Thesis for the degree
Doctor of Philosophy

By
Ilan Breskin

Connectivity in living neural networks

Regular Format

Prof. Elisha Moses
Supervisor

October 2006

Submitted to the Scientific Council of the Weizmann Institute of Science
Rehovot, Israel
Acknowledgments

I wish to express my deep gratitude to all of those who advised, supported and helped me to carry out this work.

First of all, I would like to thank my supervisor Prof. Elisha Moses for his guidance. The genuine enthusiasm he has for science, his creative and original ideas directed me toward Neurophysics and inspired me to initiate my thesis project. His support and kind advice throughout this work have been of great help.

I would especially like to thank my collaborators Dr. Jordi Soriano and Dr. Tsvi Tlusty for their efforts, assistance and contributions to the work presented here. Their support and cooperation have been much appreciated.

My sincere appreciation goes to my doctoral supervision committee members Prof. Yaron Zilberberg and Prof. Misha Tsodyks for their invaluable council and encouragement.

I would like to thank Prof. Jean-Pierre Eijkman and Leon Gruendlinger for helpful discussions and for their good advice.

I would like to thank all the past and present lab members: David Biron, Ofer Feinerman, Nava Levit-Binun, Assaf Rotem, Shimshon Jacobi, Enric Alvarez, and Nestor Handzy for their assistance, fruitful discussions and support. I would also like to thank our technician Ruti Tal for all her valuable efforts and help.

As a physicist experiencing a hard landing into the world of wet biology, I owe a lot to the group of Menahem Segal from the Neuroscience Department. I would like to thank Prof. Menahem Segal, Varda Greenberger, Ianai Fishbein, Miryam Brodt, and Yair Pilpel for all their valuable help and advice.

More thanks are sent to all the amazing staff of the Physics of Complex Systems department: Yossi Shopen, Gershon Elazar, Yossi Leibovits, Yossi Drier, Yuri Shimon, Rostyslav Baron, Malka Paz, Israel Gonen, Ssimon Perez, Perla Zalcberg and Rachel Goldman.
Abstract

This thesis is comprised of two studies performed in the interface between Physics and Neuroscience.

The first study focuses on the architecture and connectivity of living neural networks. We used a novel experimental approach to quantify statistical properties related to the connectivity of neural networks. Experiments were performed on two-dimensional neural networks of dissociated rat hippocampal neurons. The neural networks were studied by measuring the neurons’ response to a global electrical stimulation. The network was weakened by lowering the neural connectivity. We used a graph-theoretic approach to quantify the size distribution of connected components. This approach supplies a simplified picture for the highly complex neuronal culture, yielding quantitative measures of the connectivity of the entire network. The novel technique presented is general and can be extended to other cultured neural networks as well as to brain slices.

The second study focuses on the development of novel optical methods based on light scattering, for measuring neural activity from single neurons. The aim is to measure and better understand light scattering from individual neurons in culture. Measuring neural activity using light scattering from individual neurons in culture is important for understanding and for characterizing the cellular mechanisms that are responsible for the changes in the scattered light. It is an important basis for the development of new techniques for measuring neural activity both in cultures and in the brain. Although measurements of changes to the light scattered from large-scale neurons correlated to neural activity were previously studied, it is still not clear what are the biophysical mechanisms responsible for such changes. Furthermore, no such measurements from mammalian neurons in culture were ever reported.

Although no intrinsic signals were detected from mammalian neurons due to electric activity in this study, an upper bound for the optical signal was set.
## Contents

1 Connectivity of 2-D neural networks .......................... 1

1.1 Introduction ................................................. 1

1.1.1 Overview .................................................. 1

1.1.2 Complex networks and random graphs ..................... 4

1.1.3 Percolation on random graphs ............................ 5

1.1.4 Directed Networks ........................................ 6

1.1.5 Connectivity of neural networks .......................... 7

1.1.6 Percolation approach for studying connectivity .......... 9

1.2 Experimental procedures ..................................... 11

1.3 Model ......................................................... 14

1.4 Results ....................................................... 17

1.4.1 Measured network response ............................... 17

1.4.2 Giant component .......................................... 17

1.4.3 Control parameter ........................................ 20

1.4.4 Blocking the inhibitory network ......................... 22

1.4.5 Cluster distribution analysis ............................. 23

1.4.6 Finite size effect ........................................ 24

1.5 Controls ...................................................... 26

1.5.1 Discrimination between glia cells and neurons. ........ 26

1.5.2 Robustness of results .................................... 28

1.6 Simulations .................................................. 30

1.7 Simulation results ........................................... 32

1.7.1 Removing bond vs. weakening bonds and the effect of the threshold T 32
CONTENTS

1.7.2 Loops .................................................. 32
1.7.3 Study of different degree distributions ..................... 34
1.7.4 Discussion about the value of the critical exponent ........ 34
1.7.5 Cluster distribution ....................................... 36
1.7.6 Checking the cluster distribution .............................. 40
1.7.7 Inhibition .................................................. 40
1.7.8 Summary .................................................. 42
1.8 Stimulation with bath electrodes ................................ 43
1.8.1 Stimulation parameters ...................................... 43
1.8.2 Neural excitability dependance on axons’ orientation ....... 44
1.9 Methods ...................................................... 48
1.9.1 Cultures of hippocampal neurons ............................ 48
1.9.2 Imaging neural activity with calcium sensitive fluorescent dye 48
1.9.3 Pharmacology ................................................. 49
1.9.4 Data Acquisition ............................................. 49
1.9.5 Data Analysis ............................................... 49
1.10 Discussion ..................................................... 51

2 Measuring neural activity using light scattering ............... 55
2.1 Introduction .................................................. 55
2.1.1 Measuring neural activity in–vitro ........................... 55
2.1.2 Measuring brain activity ....................................... 57
2.1.3 Measuring neural activity with intrinsic optical signals .... 57
2.2 Scattering experiments from a single neuron ................. 60
2.2.1 Experimental procedures ..................................... 60
2.2.2 Results ...................................................... 61
2.3 Scattering from the frog sciatic nerve .......................... 66
2.3.1 Experimental procedures ..................................... 66
2.3.2 Results ...................................................... 66
2.4 Methods ...................................................... 68
2.4.1 Neurons on glass ............................................. 68
## CONTENTS

2.4.2 Loose-patch recordings .......................... 69  
2.4.3 Data Acquisition ................................. 69  
2.4.4 Measuring light scattering ...................... 69  
2.4.5 Sciatic nerve dissection ......................... 70  
2.5 Discussion ........................................ 71  

Bibliography ........................................... 73  

Independent colaboration .............................. 79
Chapter 1

Connectivity of 2-D neural networks

1.1 Introduction

1.1.1 Overview

In recent years, complex networks have received significant attention in a wide range of disciplines from biology and physics to social sciences [1]. Representing complex structures as connected graphs yields a simplification that retains much of their functionality. It is therefore natural that network connectivity emerges as the fundamental feature determining statistical properties, including the existence of power laws, clustering coefficients and the small world phenomenon [2; 1]. While experimental access to man–made networks such as the WWW or e–mail is feasible [3; 4], biological ones such as the genetic networks must be painstakingly revealed node by node [5]. The connectivity in living neural networks is even more difficult to uncover due to the high complexity of such networks.

Neurons in living neural networks are highly connected and form complex networks in which activity flows between neurons through synapses. The complexity of the networks is manifested not only in their size but also in their architecture and the dynamical nature of their connections. In the brain, neural networks have functions. They are able to process and integrate information from many internal and
external sources and to perform different tasks. Many questions arise concerning the connection between the networks’ function and structure. What architecture permits the broad range of functions such networks perform? What organizational principles allow cognitive processes? A major task for answering such questions is to measure the connectivity of brain networks.

Questions related to the architecture and organization of neural networks can be addressed also to networks growing in-vitro. Although different from real brain networks and non-realistic in a way, such networks still provide a simplified model of real brain networks. From a physicist’s point of view, such networks are an excellent model system for studying problems such as information coding, computing processes and the relation between structure and function.

Two preparations dominate the in vitro experiments in neuroscience, the brain–slice preparation and the dissociated cultures preparation, each having its advantages and limitations.

The main advantage of the brain–slice preparation is its resemblance to real brain tissue, at least on the local scale. The characteristic of single neurons as well as the connections between neurons are assumed to be un-altered from the dissection. Neural growth and synapse formation take place in natural environment prior to the dissection, therefore, the connection between two neurons lying within the slice is preserved. This makes the slice preparation ideal for studying the structural properties of single neurons, the local structure of brain tissue, synaptic connectivity between neurons and the distribution of neuron types in different brain areas.

The damage caused by the slice dissection is poorly understood. Distant connections as well as connections perpendicular to the slice are not preserved. Evidently, increasing the slice’s physical dimension preserves more of the structure. However, larger slices are more difficult to study experimentally. Another disadvantage of slice preparations is the nature of their activity. Being separated from their natural environment, they receive less inputs than they would in in vivo conditions. This changes significantly the activity of neurons in slices.

In primary dissociated cell cultures, neurons (and glial cells) are plated on a two
dimensional cover glass where they connect to each other and form a functional network within a week. The main weakness of these networks is the abnormal conditions under which they develop and are later active. Although nerve cells are shown to resemble in detail neurons in slices or in the brain, the connections formed between them under unnatural conditions (e.g. grown in 2 dimensions, lacking target cells, lacking sensory input during development) result in networks that are far from resembling those of the brain [6]. Even though network structure as well as its inputs are altered by this kind of preparation, cultured neurons exhibit spontaneous activity. This spontaneous activity consists mostly on bursts, governed by the presence of both excitatory and inhibitory neurons [7; 8]. Yet, such spontaneous activity is still far from being considered natural brain activity. Thus any study that uses dissociated cultures should be carefully checked to mimic the relevant features of the natural system.

What dissociated cultures lack in their resemblance to in-vivo systems, however, is gained in their high amenability to experimental manipulation. These cultures can be kept for extended periods of time, up to many months [9], allowing the study of issues such as structural or functional development [10], and long-term plasticity [11; 12]. Recording from a large number of cells simultaneously allows a more complete monitoring of network activity. This is done by the use of either activity sensitive dyes (e.g. voltage sensitive dyes, Calcium sensitive dyes) or multi-electrode recording systems.

The difference between the structure of dissociated cultures and brain slices or networks in the brain is not necessarily a disadvantage. In fact, it can be used in two different ways. First, it can help to understand what the capabilities of cultured networks are and why their repertoire of activity is limited. Second, the understanding of the structure–function relation in cultures can help understanding the underlying principles of the connectivity in real brain networks.

A major advantage of cultured networks is that their connectivity can be regarded as an experimental handle ([13; 14]). Controlling the connectivity can be useful in two different ways. First, realistic neural structures (excitatory layered networks for
example) may be modeled to facilitate the study of their activity. Second, networks may be engineered to perform specific functional tasks, thus, helping to understand the structure–function relationship.

1.1.2 Complex networks and random graphs

A variety of systems in nature are described by complex networks whose simplest realization are random graphs. A random graph is a collection of vertices with edges connecting pairs of them at random. The study of random graphs began in the 1950s with the introduction of the Erdős–Rény (ER) graph [15]. An ER graph is obtained by starting with N vertices, and connecting every pair of nodes with a probability $p$. The result is a graph with $\sim pN(N - 1)/2$ edges distributed randomly. The degree distribution of the ER graph is Poisson: $p_k(k) \sim e^{-\lambda}\lambda^k/k!$. For many years since its introduction the ER graph has been used as a model for complex networks. However, the growing interest in complex networks has led to the discovery that most networks in nature do not have a Poisson degree distribution. Moreover, intuition indicates that complex systems must have some organizational principles which are not manifested in the ER graph. Three main concepts exist for describing complex networks. The first is the degree distribution ($p_k$) mentioned earlier, describing the probability that a node has $k$ links. A large number of networks in nature seem to have a power–law degree distribution: $p_k \sim k^{-\lambda}$. Such networks are called scale-free networks since they lack a characteristic scale. The long tail of the distribution indicates that there are a few nodes (‘hubs’) having an exceptionally large number of links. Examples of scale–free networks are the World Wide Web, e-mail and metabolic networks. The structure of such networks leads to robustness since the removal of random nodes doesn’t critically damage the structure of the network. Another concept is clustering. In a large number of networks there is a tendency for clustering. Clustering is quantified through the cluster coefficient $C$. Selecting a node $i$ with $k_i$ neighbors, $C_i$ is defined as the ratio between the actual number of edges between the $k_i$ nodes: $E_i$, and the maximal number of edges between those nodes: $C_i = 2E_i/[k_i(k_i - 1)]$. The clustering coefficient $C$ of the whole network is the
average of all individual $C_i$'s. In an ER graph, $C = p$ since the nodes are distributed randomly. In real networks, the clustering coefficient is much larger than it is in a comparable ER network. A third concept is the small world \[16\]. This concept describes the fact that despite the large size of complex networks, in most cases there is a relatively short path between two nodes. The most popular manifestation of the small world concept is the "six degrees of separation" proposed by Stanley Milgram, who concluded that there is a path of acquaintance with a typical length of six between most pairs of people in the USA. The small-world property is characterized by high clustering and a few connections between clusters.

A formalism of generating functions was developed for characterizing random graphs with arbitrary degree distributions \[17; 18; 19\]. This formalism enables to calculate quantities such as the cluster size distribution, the average cluster size or the average path length.

### 1.1.3 Percolation on random graphs

Percolation theory studies the emergence of a path that percolates through the system. Usually, percolation is defined on a $d$-dimensional lattice whose edges are present with probability $p$ and absent with probability $1 - p$. For small $p$, only few small clusters form. At a critical probability $p = p_c$ called the percolation threshold, a percolating cluster appears. This cluster is also called the infinite cluster since its size diverges as the size of the lattice increases. Two main versions of percolation exist, the percolation described above is called bond percolation. A second percolation in which nodes are occupied with probability $p$ is called site percolation. Percolation may be defined also on random graphs. As for the lattice case, below $p_c$ the network comprise of isolated clusters and above $p_c$, a giant cluster spans the entire network. The main quantities of interest in percolation are the giant component size $g$, the percolation threshold $p_c$, the cluster size distribution $p_s$ and the average cluster size $\langle s \rangle$.

Close to the percolation transition the giant component can be described as a
power–law:
\[ g = \begin{cases} 
0 & p < p_c \\
(p - p_c)\beta & p > p_c 
\end{cases} \]  
(1.1)

The exponent $\beta$ depends on the connectivity of the graph. For a percolation on a lattice, $\beta$ depends on the lattice dimensionality $d$: 

<table>
<thead>
<tr>
<th>$d$</th>
<th>$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>$5/36$</td>
</tr>
<tr>
<td>3</td>
<td>0.41</td>
</tr>
<tr>
<td>4</td>
<td>0.64</td>
</tr>
<tr>
<td>5</td>
<td>0.84</td>
</tr>
<tr>
<td>$d &gt; 6$</td>
<td>1</td>
</tr>
</tbody>
</table>

The value of $\beta$ can be solved analytically for several random graphs. The case of a Bethe tree provides $\beta = 1$ [20]. A random graph with a Poisson degree distribution (ER graph) provides $\beta = 1$ as well [2]. For the case of a random graph with a power–law degree distribution: $p_k \sim k^{-\lambda}$, the value of $\beta$ is equal or larger than one depending on the value of the exponent $\lambda$ [21; 22].

Percolation theory has not been widely used for the study of neural networks. An approach that utilizes the morphology of individual neurons together with numerical simulations was introduced by Costa et al. to study the growth of neural networks [23; 24]. A percolation transition has been observed with the emergence of a giant component as dendrites and axons grow with time.

Percolation can be used to address questions such as how the removal of nodes (neurons) and bonds (synapses) affects the functionality of networks. Such questions are important for the study of neurodegenerative diseases such as Alzheimer’s.

### 1.1.4 Directed Networks

So far I have described properties of undirected networks. However, many networks including neural networks are directed. In directed graphs each vertex has a separate input/output degree $k_{\text{in/out}}$. Each node has an input cluster defined as the set of nodes that have a directed path to the selected nodes. Likewise, each node has an output cluster defined as the set of nodes that can be reached starting from the selected node. In general, a directed graph comprises of a giant weakly connected
1.1 Introduction

component (GWCC) and several finite components. In the GWCC, every site is reachable from every other site provided that the bonds are treated as bidirectional. The GWCC is further divided into a giant strongly connected component (GSCC), an OUT component and an IN component (Fig. 1.1). The GSCC consists of all nodes reachable from each other following a directed path. All the nodes that can be reached from the GSCC comprise the OUT component and all the nodes from which the GSCC is reached comprise the IN component.

![Diagram of directed random graph components]

Figure 1.1: The giant component of a directed random graph.

The most studied directed network is probably the World Wide Web [25; 26]. In general, most of the network models ignore the networks’ directionality and directed networks have been studied much less than undirected ones. However, in recent years there has been an increasing interest in directed networks and a few theories of directed networks and percolation on directed networks have been developed [21; 19; 27]. In a study of percolation on a directed scale-free network it was found that the critical exponent $\beta$ is larger than or equal to one [21]

1.1.5 Connectivity of neural networks

Two kinds of connectivities are defined for brain networks, an anatomical connectivity and a functional connectivity. The anatomical connectivity is the physical connectivity defined by the connections linking the neurons in the network. The functional connectivity is defined by the correlation between the activity of neurons or brain areas. The Anatomical connectivity in the brain is hard to access and to measure. Functional connectivity in the brain can be measured with fMRI. In several studies it was found that functional connectivity exhibits power-law distributions as well as
small-world attributes [28; 29].

Advances in technology have permitted to measure local connectivity in large neural networks and the connectivity of small invertebrates neural networks. On the smallest scale, electron microscopy is used to image single synapses. On the next scale, the local connectivity between two neurons can be mapped in detail using advanced microscopy techniques. Three-dimensional reconstructions with confocal microscopy show the connections between dendrites and axons as well as the distribution of spines. Models exist predicting the physical connectivity between two neurons derived from the measured neuronal morphology [30]. Further zooming out, the number of target neurons a neuron is connected to can be measured using dual whole cell recording. In large cortical cultures it was roughly estimated that each neuron is mono-synaptically connected to 10–30 % of all other neurons [8; 31; 32]. On the scale of small neural networks, the wiring diagram of small invertebrate can be measured. Using electron microscopy the entire network of the C. elegans nematode containing 302 neurons was mapped in details [33]. The clustering number of the C. elegans network was found to be 0.28, higher than that of a random graph of the same size and with same average degree which is 0.05 [16]. The degree distribution of the C. elegans network was found to have an exponential tail, rejecting the hypothesis of a power-law connectivity [34].

Unraveling the entire neural wiring diagram in large neural networks is presently not feasible. However, some progress has been attained in the study of statistical properties related to the connectivity of such networks and in the link between neural connectivity and information. Considerable attention has been given to scale-free networks and the small-world property since a large number of networks in nature seem to have these two properties. Scale-free connectivity was suggested as a mechanism for synchronicity and oscillations [35; 36]. Small world has been proposed for fast signal processing and coherent oscillations [37], unlike either random (ER) or regular graphs. It has been also shown that small world characteristics can be selected using graph selection, for obtaining rich functional behavior [38].

Eytan et al. suggest that the degree distribution of large cultured cortical networks is scale-free [39]. They based their claim on the relationship between the firing
rate of a neuron and the number of inputs it has, together with their experimental observation that the number of spikes a neuron fires is distributed as a power-law.

A correlation connectivity defined by the correlated activity in synchronized spontaneous firing of cortical neurons was measured and shown to be related to the physical connectivity [40]. The correlated activity in synchronized bursting events is used to define subgroups which are relate to coding storage and retrieval of specific information [41].

1.1.6 Percolation approach for studying connectivity

In this work a novel experimental approach to quantify statistical properties of neural networks is presented. The connectivity of dissociated cultures of rat hippocampal neurons is investigated. The networks are studied in terms of bond percolation on random graphs [20; 18]. The networks are considered as random graphs where vertices represent neurons and edges represent synaptic connections between the neurons. We control the connectivity of the entire network, gradually reducing the synaptic strength by means of chemical application. As more synapses are blocked, the initially connected network progressively breaks down into smaller clusters until a fully disconnected network is reached (Fig. 1.2). The weakening of the network is quantified and the distribution of sizes of connected components determined. Experimentally, this is achieved by analyzing the neurons’ response to stimulations applied simultaneously to the entire network. Viewed inversely, as the network’s connectivity increases, a percolation transition occurs at a critical synaptic strength with the emergence of a giant component. The giant component is the largest cluster in the network. Below the critical connectivity, the network comprises of small clusters. Above the critical connectivity, the giant component becomes the dominant cluster in the network. We show that at the vicinity of the critical point the size of the giant component increases as a power law with an exponent $\beta \simeq 0.65$. Performing experiments while blocking the inhibitory network, we show that the value of $\beta$ does not depend on the balance between excitation and inhibition. The experimental value of $\beta$ together with results obtained in numerical simulations suggest that the connectivity distribution in the
neural network does not follow a power-law distribution. Percolation on random graph with a power-law degree distribution gives \( \beta \geq 1 \). An analysis of the clusters that do not belong to the giant components shows that for a high connectivity the clusters are relatively big and with a broad distribution in sizes, and rapidly become smaller with a narrow distribution as the connectivity decreases.

![Figure 1.2: Concept of the giant component.](image)

Part of this work was submitted for publication:

1.2 Experimental procedures

Experiments were performed on primary cultures of rat hippocampal neurons, plated on glass coverslips following the procedure described by Papa et al. \([42]\) (Fig. 1.3). The cultures were used 14–20 days after plating. The neural network includes \(N \approx 2.5 \times 10^5\) neurons. The neural culture was placed in a chamber that contains two parallel platinum wires (0.005” diameter) fixed at the bottom and separated by 15 mm (Fig. 1.4). The neurons were electrically stimulated by applying a 20 msec square bipolar pulse through the wires, delivered by a computer controlled current source (KEITHLEY 220). The current was controlled and gradually increased between subsequent pulses, while the corresponding voltage drop \(V\) was measured with an oscilloscope. The chamber was mounted on a Zeiss inverted microscope and the culture was imaged through a 10X objective. Neuronal activity was monitored using the fluorescent calcium indicator Fluo-4. We chose to measure neural activity with a calcium sensitive marker since it enables to monitor the activity of hundreds of neurons simultaneously with relatively ease. Images were captured with a cooled charge–coupled device (CCD) camera (SensiCam QE) at a rate of 5 frames/sec, and processed to record the fluorescence intensity \(I\) of a sample of the culture including \(n \approx 400\) individual neurons as a function of time (Fig. 1.5a,c). Neural spiking activity was detected as a sharp increase of the fluorescence intensity.

The connectivity of the network was gradually weakened by adding increasing amounts of CNQX, the antagonist of the AMPA type receptors in the glutamate synapses of excitatory neurons. NMDA receptors were completely blocked with 20 \(\mu\)M of the corresponding antagonist APV, enabling the study of network breakdown due solely to CNQX. To study the role of inhibition, we performed experiments with inhibitory neurons either active or blocked with 40 \(\mu\)M of the GABA receptor antagonist bicuculine. From here on, we label the network containing both excitatory and inhibitory neurons by \(G_{EI}\), and the network with excitatory neurons only by \(G_E\).

The response of the network for a given CNQX concentration was measured as the fraction of neurons \(\Phi\) that fired in response to the electric stimulation at voltage \(V\) (Fig. 1.5b). Response curves \(\Phi(V)\) were obtained by increasing the stimulation
voltage from 2 to 6 V in steps of 0.1 – 0.5 V. Between 6 and 10 response curves were measured per experiment, each at a different CNQX concentration. Measurements were completed within 4 h, at the end of which the culture was washed of CNQX to verify that the initial network connectivity was restored.

Figure 1.3: a. A phase contrast image of a 2-D neural network comprising of neurons plated on a glass coverslip. Individual neurons appear as small spheres in the image. b. A bright-field image of the same neural network.

Figure 1.4: Sketch of the experimental setup.
1.2 Experimental procedures

Elaborated explanations of the experimental methods follow at the end of this chapter.

Figure 1.5: (a) Fluorescence image of a small region of the neural culture. Bright spots are cell bodies. Neural connections, mostly dendrites, are visible. (b) Plot of the neural response. Black lines indicate those neurons that respond to the excitation. (c) Fluorescent intensity as a function of time $F(t)$ for 3 neurons at increasing voltages. Vertical lines indicate the excitation time. Traces of responding neurons are in red.
1.3 Model

To elucidate the relation between the topology of the living neural network and the observed neural response, we consider a simplified model of the network in terms of bond–percolation on a graph. The neural network is represented by the directed graph $G$. Our main simplifying assumption is the following: A neuron has a probability $f = f(V)$ to fire as a direct response to an external excitation (an applied electrical stimulus in the experiment), and it always fires if any one of its input neurons fire. (Fig. 1.6). This ignores the fact that more than one input is needed to excite a neuron, and that connections are gradually weakened rather than abruptly removed. The model also ignores the presence of feedback loops and recurrent activity in the neural culture. However, we verified with numerical simulations that relaxing these assumptions does not affect the validity of the model.

![Diagram](image)

**Figure 1.6:** (a) The model. The neuron represented in grey fires either in response to an external stimulation with a probability $f(V)$ or if any of its input neurons fired. (b) Corresponding cluster size distribution $p_s$ obtained by counting all input clusters for all neurons.
Evidently, the firing probability $\Phi(f)$ increases with the connectivity of $G$, because any neuron along a directed path of inputs may fire and excite all the neurons downstream (Fig. 1.6a). All the upstream neurons that can thus excite a certain neuron define its input–cluster. It is therefore convenient to express the firing probability as the sum over the probabilities $p_s$ of a neuron to have an input cluster of size $s - 1$,

$$
\Phi(f) = f + (1 - f)P(\text{any input neuron fires})
= f + (1 - f) \sum_{s=1}^{\infty} p_s (1 - (1 - f)^{s-1})
= 1 - \sum_{s=1}^{\infty} p_s (1 - f)^s,
$$

(1.2)

where we used the probability conservation $\sum_s p_s = 1$. It is readily seen that $\Phi(f)$ increases monotonically with $f$ and ranges between $\Phi(0) = 0$ and $\Phi(1) = 1$. The deviation of $\Phi(f)$ from linearity manifests the connectivity of the network (for disconnected neurons $\Phi(f) = f$). Eq. (1.2) indicates that the observed firing probability $\Phi(f)$ is actually one minus the generating function $H(x)$ (or the $z$–transform) of the cluster–size probability $p_s$ [17],

$$
H(x) = \sum_{s=1}^{\infty} p_s x^s = 1 - \Phi(f),
$$

(1.3)

where $x = 1 - f$. One can extract from $H(x)$ the input–cluster size probabilities $p_s$, formally by the inverse $z$–transform, or more practically, in the experiment, by fitting $H(x)$ to a polynomial in $x$.

Once a giant component emerges, the observed firing pattern is significantly altered and Eq. (1.3) has to be modified. In an infinite network, the giant component always fires no matter what the firing probability $f$ is. This is because even a very small $f$ is sufficient to excite one of the infinitely many neurons that belong to the giant component. We account for this effect by splitting the neuron population into a fraction $g$ that belongs to the giant component and always fires and the remaining fraction $1 - g$ that belongs to finite clusters. This gives

$$
\Phi(f) = g + (1 - g) [f + (1 - f)P(\text{any input neuron fires})]
= 1 - (1 - g) \sum_{s=1}^{\infty} p_s (1 - f)^s.
$$

(1.4)
As expected, at the limit of almost no self–excitation $f \to 0$ only the giant component fires, $\Phi(0) = g$, and $\Phi(f)$ monotonically increases to $\Phi(1) = 1$. With a giant component present the relation between $H(x)$ and the firing probability changes, and Eq. (1.3) becomes

$$H(x) = \sum_{s=1}^{\infty} p_s x^s = \frac{1 - \Phi(f)}{1 - g}. \quad (1.5)$$

In reality, the giant component is not infinite and it is measured from a sample which has $n$ neurons. Therefore, it fires only after a non–zero, though small, firing probability $f_T$ is exceeded. To estimate this finite size threshold we note that when we measure a giant component of size $gn$, then the firing probability is

$$\Phi(f) \simeq g \left(1 - (1 - f)^{ng}\right) \simeq g \left(1 - e^{-fgn}\right). \quad (1.6)$$

This probability becomes significant at the threshold

$$f_T \simeq (gn)^{-1}. \quad (1.7)$$
1.4 Results

1.4.1 Measured network response

An example of the response curves $\Phi(V)$ for a $G_{EI}$ network with $n = 450$ neurons measured at 6 different concentrations of CNQX is shown in Fig. 1.7. At one extreme, with $[\text{CNQX}] = 0$ the network is fully connected, and a few neurons with low firing threshold suffice to activate the entire culture. This leads to a very sharp response curve that approaches a step function, where all neurons form a single cluster that comprises the entire network. At the other extreme, with high concentrations of CNQX ($\simeq 10 \mu\text{M}$) the network is completely disconnected, the response curve rises moderately, and is given by the individual neurons’ response. $\Phi(V)$ for individual neurons (denoted as $\Phi_\infty(V)$) is well described by an error function $\Phi_\infty(V) = 0.5 + 0.5 \text{erf}\left(\frac{V-V_0}{\sqrt{2}\sigma_0}\right)$. This indicates that the firing threshold of a neuron in the network follows a Gaussian distribution with mean $V_0$ and width $2\sigma_0$.

Intermediate CNQX concentrations induce partial blocking of the synapses. As the network breaks up, neurons receive on average fewer inputs and a stronger excitation has to be applied to light up the entire network. The response curves are gradually shifted to higher voltages as $[\text{CNQX}]$ increases. Initially, some neurons break off into separated clusters, while a giant cluster still contains most of the remaining neurons. The response curves are then characterized by a big jump that corresponds to the biggest cluster (giant component), and two tails that correspond to smaller clusters of neurons with low or high firing threshold (Fig. 1.8). Error functions describe these tails well. Beyond these concentrations ($[\text{CNQX}] \gtrsim 500 \text{nM}$ for $G_{EI}$ networks) a giant component cannot be identified and the whole response curve is then also well described by an error function.

1.4.2 Giant component

The largest cluster in the network characterizes the giant component. The size of the giant component $g$ is experimentally measured as the largest fraction of neurons that fire together in response to the electric excitation. For each response curve, $g$ is
Figure 1.7: Response curves $\Phi(V)$ for 6 concentrations of CNQX and $n = 450$ neurons. Lines are a guide to the eye except for $1 \mu M$ and $10 \mu M$ that are fits to error functions, with $V_0 = 3.96 V$ and $\sigma_0 = 0.46 V$ for $10 \mu M$. Inset: (a) the response $f$ of the disconnected network. (b) The probability $p_v$ of a neuron to have a threshold $v$.

measured as the biggest jump $\Delta \Phi$, as shown by the grey bars in Fig. 1.8. The size of the giant component is considered to be zero when a characteristic jump cannot be identified, or when the jump is comparable to the noise of the measurement, which is typically about 4% of the number of neurons measured.

In an infinite system the giant component always fires no matter what the firing probability $f$ is. This is because even a very small $f$ is sufficient to excite one of the infinitely many neurons that belong to the giant component. In the real neural network, the giant component is finite and it is measured from a sample of $n \sim 400$ neurons. Therefore it fires only after a small firing probability $f_T$ is reached as shown in Fig. 1.8.

We studied the size of the giant component in a range of CNQX concentrations
1.4 Results

Figure 1.8: Response curves $\Phi(V)$ for 6 concentrations of CNQX and $n = 450$ neurons. The grey bars show the size of the giant component. Lines are a guide to the eye except for 1 $\mu$M and 10 $\mu$M that are fits to error functions, with $V_0 = 3.96$ V and $\sigma_0 = 0.46$ V for 10 $\mu$M.

spanning almost three orders of magnitude from 0 nM to 10 $\mu$M in logarithmic scale. The results are shown in Fig. 1.9.

We define the control parameter $c = 1/(1 + [\text{CNQX}]/K_d)$ as a measure of the synaptic strength, where the dissociation constant $K_d = 300$ nM is the concentration of CNQX at which 50% of the receptors are blocked [43]. Fig. 1.10 shows the breakdown of the network for a $G_{EI}$ network. The behavior of the giant component indicates that the neural network undergoes a percolation transition, from a set of small, disconnected clusters of neurons to a giant cluster that contains most of the neurons. At the vicinity of the emergence of the giant component, the percolation transition is described by the power–law $g = A|c - c_o|^{\beta}$. A power–law fit for $G_{EI}$ networks (inset of Fig. 1.10) gives: $A = 1.7 \pm 0.1$, $c_o = 0.36 \pm 0.02$, $\beta = 0.66 \pm 0.10$. 
1.4.3 Control parameter

As described earlier, we use the control parameter $c = 1/(1 + [\text{CNQX}]/K_d)$ as a measure of the connectivity. The parameter $c$ characterizes the connectivity probability between two neurons, and takes values between 0 (full blocking, independent neurons) and 1 (full connectivity). Conceptually, it quantifies the number of receptor molecules that are not bound by the antagonist CNQX and therefore are free to activate the synapse.

The interaction of receptors (R) and drugs (D) forming a receptor–drug complex (RD) can be described as follows:

$$ R + D \overset{k_1}{\underset{k_2}{\rightleftharpoons}} RD $$

where $k_1$ is the rate at which $R$ and $D$ bind and $k_2$ is the rate at which the complex
1.4 Results

Figure 1.10: Size of the giant component $g$ as a function of the synaptic strength $c$, averaged over 18 $G_{E1}$ networks (•), and 6 $G_{E}$ networks (□). Lines are a guide to the eye. Some CNQX concentrations are indicated for clarity. Inset: Log–log plot of the power law fits $g \sim |1 - c/c_0|^{\beta}$. The slope 0.65 corresponds to the average value of $\beta$ for the two networks.

$RD$ disintegrates. The interaction can be formulated as:

$$\frac{d[RD]}{dt} = k_1[R][D] - k_2[RD]$$

(1.9)

where $[R]$ is the number of free receptors, $[D]$ is the amount of free drug and $[RD]$ is the number of receptor–drug complexes (or the number of occupied receptors). At equilibrium, the rate at which the $RD$ complex forms is equal to the rate at which it disintegrates. Therefore:

$$k_1[R][D] = k_2[RD]$$

(1.10)

The dissociation constant is defined as:

$$K_d \equiv \frac{k_2}{k_1} = \frac{[R][D]}{[RD]}$$

(1.11)
The total number of receptors \([R_T]\) is equal to the sum of the free receptors and the occupied receptors: \([R_T] = [R] + [RD]\). The fraction of occupied receptors is then given by:

\[
\frac{[RD]}{R_T} = \frac{[D]}{K_d + [D]}
\]
(1.12)

Thus, \(k_d = [D]\) when half of the receptors are occupied.

The fraction of free receptors is given by:

\[
\frac{[R]}{[R_T]} = \frac{1}{1 + \frac{[D]}{K_d}}
\]
(1.13)

Which is exactly the definition of the control parameter \(c\) we use to define the network’s connectivity.

To check whether \(c\) is a good measure of the connectivity, experiments were performed blocking the AMPA receptors with DNQX instead of CNQX. The affinity constant \(K_d\) of DNQX is 500 nm [43]. Experiments with DNQX yielded response curves similar to those obtained with CNQX. Plotting the giant component size as a function of the CNQX/DNQX concentration (Fig. 1.11) one can see that with DNQX, the giant component disintegrates slower than with CNQX. When plotting the giant component size as a function of the order parameter \(c\), the two curves collapse. This indicates that the order parameter \(c\) is a good measure of the connectivity in the network.

### 1.4.4 Blocking the inhibitory network

To check the behavior of the network with no inhibition (\(G_E\) networks), the experiments were repeated while blocking the inhibitory synapses with bicuculine (Fig. 1.10). The giant component for \(G_E\) networks breaks down at much higher CNQX concentrations compared with \(G_{EI}\) networks, and one can think of the effect of inhibition on the network as effectively reducing the number of inputs that a neuron receives on average. For \(G_{EI}\) networks, the giant component vanishes at \([CNQX] \simeq 600 \text{ nM}\), while for \(G_E\) networks the critical concentration is around 1000 nM. A power–law fit for \(G_E\) networks (inset of Fig. 1.10) gives: \(A = 2.1 \pm 0.1, \ c_o = 0.24 \pm 0.02,\)
1.4 Results

Figure 1.11: (a) The size of the giant component $g$ as a function of the CNQX concentration (black curve), averaged over 14 $G_{EI}$ networks and DNQX concentration (blue curve), averaged over 5 $G_E$ networks. (b) The size of the giant component $g$ as a function of the order parameter $c$ for experiment with CNQX (black curve) with $K_d = 300$ nm and DNQX (blue curve) with $K_d = 500$ nm. The two curves collapse indicating that $c$ is a good measure of the connectivity.

$\beta = 0.63 \pm 0.10$. The exponent $\beta$ for $G_{EI}$ and $G_E$ networks is the same within the experimental error indicating that is an intrinsic property of the network.

1.4.5 Cluster distribution analysis

The construction of the experimental function $H(x)$ defined in Eq. (1.5) allows the fit of a polynomial $\sum p_s x^s$ to determine the size distribution $p_s(s)$ for clusters that do not belong to the giant component. Since $f \equiv \Phi_\infty(V)$ is the response curve for individual neurons (Fig. 1.8) and $x = 1 - f$, the function $H(x)$ for each response curve is obtained by plotting $1 - \Phi(V)$ as a function of $1 - \Phi_\infty(V)$. For curves with a giant component present, its contribution is eliminated and the resulting curve normalized by the factor $1 - g$.

Fig. 1.12 shows $H(x)$ for the response curves shown in Fig. 1.8. The corresponding $p_s(s)$ distributions, shown in Fig. 1.13(a), are obtained from polynomial fits up to order 20. Since the cluster analysis is sensitive to experimental resolution, it is limited
to \( g \lesssim 0.8 \), which corresponds to \([\text{CNQX}] \gtrsim 200 \, \text{nM}\) for \( G_{EI} \) networks. Experimental resolution also limits the observation of very small clusters for \([\text{CNQX}] \lesssim 500 \, \text{nM}\), since they are associated with values of \( \Phi(V) \) close to 1. Overall, as shown in Fig. 1.13b, the clusters start out relatively big and with a broad distribution in sizes, to rapidly become smaller with a narrow distribution for gradually higher concentrations of CNQX. Isolated peaks in \( p_s(s) \) indicate non tree–like clusters outside the giant component, in contrast to the model. This hints at the persistence of loops and at a strong local connectivity. While our sample covers only part of the culture, it does represent the statistics of the whole population. Sample size will affect the noise level, but the overall cluster size distribution of Fig. 1.13a is representative of the whole network. Our assumption that one input suffices to excite a neuron leads to an under–estimation of the cluster sizes, probably in direct proportion to the number of inputs actually needed to excite a neuron, which is on the order of ten [44].

### 1.4.6 Finite size effect

Finite size effects are observed in the behavior of the firing threshold for the giant component \( f_T \), which increases linearly with \( 1/g \), as predicted in Eq. (1.7). \( f_T(g) \) is measured for each concentration as the value of \( f \) at which the giant component

\[ H(x) \text{ functions corresponding to the response curves shown in Fig. 1.8.} \]

The bar shows the size of the giant component for \( 300 \, \text{nM} \).
Figure 1.13: (a) 3D representation of the cluster size distribution $p_s(s)$ for the experiment shown in Fig. 1.8. (b) Average cluster size, $\langle s \rangle$, and variance of the cluster size distribution, $\sigma^2 = \langle s^2 \rangle - \langle s \rangle^2$, as a function of the concentration of CNQX, averaged over 15 $G_{EI}$ networks. (c) Giant component firing threshold $f_T$ as a function of $1/g$ for two groups of experiments with 90 (□) and 400 (■) neurons measured, and for CNQX concentrations between 0 and 500 nM. The lines are least square fits.

Fig. 1.13c shows the results for two groups of experiments with $\langle n \rangle = 90$ and $\langle n \rangle = 400$ neurons measured. Linear fits provide respective slopes $\simeq 0.02$ and $\simeq 0.005$, which are of the same order of magnitude as $1/n$, with $n$ the number of neurons measured.
1.5 Controls

1.5.1 Discrimination between glia cells and neurons.

We are interested in recording only from neurons, but as the picture shown in Fig. 1.5a reveals, neurons as well as glia are stained by the Fluo-4 dye. Glia are cells that grow with the neurons and support their growth in culture as well as in the brain, and can have electric activity despite the fact that they are not spiking cells. To avoid recording fluorescence from both cell classes, the responses of neurons and glia must be distinguished from each other. Based on visual inspection, we can give an initial estimate of the number of glia that fluoresce with Fluo-4, which is under 10% of the neurons. To further check this, we first identified by the time course of their firing response those glia cells that are fluorescent with Fluo-4 and may be confused with regular neurons. The group stands out clearly, responding with a noticeable delay as compared to the stimulation of regular neurons (Fig. 1.14a). It is important to note that such cells, which indeed comprise about 7% of the culture, are excluded from the sample chosen during the experiment.

Analysis of the cells’ fluorescent signal is shown in Fig. 1.14a, demonstrating that glia and neurons indeed have a different firing pattern in response to the external excitation voltage. Glia exhibit a significantly retarded response compared with that of the neurons, typically 3 sec after neurons’ response. This time difference is used to discriminate between neurons and glia, and all cells with delayed response are rejected. This process eliminates almost all glia from further analysis. For example, in one experiment with 400 Fluo-4 stained cells in the field of view, 27 had a clearly delayed response to the external excitation and were identified as glia. Of the later, 14 had weak fluorescence and were immediately rejected, while the remaining 13 were rejected during data analysis.

To further verify this identification we stained the culture with a neuron-specific dye (NeuN), and identified all cells that are observed with Fluo-4 to fire, but are not stained by NeuN (Figs. 1.14b to 1.14d). This group consists of glia and a (small) percentage of neurons that failed to be stained by the NeuN. In total, we found
Figure 1.14: (a) Response of two neurons and one glia cell to the external excitation. The glia’s response is delayed by \( \sim 3 \) sec with respect to the neurons. The initial small increase in fluorescence of the glia cell is due to activity in the processes of neighboring neurons that pass near the glial cell. The inset shows the corresponding neurons and glia analyzed. (b) Fluorescence image of cells stained with Fluo-4, shown in green for clarity. Cells that were identified as glia through analysis of the time course of their fluorescence signal are shown in blue. Scale bar, 100 \( \mu \)m. (c) The same sample as in (b), with fluorescence image of NeuN-labeled cells shown in red. (d) Combination of (b) with (c). Neurons that are stained both with Fluo-4 and NeuN, are shown in yellow.

slightly more than 10% of the culture, and among this group are all the cells we identified as glia by their firing pattern. Incidentally, there is also a small percentage (about 3%) of neurons that stain with NeuN but fail to stain with Fluo-4.

Figs. 1.14b to 1.14d illustrate which cells were stained with Fluo-4 (Fig. 1.14b), NeuN (Fig. 1.14c), and the identification of glia (Fig. 1.14d). Using the combined image (Fig. 1.14d) we can identify the cells that are stained by Fluo-4 but are not neurons. Obviously, the staining permits to clearly identify both types of cells, and
verifies the results obtained by the time course of their firing.

### 1.5.2 Robustness of results

To check the reproducibility of the results and the network’s fatigue, the response curve for no CNQX was consecutively measured for the same neural network several times. Measurements were carried out as explained previously. Since the measured network was fully connected, a giant component appeared in all of the response curves. The threshold voltage $V_0$ at which the giant component emerged and its size $g$ were measured for each response curve; the results are shown in Fig. 1.15, for a sample size of $n = 90$ neurons. As shown, the size of the giant component was $\sim 1$ for all the measurements. The deviation from 1 defines the measurements error to be $5\%$, which corresponds to $\sim 5$ neurons. The threshold voltage $V_0$ increased slightly between runs, however this increase is negligible compared to the voltage shift of the response curves caused by the breakdown of the network as sown in Fig. 1.8. It is concluded that the response curve measurements are reproducible, the exposure of the neurons to the fluorescence illumination and the stimulation with the bath electrodes do not change the results along $3hrs$ of measurements, which is the duration of one experiment.
Figure 1.15: (a) The giant component size $g$ as a function of time. (b) The threshold voltage $V_0$ as a function of time.
1.6 Simulations

The model presented was derived from classic bond percolation theory and has an analytic solution that yields precise results. However, some of the simplifying assumptions in it may have an effect on the results. To investigate the effect of removing or relaxing these assumptions, numerical simulations that describe the firing neural network were performed. The numerical simulations provided response curves that are qualitatively similar to the ones observed experimentally. In particular, we observed the existence of a giant component that undergoes a percolation transition at a critical connectivity.

Three assumptions of the model are unrealistic. First, we assume that one input suffices to activate a neuron, while in reality a number of input neurons must spike for the target neuron to fire. Second, the effect of CNQX is to bind and block AMPA glutamate receptor molecules, and consequently to continuously reduce the synaptic strength, so that bonds are in reality gradually weakened rather than abruptly removed. Third, there are no feedback loops within finite clusters in the model, while in the living culture they may exist. We performed numerical simulations of the model to test that none of these assumptions change the main results. We have also tested different degree distributions and showed that a Gaussian distribution provides $\beta = 0.66 \pm 0.05$, the same value as the experiments, while a power–law distribution yielded a significantly different value.

The neural network was simulated as a directed random graph $G(N, k_{I/O})$ in which each vertex is a neuron and each edge is a synaptic connection between two neurons [18]. The graph was generated by assigning to each edge an input/output connectivity $k_{I/O}$ according to a predetermined degree distribution. Next, a connectivity matrix $C_{ij}$ was generated by randomly connecting pairs of neurons with a link of initial weight 1 until each vertex was connected to $k_{I/O}$ links. The process of gradual weakening of the network was simulated in one case by removing edges and in the second case by gradually reducing the bond strength from 1 to 0. The connectivity is defined for the case of removing bonds as the fraction of remaining edges, and for the case of weakening bonds as the bond strength.
Each neuron had a threshold $v_i$ to fire in response to the external voltage, and all neurons had a threshold $T$ to fire in response to the integrated input from their neighbors. Since the experiments show that the probability distribution for independent neurons to fire in response to an external voltage is Gaussian, the $v_i$'s were distributed accordingly. For the simple case of removing links, the global threshold $T$ differentiates networks where a single input suffices to excite a target neuron from those where multiple inputs are necessary. When links are weakened, $T$ plays a more subtle role, and determines the variable number of input neurons that are necessary to make a target neuron spike.

The state of each neuron, inactive (0) or active (1) was kept in a state vector $S$. In the first simulation step, a neuron fired in response to the external voltage if the ”excitation voltage” $V$ was greater than its individual threshold $v_i$, i.e. $V \geq v_i \rightarrow S_i = 1$

In the subsequent simulation steps, a neuron fired due to the internal voltage, if the integration over all its inputs at a given iteration was larger than $T$: $\sum C_{ij} S_j \geq T \rightarrow S_i = 1$. The simulation iterated until no new neurons fired. The network response was then measured as the fraction of neurons that fired during the stimulation.

The process was repeated for increasing values of $V$, until the entire network was activated. Then, the network was weakened and the exploration in voltages was repeated.
1.7 Simulation results

The simulations provided response curves $\Phi(V)$ similar to the curves measured experimentally (Fig. 1.16). A giant component was clearly identifiable, and its analysis was performed as for the experimental data.

1.7.1 Removing bond vs. weakening bonds and the effect of the threshold $T$

We ran simulations considering 4 different combinations of removing or weakening edges, and for $T = 1$ or $T = 5$, with the connectivity set to be Gaussian for both input and output degree distributions. The results of the simulation are presented in Fig. 1.16. All 4 studied cases yielded qualitatively similar results, with a giant component clearly identifiable. The analysis of the percolation transition yielded $\beta = 0.66$ in all 4 cases, in striking agreement with the value measured experimentally.

1.7.2 Loops

Although loops are very rare in a random (Erdös–Rény) graph, our neural culture is not random; consequently, locality and neighboring probably play important roles. It can therefore happen that there are feedback loops in our culture, and they may have an effect. However, graph theory suggests that most loops will be found in the giant component, where all neurons anyway light up and their effect is therefore irrelevant to our analysis. Clusters outside the giant component are in general tree-like. If any of the finite clusters do have loops, then their effect will be limited to higher order effects. The initial firing is determined by direct connections rather than by indirect feedback. The effect of feedback loops could only be in stimulating a second or third spike from a neuron that has already spiked.

We checked in the simulations we ran that the results are not significantly altered if no loops are allowed. Since the networks in the simulations naturally include feedback loops, we can conclude that their existence in the neural network does not alter the results that we present.
Figure 1.16: Numerical simulations for four different conditions. Shown are the response curves $\Phi(V)$ (left), the corresponding $H(x)$ functions (insets), and the characterization of the percolation transition (right) for (a) removing edges, $T=1$; (b) weakening edges, $T=1$; (c) removing edges, $T=5$; and (d) weakening edges, $T=5$.
1.7.3 Study of different degree distributions

The behavior of the giant component was examined for other than Gaussian degree distributions. First we studied a Poisson degree distribution: \( p_k \sim e^{-\lambda} \lambda^k / k! \). Both directed and undirected graphs were simulated. For both graphs, the response curves and the giant component behavior were found to be qualitatively similar to the results obtained for the Gaussian distribution. An undirected graph with a Poisson degree distribution provided \( \beta = 1 \), in agreement with the theoretical value. A directed graph with a Poisson degree distribution provided \( \beta = 0.75 \pm 0.05 \).

The results for a power–law degree distribution, \( p_k(k) \sim k^{-\lambda} \), are shown in Fig. 1.17. The disintegration of the giant component looks qualitatively very different compared with the behavior of the giant component for the Gaussian or Poisson degree distributions. The critical exponent \( \beta \) obtained is larger than 1 as predicted by theory [21].

![Numerical simulations for a power–law degree distribution](image)

**Figure 1.17:** Numerical simulations for a power–law degree distribution. Shown are the response curves \( \Phi(V) \) (left), the corresponding \( H(x) \) functions (inset), and the characterization of the percolation transition (right).

1.7.4 Discussion about the value of the critical exponent

The size of the giant component \( g \) undergoes a percolation transition at a critical connectivity \( c_0 \). Below \( c_0 \), the value of \( g \) is zero. Above \( c_0 \), but close to the transition,
1.7 Simulation results

$g$ is described by the power–law $g \sim (c - c_0)^\beta$. In the simulations, the phase transition is smooth near $c_0$ due to finite size effects, rather than being a true sharp transition (Fig. 1.18). The smoothness depends on the number of nodes $N$ in the simulation as illustrated in Fig. 1.18. Due to this smoothness, $\beta$ was calculated only for values of $c$ away from $c_0$. For the Gaussian degree distribution, the value $\beta = 0.66$ was obtained from a power–law fit in which the giant component was studied in the range $0.15 \leq g \leq 0.6$ only. This is the same range of $g$ used in the experiments to determine $\beta$. Hence, due to the lack of accuracy in approaching $c_0$, it is possible that the real value of $\beta$ is larger than 0.66. To verify this, simulations in which the giant component can be measured closer to $c_0$ should be carried out.

For an undirected graph with a Poisson degree distribution and $N = 40,000$ nodes, we obtained $\beta = 1 \pm 0.05$ for $g$ between 0.03 to 0.25. When calculating $\beta$ in the range $0.1 \leq g \leq 0.5$, a value of $\beta \sim 0.7$ was obtained, similar to that obtained for the Gaussian degree distribution. For a directed graph with a Poisson degree distribution and $N = 40,000$ nodes, a value of $\beta = 0.75 \pm 0.05$ was obtained for $g$ between 0.06 to 0.25. For the undirected graph, the simulated results for $g(c)$ are sharper and more faithful to theoretical predictions for the transition near $c_0$, since its number of edges is twice the number of edges of a directed one.

In conclusion, the value of $\beta$ obtained in the simulations for a Gaussian degree distribution is in agreement with the one obtained experimentally. However, the transition in the simulations is smooth due to finite size effects and the real value of $\beta$ may be larger than 0.66, in fact, $\beta$ can reach 1. The disintegration of the giant component of a graph with a power–law degree distribution is qualitatively different than the behavior of the giant component for a graph with a Poisson or a Gaussian degree distribution. It is also very different from the experimentally measured behavior of the giant component.

It is therefore concluded that the degree distribution of the real neural network is not scale–free, but rather Gaussian or Poisson.
Figure 1.18: Smoothness of the phase transition. The giant component size $g$ as a function of the fraction of remaining edges $c$ close to $c_0$, for increasing number of nodes $N$ in the graph. The smoothness of the transition decreases as $N$ increases. Inset: The entire $g(c)$ curve. Away from $c_0$, $g(c)$ looks similar for all values of $N$.

1.7.5 Cluster distribution

We have undertaken a number of simulations to reproduce the cluster distribution measured experimentally (Fig. 1.13a).

The first, and simplest, graph we analyzed was one with an artificially induced highly clustered connectivity. We generated a network where most of the links are located in highly connected clusters, with only weak connections between clusters. Fig. 1.19 provides an example of the kind of connectivity matrix that can be obtained using clusters of size $n=15$ and $n=25$. In the simulation we used three cluster sizes $n=15$, 25 and 40, each located on different areas of the diagonal.

The $p_s$ distribution obtained from the breakdown of connectivity in such a clustered network is shown in Figs. 1.20a-c. As expected, a high connectivity between neurons ($c = 0.3$) reinforces the clusters, and thus $s=15$, 25 and 40 are the dominant peaks (Fig. 1.20a). At lower values of the connectivity, the peaks at $s=15$, 25 and 40 decrease in amplitude, while other peaks appear (Figs. 1.20b-c).
1.7 Simulation results

Having demonstrated the importance of highly connected clusters, we went on to consider realizations of a graph that would be closer to the experimental network. We therefore introduced the notion of distance, placing all vertices on a spatial grid. Three different configurations were used: (i) a Gaussian degree distribution with no locality, (ii) a Gaussian connectivity with local connections and (iii) a Gaussian connectivity with distance dependent link strength.

The results are presented in Fig. 1.21. For the non-local Gaussian (Fig. 1.21a), the distribution has no dominant peaks. The network comprises of small clusters and $p_s$ decreases rapidly showing no significant clusters above $s = 10$. For the local Gaussian (Fig. 1.21b), the existence of isolated peaks becomes apparent. However, these peaks are small. In the case of a local Gaussian in which the link strength decreases with distance (Fig. 1.21c), most of the clusters are still small, but the reinforced connectivity significantly increases the probability to have isolated input-clusters of sizes larger than $s = 10$.

In conclusion, the numerical simulations demonstrate that high locality is important for the presences of isolated peaks in $p_s(s)$. The strong local connectivity
Figure 1.20: $p_s(s)$ distribution for a highly clustered network with three different sizes of dominant clusters, $n=15$, 25 and 40. (a) Connectivity $c = 0.3$. (b) $c=0.25$. (c) $c = 0.2$. 
Figure 1.21: $p_s(s)$ distribution for (a) Gaussian degree distribution without locality, (b) Gaussian connectivity with locality, and (c) reinforced local Gaussian connectivity. The distributions are shown for different values of the connectivity $c$. 
substantially increases the presence of loops in the network. We can conclude that the $p_s(s)$ distribution shown in Fig. 1.13 for the real neural network is characteristic of a local connectivity, and that many loops could still be present even for high concentrations of CNQX.

### 1.7.6 Checking the cluster distribution

According to the model, the cluster distribution of the network, $p_s$, is extracted by fitting $H(x)$ to polynomials in $x$. To verify whether the model provides the correct cluster distribution $p_s$, the results for $p_s$ obtained by fitting $H(x)$ to polynomials were compared to the ”real” cluster distribution extracted directly from the connectivity matrix $C_{ij}$. Three different cases were analyzed. In the first case, a connectivity matrix with predefined clusters of sizes 2 to 7 with no loops was generated. In the second case, a connectivity matrix with predefined clusters of sizes 2 to 7, containing loops, was generated. In the third case, a random connectivity matrix was generated according to a Gaussian degree distribution. Fig. 1.22 shows $p_s$ for the three different cases. As shown, for all three cases, $p_s$ obtained by fitting $H(x)$ to polynomials is in a good agreement with $p_s$ extracted directly from the connectivity matrix.

### 1.7.7 Inhibition

To simulate a neural network with inhibitory neurons, a connectivity matrix was generated according to a Gaussian distribution. A subgroup of nodes was randomly selected to be inhibitory neurons. All outgoing edges of these neurons were assigned with a weight of $-1$. The simulations were carried out as described previously, with edges removed and $T = 4$. The simulations were repeated for different fractions of inhibitory neurons ranging between 0 to 50%. In all cases a phase transition occurred with the same critical exponent $\beta = 0.66$. The critical connectivity $c_0$ at which the giant component emerged, depended linearly on the number of inhibitory neurons as shown in Fig. 1.23. As expected, more connections were required for the presence of a giant component, to compensate for the inhibitory connections as the number of inhibitory neurons increased.
1.7 Simulation results

Figure 1.22: Comparison between the cluster distribution obtained from $H(x)$ and the "real" cluster distribution extracted directly from the connectivity matrix $C_{ij}$ for: (a) defined clusters containing no loops, (b) defined clusters with loops and (c) random connectivity matrix built according to a Gaussian degree distribution.

Figure 1.23: Linear dependence of the critical connectivity $c_0$ on the number of inhibitory neurons. A linear fit provides a slope of 0.28.
1.7.8 Summary

In summary, the simulations show that the assumptions made in the graph theoretic model do not detract from the validity of the results in describing the experimentally observed percolation transition. Simulations of the modified model qualitatively reproduce the response curves, with the presence of a giant component that undergoes a percolation transition at a critical connectivity. The simulations also show that a Gaussian connectivity yields a value of \( \beta = 0.66 \), and suggests that the connectivity in the real network is Gaussian rather than power–law. Also in agreement with the experiments, the inhibition changes the value of the critical connectivity \( c_0 \) but the exponent \( \beta \) remains the same. The agreement of the critical exponent obtained from the simulations with the one reported experimentally indicates that the model contains the essential ingredients necessary for interpreting the experimental results.
1.8 Stimulation with bath electrodes

The basis for the novel experimental approach presented is the simultaneous stimulation of the entire neural culture with the two parallel bath electrodes. The electrodes are made of platinum wires and the chemical reaction describing their function is:

\[
e^{-} + H_{2}O \rightarrow OH^{-} + \frac{1}{2}H_{2}
\]

\[
H_{2}O \rightarrow 2H^{+} + \frac{1}{2}O_{2} + 2e^{-}
\]

As described earlier, the platinum wires are fixed at the bottom of the recording chamber. The platinum wires are inexhaustible and can be used for many experiments.

The global stimulation of neurons with bath electrodes is mostly used in the study of exocytosis for the loading of fluorescent markers into vesicles in the synapses (see for example [45]).

1.8.1 Stimulation parameters

The neurons were stimulated by applying a bipolar pulse through the wires. Applying a unipolar pulse would result in the accumulation of ions next to the electrodes, causing local changes to the pH. Such a change may damage the neurons and bring to their death. The pulse duration for the stimulation of the neurons was set to 20ms (10ms positive and 10ms negative) and its amplitude ranged between 2V to 6V. With shorter pulses, higher stimulating voltages were required for activating the neurons. An example of the response of a neuron to a stimulation with the bath electrodes is shown in Fig. 1.24. In this example, the neuron fires 5 action potentials in response to the stimulation, followed by 2 spikes due to spontaneous activity. The neural activity was simultaneously measured electrically with an extracellular electrode (using the loose–patch technique) and with the fluorescent calcium–sensitive dye Fluo-4.

When a fully connected network ([CNQX]=0) is stimulated, the neurons respond with a burst of spikes, causing synaptic depletion. A recovery time of $\sim 10sec$ is then needed before another stimulation can be applied. This recovery time limits the
rate at which stimulations can be given. In our experiments, the interval between two consecutive stimulations was set to be larger than 20 sec. With such intervals, the fluorescence response remained constant along many stimulations. Smaller time intervals resulted in a decrease of the fluorescence response and to an increase of the baseline fluorescence due to the cell’s inability to properly regulate its calcium level.

1.8.2 Neural excitability dependance on axons’ orientation

Linear neural cultures were used to check whether physical geometry such as the axon’s orientation relative to the external applied electric field affects the neuron’s excitability.

Experimental setup

Experiments were performed on linear cultures of rat hippocampal neurons prepared as described by Feinermann et.al. [44]. In such cultures, the axons grow mainly parallel to the lines. The samples utilized in these experiments consisted of three lines: two perpendicular lines and one line at 45° between them as shown in Fig. 1.25. The cultures were placed in the recording chamber containing two parallel platinum wires.
as described earlier. The lines orientation relative to the electrodes could be changed during the experiments. The cultures were stimulated with increasing current pulses and the threshold of each line to fire was measured. The threshold of a line is determined as the lowest stimulating voltage required for the activation of the line.

**Figure 1.25:** A bright field image of the linear cultures utilized for measuring the neurons’ threshold dependence on their orientation relative to the electrodes. The cultures’ line width is $\sim 180\mu m$.

**Model**

Activation of a cell with an extracellular electric field can be modeled with cable theory. For a cable of length $2L$ placed in a constant uniform electric field $E$, parallel to the cable, the membrane potential is given by ([46])

$$V_m(x) = \lambda E \frac{\sinh \left( \frac{x-L}{\lambda} \right)}{\cosh \left( \frac{L}{\lambda} \right)} \quad (1.14)$$

where $\lambda$ is the cable’s space constant defined as $\lambda^2 = R_m/R_i$. $R_m [\Omega \times m]$ is the membrane resistance and $R_i [\Omega/m]$ is the internal resistance of the cable. The maximal membrane polarization occurs at the end of the element where:

$$V_m = \lambda E \tanh \left( \frac{L}{\lambda} \right) \quad (1.15)$$

This term tends to $EL$ for $L \ll \lambda$ and $\lambda E$ when $L \gg \lambda$. 
Results

The threshold of a line $V_T$ as a function of its angle $\alpha$ for $\alpha = 0^\circ$, $45^\circ$ and $90^\circ$ is shown in Fig. 1.26. The line perpendicular to the electrodes ($\alpha = 0^\circ$) has the lowest threshold, the next line to fire is the $\alpha = 45^\circ$ line and the line parallel to the electrodes has the highest threshold. The line’s threshold is described by $V_T(\alpha) = V_o/(1+b \cos \alpha)$ with $V_o = 7.5$ V and $b = 0.37$. The results indicate that two factors contribute to determine the neurons’ threshold. One is an isotropic contribution due to the dendritic tree and the second is a directional contribution set by the axon orientation. The polarization of the soma is given by $V_s = E d_s$ where $d_s$ is the soma’s diameter. This polarization is negligible compared to the polarization of the axon or dendrites, which is of the order of $EL_{A/D}$ where $2L_{A/D}$ is the axon’s or dendrites’ length.

The threshold electric field $E_{A/D}$ of an axon/dendrite is given from Eq. 1.15 to be: $E_{A/D} = U_t/\lambda_{A/D} \cdot \coth(L_{A/D}/\lambda_{A/D})$, where $U_t$ is the membrane threshold for firing. The ratio $E_A/E_D$ is then:

$$\frac{E_A}{E_D} = \frac{\lambda_D \tanh(L_D/\lambda_D)}{\lambda_A \tanh(L_A/\lambda_A)} \quad (1.16)$$

The typical diameters of axons and dendrites are $d_A = 0.5 \mu m$ and $d_D = 1 \mu m$ respectively. The length of an axon in a line is $2L_A = 300 \mu m$ ([44]) and the dendrites’

![Figure 1.26](image_url)

**Figure 1.26:** The lines’ threshold $V_T(\alpha)$. Data is the average over $N=12$ experiments. The red curve is the function $V_T(\alpha) = V_o/(1+b \cos \alpha)$ with $V_o = 7.5$ V and $b = 0.37$. 

lengths is assumed to be equal to the lines width which is 180µm. Substituting these values into Eq. 1.16 provides $E_A/E_D \approx 0.6$. This is of the same order as the lines’ threshold ratio $V_T(\alpha = 0^\circ)/V_T(\alpha = 90^\circ) = 0.7$.

In the 2-dimensional cultures, the axon of each neuron follows a complex path rather than a simple linear trajectory in an arbitrary direction. Therefore, each axon has segments lying in different orientations relative to the stimulating electrodes. This implies that individual neuron’s threshold doesn’t depend on its orientation relative to the electrodes; therefore, the sample’s orientation in the experiments, carried out with 2-D cultures, is of no importance.

It is concluded that the neurons’ threshold distribution when stimulating 2-D cultures with bath electrodes (Fig. 1.8 $[CNQX] = 10\mu M$ curve), is an intrinsic property of the neurons and not caused by the directionality of the electric field.
1.9 Methods

1.9.1 Cultures of hippocampal neurons

Primary cultures of rat hippocampal neurons were prepared following Papa et al. [42]. Briefly, 19-days-old embryos were taken from Wistar rats. The brains were removed and the hippocampi dissected out and dissociated by mechanical trituration. The dissociated cells were plated onto 13-mm glass cover slips (#1 Menzel-Glaser) coated with Poly-l-lysine (Sigma P2636) at 750,000 cells/ml. The cells were incubated at 37°C, 5% CO2 in plating medium [Eagle’s MEM supplemented by 5% heat-inactivated horse serum, 5% heat-inactivated fetal calf serum, 0.6 % glucose, gentamicin (20µg/ml), glutamax (Gibco) and B27 supplement (1µl/ml)]. After 3 days the medium was switched to one that contains, apart from 10% heat-inactivated horse serum, FUDR (5-fluoro-deoxy-uridine) and uridine to inhibit further division of glial cells. The final change of medium 3 days later included 10% heat-inactivated horse serum, glucose, gentamicin and glutamax.

1.9.2 Imaging neural activity with calcium sensitive fluorescent dye

14 to 21–days–old cells were incubated for 60 min in recording solution (128 mM NaCl, 1mM CaCl2, 1mM MgCl2 45 mM sucrose 10 mM glucose and 0.01M Hepes; pH is titrated to 7.4) in the presence of 2µg/ml cell-permanent Fluo4-AM (Teflab), a calcium–sensitive dye. Cells were then washed with recording medium and placed in a recording chamber, maintained at room temperature. The culture was imaged through a Zeiss Axiovert 135 TV microscope with a 10X objective. Neural activity was measured through EPI-fluorescence. The sample was illuminated with an Arc lamp (Solarc light) through an excitation filter of 475nm and the fluorescence light was collected through a 535nm filter. Images were captured with a cooled charge–coupled device (CCD) camera (Sensicam QE) at a rate of 5 frames/s.
1.9.3 Pharmacology

Synaptic strength was reduced by bath application of several antagonists. The excitatory network was gradually weakened by blocking the AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors with increasing amounts of either CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) or DNQX 6,7-Dinitroquinoxaline-2,3(1H,4H)-dione). NMDA (N-methyl-D-aspartate) receptors were completely blocked with 20 µM of the corresponding antagonist APV (2-amino-5-phosphonovalerate), enabling the study of network breakdown due solely to CNQX. To study the role of inhibition, we performed experiments with inhibitory neurons either active or blocked with 40 µM of the GABA (Gamma-aminobutyric acid) receptor antagonist bicuculline.

1.9.4 Data Acquisition

As described earlier, neural activity was measured with the calcium sensitive dye Fluo-4. Measurements of the fluorescence intensity were conducted on-line with a self written LabView software. ~ 90 region of interest (ROI’s) were chosen on the acquired image, each ROI surrounding a single neuron. The average fluorescence intensity of each ROI was measured as a function of time at a rate of 5 frames/sec. The fluorescence intensity of the ROI’s was displayed on-line on the computer’s screen for monitoring and saved onto the computer’s disc for further off-line analysis. The captured fluorescence images were also saved onto the computer’s disc for further off-line analysis, enabling to track the activity of a larger number of neurons (~ 400). The stimulating voltage was simultaneously measured with a data acquisition board (National Instruments PCI-6052E) at a rate of 1KHz and saved onto the disc.

1.9.5 Data Analysis

The data analysis was performed off-line. The fluorescence intensity of all of the neurons (~ 400) was measured as a function of time by selecting ROI’s around single neurons and measuring their average fluorescence intensity. An estimate of the
mean fluorescence and standard deviation (SD) was calculated for each neuron and the baseline $f_0$ was calculated from the fluorescence intensity during the non-firing periods. A neuron was considered as if it fired if its fluorescence signal $f - f_0$ was larger than $4 \cdot SD$ (see for example Fig. 1.5c).
1.10 Discussion

Neurons in primary dissociated cell cultures form complex neural networks that differ from real brain networks both in structure and in function. From a physicist’s point of view, studying the self assembly of living neurons into complex functioning neural networks is of great interest. Exploring such networks and understanding the underlying principles behind their structure is a challenge toward the understanding of how such networks function and what are their capabilities. Although different from the real brain, cultured networks constitute a minimal model in which computational and physical processes can be investigated in details.

In this work, a novel experimental approach for quantifying statistical properties related to the connectivity of neural networks was developed. The neural networks are studied by measuring the neurons’ response to a global electrical stimulation. The network is weakened by lowering the neural connectivity and the size distribution of connected components is quantified using a graph theoretic approach. The major advantage of this novel experimental approach is that it enables to measure and quantify properties related to the connectivity of the entire network in a single experiment. Another advantage of this approach is its relative simplicity. The stimulation of the neurons with bath electrodes and the recording of the activity with a camera are both relatively easy to implement.

Instead of utilizing the global stimulation, one could think of performing percolation experiments while stimulating single neurons with a local electrode and exploring how the activity propagates along the network. Such an approach suffers from two main drawbacks. First, stimulating one neuron may not suffice to generate activity in the network since neurons need to receive more than one input in order to fire. Second, the stimulation of a single neuron or even of a small group of a few neurons would provide information only about the cluster to which the stimulated neuron belongs and not of the entire network.

Measuring the network response to a global stimulation while looking at the network’s connectivity as a control parameter turns out to provide relevant information about the connectivity of neural cultures.
Performing experiments with a completely disconnected network provides the probability distribution of individual neurons to respond to the stimulation applied with the bath electrodes. The neurons’ threshold distribution turns out to be Gaussian as demonstrated in Fig. 1.8.

Experiments performed while controlling the network’s connectivity showed that two–dimensional neural cultures are an experimental system in which a clear percolation transition can be mapped in detail. At low connectivity, the network consists of small clusters. As the network’s connectivity increases, a percolation transition occurs at a critical synaptic strength with the emergence of a giant component. Next to the critical connectivity, the emergence of the giant component is characterized by a power–law with a critical exponent of $\beta = 0.66$. Experiments with cultures in which inhibition was completely blocked resulted with the same value of $\beta$ indicating that it is an intrinsic property of the network and doesn’t depend on the balance between excitation and inhibition. The value of the critical connectivity $c_o$ at which the transition occurs does depend on the balance between excitation and inhibition. With no inhibition the giant component vanishes at a much lower connectivity. Therefore, one can think of the effect of inhibition as effectively reducing the number of inputs that a neuron receives on average.

It is possible to estimate the degree distribution of the network by comparing the experimental disintegration of the giant component $g(c)$ and the value of $\beta$, with the behavior of the giant component and the value of $\beta$ obtained from simulations or theoretical models. Numerical simulations of our model with a Gaussian degree distribution provided a $g(c)$ behavior that strongly resembles the experimentally one. The value of $\beta$ obtained for these simulations was $0.66 \pm 0.05$, as in the experiment. Percolation on a random graph with a power–law degree distribution yields a $g(c)$ that differs qualitatively from the one obtained experimentally. These simulations give $\beta \geq 1$, where its exact value depends on the degree exponent $\lambda$. Since both the behavior of the giant component and the critical exponent $\beta$ are clearly different from the values obtained experimentally, we conclude that the degree distribution in the cultured network is not a power–law.
1.10 Discussion

Analyzing the cluster distribution $p_s$ of clusters not belonging to the giant component showed that the distribution is peaked rather than a broad one. Such a peaked distribution hints for the presence of loops and for a high local connectivity. High locality substantially increases the number of loops in the network. Numerical simulations demonstrated that indeed, a strong local connectivity increases the presence of peaks in $p_s$. It is concluded that the peaked cluster size distribution obtained experimentally for the living neural network is characteristic of a local connectivity, and that many loops could still be present even for low connectivity (high concentrations of CNQX).

In the experiments, we defined $c = 1/(1 + [CNQX]/K_d)$ to be the parameter describing the connectivity of the network. The parameter $c$ takes values between zero and one and conceptually, it quantifies the number of receptor molecules that are not bound by the antagonist CNQX and therefore are free to activate the synapse. To check whether $c$ is a good parameter for describing the connectivity, experiments were carried out both with CNQX and DNQX, both antagonists of the AMPA receptor. CNQX and DNQX differ in their affinity constant $K_d$. The size of the giant component for experiments performed with CNQX and with DNQX when plotted as a function of the parameter $c$ collapsed into a single curve, indicating the $c$ is a good measure for the connectivity.

The presented approach for studying the connectivity of neural networks supplies a simplified picture for the highly complex neuronal culture, yielding quantitative measures of the connectivity. This experimental approach can be further extended in several different directions. First, experiments can be carried out with different kinds of neural cultures. This research was carried out on hippocampal cultures but experiments can be performed also on cortical cultures for example. Questions such as whether neurons from different brain areas connect differently and form different networks can be addressed. Second, neural networks can be grown in specific patterns and with a controlled design, using specialized patterning techniques. An example of such a network would be a network of clustered aggregates of neurons connected by bundles of axons. The connectivity of such networks can be explored and compared
to the connectivity of regular networks. Third, this research may be extended to brain slices. Since the wiring in brain slices remains unaltered and resembles that of real brain, exploring the connectivity of slices may hint on the connectivity of real brain networks. The differences between real brain networks and cultured networks can be explored, helping to understand the limitations of cultured networks and what capabilities they have. In a fourth direction, the percolation approach can be used to study the growth of neural networks. From the time of plating of the neurons, the network’s connectivity increases with time as axons grow and connect to an increasing number of neurons. Performing experiments at increasing times after the plating would provide information about the increase of connectivity with time and about the growth of the network.
Chapter 2

Measuring neural activity using light scattering

2.1 Introduction

This part of the research work aimed at developing novel approaches and tools for measuring neural activity \textit{in-vitro}. Measuring neural activity both \textit{in-vitro} and non-invasively from the intact brain is a major task for understanding brain functioning. In both approaches, a major barrier encountered is the lack of an "ideal" measuring tool for neural activity. In order to investigate the activity of neural networks consisting of hundreds of thousands of neurons working in synchrony, measuring tools of high spatial and temporal resolutions that allow for measurements from such large neural populations are required.

2.1.1 Measuring neural activity \textit{in-vitro}

Several methods already exist for measuring neural activity \textit{in-vitro}, each with its own advantages and limitations. Single electrode techniques, such as patch clamp or intracellular recording, serve for gathering high resolution data from a single cell or from a small number of cells [47]. The Patch clamp enables the measurement of action potentials as well as sub-threshold activity with high sensitivity. The main drawback of these techniques is that the activity of only a small number of neurons
can be tracked simultaneously. This is because the manipulation of more than a few micro-pipettes becomes very difficult. The highest number of neurons recorded simultaneously using multi site patch clamp recording is 12.

For studying the activity in neural networks, electrical recording from a larger number of neurons is required. Multi Electrodes Arrays (MEA’s) \[48\] can then be used for the stimulation and for recording the neural activity. With MEA’s, the neurons are positioned on top of an array of electrodes and the extracellular potential of roughly 100 neurons is measured. Each electrode records the potential of approximately 3 neurons, and the activity of individual neurons is obtained through spike shape discrimination. The main drawback of MEA’s is that only the activity of neurons positioned close to an electrode, which is a small fraction of the total number of neurons in the network, can be tracked. Another limitation is that only spikes can be measured while sub-threshold activity is not measured.

A more reliable electrode array technique is the Open gate FET micro-arrays developed by Fromhertz et al. \[49\]. Here the neurons are placed above open-gate FET transistors that are used both for stimulation and recording. The electric contacts here are more reliable, however the number of such electrodes is limited compared to the MEA’s. The open gate FET arrays are utilized mainly for measuring the activity of invertebrate neurons.

Advances in optical methods and fluorescent dyes have lead to several optical techniques such as fluorescence microscopy and two–photon microscopy. These methods use fluorescent markers such as calcium–sensitive dyes or voltage–sensitive dyes which change their properties due to electric activity. Most of these markers are toxic and cause cell death after several hours. The use of fluorescent markers enables the monitoring of numerous neurons simultaneously. The calcium–sensitive dyes have a high signal–to–noise ratio (SNR), but since their signal follows the calcium level in the cell, their time course is much slower than the duration of a single spike. Therefore, the activity measured with those markers is from the integration of several action potentials and the exact timing of each individual spike can not be determined. Voltage–sensitive dyes are much faster than the calcium–sensitive dyes and they can
be used for measuring single action potentials. However, the SNR obtained using voltage-sensitive dyes is much lower than that obtained with calcium-sensitive dyes. The usage of fluorescent proteins such as the Green Fluorescent Protein (GFP) [50] is slightly more complicated since it involves some genetic engineering. Most of the fluorescent markers suffer from bleaching, which is a decay in the fluorescence caused by oxidation.

2.1.2 Measuring brain activity

The drawback of the existing methods for non-invasive measurements of whole-brain activity is the inability to combine good temporal and spatial resolutions. Methods based on hemodynamics such as fMRI and PET suffer from poor temporal resolution. These techniques measure only "slow" activity associated with blood flow. Methods that measure neuronal electric or magnetic fields such as EEG and MEG, have excellent temporal resolution but poor spatial resolution. Techniques that combine both fMRI and EEG are difficult to implement since the fMRI's intense electromagnetic pulses interfere with the EEG measurements.

2.1.3 Measuring neural activity with intrinsic optical signals

Advanced optical methods utilize intrinsic optical signals of neural tissue to measure neural activity. Such methods measure processes related to changes of tissue absorption or scattering due to electric activity. The slow optical changes are related to changes of the absorption properties governed by hemodynamics. The fast optical changes are related to the scattering properties of the tissue and they are often tightly coupled to electrophysiological response.

Initial evidence for the relation between neural activity and light scattered from axons was reported by Cohen [51] in 1973. Cohen performed scattering experiments on the giant squid axon and reported both changes of birefringence and scattering changes, correlated with changes in the membrane potential. He concluded that the retardation changes are caused by the re-orientation of dipoles in the membrane due to the electric field induced on it. The changes to the scattered light were thought
to be related to movement of ions causing changes in osmotic pressure, resulting in
volume changes and physical changes of the membrane shape. In 1991, Steponski et al. \[52\] were able to show a correlation between the light scattered from a neuron of the Aplysia California sea slug and its action potential. They presented a model in which reorientation of electric dipoles in the membrane resulting from changes of the membrane potential, is the source of the intrinsic optical signal measured from the axon. Salzberg et al. \[53\] related changes of scattered light from a population of nerve terminals in the neurohypophysis of a mouse to secretion events in the synapses. They suggested the swelling of secreted vesicles, changes in the cytoskeleton or rapid changes in the state of intracellular calcium stores, following calcium entry prior to secretion, as mechanisms responsible for the changes in light scattering. In recent work by Yao et al. \[54\] on the nerve of a lobster, polarization changes due to electric activity were measured using cross polarized reflected light. In their work, the optical signal was related to axonal swelling associated with neural activation.

Optical methods for measuring fast brain activity from the exposed cortex exist. Among them are the usage of voltage-sensitive dyes and light scattering \[55\]. Non-invasive optical methods for measuring ”slow” brain activity, based on hemodynamics, exist and are not far from implementation \[56\]. Non-invasive optical methods for measuring ”fast” brain activity using intrinsic optical signals were reported \([57; 58; 59]\), however they are far from being accepted, understood or widely used.

Although measurements of changes to the light scattered from large scale neurons or axons correlated to neural activity were previously reported as described above, it is still not clear what the physiological mechanism responsible for such changes is. Furthermore, no such measurements from mammalian neurons in culture were ever reported.

The aim of this work was to measure and better understand light scattering from individual neurons in culture. Measuring neural activity using light scattering from individual neurons in culture is important first for the understanding and for the characterization of the cellular mechanisms that are responsible for creating changes
2.1 Introduction

in the scattered light. Second, it is important for the development of new techniques for measuring neural activity both in cultures and from the intact brain.

Measurements of neural activity from a single neuron using light scattering can be extended to measure the activity of a large number of neurons in a network using multiple beams or beam–scanning techniques. Such a configuration can be utilized to measure the neural activity in the experiments described in chapter 1. There are several advantages for measuring the activity using intrinsic optical signals rather than with fluorescent markers in these experiments. The first advantage is related to the toxicity of the fluorescent markers. This toxicity limits the duration of the experiments to 3 – 4 hours. Using intrinsic optical signals, the duration of the experiments can be extended. This would then enable the measurements of a larger number of response curves for each sample or to measure the response curves with a higher accuracy. The second advantage is the ability to measure single action potentials. When the network’s connectivity is low, each neuron fires only once in response to the applied stimulus resulting in a low fluorescent signal which is hard to detect. The detection of single spikes would enable the measurements of the neurons’ response with a higher accuracy especially when measuring their response with high concentrations of CNQX.

In this chapter, an attempt is presented to measure neural activity from cultured rat hippocampal neurons by correlating light scattering and electric measurements. Light scattering experiments were performed on single neurons while electric activity was simultaneously measured through the loose–patch technique. We also present an attempt to measure neural activity from a much larger nerve fiber: the frog sciatic nerve.


2.2 Scattering experiments from a single neuron

In experiments performed by Steponsky et al. [52] on the axon of the Aplysia California sea slug, changes to the light scattered from the axon were measured and correlated to neural activity. The magnitude of the optical signal reported by them was \( \Delta I/I \sim 10^{-4} \) where \( I \) is the intensity of the scattered light. The signal was related to changes of the index of refraction due to re-orientation of dipoles in the axon’s membrane. It is therefore expected that the scattering signal, when performing experiments on smaller neurons, will be equal to or smaller than \( 10^{-4} \). This value sets the highest acceptable experimental noise.

2.2.1 Experimental procedures

To correlate light scattering measurements from neurons with neural activity, we devised an experimental setup (Fig. 2.1a) for performing neural activity measurements using calcium fluorescence or electric recording, and light–scattering measurements simultaneously. The scattering light source was a 830\( \text{nm} \) laser diode. The laser beam entered the microscope’s objective (40X) and was focused onto one of the neuron’s processes as shown in figure Fig. 2.1b. The focused light spot could be visualized with a CCD camera. The forward scattered light was collected through a 0.55 NA condenser and focused onto an avalanche photo-diode (APD). The laser was modulated and the APD signal was measured with a Lock-in Amplifier. Different scattering angles could be measured by using different beam-stops placed on the condenser. Neural activity was measured either optically using the calcium-sensitive fluorescent dye Fluo-4 or electrically with an extracellular electrode. Fluorescence images were obtained through EPI-fluorescence and captured with a cooled CCD camera at a rate of 5 frames/sec. Neural activity was detected as a sharp increase of the fluorescence signal. Electric spikes were measured using the loose–patch technique (Fig. 2.1c). The electric signals measured with the patch–pipette were amplified and sampled. Experiments were performed on primary cultures of rat hippocampal neurons, plated on glass coverslips following the procedure described by Papa et al. [42]. The cultures were used 14–20 days after plating. The neural culture was placed in a chamber that
2.2 Scattering experiments from a single neuron

contains two parallel platinum wires fixed at the bottom, separated by 15 mm (Fig. 1.4). The neurons were electrically stimulated by applying 20 msec bipolar pulses through the wires.

Elaborated explanations of the experimental methods follow at the end of this chapter.

2.2.2 Results

Correlating light scattering to fluorescence signals

In the first experiments performed, we tried to correlate the light-scattering signal to the neural activity detected with the fluorescent marker. We realized that these measurements have several drawbacks. The noise of the scattering measurements

Figure 2.1: (a) Experimental setup of the scattering experiments. (b) IR laser beam (white spot) focused on one of the neuron’s processes. (c) Loose-patch recording from a neuron, the patch-pipette connected to a neuron. (d) IR laser beam (white spot) focused on the sciatic nerve.
Figure 2.2: Electric spikes (blue traces) and the corresponding fluorescence response (red traces) of two firing events of the same neuron. The rising of the fluorescence signal does not occur at a precise time relative to the electric spikes, therefore, it cannot be used as a trigger for averaging the light-scattering signal.

defined as $\text{std}(I)/I$ where $I$ is the intensity of the detected light, was $\sim 1.5 \times 10^{-3}$ for a single trial, which is larger than the expected signal by one order of magnitude. This implies that for reducing the noise, an averaging over many events should be performed.

Averaging the scattering signal is performed by summing the scattered light intensities over successive time intervals defined by the neuronal activity as measured with the fluorescence. In an experiment performed, averaging over $\sim 50$ events, the noise was reduced to $2.5 \times 10^{-4}$. Nonetheless, no scattering signal was detected. In fact, using the fluorescence signal as a trigger is wrong because of the time difference between the onset of the fluorescence signal and the precise timing of the action potential as demonstrated in Fig. 2.2.

Another limitation imposed is that averaging over the fluorescence signal is limited by the slow rate at which stimulations can be applied with the bath electrodes, which is lower than 5/min.
2.2 Scattering experiments from a single neuron

Correlating light scattering to electric spikes

To overcome the limitations imposed by measuring the neural activity with the calcium–sensitive dye, neural activity was next monitored by measuring electric spikes using the loose-patch technique. The higher temporal precision of the electric measurement enabled to average events with a higher temporal accuracy compared to averaging events using the fluorescence signal. As described earlier, averaging the light–scattering signal was performed by summing the scattered light intensities over successive time intervals defined by the electric activity as measured with the electrode. Each stimulation was followed by a series of action potentials enabling the averaging over numerous spikes. Several experiments were carried out while using the electric spikes as a trigger for averaging. An example of the averaged electric activity measured with an electrode and the light scattering traces are shown in Fig. 2.3. When averaging over ~ 150 action potentials the noise was reduced to $8 \times 10^{-5}$. Even with this low noise level, no changes to the intensity of the scattered light correlated to the electric spikes were observed.
Changing the laser wavelength

The scattering cross section depends on the light wavelength $\lambda$. For particles much smaller than $\lambda$ (Rayleigh Scattering), the cross section is proportional to $1/\lambda^4$. In this case, changing $\lambda$ from 830 nm to 488 nm (Ar laser) will increase the scattering by a factor of 8. For particles whose size is of the order of the wavelength, numerical solutions of Mie scattering are utilized. For 1 $\mu$m particles, the scattering coefficient increases by 3 when changing $\lambda$ as described above.

Trying to improve the scattering, the IR laser diode of the experimental setup was replaced by an argon laser whose light was modulated with an acousto-optic modulator. Averaging over electric spikes as described above, no changes to the intensity of the scattered light correlated to the electric spikes were observed, as shown in Fig. 2.4.

Neurons on glass

The neurons utilized in the experiments were grown on a layer of glia cells. Glia are cells that grow with the neurons and support their growth in culture as well as in the brain. Therefore, in the experiments conducted with these cultures, the incident light beam interacted first with the glia before reaching the neuron’s processes. To overcome this problem we utilized a culturing technique in which the neurons grew directly on glass [6]. Briefly, neurons were plated on glass and a glia layer was placed above the neurons during their growth. The glia layer was removed when performing the experiments. With such a technique, one could measure scattering from the neurons without the glia interfering. Experiments were carried out with neurons growing on glass and still no changes to the intensity of the scattered light, correlated to the electric spikes, were observed. An example of the averaged electric signal and averaged light-scattering trace of an experiment carried out with the argon laser, performed on neurons grown on glass is shown in figure 2.4.

In conclusion, no changes to the scattered light due to electric activity were measured from single neurons. The low noise achieved should suffice for detecting small signals of the order of $10^{-4}$. The small size of the axons’ radius ($\sim 0.5\mu$m)
Figure 2.4: Experiment conducted with an argon laser, performed on neurons growing on glass. The averaged trace of the electric measurements (bottom) and the corresponding averaged scattered light measurements (top). The data shown is the average over 400 action potentials. No changes to the scattering signal are observed although the noise is as low as $2 \times 10^{-4}$.

compared to the large radius of the squid axon ($\sim 10\mu m$) may be crucial for detecting small changes to the scattered light due to electric activity. Since the mechanism responsible for the changes to the light scattered from a neuron is still not understood, it is not known whether the largest signal should be measured from the neuron’s axon, soma or dendrites. Experiments were carried out focusing the laser beam on different neural processes as well as on the soma. To better identify the axons, an axon-specific dye should be utilized. To further improve the measurements, experiments should be carried out with the patch clamp technique which enables to control the membrane potential in a precise manner.
2.3 Scattering from the frog sciatic nerve

Since the axon’s size may be a crucial factor for measuring light scattering changes due to electrical activity, we performed experiments on a much bigger sample: the frog sciatic nerve. The frog sciatic nerve is a bundle of aligned axons. It is the main nerve trunk from the spinal cord to the leg. Its diameter is $300\mu m$ and it comprises hundreds of axons. The axons of the frog sciatic nerve are myelinated. Myelin is an isolating layer wrapping the axons that speeds the conduction along the axon.

2.3.1 Experimental procedures

Experiments were performed on the sciatic nerve of the *Rana Ridibunda* frog. The nerve was dissected and placed on top of two stimulating electrodes in a recording chamber containing a ringer solution. The nerve was stimulated at a rate of $0.5Hz$. Experiments were performed within a few hours of dissection. The IR laser beam was focused on a segment of the nerve bundle through a 5X objective (Fig. 2.1(d)). The scattered light was collected through the microscope condenser and sampled as described above. In experiments where the muscle was left connected to the nerve, a contraction of the muscle was observed.

2.3.2 Results

When performing experiments on the frog sciatic nerve, the noise was $\sim 5 \times 10^{-4}$ for a single trial. As described previously, the light scattering traces were averaged for reducing the noise. Averaging over 400 stimuli the noise was reduced to $2.5 \times 10^{-5}$. Even with this low noise, no changes to the intensity of the scattered light correlated to the electric activity were observed as shown in Fig. 2.5.

The low noise level achieved should suffice for measuring signals of the order of $10^{-4}$. In experiments performed by Yao *et al.* [54] on the nerve of a lobster, polarization changes due to electric activity were measured using cross polarized reflected light. The magnitude of the optical signal reported by them was $\sim 1 \times 10^{-4}$. In their work, the optical signal was related to axonal swelling associated with neural
2.3 Scattering from the frog sciatic nerve

![Graphs showing scattered light measurements](image)

**Figure 2.5:** (a). The averaged trace of the scattered light measurements, averaged over 400 action potentials. (b). An enlargement of the trace shown in (a). No changes to the scattering signal are observed although the noise is as low as \(2.5 \times 10^{-5}\). The red bars indicate the time at which a stimulus was applied.

activation. Both the lobster nerve and the frog sciatic nerve comprise of a bundle of axons. Despite this similarity, there might be several reasons why such a signal was not detected from the sciatic nerve. First, axons’ diameters are larger in the bundle of the lobster than in the frog sciatic nerve. While the average radius of an axon in the lobster nerve is \(\sim 50\mu m\), the average radius of an axon in the sciatic nerve is only \(\sim 10\mu m\). Second, while the axons in the lobster nerve are unmyelinated, the axons in the frog sciatic nerve have myelin. If the reason for the changes in the optical properties of the axons is due to swelling as proposed by Yao *et al.*, then myelin surrounding the axon may reduce this effect by a large factor.
2.4 Methods

The preparation of cultured rat hippocampal neurons and the imaging of neural activity were previously described in the Methods section of chapter 1.

2.4.1 Neurons on glass

Cultures of low density (LD) hippocampal neurons growing on glass were prepared in two stages. In the first stage, glia cultures were prepared. 19-days-old embryos were taken from Wistar rats. The brains were removed and the cortices dissected out and dissociated by mechanical trituration. The dissociated cells were plated onto 13-mm glass cover slips (♯1 Menzel-Glaser) coated with Poly-l-lysine (Sigma P2636) at 500,000 cells/ml. 3 dots of wax serving as spacers were placed on the perimeter of each coverslip prior to the coating. The cells were incubated at 37°C, 5% CO₂ in plating medium [Eagle’s MEM supplemented by 105% heat-inactivated fetal calf serum, 0.6 % glucose, gentamicin (20µg/ml) and glutamax (Gibco). After 10 days the medium was switched to one that contains, apart from 10% heat-inactivated fetal calf serum, FUDR (5-fluoro-deoxy-uridine) and uridine to inhibit further division of glial cells.

In the second stage, 19-days-old embryos were taken from Wistar rats. The brains were removed and the hippocampi dissected out and dissociated by mechanical trituration. The dissociated cells were plated onto 13-mm glass cover slips (♯1 Menzel-Glaser) coated with Poly-l-lysine (Sigma P2636) at 40,000 cells/ml. The cells were incubated at 37°C, 5% CO₂ in LD plating medium [Eagle’s MEM supplemented by 10% heat-inactivated horse serum, 0.6 % glucose, gentamicin (20µg/ml), glutamax (Gibco) and B27 supplement (1µl/ml)]. After 3 hours the medium was changed to maintenance medium [Eagle’s MEM supplemented by 0.6 % glucose, gentamicin (20µg/ml), glutamax (Gibco), B27 supplement (20µl/ml), B27 supplement (20µl/ml), 0.1% ovalbumin and pyruvate (10µl/ml)] with FUDR (5-fluoro-deoxy-uridine) and uridine, to inhibit the division of glial cells. A coverslip with 2-weeks old glia culture was placed above each of the coverslip. After 3 days 1/3 of the medium was replaced by fresh maintenance medium and after a further 4 days, 1/3
2.4 Methods

of the medium was replaced again by fresh maintenance medium.

2.4.2 Loose–patch recordings

Electric spikes were recorded using the loose–patch technique. The recording pipette (tip diameter \( \sim 2 \text{ m} \)) filled with recording medium was connected to an extracellular amplifier (A-M systems 3000). The pipette was lowered onto a cell and loose seal was obtained with slight suction. The reference electrode was an AgCl wire immersed in the recording bath.

2.4.3 Data Acquisition

As described earlier, neural activity was measured either with the calcium–sensitive dye Fluo–4 or with an extracellular electrode. Measurements of the fluorescence intensity were carried out on–line with a self written LabView software. A region of interest (ROI) was chosen on the acquired image, surrounding a single neuron. The average fluorescence intensity of the ROI was measured as a function of time at a rate of 5 frames/sec. The fluorescence intensity of the ROI was displayed on–line on the computer’s screen for monitoring and saved to the computer’s disc for further off–line analysis. The electrode’s measurements were simultaneously measured with a data acquisition board (National Instruments PCI-6052E) at a rate of 0.2 KHz and saved to the computer’s disc.

2.4.4 Measuring light scattering

Light scattering experiments were carried out with either an IR laser diode or with an Argon laser.

Measuring light scattering with an IR laser

The light source for the scattering experiment was a 830 nm laser diode (SDL 5432-H1). The collimated laser beam entered the microscope’s objective, either a 40X Zeiss Plan-Neofluar objective for experiments carried out on single neurons, or a 5X
Zeiss A-Plan objective for experiments carried out on the frog sciatic nerve. The backscattered light was collected through the objective and could be visualized with a CCD camera. The forward scatter light was collected through the microscope’s condenser (0.55 NA) and focused onto an Avalanche Photo Diode (Hamamatsu C5460). The laser beam was electrically modulated through the laser driver at a frequency of 47 KHz and the APD signal was measured with a Lock–in Amplifier with a time constant of either 1 msec or 0.3 msec. The lock–in amplifier was sampled with the data acquisition board at a rate of 0.2 KHz.

Measuring light scattering with an Argon laser

The light source for these scattering experiment was an Argon laser (Coherent Inova-70, 488 nm line). The collimated laser beam entered the microscope’s objective (40X Zeiss Plan-Neofluar). The backscattered light was collected through the objective and could be visualized with a CCD camera. The forward scatter light was collected through the microscope’s condenser (0.55 NA) and focused onto an Avalanche Photo Diode (Hamamatsu C5460). The laser beam was modulated at a frequency of 47 KHz with an acousto-optic modulator (Brimrose MTED-60-20) and the APD signal measured with a Lock–in Amplifier with a time constant of either 1 msec or 0.3 msec. The Lock–in Amplifier was sampled with the data acquisition board at a rate of 0.2 KHz.

2.4.5 Sciatic nerve dissection

Rana Ridibunda (Marsh Frog) was pithed and its spinal cord severed. The sciatic nerves were isolated and removed with the Gastrocnemius muscle intact. Proximal end of nerve was ligated. The nerve and muscle were immersed in Frog Ringer solution (NaCl 116mM, KCl 2mM, CaCl2 1.8mM, HEPES 5mM, Ph7.4 with NaOH).
2.5 Discussion

The detection of fast neural activity through intrinsic optical signals could be a method that combines a good temporal and spacial resolution for exploring both the real intact brain and neurons growing in neural networks in-vitro. Although they look promising, such methods are not completely understood and they are far from being applied to measure neural activity both from the brain and from single neurons.

Intrinsic optical signals resulting from neural electric activity have been measured from large scale axons of several experimental preparations since the pioneering work of Cohen in 1973 [51]. In all those studies, the measured signal was found to be of the order of $\Delta I/I \sim 10^{-4}$. Such small signals are hard to detect in a noisy biological environment. The biophysical origins of the intrinsic optical signal are not well understood. It is still under debate whether the signals result from the swelling of the axon, re-orientation of dipoles in the membrane or from secretion events in the synapses. It is also not clear whether this signal has a single biophysical origin for all species and preparations.

Fast intrinsic optical signals have never been measured from mammalian neurons. In this work, an attempt was made to measure intrinsic optical signals from cultured rat hippocampal neurons. Although many experimental configurations were tested and the noise could be reduced to $1 \times 10^{-4}$, no intrinsic signals were detected, setting an upper bound of $\Delta I/I \sim 10^{-4}$ for the optical signal.

It is not clear why intrinsic optical signals were not detectable in small mammalian neurons while they were measured in large axons. The small size of the axons’ radius ($\sim 0.5 \mu m$) compared to the large radius of the squid axon ($\sim 10 \mu m$) may be crucial for detecting small changes to the scattered light due to electric activity. Since the mechanism responsible for the changes to the light scattered from a neuron is still not understood, it is not known whether the largest signal should be measured from the neuron’s axon, soma or dendrites. To better identify the axons, an axon-specific dye such as $\tau – GFP$ should be utilized. To further improve the measurements, experiments should be carried out with the patch clamp technique, which enables better control of the membrane potential.
An attempt to measure a fast intrinsic optical signal from a large system, the frog sciatic nerve, also yielded no results. The noise achieved in this case was as low as $2.5 \times 10^{-5}$. The main difference between the frog sciatic nerve and the lobster sciatic nerve (from which an intrinsic signal was measured [54]) is the myelin sheet that surrounds the frog’s nerve but is absent in the lobster’s nerve. This may hint that measuring neural activity through intrinsic optical signals could be limited to non-myelinated nerves only.
Bibliography


**Independent Collaboration**

- The theoretical model that explains the experimental results in chapter 1 was developed together with Dr. Tsvi Tlusty.

- Experimental setup in chapter 1 was designed and built exclusively by Ilan Breskin.

- Experiments in section 1.4 were carried together with Dr. Jordi Soriano–Fradera.

- All the experimental work and its analysis in chapter 2 were done exclusively by Ilan Breskin.
למרות שהמيدة את אל הגיבחון המושמע הניח, וה מונדיה סף עלינו
לслав המוסי של מaurant ואור מונרונים של הנקים כנותאת מעילית
חשפולות.
הматור

בעדך והיה שיכומך של שני מחק蕊ים משולבבים פיסיקה והקר מוה שמתורו ומתחפפ מציון כל מחקר ושישה 활מן להקר שורחה.

גיור됭.

המקר יראוש עוסק בראכיטוקורטט בקודוורית של רשות הגרוז.

המקר מבצע עשת בסיוריה ודרישות ומדידת תכולות סטטיסטיות הקושה להבורה של רשות הגרוז. הניסיון בוצע על רשות גיורדון-ד-מיידות של חברות ואחרים הקפסים. המקר עשה על דיר

מידיון מונחת גיורדון בﴩ עזוריו שעון גלובלי ה(recipe לכל החשב-ב

מтяז רשות הגרוזים ממוחשב על ידי התלוי הסטריס של

התקנון הנ **************************************************************************

ברשת. התלויות חד及び הדרכים המקרהirement בﴩ וגיורדון

משויכת באתה ו논ז ו فِ רגיס טרוקורטט. גישה ומחוז השפה

למראו מרכבה מואד ולספסיו הורכות מחנה לבקишוריית של

רשות הגרוזים. השיטה המנוסחת היא לכלל כל פיתוחה להקר ר捽

גיוורדון סופות כל קשר וברית Grikk Liên. ירה על ק שיחות ומדאמה גו

לקר הקשוריית של פורטוש מוה.

המקר השתי עוסק בפיתוח של שיווט חישה על מדידה שלפלט עצבית

של גיוורדון בודידי באמת עשת פיוור. אר מתרח המקר היה למדד

שימו בפור אוי גיוורדון של תולטו 2046. את הבסיס בני-פיסיקל הש,strong מאמץ-שפייםール.

מדידת פועלת שלמות גיוורדון באמדעת פיוור אוי חשב ר מעולה

למרכך המנהגונן הפור-אני גיוורדון שלימין חברה המפור-

שנתון. ניסיון,شورו על המקר היא בפיתוח של אמצעה מדידה

השימים של מדידת פועלת עצבית של גיוורדון חברה והקר

מדידת פועלת מ增至ת ברוזה אלא פלישה.

על אם שיוויים בפור או גיוורדון בדיל של חסרי חולות מחלה עבור

פועלת שלמות נמוכה ב’yём, ההסיס בני-פיסיקל הגרוז שליםינוール

עדכי אל מנובו. ירה על ק, מעשה לא נמדו שיווי בפור או גיוורדון

של יניקום חותצאה של פועלת שלמות.