NUDGING NEURONS

Characterizing the behavior of single neurons and small networks in the presence of weak electric fields

> Elles Raaijmakers Public summary





Preface

Of all the PhD theses that have filled up my bookshelf over the years, I have only read a few: the ones that had a direct link with my own research. I have come to realize a few things in the process. One, I need a bigger bookshelf, and two, many researchers pour their heart and soul into a piece of literature that is not even read.

The idea that my thesis would await the same fate and end up under a wobbly table-leg filled me with regret. Because I worked in Amsterdam at the time, I had a lot of time to contemplate my sins during the long train journeys. That is how I came to the idea to write another booklet. One where my ideas and research outcomes would be presented in a way that can be understood by people who are not already world-renowned experts in the exact field of my research.

So this booklet is for you, dear reader. It delights me to know that you have at least read half a page by now, which is more exposure than I expect for my actual thesis. I hope you like my illustrations – I always had a soft spot for comic books – and that you catch some of the ideas that have driven my investigations.

Further, Ionica Smeets pointed out in one of her papers that science communication is often a monologue. Scientists present their findings, but the actual meaning gets lost in translation. I have to agree with her. So if you have further questions regarding my findings or the stories in this booklet, come find me. We can have a drink and a good discussion.



Historical Background

"There is something at work in my soul, which I do not understand." Mary Shelley

Abstract

To understand where we are going, I firmly believe you have to know where we come from. So I wrote this chapter to summarize the journey of electrophysiology to today. I hope it helps you to see the broader picture of the researches I have conducted: the "Why?" behind it all.

Note that this chapter extends beyond the topics covered by my research. As many PhD researchers, I conducted more investigations than were included in my final thesis; some researches were abandoned because they took too much time, some yielded inconclusive results. It happens a lot, unfortunately.

Because I have been working on this chapter during almost my entire PhD, I included things that seemed relevant at the time but ultimately do not support the stories behind my thesis.

In the end, rather than shortening and cleaning the historic background chapter, I decided to leave it as it is. Because, just maybe, a chapter that includes many wrong turns, philosophical debate, irrelevant information and extraordinary amounts of good and bad luck is quite representative for the search for the truth that we call scientific research.

I hope you enjoy reading it.



It therefore took about 1400 years to prove Galen's theory. Fast-forward to 1662, when Dutch researcher Jan Swammerdam performed some ethically dubious experiments on frogs.



The outcome of Swammerdam's experiments showed that a beating heart alone is not sufficient to make motions, and that the nervous system plays a key role in processing information and activating muscles.

Another breakthrough in the field of neuroscience came in 1781 in Italy, by the scientist Luigi Galvani, in what must have been a very, very messy laboratory. His discovery was made like this:











Helmholtz's students also rose to fame: amongst many notable others, he was the supervisor of 4 later Nobel Prize winners and Heinrich Hertz, who died young, before he could receive one.



Back in the 1840's, the search for nerve signals continued without the accurate measurement equipment we have today. Electric currents were measured with a compass. Normally, the needle would be

compass. Normally, the needle would be kept in place by a magnet. The needle would shift, however, if currents would pass through a nearby wire.



Winding the wire around the core multiple times would help to amplify the signal, hence to capture smaller currents.



For his research, the German Emil du Bois-Reymond single-handedly wound more than 24.000 turns ...











The signal Bernstein reconstructed indeed showed the negative current spike that was predicted. Later, these spikes were named "action potentials" or APs.



By the late 1800's, it was known that organic tissue consists of cells – living units embedded in enclosed by a tiny layer called a membrane, embedded in salty liquid. Brain, nerve and muscle cells are called neurons.



Reconstructing the action potential is only Bernstein's second most famous contribution to science. His major achievement is his "membrane hypothesis".



Bernstein combined this with the ideas of Walther Nernst. The Nernst theory - again related to energy storage in batteries - helped to explain how neurons create electric signals.

















Glass and fat are electrical insulators. So the electric currents you measure have to have passed through a hole in the tiny piece of clamped membrane. This way, you can see ions passing through the ion channels.







Neher and Sakmann kept on improving the patch clamp With the patch clamp method, you can measure technique, and discovered multiple new ways to use it. A popular action potentials in any cell that you can patch - you variation is the whole cell method, which is used in this research. are no longer dependent on exceptionally large cells. When patching, you can use a bit of extra Cell force to make a hole in the membrane at the location of the pipette. You can now see the electric Pipette signals flowing through the whole cell. Neher and Sakmann also developed theories about the functioning of ion channels; a theoretic sodium channel is shown here. Sensor: the channel changes shape when the sensor is triggered, such that it opens. Filter: negative ions are repelled, only positive ones can pass. S 2222 Channel: Ions can pass through this tunnel.

The theories of Neher and Sakmann left another riddle. Sodium channels simply filter based on size; only very small, positive ions can pass.

But how is it possible that potassium channels allow the passage of the positive potassium particles, but not the only slightly smaller positive sodium ions?



The puzzle was solved in 1998 by Roderick MacKinnon and his team. He would win the 2003 Nobel Prize in Chemistry for it.

Neher and Sakmann received

the Nobel prize in Physiology

or Medicine in 1991 for their discoveries



You see, charged particles attract water molecules, which are polar. A sphere of water forms around any charge in a watery solution.



Polar molecules are like magnets. They have a positive and a negative side, but their net charge is zero.

To pass through a potassium channel filter, an ion has to break its connections to water molecules. It has to connect to the channel filter molecules instead. These filter molecules are spaced such that they replace a water sphere of a certain size; sodium has a smaller hydration sphere and is less likely to pass.





The team of MacKinnon used, amongst a variety of other experiments and models, X-ray crystallography to determine the workings of the potassium channel. This method uses reflected X-rays to reconstruct the 3D structure of molecules. By today, a.o. the channel structures of the sodium and potassium channels have been revealed by this method.



At this point in the story, it should roughly be clear which electrophysiological processes form the base of neuronal communication. Note, however, that the anatomical structure of neurons is at least as essential to their functioning. The Dutch Antoni van Leeuwenhoek was around 1674 among the first to study the fine details of nerve tissue with his home-made microscopes.



Van Leeuwenhoek's best microscopes magnified a staggering 267 times. Van Leeuwenhoek managed to describe the rough structures of nerve tissues.



Antoni invented staining: he used saffron to colour the tissues he studied.

But brain cells are densely woven with their surroundings. Even with modern illumination techniques, single neurons appear as vague blobs.



So 150 years later, still not much was known about the finer structures of the brain.

As microscope techniques progressed, rough descriptions of cells were made. The first drawing of a nerve cell in 1836 is attributed to the German Gabriel Valentin.



Otto Deiters, also a German, described the neuronal outgrowths in 1865. He differentiated between outgrowths that branch many times (later named dendrites) and a single long neurite that does not branch (the axon).









But his father, a doctor himself, decided that the boy should become a doctor instead.

There was only one tiny thing in the way of dad's ambitions: Cajal had a HUGE problem with authority.



He got kicked out of multiple schools for rebellious behaviour.

On one boring afternoon, Cajal engineered a canon and shot his neighbour's new wooden gate to smithereens.





Years of rebellion followed, as Cajal stood by his dream to become an artist. But when he was 16, his father took him to a graveyard to search for remains for anatomical studies. Sketching bones intrigued the rascal.



His new-found ambitions helped Cajal to eventually graduate as a medical doctor under his father's supervision.



It is interesting to note that, by the time he graduated, Santiago was the calmer son. Little brother Pedro ran away from home at seventeen, hid on a ship to South-America as a stowaway, was keelhauled, became a soldier in the Uruguayan civil war, worked for the revolutionaries for years and ultimately got caught stealing a gun and a horse from the army's general. He was sentenced to death, but the Spanish consulate managed to interfere just in time. Pedro returned to Spain after 7 years of adventures, narrowly escaping death at least 3 times, to later become a successful doctor and professor.









Cajal focused on academics and became professor at the University of Barcelona in 1887. There, he learned of the research of the Italian Camillo Golgi.



Golgi's staining method finally helped to reveal the structures of the brain. Cajal improved the method and, helped by his trained artist's eye and keen intuition, started drawing and describing the things he saw.



Cajal made many notable discoveries. He found that neurons receive information from other cells through their dendrites and send information through their axon, that neuron fibers are not smooth but have little stumps and he discovered and described a number of new cell types and structures.







His most famous work is the 'neuron doctrine'. He proved that each nerve cell is an independent unit, which went against the then-popular theory that all nerve cells function as one.



Santiago Ramón y Cajal and Camillo Golgi shared the 1906 Nobel prize in Physiology or Medicine.



Today, Santiago Ramón y Cajal is honored as the father of modern neuroscience. His art can be found in museums and is as famous as his scientific ideas, so you can argue that the stubborn Cajal did become a world-famous artist after all.













The brain truly is like an electric circuit. Albeit a very, very complicated one.







Study I: Electric fields in neuron-related experimental settings

Modern brain stimulation research partly relies on experiments to predict the effects of electric fields on the brain, and partly on computer models. The data gathered from experiments is used to set up the models, and the models can then be used to further study the behaviour of for example neuronal networks. Experiments are often performed on small patches of brain tissue (1 to 2 cm wide).



During an experiment, an electric field is usually generated from two charged plates, the so-called electrodes. It is often assumed that these electric fields are uniform. This means that the field is equally strong everywhere between the electrodes.

The electric field strength is an important parameter that you need to measure. In a uniform field, it does not matter where you place sensors to measure the field, since it is the same everywhere anyway. Just placing two sensors somewhere between the plates is enough to calculate the fields. This seems to be the current industry standard for measuring fields.

But generating a true uniform field is extremely difficult. In real life, electric fields quickly lose strength at a distance from an electrode. Unfortunately, non-uniform field strengths are more difficult to measure.





A piece of brain (the hippocampus for connaisseurs) in a typical measurement setup. Sometimes single cells are probed and measured, other investigations study the effect of fields on a whole brain slice.

Because these models are used to understand and develop medical treatments, the model details should be as complete and correct as possible. Another reason that the parameters fed to the models should absolutely be correct is the stability of the models. Neuronal models are incredibly sensitive. A small change in a parameter can already lead to big changes in the outcomes.



Top view of a typical experimental setup. Two electrodes, one positively charged and one negatively, are placed in salty water that also contains a reference ground. The electric field that forms between these electrodes is often assumed to be equally strong everywhere.

Furthermore, generating a high-magnitude electric field in salty water is challenging. The liquids surrounding the neurons contain charged particles. Negative charges travel to the positive electrode (and vice versa) and partly counterbalance the potential of the electrodes: a process that is called screening. Screening greatly decreases the electric field strength that you can achieve.

This is a serious issue for some types of experiments. While some brain stimulation strategies use fields of only I milliVolt per millimetre, others go up to a few hundred mV/mm.

The goal for this research was to determine the uniformity and field strength of the electric fields that are generated in different experimental setups. Different conditions such as the temperature, the distance between the electrodes and the electrode shape were investigated.



To predict the electric field distribution and the field strength, we can make use of multiphysics software to calculate the effect of the interdependent physical processes.

Firstly, we modeled the screening losses through a limitation of the electric current. The Butler-Volmer model calculates the electric currents that effectively flow from the electrodes into the solution. These currents are influenced by the electric potentials of the electrodes and the material properties of both the electrodes and the solution.



$J = J_{o}[exp(\alpha_{a}F\eta/R_{g}T)]$ $exp(\alpha_{c}F\eta/R_{g}T)]$

You can calculate the electric fields from the electric currents in the solution. So as a second step, we used the current flow from the electrodes and the material properties of the solution to find these currents and we used this information to calculate the electric fields.

The field shapes are dependent on the electrode shape and the distance between the electrodes. The field strengths depend on the temperature of the solution and the electric potential that is applied to the electrodes. So we calculate the fields for all reasonable values. Reasonable here means temperatures that do not kill the cells (20 to 37 °C) and voltages that do not electrolyse the water (≤ 1.2 V).



The six electrode shapes that were tested: square plates circular plates, thin patches, long wires, short wires, and thick wires. All of these shapes have been used in literature.

We checked the field uniformity with a new method that we developed. Basically, we determine what the field would look like in 3D if it were perfectly uniform. Then we check how much the actual field differs from that. We scale this to a scale of 0 to 1. This scale is not linear so in practice, only really high uniformity values are acceptable.



A heavily simplified plot of the difference between the field and a perfect uniform field vs. the outcome of the uniformity metric. For example, a difference value of 10 means that, considering all points in space, the actual field strength is roughly 10 times lower than for a uniform field (or 10 times higher, theoretically). But again, the actual situation is much more complex.



Not surprisingly, we found that even the most favourable electrode setup generates non-uniform electric fields. The field strengths are much higher near the electrodes. Still, the fields deviate much less at a distance from the electrodes. So if you consider a small patch of tissue far away from the electrodes (which is common with single cell experiments), you are okay. It is still better to include the non-uniformity in the models though. Experiments that intend to stimulate a large patch of brain tissue (full network experiments) do really have to take the non-uniformity of the fields into account, as the fields can be factors higher near the electrodes than midway in between.



Aside from the non-uniformity, note that the maximum field strength is 75 mV/mm rather than 1200 mV/10mm = 120 mV/mm. This loss is mostly due to screening.

The non-uniformity of the fields also means that it <u>does</u> matter where you position the sensors that measure the electric field. Placing them near the electrodes means that you overestimate the field strength of the middle areas; this likely happens occasionally in experimental research. It also means that two sensors are not enough to find the field shape. You need to place multiple sensors between the electrodes: the more, the better.





Furthermore, it was found that roughly 40% of the input energy is lost to screening effects ... in the bestcase scenario. In the example to the left, which shows the most favourable setup, fields of only 75 mV/mm are generated near the electrodes. The field strength even falls to 45 mV/mm midway between the electrodes. This is way below the level of high-intensity brain stimulation research. You can place the electrodes closer together for higher field strength or only measure cells near the electrodes, but that will come at the cost of reduced uniformity.



The local field magnitude and shape estimates for different numbers of sensors. The sensors are assumed to be spaced evenly. Note that the field estimate becomes better for more sensors.

In conclusion, neuronal models are often built upon some (over)simplified assumptions regarding the electric fields. Both the electric field strength and shape should be measured more carefully during experiments and included in follow-up modeling studies. Because neuronal models are so sensitive, the experimental details that are used to create them should be as correct as possible: we all want to predict the effects of brain stimulation, a medical treatment for complex, high-impact diseases, as accurately as possible.







Neurons talk in the form of action potentials, or spikes. Currently, state-of-the-art science only has a vague idea of what they are saying, or where the information is coded. It could be in the number of spikes, it could be the amount of time between the spikes, it could be the exact timing, it likely differs between cell types, a part of the information could be stored in the cell network ... There are many unanswered questions.



A first issue when studying patterns is that neurons are living beings. They often adapt during an experiment. The resistance of the measurement instrumentation can also change as it e.g. gets dirty. You can either do experiments until you find a neuron whose behaviour does not change much (this is done sometimes), or find a way to circumvent these effects.



Metrics often compare two spike patterns by giving a total score of the differences. This means that patterns that e.g. only include spike movements may end up with the same score as patterns with only deletions. We chose a different system, as we saw no way to weigh the different pattern mutation types fairly without knowing what they mean.



But before we get stuck in a loop of philosophy and existential questions: we are not neurobiologists, so we simply cannot interpret the neuronal signals. However, we do want to know whether an electric field interferes with the signal processing of a neuron. So we are just going to describe that something is changing to the spike patterns a neuron produces, without knowing what

it means. Just in case somebody else cracks the neuronal code some day.



The electric resistance of a normal, healthy cell attached to standard measurement instruments over nearly 10 minutes. The resistance changes by tens of percents over only a short time. These changes heavily influence the patterns that a cell produces.

The study of pattern changes itself is also up for debate. There are already some metrics that study changes in the spike activity, but these often only describe the total number of differences between full measurements. But we want to know exactly what these changes are: spike deletions and spike movements might mean something totally different. So we decided to study the alterations per spike instead of all changes at once.

So we had two goals for this research: to stabilize the spike patterns and to construct a way to describe the changes between spiking patterns. For the latter topic, we developed a new metric. To demonstrate the stabilization and the new metric, we used it on a data set of measurements on neurons stimulated with weak (I-5 mV/mm) electric fields.



To gather the data and to demonstrate our methodoly, we performed patch clamp measurements on neurons of the CAI type. These neurons have a role in memory processes and are known to be sensitive to electric fields.

We countered the first problem, the stabilization of the patterns, by regularly measuring the system's electric resistance and scaling the injected currents accordingly.

This seemed to work: generally, about 70% of the patterns overlapped between measurements. This overlap fraction was estimated through a statistical analysis.



36 measurements in the same cell: the redder the trace, the more the patterns overlap. The introduction picture of this chapter even shows 108 measurements in the same cell. You can see that the patterns are quite alike.

With a method to generate reliable patterns in place, you can investigate the effect of the fields. To uncover some fine details of the pattern changes, we compared spike patterns in sets of 3 measurements. Time-wise, neuronal spiking is not infinitely accurate. So we first have to determine which spikes are the same spike. By using a bunch of statistics, we defined windows within which spikes were deemed the same.



We now know which spikes are the same, so we can also find which spikes are different from before. We use this to compare measurements with electric field to measurements without electric field. This way, we can determine what changes in the pattern an electric field introduces. We checked per spike whether the chance of it occurring increased or decreased, whether the spike moved or whether a spike was even completely new.



We found that a negative field (example pattern in blue) decreases the number of spikes by a relatively large amount compared to a corresponding no-field pattern (white pattern). A negative field mainly suppresses the spikes of the original pattern. It does not introduce a lot of spikes at new positions nor does it move spikes.



A positive electric field (red example pattern) increases the number of spikes by a relatively small amount. It increases the number of spikes by increasing the spike chance everywhere. This means that spikes are introduced at new positions, but also that existing spikes have a higher chance of occurring (at some positions, spikes do not occur each time a measurement is performed, see the figure on the previous page).

Positive fields also move spikes. The movements can be explained by the rest period of a neuron. After spiking, a neuron needs to rest for some time before it can generate another spike. It gets tired. So introducing a new spike at a certain time may suppress a spike that would originally follow a short time later. Hence the spike movements. Furthermore, by increasing the overall spike chance, spikes will occasionally show up at entirely new locations. This of course scrambles the pattern.

Overall, a negative field changes the spike pattern by a larger number of spikes and in a more predictable way than a positive field does. It is much more difficult to predict where the extra spikes that are introduced by a positive field end up.

These preliminary results suggest that brain suppression would be an interesting topic to look at. Brain

stimulation is often based on stimulating certain brain areas or cells. Neuronal networks continuously interact and balance each other. Since negative fields appear to yield more predictable results using less intense fields, suppressing the right brain areas or cells might give one more control over the treatment effects. Still, this thought is based on initial results in a small data set for specific cells and would need to be investigated much further.







Study 3: Calcium fluorenscence signal processing software



The third research focused on calcium signals. Like stated in the history chapter, the calcium concentration inside of a cell is an indication of the cell's electric activity, metabolism and ongoing learning processes. Note that the calcium signals vary in shape and duration for these different processes. After an action potential, the calcium concentration rises only for a few seconds. There are also calcium signals that vary on the minute scale.



Calcium fluorescence imaging is a pretty cool technique that makes the calcium concentrations in the cells visible. Basically, you pour a tiny bit of dye on the neurons, after which they light up when a special light shines on them.



The cells glow more brightly when their calcium concentrations rise. Because calcium concentrations rise directly after a cell spikes, you can study the spiking activity of a neuron through calcium imaging.

You can track the fluorescence in large groups of cells at the same time to investigate the communication within a neuronal network. While calcium is only an indirect measure of a neuron's inner processes, it still gives valuable insights regarding the cell's health and state.

The processing of the data related to calcium signal processing is quite intensive. The signals need to be extracted and calculated per cell. There are some software tools that can do this, but these were often based on existing (read expensive) supporting software. Moreover, most tools only track the calcium activity, but do not use this data to study cellular communication. We want to see how information spreads through a network.



An example of likely communication paths between cells in a network, processed by the software that was written by us.

The goals for this research were to develop a software tool to process calcium fluorescence image data. Processing speed and ease of use were essential, as the real-time study of a network of neurons was the goal of future researches. Furthermore, the performance and possibilities of existing software tools were checked out in the process.



With a team including my friend and colleague Fer, we developed a software tool named Calima to help with the calcium imaging processing. To this day, I am very disappointed that a tool to process light-based data for which the software was mainly written by Fer was not named LuciFer (somehow, my suggestion was voted out by all other co-authors).

We thought it was a bad idea to try to fully automate the data processing for something as stubborn as a group of cells. We wanted to give the user some control to fine tune the results. Therefore, we made all data processing steps depend on parameters that are set by the user. Hence, Calima is semi-automated. Still, we made some suggestions for the minimum and maximum expected values of these parameters.

The first data processing step of Calima was the detection of cells. To that end, the calcium imaging data that was studied was first contrast enhanced. Then, a Difference of Gaussians (DoG) filter was applied. A DoG filter blurs the image two times with different settings and then checks the differences between both results. Regions with a stark contrast (so bright cells against a dark background) react differently to the changed blurring settings, so you can find them easily. The edges that were found were then filled up to regions.

Secondly, the data was analysed per region. The signal intensity was found by averaging it per frame over all pixels of the region. Then, a standard formula was applied to calculate the (relative) calcium concentrations from the signals.

A peak in calcium activity can indicate that the cell has just made a spike. So the peaks of the calcium concentration signals were detected next. A so-called sliding z-score was used for this. Basically, you estimate how much noise a signal contains. If a signal rises a certain amount above that standard noise level, it must be a peak.

From the peak activity, the calcium activity could be compared between cells. A Pearson correlation coefficient was used to check if a linear correlation existed between cells. A strong Pearson correlation suggests (but certainly not proves) that cells could be communicating because their activity is aligned. A map was then drawn between the cells with the strongest correlations.

The overall activity of the network was displayed in the form of heat maps.





An analysis on existing data sets and a comparison with other software tools showed that Calima, using the suggested values for the parameters plus some fine tuning, performs nicely regarding cell detection, peak detection and network correlation.



The left view shows a snap shot from a calcium imaging data set, the right view shows the cells found by Calima in blue. Only a single cell (red) in this example was not found, but some cells were merged (grey).



Calima was also found to be sufficiently fast. On a low-performance computer, it can track the activity in tens of cells for data acquisition rates of 10 samples per second. This is the sample rate that is needed to track the spiking activity in cells. The calcium waves that are related to growth and learning processes vary more slowly. To track these, one sample per 10 seconds usually suffices. Calima can track hundreds of cells for that purpose.



This leaves us with some exciting possibilities for future research: to track the activity and learning processes in neurons in real time and stimulate a cell culture accordingly, for example.

In the meanwhile, some new algorithms have been developed by other researchers. There are now faster and more accurate ways to detect cells, means to find a calcium spike and directly analyse whether it is related to cellular metabolism or action potentials, and better ways to follow the information transfer between cells. For a future project, it would also be interesting to update Calima with the state-of-the-art techniques to improve the tool further.



A culture of neurons treated with calcium fluorescence dye Note that there are thousands of cells in such a culture.





Ethical declaration

Some research can, unfortunately, not be performed without the use of laboratory animals. The neurons that were used to obtain the results presented, which involved determining the neuronal firing pattern changes that were induced by weak electric fields, were harvested from mice. The goal of this research was to detect very subtle pattern changes to ultimately further develop brain stimulation treatments for humans. An ex-vivo setup was chosen rather than a setup using cultured neurons because, at the moment of performing said experiments, there was no guarantee that cultured neurons would respond sufficiently similar to either frozen noise current clamp injections or to weak electric fields. Hence, the conclusions drawn from experiments performed on cultured neurons would not be reliable. Furthermore, the neurons had to be measured within hours after harvesting; brain tissue from e.g. deceased humans who donate their body to science has decayed beyond measurement quality by the time it reaches a lab. Brain samples that are removed during surgery can be preserved for experiments, but these samples are usually epileptic or tumorous. In conclusion, samples from other sources than fresh ex-vivo animal samples were deemed unfit for various reasons.

To reduce the number of animals required for the experiments, some measures were taken. Firstly, all experiments were performed with the approval of the committee on animal bioethics of the University of Amsterdam, which oversees that the experiments are performed according to law and according to the newest insights regarding the reduction of animal suffering.

Secondly, the animals were shared between researchers as much as possible. A dozen brain slices could be harvested from a single animal, which is more than a single researcher could possibly investigate during a measurement day. By distributing the brain slices between multiple scientists, up to four parallel sets of experiments could be performed on the brain slices harvested from a single mouse.

Additionally, different standard checks were performed on the patched cells and quite some residual, at first glance seemingly irrelevant, data was saved. Some new questions that would arise in later stages of the investigation could be answered by this extra data, which often prevented the need for new experiments.

Still, while the experiments have been designed to reduce the amount of animal suffering, one can only go so far in minimizing suffering while still using lab mice and rats. Cultured neurons, as stated in the preceding section, do not behave the same as animal brain cells. But that does not mean they never will. The final investigation of my thesis was dedicated to an analysis tool to support Brain–on–a–Chip (BoC) research. This research field intends to recreate the physiological microenvironment of the brain such that cell cultures can operate under "natural" conditions. Recently, papers have been reporting the existence of communication networks in BoC cultures, and, hopefully, BoC setups will eventually replace animal testing.



Personal remark

My research required the sacrifice of tens of animals – I do not even know the exact number, as we shared animals between experiments and the samples were provided by the University of Amsterdam. Still, it fills me with emptiness thinking about how a living creature can be reduced to just a number in the name of science.

I genuinely felt sad every time an experiment failed for whatever reason, because it felt like the stakes were much higher than for any purely engineering measurement. A fellow researcher once told me that you get used to it after a while, but I do not think I ever really did. Maybe it is just me, but I still felt I was not the only one carrying extra weight during my time in the lab: most of the researchers I spoke to said they wished there were good alternatives to animal testing.

I am not against animal testing per se, as it has brought humankind knowledge we could not have gathered otherwise. But the valuable information that was gathered came at a price. I sincerely, deeply hope there will soon be viable alternatives to animal testing. Alternatives like BoC, computer-aided simulations, or maybe an entirely different approach; who knows what human creativity will come up with.

Still, I would like to dedicate a closing gesture to the animals that were used for my research. There is no such thing as a minute of silence on paper, but I think that a moment of reflection is in place. So that is why I will leave the next page empty.





Sources

The story of how our ideas about the brain developed throughout the ages is not a simple one to tell. I had to take some short turns to make things comprehensible. In the process, I had to skip more than a few interesting scientists, explain years of development in the scientific community in one or two words and simplify some of the brilliant breakthroughs to layman terms. So please realize that the story I told you is far from complete.

In this overview of the sources I used, I will try to offer some context or additional information where possible. If you are interested in reading more about the fascinating history of neurophysiology, I recommend you check out the sources I have used to write this chapter. If you only have half as much fun reading them as I had, there is a good chance you end up with an interesting new hobby.

The sources are sorted per page. Image counting goes from the upper left corner to the lower right corner.

Pages

Page 1:[1-5]. Background image 3-5: Old Egypt Glyphs Font [6], displaying the Linux Kernel Init() void [7]. Page 2: [8-11]. Image 5 "Galvani's experiment during thunderstorm" (public domain) [12].

Page 3: [11], [13], [15-17]. Image 2 "experiment at execution" (public domain) [14].

Page 4: [13],[16], [18-26]. Image I "photograph of flowers by Gabriel Lippmann" (public domain) [27].

I could not resist leaving a little homage to Helmholtz, a scientist who made contributions to both physiology and electromagnetism, my specialization. Furthermore, I really liked that his students specialized in heat radiation, the topic of my Master thesis, light propagation and electromagnetic radiation, both closely related to my area of expertise. And I needed a little excuse to draw Gabriel Lippmann, the scientist with the world's most awesome moustache.

Page 5: [25-26], [28-30],[32, Chapter 3]. Image 2 "the action potential as reconstructed by Bernstein" 'Reproduced with permission from Springer Nature' [32].

Page 6: [11] [13] [25] [32] (Chapter 1, 18), [33]

Page 7: [32] (Chapter 2), [34-35]. Image 2 "measurement probe in squid axon" ('Reproduced with permission from John Wiley and Sons') [37]. Image 3 "AP measured by Hodgkin and Huxley" 'Reproduced with permission from Springer Nature' [38].

Note that Bernstein also initially measured the overshoot that was reported by Hodgkin and Huxley, as did some other researchers. After developing the membrane theory, Bernstein determined that the overshoot had to be a measurement error, and the overshoot was overlooked for about 35 years.

Page 8: [32] (Chapter 3, 4), [40]. Image 6 "formulas describing HH-model" [40].

Fatt and Katz later went to win an Nobel Prize in 1970 for other discoveries regarding synaptic transmission. Page 9: [32] (Chapter 4), [41-49]. Image 1 "icons telephone [51] (CC BY 3.0), settings [52] (CC BY 3.0) and learning [53] (CC BY 3.0)". Image 8 "recording of ion channel opening and closing" 'Reproduced with permission from Springer Nature' [50].

Note here that the role of calcium in other nerve cells, like heart and muscle, can differ from the functions that it has in brain neurons.

Page 10: [43], [45], [46], [51]. Image 1 "Differential interference contrast microscopy picture of mouse CAI pyramidal cells" [52]. Image 2 "Action potential in mouse CAI pyramidal cell" [53]. Images 3 and 4: "Neher theory of ion channels" based on [43]. Image 6 "Sodium ion with first layer of hydrogenation shell" based on image [54]. Image 7 "Potassium entering potassium channel" and "filter selectivity" based on [51],[55-57].

Page II: [50], [52], [53], [55-59]. Image I "Sodium channels rendered from x-ray structure SEKO PDB" [60] and "Potassium channels rendered from x-ray structure 3LUT PDB" [61], 3D structure generated by PyMol 1.74 [62], renders by 3DSMax 2016 [63].

Page 12: [58], [59], [64-66]. Image 7 "Golgi stained pyramidal cell" [80] (CC BY-SA 3.0 license). Encouraged by his invention, this was only the beginning of Cajal's fascination for canons. He built many more, as he writes in his memoirs.

Page 13: [31],[79-85]. "All drawings by Cajal now in the public domain".

Page 15: [31], [86]. [87] 'Reproduced with permission from Springer Nature'. [88] Picture with CC-BY open access license.

Page 16: Image 1 : Reprinted with permission from [89]. Image 2: CC-BY image from [90]. Image 4: Image with CC-BY license [91]. Image 5: Image reproduced with permission from Springer Nature [92].

Page 20 – 23: based on Chapter 3 of [93].

Page 24 – 27: based on Chapter 4 of [93].

Page 32 - 34: based on [93].

Page 28 – 31: based on Chapters 5 and 6 of [93] and [94], [95].

39

Visual impressions

Visual impressions have been created for the chapters of this booklet. All graphics were generated using Blender 3.5 [96]. Several pre-existing models have been used to render these impressions. The original sources are mentioned here below. Note that most models have been slightly modified (materials, number of vertices, minor changes of 3D shape) compared to the original sources.

For the cover, 3D shapes were calculated with UCSF ChimeraX 1.5 [97]. The shapes were exported to Blender 3.5 and rendered there. Protein Data Bank [98] shapes SEK0, IK4C, SGJV (sodium, potassium and calcium channels) [66], [99], [100], and 3GD8, 3KJ6, 2KPF, 2JWA (other membrane structures) [101], [102], [103], [104] were used.

The 3D brain renders on page 2 and 42 use a 3D model created by my colleague Steven Beumer. It encompasses a scan of his own brain. The model was used with permission of Steven.

Page 46 uses a 3D model of a hippocampus CAI pyramidal cell [105].

Page 24 was made from 108 measurements in the same cell from the data collected for Chapter 4 of [93]. The graphics were made using a preexisting script [106] and Blender node tree [107].

Page 28 was made from scratch. The code is a small snippet of CALIMA's utils.cs file.

Page 32 uses a preexisting mouse model [108].

Page 36 uses three theses from colleagues [109], [110], [111].

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Elles Adriana Lamberdina Raaijmakers was born on November 16th in 1989. Please do not confuse her with Elles Adriana Van Megen, a woman from Limburg with whom she shares her first two uncommon first names through a cosmic amount of coincidence and who, through even more coincidence, is Elles' mother-in-law.

Elles graduated from the Maria ter Heideschool in 2002 and the Zwijsen college in 2008, coincidentally the same schools her first promotor Martijn van Beurden attended. Some people would find it typical that Martijn's father, who used to be a teacher, once taught a student named Antoon Raaijmakers who is Elles' father – history repeats.

Elles continued the same educational path as her first promotor and obtained her bachelor and master degrees in electrical engineering from Eindhoven University of Technology in 2012 and 2015. During her studies, she became a member of the S5th board of the study association for electrical engineers Thor in the first-ever Thor board with a girl/boy ratio of 50% - a milestone previously deemed impossible. Another milestone during her studies was organizing a study tour to Brazil which was supervised by official-faculty-member-butstudent-at-heart Rob Mestrom. Unbeknownst to Elles and Rob at the time, Rob Mestrom would (weeks) later be appointed as Elles' graduation thesis supervisor and even later supervise her PhD project as well.

During her studies, Elles specialized in biomedical engineering through her minor and electives. During her PhD in electromagnetic brain stimulation at Eindhoven University of Technology, Elles worked part-time at the University of Amsterdam at the Swammerdam Institute of Life Sciences to conduct cell-related experiments. She discovered there that engineering, designing a product where you make the rules, and biology, studying the badly-commented designs of mother nature, are two entirely different things. Still, she considers the new ideas related to biochemical processes and statistics which she encountered at the UvA an invaluable enrichment.



Currently, Elles is working on a research project focusing on integrated circuits with professor Peter Baltus. As the universe would have it, the parents of Elles' partner Edgar got together at the wedding of prof. Baltus, whose wife turned out to be an old classmate of theirs. But before you accuse us of some form of nepotism - we only recently found out.

Other major achievements of Elles consist of catching 630 species of Pokémon and so far completing 3 of all series and spin-offs of Star Trek. In her free time, Elles likes listening to French eighties new wave music, reading ancient biblical scriptures and eating potato chips right out of the bag. Elles has a younger brother and sister, the latter of whom shares her date of birth with Elles' partner Edgar. She has three nephews, who were all born within the same week. Elles herself has one daughter and is expecting a second daughter on the birthday of the first one.

Unfortunately, the statistical methods described in this thesis have thus far proved insufficient to calculate the odds of the coincidences described here occurring. Elles is not planning on naming any future daughters Elles Adriana to avoid further confusion at family gatherings, is considering a career in teaching which leaves the option open to teach Martijn's daughters one day, is not planning on organizing study trips for her other supervisors, is mildly disappointed that there is no unlikely statistical link with second promotor Regina Lüttge, and is delighted she only has to remember a few family birthdays.



Publications

Journals

JI. Elles A. L. Raaijmakers, Rob M.C. Mestrom, Kemal Sumser, Ghassan Salim, Gerard C. van Rhoon, Jeroen Essers, Margarethus M. Paulides, "An MR–compatible antenna and application in a murine superficial hyperthermia applicator," August 18, 2018, International Journal of Hyperthermia. 34, 6, pag. 697–703. Based on work unrelated to thesis.

J2. Fer D. W. Radstake, Elles A. L. Raaijmakers, Regina Lüttge, Sveta Zinger, Jean–Philippe Frimat, "CALIMA: the semi–automated open–source calcium imaging analyzer," I okt 2019, Computer Methods and Programs in Biomedicine. 179, 10 pag., 104991. Shared first authorship.

J3. Elles A. L. Raaijmakers, Nikki Wanders, Rob M. C. Mestrom, Regina Lüttge, "Analyzing Developing Brain–On–Chip Cultures with the CALIMA Calcium Imaging Tool," April 8, 2021, Micromachines. 12, 4, 412.

J4. Elles A. L. Raaijmakers, Rob M. C. Mestrom, Dmitri N. Yousef Yengej, Martijn C. van Beurden, Wytse J. Wadman, "Changes in neuronal firing patterns induced by weak DC electric fields," Manuscript in preparation.

Conference talks

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C2. Debby C.W. Klooster, Elles A. L. Raaijmakers, Maarten J.R.A. Van Rossum, Martijn C. Van Beurden, Paul A. J. M. Boon, Rob M. C. Mestrom, "Non–invasive brain stimulation: From field modeling to neuronal activation," 12 Jan 2021, 2020 50th European Microwave Conference, EuMC 2020. Institute of Electrical and Electronics Engineers, p. 1059–1062 4 p. 9338077.

C3. Elles A. L. Raaijmakers, Zeph M. C. Van Berlo, "Billions of blue blistering barnacles! What we can learn from comics about the visualization of complex ideas," 32nd Annual Conference of the European Association for Education in Electrical and Information Engineering (EAEEIE), June 14–16, 2023, Eindhoven. Manuscript accepted. Based on work unrelated to thesis.

Posters

PI. Elles A. L. Raaijmakers, Rob M. C. Mestrom, Margarethus M. Paulides, "Murine head & neck applicator: hyperthermia prototype development," Joint URSI – NARF – AP–S Symposium, Dec 7, 2015, Drienerburcht, Twente, Nederland. Best poster presentation. Based on work unrelated to thesis.

P2. Elles A. L. Raaijmakers, Maarten J. R. A. v. Rossum, Rob M. C. Mestrom, René M. H.
Besseling, Anton G. Tijhuis, Bert P. Aldenkamp, "The Spectral Content of TMS Signals: An Analysis of Frequency–Related Neurostimulation Mechanisms," Kempenhaeghe congress, March 11, 2016.
P3. Elles A. L. Raaijmakers, René M. H. Besseling, Rob M. C. Mestrom, "Neuronal Targeting with Electrical Stimuli: A description of a setup for an experimental evaluation," Kempenhaeghe congress, March 24, 2017.

P4. Elles A. L. Raaijmakers, Dmitri N. Yousef Yengej, Wytse J. Wadman, "Measuring the Sub– Threshold Influence of an Electromagnetic Field on Neuronal Spiking Behavior," Swammerdam Institute of Life Science congress, June 14, 2018.

P5. Elles A. L. Raaijmakers, Dmitri N. Yousef Yengej, Martijn C. v Beurden, Wytse J.
Wadman, Rob M.C. Mestrom, "Zapping Neurons: Measuring the Sub-Threshold Influence of an Electromagnetic Field on Neuronal Spiking Behavior," Kempenhaeghe congress, June 14, 2019.
P6. Nikki Wanders, Maaike F.J. Fransen, Elles A.L. Raaijmakers, Sija Xie, Regina Lüttge,
"Substrate stiffness effects on neuronal cell culture in vitro," Nanobiotechnology for Cell Interfaces seminar, March 17, 2021.

P7. Elles Raaijmakers, Marco Barros Barreto, Peter Baltus, "Integrated circuits designed by the public," Joint ProRISC – SAFE conference, July 14–15, 2022, Twente, Nederland. Best poster presentation. Based on work unrelated to thesis.





Acknowledgments

Writing a thesis about brain stimulation can – in a way – be summarized as thinking about thinking. I hereby want to thank the many great minds who have shared with me their thoughts and insights. Of course there are some people who deserve a special thanks for their contributions. I will mention them here in semi–alphabetical order.

First and foremost, I wish to mention my promotors and co-promotor. I want to thank Martijn, my first promotor, for the radiant discussions we always had. You are without a single doubt the very best in numerical mathematics, but due to circumstances my project initially deviated far away from the physics and numerical methods that are so clearly your field of expertise. It was very challenging for the both of us to explore the world of physiology from the engineering perspective. You have high standards and really want to understand things, so I am delighted we were able to finish the thesis without diminishing the quality of our work. I am especially proud of Investigation I, where we found our common ground.

Regina, my second promotor, I am always happy to work with you. You see the possibilities in every situation, even when I think we have arrived at a dead end. I have learned a lot from your positive energy. Especially important to me where the personal talks where we discussed the opportunities and the difficulties of being women at a man-dominated and competitive technical academical world. You taught me that it is better to find a way to be me than to see competition in everything. Thank you, I really needed to hear that.

I have always admired the sense of humor of *Rob*, my co-promotor. You have the tendency to say the right thing at the right time, often in the form of complex many-layered jokes with a hidden meaning. You also have a great detailed technical knowledge about a plethora of topics, but it is really your quick wit that makes you the great mentor than you are. I learned from you to be proud to be a teacher, a role of great importance that is too often overlooked in the academic world.

Giving the presentation of your life is a challenge. I was not really looking forward to my defense itself until I got your help, Karin Herrebout. Thank you for your patience, your fantastic advice and your help in shaping my presentation.

I learned electrophysiology and statistics from Helmut Kessels and Wytse Wadman, both of whom contributed greatly to my research. Wytse, thank you for giving me the opportunity to study electrophysiology in the lab. From you I learned that engineering is where you yourself decide the framework of the invention you are developing, and biology is where you study the framework already created by mother nature – and that both sciences require totally different mindsets. I learned a lot from your never–ending flow of new ideas. Helmut, I am glad that you were willing to thoroughly check the methods and statistical analyses of my thesis. I am especially thrilled that you are a part of the promotion committee, as your expertise and advise certainly helped to improve my science.

I also want to mention the many nice colleagues from the Wadman (later Kessels) lab of the University of Amsterdam, especially Dmitri, Michel, Janna, Xante, Cato, Tamar, Niels, Femke, Aile, Hui, Taco, Jan, Natalie, Pascal and Karin. I learned so much about life around an electrophysiology lab from you, an experience I would not have wanted to miss.



My colleagues from the Electromagnetics research group should not be forgotten. First of all, I want to thank my fellow PhD-students, especially TVN, Niels, Anouk, Steven, Martijn, Leroy, Ellen, Teun and Roel. Thank you for sharing memes, stories and good advice. I also received a lot of good advice from my other colleagues, senior fellow researchers and professors, Bas, Roeland, Maarten, Suzanne, Bart and Mark.

René and *Ad*, I am thankful that you both took the time to read and comment on early versions of this booklet.

My new research group, the IC group, also deserves a praise for welcoming me so kindly. So thank you Peter, Margot, Rainier, Remco, Kevin, Erik, Kyle, Martijn, Stijn, Jan, Marco, Eugenio, Marion, Pieter, Georgi, Dusan, Vojkan, Antonio, Piyush, Johan, Corné, Yijing, Irena and the others. I have been working with you with great pleasure, and I am looking forward to working on our future projects.

Pierre and Tim, my fellow graduate students from the hyperthermia group of the Erasmus MC; I always have great fun during our get—togethers. I hope our PhD trajectories will end soon, but that our occasional drinks will last.

My old fellow students, many of whom I met through study association Thor, also mean a great deal to me. I am always happy to join the annual Treasurer and Commissioner of Education dinners, or go to the Walhalla to have a drink with some of my old buddies such as Fer or Daan. But I must admit that I am starting to feel pretty old out there. To my family, my parents, my brother and sister, and my in-laws: thank you for your support. Edgar, my partner, I hope we can always keep on doing inventions and keep on making all kinds of art projects together. And I am looking forward to the day that our children will join our schemes.



Disclaimer

This booklet was created to summarize and support the scientific research presented in the thesis "Nudging neurons: Characterizing the behavior of single neurons and small networks in the presence of weak electric fields".

If you are interested in the details of the thesis, please check it out. A catalogue record is available from the Eindhoven University of Technology Library ISBN: 978–90–386–5727–1. NUR: 959.

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Related research

Page 19 already revealed that quite some exciting research is currently ongoing at Eindhoven University of Technology. In case you are interested in other neuron or Alrelated topics, please visit one of the following websites:

https://www.tue.nl/en/research/research-groups/electromagnetics/ https://www.tue.nl/en/research/research-groups/integrated-circuits/ https://www.tue.nl/en/research/research-groups/signal-processing-systems/ https://www.tue.nl/en/research/research-groups/microsystems/

The research group that I cooperated with during my research at the University of Amsterdam:

https://sils.uva.nl/content/research-groups/cellular-and-computational-neuroscience/ research-lines/research-lines-csn.html

The research pages of my supervisors:

Martijn van Beurden, computational electromagnetism https://www.tue.nl/en/research/researchers/martijn-van-beurden

Regina Lüttge, neuro-nanoscale engineering https://www.tue.nl/en/research/researchers/regina-luttge/

Rob Mestrom, multiphysics and clinical electromagnetism https://www.tue.nl/en/research/researchers/rob-mestrom



