 2013).
- Career planning and self-marketing skills (Life Science Zurich Graduate School, Switzerland, 2012).
- Zurich life science week (Life Science Zurich Graduate School, Switzerland, 2012).
- Introductory course in laboratory animal science (FELASA B, Institut für Labortierkunde, Zurich, 2009).
- Neuroninformatics (ETH Zurich, Institute of Neuroinformatics, Switzerland, 2009).
- Introductory course in neuroscience (ZNZ Neuroscience Center Zurich, Switzerland, 2009).

Language Skills

- Italian: mother tongue.
- English: fluent, proficient level.
- German: basic level.
- Spanish: basic level.

IT Skills

- Programming, statistical analysis and modelling of biological data: Matlab (advanced), Python (basic).
- Histological image analysis: Imaris, ImageJ.
- Adobe softwares: Illustrator, Photoshop.
- Documentation databases: EndNote.
- Office: Word, Excel, Powepoint.
- OS: Window, Mac, Unix.

Extracurricular Activities and Hobbies

Football, gym, hiking, travelling, reading, foreign food and wine tasting, going to concerts.

References

Prof. Andreas Hierlemann, ETH Zurich, Mattenstrasse 26, 4058 Basel, Switzerland.
Phone: +41 61 387 3150
E-mail: andreas.hierlemann@bsse.ethz.ch

Dr. Botond Roska, Friedrich Miescher Institute, Maulbeerstrasse 66, 4058 Basel, Switzerland.
Phone: +41 61 697 8575
E-mail: botond.roska@fmi.ch