In Vitro Platform for Basic Testing of Micro- and Nanoelectrodes Intended for Chronic Implantation

Daniel Henriksson

Master´s Thesis

Faculty of Engineering, LTH Department of Measurement Technology and Industrial Electrical Engineering Division of Electrical Measurements

Supervisor: Martin Bengtsson

Contents

Contents 2

Abstract 4

Abbreviations 4

1. Introduction 5

1.1. Deep Brain Stimulation Today 7

1.2. A Clinical Example; Parkinson's Disease 9

1.3. On *in vivo* and *in vitro* 11

1.4. On the PC12 Cell Line 11

1.5. The Project 13

2. Method and Materials 14

2.1. The Setup 15

2.1.1. The Faraday cage 15

2.1.2. The recording equipment 17

2.1.3. Miscellaneous equipment 18

2.2. The Cell Cultures' Protocols 18

2.2.1 Divergences from the protocols 18

2.3. Potassium Chloride 19

2.3.1. KCl for PC12 cells 19

2.3.2. KCl for cortical neural stem cells 20

2.4. Control Measurements 21

2.5. Electrodes 21

2.5.1. Rudimentary metal electrodes 22

2.5.2. Glass capillaries 22

2.5.3. Manufactured quartz/metal microelectrodes 23

3. Results and Discussion 25

3.1. The PC12 Cell Line 25

3.2. The Cortical Stem Cell Line 27

3.3. Possible Improvements of the Setup 29
3.4. Evaluation of the Project 30
4. Conclusions 32
References 33
Appendix I.. 37
Appendix II 40

Abstract

Microelectrodes today and nanoelectrodes tomorrow further studies in fields of science such as neurobiology and electrophysiology, and are already in use as neural prosthetics. When electronic devices are used in biomedicine, especially when needed for chronic implantation, their ongoing downscaling is largely motivated by two aspects: biocompatibility and specificity. It has been shown that downscaling is a way to significantly decrease the damage done by such devices.

In the research and development of new, complex devices, these need be continuously evaluated in various ways. *In vivo* experiments are one necessary part, and *in vitro* cell cultures are far from a complete substitute to an entire animal, but they constitute a very useful model nonetheless. There are three main drawbacks to *in vivo* testing: the cost, the complexity of the living organism – which can be an obstacle in routine tests – and animal suffering – the main reason why animal testing should only be used if there is no other sufficient option. Consequently, cell cultures can be much more appropriate in some instances.

The aim of this project was to set up a quick-stop *in vitro* test station at which different electrode designs can be tested against one another. An existing setup originally assembled for patch clamp experiments was used with cell cultures chosen for other experiments primarily. PC12 cells and later mouse cortical neural stem cells were stimulated with KCl to produce action potentials. For PC12 cells no action potentials were registered, and for the cortical stem cells the consistency sought was not achieved. It is possible that this setup, using cortical stem cells, can achieve the intended practical use, but this project cannot assess this finally.

Abbreviations

AP – Action Potential
BMI – Brain Machine Interface
CNS – Central Nervous System
DBS – Deep Brain Stimulation
EEG – Electroencephalography
MP – Membrane Potential
NGF – Nerve Growth Factor
NRC – Neuronano Research Centre
PD – Parkinson's Disease
PLL – Poly-L-Lysin
VGCC – Voltage-Gated Calcium Channels
NB: PC12 is a designated name, not an abbreviation.

1. Introduction

The role of nanoscience in biomedical engineering is increasingly one of concrete utility over abstract concept; science rather than science fiction; and real more than ideal. The nanoscale has much to offer, but many challenges are inevitably involved when working on a scale largely too small for us to directly interact with – making us even further dependent on our tools, practices and theories than within the macro- or indeed microscale. Yet the field's enticing potential continuously drives research and development.

As our base of knowledge grows and cross-scientific projects and collaborations enhance the foundations of much half excavated understanding, along with the emergence of new tools and improved equipment, the boundaries of what we perceive as achievable are continuously pushed back. Within the broad scope of nanoscience, electronic devices find their place and application in biomedicine. Electrodes can be used for communicating with neurons in living organisms, both to the end of stimulating adjacent neurons and to gather information from the living environment, to the benefit of many adjacent scientific disciplines.

Microelectrodes today and nanoelectrodes tomorrow further studies in fields of science such as neurobiology, where they increase our understanding of increasingly specific aspects of the single neuron or the central nervous system (CNS). In electrophysiology they find themselves put to obvious use, and nanoelectrodes have already played an important role as electrical or chemical sensors. One might, for example, use them as amperemeters or voltammeters to observe (changes in potential at) and study exocytosis (the process of neural transmitter release).[1]

When referring specifically to brain-machine interfaces (BMIs, alternatively named brain-computer interfaces, or sometimes (cortical) neural prosthetics) used for example in deep brain stimulation (DBS), the ongoing downscaling of the apparatuses involved is largely motivated by two aspects: biocompatibility and specificity. It has been shown that downscaling is a way to decrease (sometimes drastically) the damage done by such devices, though it is not the only important aspect (others include surface structure, shape, rigidity).[2,3]

In constructing neural probes, reducing damage from the implant is one of the major challenges – and one of the major subjects of research at the Neuronano Research Center (NRC), among other places. The NRC has the explicit view that "The goal of NRC is to develop highly advanced BMI that will enable us to record and/or stimulate hundreds or more neurons with a high spatial and

temporal resolution in freely moving animals. As these electrodes are meant to be implantable in animals and later on in humans for long periods of times, they need to be fully biocompatible, i.e. 'tissue friendly'".[4] Nanowires might have the added benefit of being biodegradable (to some extent). That is, if a nanosize semiconductor wire – a part of a larger BMI complex – breaks off from its substrate, the brain's normal defense mechanisms might handle the alien matter, reducing it to small, containable and removable fragments.[5] This is a very useful step towards complete biocompatibility and reduced medical concerns about chronic neural implants. Especially since biodegradability achieved by choosing a material specifically for that aspect might be more likely to be degraded also when and where it should not be (*i.e.* when working at its intended site). This degradation of implants over time is another issue when developing chronic implants.[6]

To meet the new or improved requirements, novel materials are sometimes chosen. One prolific example is a material used in BMIs at the NRC: the negative resist SU-8. Beyond its biocompatibility, the relative ease of manipulation (by UV-light, neglecting more advanced and expensive techniques) is lauded, and wherefore it has conventionally been used as a pattern transfer substance. But the material also has low Young's modulus (*i.e.* it is flexible), high chemical resistance (durable in a biological environment), is transparent in the visible spectrum and has dielectric properties (offers some interesting possibilities for fine manipulation). At the NRC, SU-8 has seen use together with gold leads to create a flexible multielectrode BMI intended for recording in the cerebellum.[7,8]

To reduce the tissue response from electrodes, one might also opt to improve the implantation method. At the NRC, gelatin embedding has shown promise both in reducing the tissue damage at implantation and at protecting small, sensitive electrodes at that crucial stage. The gelatin will also provide protection from degradation (both from mechanical stress and the biochemical environment) over time.[3]

Naturally, the more and more distinguished ability to in detail control the production of these devices is tantamount to practical advance. While BMIs are unlikely to become a simple, low-cost solution to mundane diseases or conditions – largely because of the cost and risk of human neurosurgery – the ability to produce the devices as effectively as possible need be developed for BMIs to become a more prominent occurrence on treatment level.

But to what extent are BMIs a real treatment option *today*? Well, DBS electrodes have been implanted for decades now, and mechanical limbs exist, though still rare. Cochlear implants are another type of neural interfaces which has achieved mainstream medical acceptance, although they are not CNS implants.[6] The following part will try to provide picture of where we stand today.

1.1. Deep Brain Stimulation Today

DBS surgery has been performed in over 75'000 people worldwide (as of 2010 [9] – 40'000 in 2008 [10]), and has been shown to be an effective treatment for Parkinson's disease (PD), essential tremor, dystonia and obsessive compulsive disorder, conditions for which DBS is a treatment approved by the United States Food and Drug Administration.[9,10] Indications exist that DBS can find further use in epilepsy, depression, Tourette's syndrome, chronic pain and more.[9,10,11] In addition, connecting neural implants to external robotic manipulators might allow people to regain lost muscle control, or to control entirely prosthetic limbs or some device (*e.g.* a wheelchair). In an optimistic future, ALS patients are able to walk and amputees regain all but the finest touch.[6,12]

While we are far from such perfection, the current applications are not essentially different; merely noticeably imperfect. Prosthetic limbs that obtain their commands from either non-invasive (*e.g.* electroencephalography (EEG) recording from the surface of the head – a method with distinct restrictions, largely concerning discernability and depth of recording; not to mention that EEG is for recording only – or functional magnetic resonance imaging, which is much too cumbersome to be of everyday use) or invasive neural recordings do exist, though they are very expensive – in production and in implementation; requiring extensive, laborious efforts from both patient and doctor to be made useful – and thus, tragically, unlikely to be readily available in the near future.[9,10] They are also far from a perfect substitute to our exceedingly complex biological body parts. When looking to improve control – in any BMI-application – invasive methods prevail, and furthering the surgical techniques and the implants' characteristics are necessary steps toward making BMIs less exotic.[9,12] Arguably, the more effective the final product is, the more likely production costs and implementation methodology will be (of interest to be) optimized.

A significant and possibly imminent change could be the development of closed- rather than open-loop BMIs. A closed-loop BMI would not require the abovementioned, meticulous – often trail-and-error – manual adjustment long subsequent to implantation, but instead relies on incorporated continuous feedback systems and the input from the patient. Most importantly, the closed-

loop BMI reacts to its real-time environment, not to the calibrated and presumed state. Trails in primates are ongoing, and closed-loop hard- and software is being developed. It might be necessary – not merely desirable – for a closed-loop BMI to include chemical as well as electrical sensors, to better act as one with its neural environment.[9,13]

While DBS implants have traditionally been of the millimeter or hundreds of micrometer scale, the benefit of having smaller electrodes (and more complex BMIs) is well documented. Among the several advantages are higher specificity and less tissue damage, with fewer and lesser side effects deriving from both previous aspects, and concerning both the short and the long term. In addition, the inflammatory response appears to increase the risk of autoimmune disease later on (including but not limited to Alzheimer's disease); further reason to avoid or reduce it as far as possible.

At the NRC, much effort is dedicated to addressing these issues; partially in developing new electrodes (smaller, new structures, bio-friendly materials), and partially in focusing on the overall design of the implanted components (including anchoring and transmitter).

Biocompatibility is most important for chronic implants, where a foreign object over time will give rise to normal but disruptive tissue responses, such as inflammation, scarring and neural rerouting. One of the most widely acknowledged concerns with traditional, macro-scale DBS electrodes is the glial scarring that develops around the implant over time. Even with improving surgical procedures, the implantation itself will give rise to *some* scarring, but not – today – an amount which cannot be accepted, and worked "around." However, the scarring worsens over time, especially for larger, stiffer electrodes, although indications are that the anchoring mechanism might be almost as important as the BMI itself. With every movement and every breath – indeed, with every heartbeat – the brain moves ever so slightly, seen from the macro-scale; movements which rub the living tissue against the rigid electronic device, damaging and irritating the tissue and giving rise to its protective responses. To the electronics, the glial scarring is the most prominent response. Glial scarring twice decreases the effect of the implant; firstly as the glial cells replace neurons (possibly damaged or dead) from which one would record, secondly as they constitute isolation to and from the neurons beyond.[2]

1.2. A Clinical Example; Parkinson's Disease

PD is a prominent example when discussing practiced DBS. This is largely due to the comparatively specific location of the disease. PD is characterized (primarily) as neurodegeneration in the *substantia nigra* (especially the *pars compacta* region), with a loss of dopaminergic motor control neurons (innervating the *basal ganglia*). Pharmacologically elevating the levels of dopamine at the site is yet the prevailing treatment. Because the blood-brain barrier does not let dopamine itself past, the precursor L-dopamine (levodopa) is administered. However, the systemic administration causes elevated levels throughout the body (albeit suppressed with co-administered substances) and in the CNS (where those co-administered substances cannot – and should not – go). The elevated levels cause a number of side effects, of both systemic and CNS origin; including but not limited to nausea and dyskinesia (involuntary, "faulty" movement; increasing over time).[14]

Early surgical approaches to PD included (irreversibly) lesioning parts of the brain. In Lund in 1960 for example, Svennilson *et alia* developed a procedure for pallidotomy (lesioning at *globus pallidus*, GPi). While these methods were variably effective in removing PD tremor, the side effects were often severe – mainly relating to motor control – and akinesia and rigidity from PD prevailed.[10,11]

For PD, DBS uses high-frequency pulse trains of up to 200Hz, and was all but stumbled upon in 1987.[15] Since then, the target areas have been the same few, which has allowed for some practice, and has inferred understanding about the proximate areas of the brain (partially due to observing side effects). Today, using DBS, the subthalamic nucleus (alternatively the ventrointermediate nucleus (for tremor symptoms only) or possibly the GPi (for which similar direct results have been recorded, yet secondary and side effects need be further investigated)[11]) is often recognized as the point of interest: a small cluster of specific glutaminergic neurons found below the thalamus. However, for all neurodegenerative conditions – even the relatively limited and straightforward PD – the mechanisms are not entirely clear, and alternative theories for the mechanisms of action exist. Beyond the prevailing major hypothesis – silencing of hyperactive (due to lack of inhibiting nervous activity) neurons – several complementary systems (some of which are directly contradictory) might or might not be involved, to degrees of importance not easily determined.[11,14] The complexity of the system(s) involved is awe-inspiring.

Because of the complexity of the situation, undesired effects do arise. One not uncommon side effect of conventional DBS for PD is speech impediment (dysarthria), believed to be connected to breathing muscle control (which give rise to fears of worse possible side effects). There are also cases where DBS is believed to have been directly responsible for depression, even to a suicidal level. Obviously undesirable, such side effects are largely hoped to disappear with higher spatial specificity in stimulation.[11,14]

Side effects become more and more difficult to avoid with progressing glial scarring around the DBS electrode (largely because specificity is lost, and unwanted areas stimulated). Thus, this is an area where smaller electrodes have greater appeal. Microelectrodes have the ability to stimulate a small volume of quite specific nerve cells, whereas nanoelectrodes can stimulate a single neuron, and could potentially – using an array of electrodes with different functions – exert control even greater than 1:1 neuron-electrode interaction.[1,2,5]

It might be worth noting that neural implants are not necessarily a competitor to conventional pharmaceuticals, but rather a compliment. PD is still a good example, and indeed; it is commonplace to treat the disease with complementary implant and pharmaca whenever possible. Among the reasons for this is a limitation of the implant: in contrast to the advantages of the highly specific neural implant, it will not – unlike what is desired and to some extent achieved with drug therapy – protect from disease propagation (unless the resumption of normal function in specific, targeted areas offer such effects secondarily (possibly the case with neurodegeneration)).[11] But even if DBS and neural prosthetics cannot fully take the place of pharmaceutical treatment, possible lowered drug doses could help minimize side effects from the pharmaca.[11,14] One may well hypothesize that future combinations of compensatory or interfering DBS together with pharmaceutics intended to retard or retract disease propagation might prove widely effective, in more cases than PD.

A side note: another use of nanotechnology in PD (or indeed most neural diseases, as well as others) is in constructing biochemical transport vessels which allow drugs to cross (more readily or at all) the blood brain barrier. This too lowers side effects – systemically – as lesser amounts of a drug is needed to give the correct concentration at the site of action, potentially all but completely removing certain (in CNS conditions: systemic ones) side effects.[6]

If the use in PD is relatively straightforward, then the use of DBS and chronic implants in the treatment of psychological conditions is far more controversial.

Advocates sometimes say the ethical issues are not intrinsically distinct from those faced by pharmacological treatments; for which clear guidelines exist. And in major depressive disorder, for example, its use has been approved in severe cases unresponsive to pharmacological treatment. A lot of the concern centers on the concept of personality alteration and the forced divergence from a “true self”, a very complex issue, naturally.[16] More outlandish (or even paranoid concerns) include fears that neural implants could be used for sinister mind-control purposes, or that brain-enhancement will constitute the foundation for a distinct rift between rich “superhumans” and their subjects.[17] While such objections might not be directly relevant to the scientific process, a lot of concern about the ethical implications (and indeed public fears, substantial or not) of emerging scientific disciplines, along with how they are researched, must be taken.

1.3. On *in vivo* and *in vitro*

Ethical concerns can be a strong argument for setting up cell cultures. There are three main drawbacks to *in vivo* testing: the cost, both direct and indirect, as animal facilities and handling are both costly, the complexity of the living organism which – while it is naturally necessary to accept and work in accordance with – can be an obstacle in more routine tests, and animal suffering. Animal testing should only be used if there is no other sufficient option – especially important if tests are intended to be continuously repeated. So while *in vitro* cell cultures are far from a complete substitute to an entire animal, they nonetheless constitute a very useful model which in some instances is much more appropriate.

1.4. On the PC12 Cell Line

PC12 cells are a cell line derived from the pheochromocytoma – a nuroendocrine tumor – of the rat adrenal medulla. The cell line is capable of differentiating into either adrenal chromaffin-like cells – neuroendocrine endothelial-like cells – or more sympathetic neuron-like ones.[18,19,20] Since the 1970’s, this cell line has been used as paraneurons – not true neurons, but closely related and sharing functions – mainly to study neurotransmitter release; exocytosis.[18,21,22] A neuron-like phenotype is often achieved by stimulating the cells with nerve growth factor (NGF), the archtypical neurotropic substance. In either form, the cells synthesize and store catecholamines (dopamine and sometimes norepinephrine), which can then be released through exocytosis.[18,20,23]

Exocytocis is normally preceded by internal signalling within a cell, and (local) shift in membrane potential (MP); with the opening of voltage-gated Ca^{2+} channels (VGCCs) intended. By raising the extracellular KCl concentration and thus creating a depolarization (of the entire cell membrane), VGCCs (especially N-type and, to a lesser extent, L-type channels) are activated in such PC12 cells grown in the presence of NGF, resulting in an influx of Ca^{2+} and leading to exocytosis.[24] The resulting in-surge of Ca^{2+} and subsequent resumption of normal MP (after vesicle deployment) can potentially be registered by voltammetry.[23]

An even more favourable development – for this intended *in vitro*-system – would be if the cells were enough like neuron that they could be prompted into giving off action potentials (APs), or AP-like signals. This could be beneficial because the probability of registering such signals ought to be grater, and also because, possibly, different signals and their amplitudes would be more uniform. Although, as such signals are less sought after in the PC12 cell line, they are both less likely to occur and less well proposed how to look for. They have however been observed (after electrical stimulation).[25]

While neurite growth could likely occur, and can be taken for correlation to the intended differentiation (which is required for the cells to be able to register and respond in the way we are looking to register), the link to the level of symphatetic neuron-likeness – neurite sprouting and branching – does not absolutely and directly correlate to the responsiveness to KCl, the chosen stimulant. And indeed does not guarantee the ability to produce APs (in response to KCl).[20]

When treated with NGF, PC12 cells stop proliferating (dividing) as they differentiate into their neuron-like state. The differentiated cells enter a static G1 state of their cell cycle, which is why – while proliferation can resume some days after discontinued NGF stimulation – they should have been allowed time to fully proliferate, approaching confluency, before its addition.[26,27]

In conclusion, the use of PC12 to model interneurons for the study of signal propagation or AP-like signals is not prominent. However, while such signals might have been desirable – due to the intended application of the electrodes intended for testing, if nothing else – the property demanded from the cells' signals, at this point, is constancy. The so called "release signals" related to the initiation of exocytosis could potentially be registered instead.

During the course of the project, the cell type was switched to mouse cortical stem cells, which are direct precursors to cortical neurons. They should be differentiated into very neuron-like states with the help of NGF.

1.5. The Project

The aim of my project was to set up a quick-stop *in vitro* test station, at which different electrode designs can be tested against one another. (It was also my intention to test some of NRC's electrodes. This aim changed during the course of the project.) While cells would have to be grown in advance, and tests scheduled in accordance with that supply, the actual testing was to be simple and quick. No special training; just enough to familiarize oneself with the equipment and the rigid hygiene regulations prescribed within a cell lab. I used an existing setup originally assembled for patch clamp experiments. The cell cultures were chosen for other experiments primarily. After, and possibly during, the project, I was to appreciate the functionality of the current equipment, as well as the cells' suitability.

2. Method and Materials

The equipment I was to work with was a setup the NRC had assembled for patch clamp experiments, but which was now unused. The cell cultures were chosen for other experiments primarily. While I was to evaluate the usefulness of the existing setup, purchasing new equipment would, beyond the high cost, benefit the project little, as most relevant apparatuses would take months for delivery. Hence, working with what I had, and using a very rough trail-and-error approach, I started (see *2.1 The Setup*).

Early on, a practical problem had to be resolved relating to the micromanipulators – how are they to hold the electrodes? While this could surely have been done several ways; the solution I chose was adequate because it resolved two aspects of the problem: how to hold the wire still and how to hold it close to the sample. With the help from the NRC workshop I copied the one "holder" (a removable plastic piece of which, presumably, there ought to have been two) and held with it a glass tube just wide enough to snugly hold the cable to the electrode. This allowed for a much better angle of approach, compared to if the cable had been held directly at the micromanipulator. This was done for the ground electrode as well as the measuring electrode, although it is not of the same importance here (as the ground does not need to be finely adjusted). I did, though, want to avoid having the ground resting on the edge of the Petri dish, as this could disturb (move or shake) the sample. Also, one would want the surroundings of the ground and the recording electrodes to be as similar as possible, so the ground too ought to rest close to the cells. Movement on the micrometer scale would be of significance – a reoccurring problem to solve or factor to consider. The air pockets on the table were filled only some time into the experiments, and by then the routine was to work around vibrations (*e.g.* form closing the cage or moving about outside) as much as possible. Besides, the cells would move slightly at the administration of stimulating solution anyway.

At this point, the PC12 cells were grown in strict adherence to recommendations (cell growth protocol). This included a collagen-IV substrate. The cells did not appear to like this, and confluence was far from obtained. Nor did the cells adhere well to the substrate, resulting in them being easily dislocated. In fact, the cells grew in clumped colonies several layers thick. The cells also did not display any significant neurite growth. Interestingly, the cells' unintended unwillingness to form a confluent layer might have been the most useful part of the whole PC12 endeavor. While this disguised the problem with using the wire electrode type I was using at the time (the electrode was much too large compared to the

individual cells), it hinted at the possibility of an improved setup using 3D cell cultures.

2.1. The Setup

To start with, I spent some time getting used to the equipment; the differential amplifier, an EX4-400 (Dagan Corporation®), in particular. I read the instruction manual to ensure the specifics were (in theory) proper (especially the internal impedance). I also read about the data acquisition software – Signal 4 (Cambridge Electronic Design (CED)) – and its hardware (Micro 1401 mk II, CED), before looking at the background noise (see *2.4. Control measurements*). Primarily, I attempted to appreciate whether or not the setup's shielding (a Faraday cage) gave adequate reduction of background noise and disturbances from surrounding electronic equipment. The cell culture room was in this aspect suboptimal for the setup, as nowhere could the cage be placed far from potential sources of noise (50Hz wiring in walls and roof, 200Hz fluorescent lights above), although my corner was mostly free from electronic equipment (apart from that of the setup, which – except the computer screen (see *fig.2*) – was within the adjacent electronics cabinet). I also wanted to further familiarize myself with the amplifier and software. This was consequently when I assembled the first, simplest possible test-electrode (see *2.5.1. Rudimentary metal electrodes*).

By now, the PC12 cell cultures are up and running. I used my first batch to simply look at, as the microscope (Olympus AX70) too required some practice. The attached camera made it possible to see and adjust for the small but significant dislocations which almost always occurred when one added the KCl to the sample. After some tuning, the ancient screen would display enough detail that depth could be perceived, although details were never as clear as when looking directly via the microscope within the Faraday cage. Unfortunately, I could not take pictures using this setup.

2.1.1. The Faraday cage

The laboratory room, designed for cell culturing and not for electrophysiological measurements, was far from free from potential sources of noise, but the setup was of course in a Faraday cage. The electronics cabinet was connected to the house's main ground. However, I only later (after the PC12 cell line) connected the Faraday cage to the house's ground wiring. This cut off some additional interference, but not enough to reevaluate the entire PC12 setup.

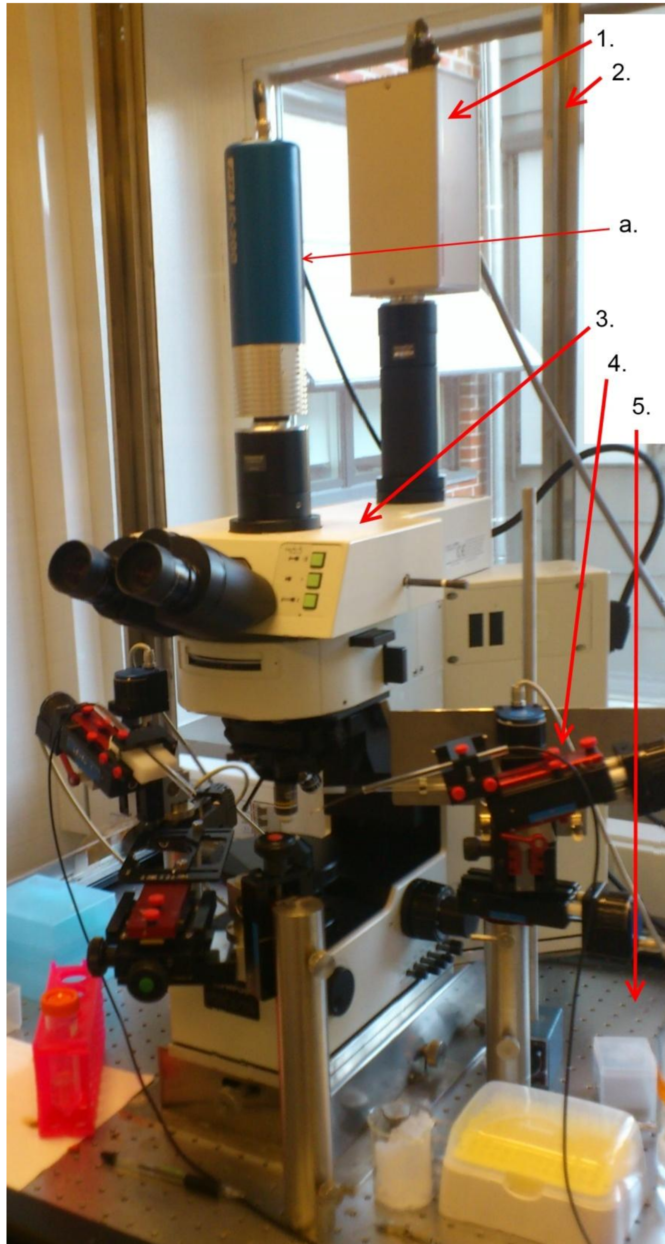

Figure 1. The Faraday cage:

1. C2400 (Hamatsu) real-time observation camera;
2. Faraday Cage (TMC);
3. AX70 (Olympus) research system microscope;
4. Mini 25 (Luigs-Neumann) micromanipulators;
5. Breadboard (TMC) vibration control table.

a. IC-200 (PTI) CCD low-light/fluorescence camera.

2.1.2. The recording equipment

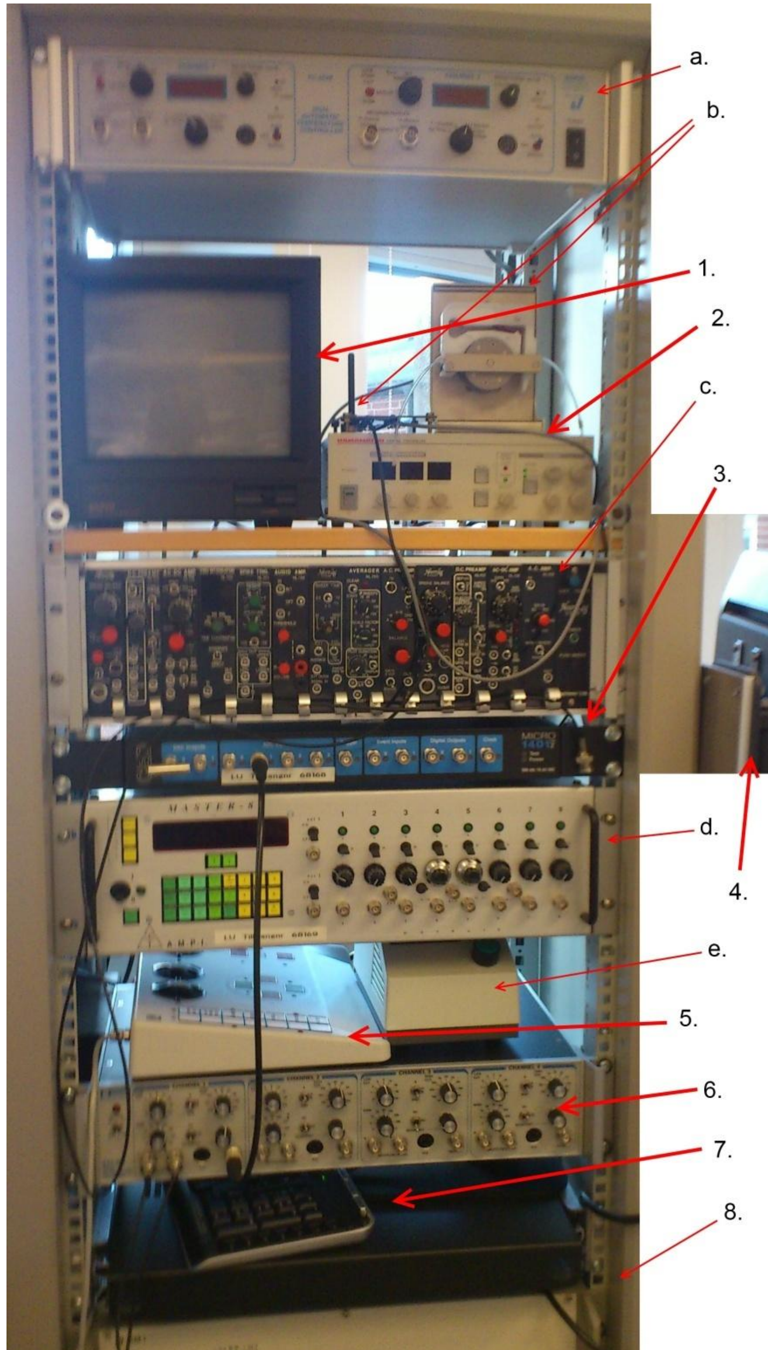

Figure 2. The recording equipment:

1. Video monitor (Sanyo);
2. C2400 (Hamamatsu) camera controller (for *Fig.1.1*);

3.Micro 1401 mk II (Cambridge Electronic Design) data acquisition unit;
4. Computer screen (NB: outside of cabinet);
5. Remote Control SM1 (Luigs & Neumann);
6. EX4-400 (Dagan Corporation) quad differential amplifier;
7. Computer keyboard and mouse (not in picture: PC);
8. Shielding electronics cabinet (Rittal CSM ltd).
a. NeuroLog System (Digimer Ltd);
b. REGLO-Analogue MS-4/6-100 (IsmaTec) peristalic pump & controller;
c. TH3 Halogen Light Power Controller (Olympus);
d. Master-8 (A.M.P.I.) stimulator.

2.1.3. Miscellaneous equipment

ISO-Flex (A.M.P.I.) stimulus isolator;
TDS2002C (Tektronix) oscilloscope;
MCO-20AIC (Panasonic/Sonyo) cell culture incubator;
Centrifuge 5810R (Eppendorf).

2.2. The Cell Cultures' Protocols

I was not the main practitioner of the cell culturing, though I tried my hand at four major steps:

- Flask coating
- Cell thawing and sowing
- Cell expansion/proliferation
- Cell differentiation (subsequent to moving into test receptacle (Petri dish))

I did not do any cell counting (nor were specific numbers relevant to the project) or cryopreservation. The cell culturing followed the same basic format for the two cell types. Protocols found in Appendices I and II for PC12 cells and Neural cortical stem cells respectively.

2.2.1 Divergences from the protocols

PC12 medium used: 86,5% F12-K; 10% Normal Horse Serum; 2.5% Fetal Calf Serum; 1% Penicillin-Streptomyicin; 50ng/ml NGF.

The substrate was changed (from collagen-IV) to poly-L-lysin (PLL; 10 microgram/ml in distilled H_2O) for better cell proliferation and neurite growth.

Plates were coated with PLL for 1 hour at 37°C, and rinsed once with distilled H_2O.

Towards the end of the project, a cover slip (Thermanox plastic cover slips, Nunc) which significantly enhanced the proliferation and differentiation of the cortical stem cells was taken into use. The substrate, PLL, remained the same.

During the last week of the project, due to imminent vacations, there was no time to order more Stem Cell Growth Medium (GIBCO), wherefore the last batch of cortical stem cells was grown and measured on in Dulbecco's Modified Eagle Medium (GIBCO). This medium has similar if not exactly the same ion composition, and I did not alter the applied KCl concentrations (since the approximation of the cells receptivity ought to be greater than the difference between the mediums' ionic concentrations). However, there are no registrations of note from this batch.

2.3. Potassium Chloride

KCl was used as stimulating agent throughout the project. This was primarily based on a number of sources using it to stimulate PC12 cells (see *1.4 On the PC12 Cell Line*), along with how an AP emerge due to depolarization in true neurons. Note that due to difference in permeability and relation between intra- and extracellular concentrations, addition of the same amount of K^+ and Cl^- ions will affect the cells MP to higher and lesser degree respectively, causing an effective depolarization (in a functioning neuron).

A too high concentration of KCl added will give an osmotic gradient capable of inducing lysis in the cell (*i.e.* the cells burst from the pressure).

2.3.1. KCl for PC12 cells

For the PC12 cells, exactly how similar to neuronal activity one might expect them to have was unclear. Exocytosis was possible to achieve in connection to AP-like responses to depolarization, according to references. However, whether the concentration required for exocytosis was actually appropriate could not be elucidated, but most likely an exocytosis inducing concentration would be above the one required for AP-like signals. In addition, if the cells were not capable of producing such signals, exocytosis associated potential shifts might be registered this way.

Since the cells progressively lose their functionality when in room temperature and lacking CO_2 (wrong pH), an estimate is that I needed be done with the measurements after a half-hour. Conversely, the cells need be given time to respond, and too high osmotic pressure (extracellular ionic concentration) would rupture the cells. This is why I add KCl in portions. Ambition was naturally to assess a more exact level of stimulant to be administered -- unfortunately, due to unresponsive cells throughout, this was not achieved.

I started out on the PC12 cell using lesser concentrations than the prescribed amount, but the finishing total – after three additions – was above. But with the lack of response the starting amount was increased, and the end concentration too was raised. I started out using 3 times 100μl of 3M KCl, rising to (200+100+100)μl for later weeks of PC12 trials.

2.3.2. KCl for cortical neural stem cells

Whereas for the PC12 cells I had used a combination of references and trial and error, for the cortical stem cells I made the assumption that they were enough like neurons that I might calculate an approximate amount of stimulant to add. I therefore assume the internal and external concentrations of ions are at natural levels. I also say that the volume does not notably change as I add the KCl (1000:1 ratio); hence other ion concentrations remain. Under these presumed conditions I apply the Nernst equation, referring to the resting potential:

$$-70\text{eV} = K * \ln\frac{[K^+]_{out}}{[K^+]_{in}} \quad \text{(eq.1)}$$

Assuming the potassium concentration outside of the cell is 4,5mM, inside the cell 140mM, and that the temperature is 37°C (which it will start at):

$$K \approx 20{,}36\text{eV}$$

This can then be used to calculate the (outside) concentration needed to achieve a specific MP. As -55 eV is a common threshold value (where an AP might emerge) for neurons, that is where I start:

$$[K^+]_{\text{out-new}} = 140mM * e^{\frac{-55}{K}} \approx 9{,}40\text{mM} \quad \text{(eq.2)}$$

Subtract the amount presumed to already exist in the fluid to arrive at the amount which should be added:

$$9{,}40\text{mM} - 4{,}5\text{mM} = 4{,}9\text{mM}$$

The volume this represents is given by:

$$v_1 * n_1 = v_2 * n_2 \qquad \text{(eq.3)}$$

$$v_1 * 3M = 10ml * 4{,}9mM$$

$$v_1 \approx 16\mu l$$

I used this as a starting point, intending, as before, to more specifically determine the amount needed by adding KCl in portions. From the PC12 cells I made the assumption the osmotic gradient would not be too disruptive if I went somewhat above the calculated amount. I regularly added 16+10+10µl.

2.4. Control Measurements

I conducted initial background noise level measurements in 0.9% NaCl solution. When the cell cultures were on their way, I tried the same in the cell growth medium – Ham's F-12K Nutrient Mixture (GIBCO), to ensure the medium itself would not be an influential source of noise (already at this point was the growth medium, correctly, assumed to be the medium in which recordings were to be conducted, since replacing it without disturbing or damaging the cells appeared tricky; however, this might have had to do with bad cell adhesion, which was later changed). Background noise levels were at this point assessed to be in the region of tens of µV, which is acceptable (would not likely drown out AP-like peaks) but not excellent.

Using a stimulus isolator and an oscilloscope I tested the setups basic ability to reproduce a distinct signal. This test was conducted several times (after major changes -- in setup (electrode) or cell culturing (cell type/medium)). The result was good representation with varying loss in amplitude (varying primarily dependant on the electrode's impedance).

2.5. Electrodes

When the project started, the explicit ambition was to test a number of different electrode designs on the finished platform. However, due to the slow progress and prevailing uncertainty with regards to cell response, the focus was shifted, simplified. Consequently, the electrodes used were not the designs currently being

developed at the NRC, but ranged from the most basic I could construct to purchased products with reliable specifications.

2.5.1. Rudimentary metal electrodes

When I started to familiarize myself with the equipment (see *2.1. The Setup*) I assembled the first, simplest possible test-electrode: a relatively thick (125 µm), non-insulated silver wire connected via a shielded cable and BNC connector to the amplifier. The prime requirement for this electrode was low impedance (ca 100kΩ), as to rule it out as a (significant) source of noise.

From here on, I used the same ground electrode, a (mm) thick silver wire connected to the chassis ground of the amplifier with a banana connector. Hence, the cable was unshielded.

The first alteration to make when the recordings proper were to begin was to change the electrode to an insulated one. When I at first forgot this, I found the recordings all but identical to the noise recordings. I switched to a wire of tungsten 33µm with 7µm formvar insulation (the California Fine Wire company). The formvar was removed where the wire was to be soldered on, using a scalpel; a principally simple procedure yet somewhat tricky to manage without damaging or bending (or losing sight of) the thinner-than-hair (well, most human hairs at least) metal wire.

I continuously progressed towards smaller wires, but the smallest wire electrode I used before the change to cortical stem cells was 12,5µm, which was yet too wide.

2.5.2. Glass capillaries

The change from PC12 cells to cortical neural stem cells coincided with the revelation that a *significantly* smaller electrode was needed to record from the cells. The individual PC12 cells had all been smaller than the hereto used electrodes. Though the cortical cells are larger (approximately 10µm soma width, and elongated), this change would still be necessary.

We opted that a pulled glass capillary with a thin metal (possibly tungsten) wire in the center would be a good option. However, it turned out that such a thing is not easily produced, and requires special skill (which there was no time for me to be taught). There were also no such electrodes ready-made and without designation. Hence, we decided I should try using pulled glass capillaries filled with a high concentration (3.0M) NaCl solution. I was to produce these glass capillaries myself, as the puller was quite simple to work, in principle. Still, it was

a bit tricky to get the opening small enough. In addition to taking some time to construct, the capillaries were rather delicate, and I dispatched of quite a few in the lab, thus needing a few ready before any recording.

I settled, after testing different settings for different types of glass, for Clark Electromedical instruments GC120-15 (*i.e*. Clark Borosilicate Standard Wall):

Composition	80.9% SiO_2, 4.4% Na_2O, 12.9% B_2O_3, 1.8% Al_2O_3	**Softening Temperature**	815°C	**Dielectric Constant**	4.7
OD [mm]	**ID [mm]**	**Wall Thickness [mm]**	**Length [mm]**	**Qty/Pkg**	**Model**
1.2	0.69	0.255	150	350	GC120-15

Approximate settings on the puller: Heat, 80; pull, 110 or max; jerk, 15. Most capillaries still required some grinding of the tip; it was quite tricky to blindly grind the exact amount needed for µm precision.

At first, I approximated the opening needed be, at most, about 5µm wide. However, this turned out to be too wide. Secondly, I managed to go to 4µm, or just below; which was *significantly* more difficult. Quite soon, it appeared apparent that < 3µm or so was a necessary size to get down to. However, when I, after some effort, managed to produce a few of these; it turned out the 1-2µm capillaries had too high impedance. At this point I considered the endeavor too time-consuming, and I continued using the TRec electrodes (2.5.3).

2.5.3. Manufactured quartz/metal microelectrodes

Before I had the level of practice needed to continuously and efficiently produce thin glass capillaries, I was borrowed two units of a reliable, manufactured and well tested type of electrode. These were quartz-glass covered pulled metal (platinum/tungsten) with an opening of a few µm and an impedance of around 1,5±0,3MΩ (ThomasRecording).

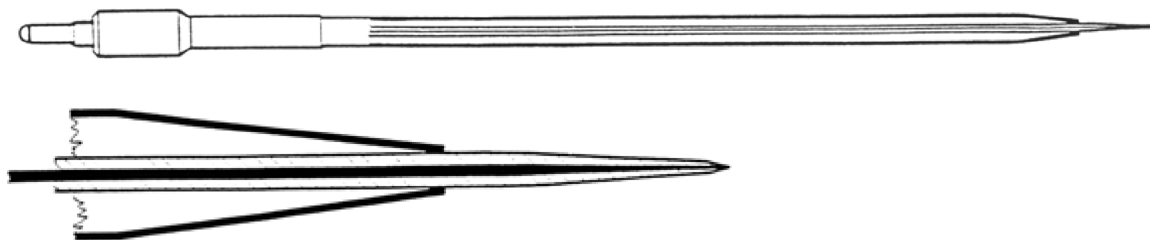

Figure 3 Schematic pictures of the electrode and its tip. The connector is gold-plated male, the protective tubing quartz. (Thomas Recording, Single Electrode Product Information.)

The specifications, including the impedance and tip size, are quite viable in the setup, and the recordings presented in the following part (*3. Results and Discussion*) were made using this electrode type.

3. Results and Discussion

The results are not given in quantitative form because I did not arrive at such. To make this section more productive I shall hence try to interweave the results, such as they are, with introspection and discussion.

3.1. The PC12 Cell Line

While the PC12 cells were grown on a collagen substrate, they did not do well, and appeared as seen in *Figure 4*. They did not approach confluence, and they did not appear to differentiate. Observe the prominence of clusters of cells; cells seemingly prefer to grow atop one another rather than on the substrate.

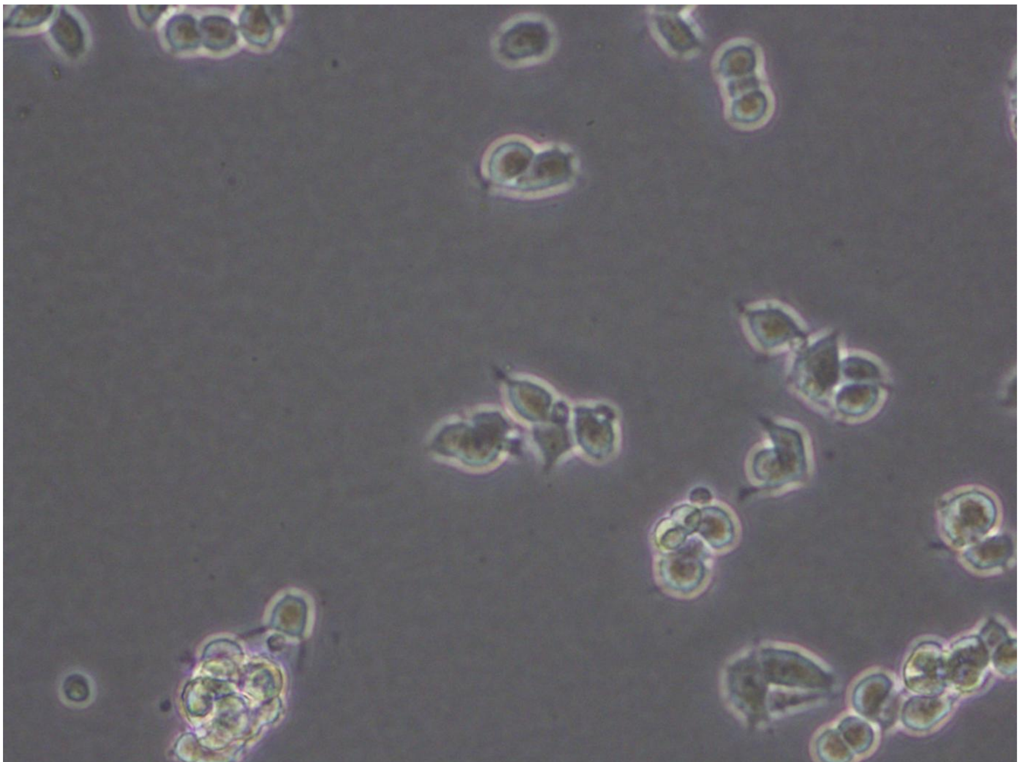

Figure 4. PC12 cells grown for two days *in vitro* (without NGF)

The change of substrate increased proliferation and the ability to grow neurites. The change was almost as drastic as the difference between *Figure 4* and *Figure 5*. After this change, the cells were used after three to six days exposure to NGF, when further confluency had been achieved.

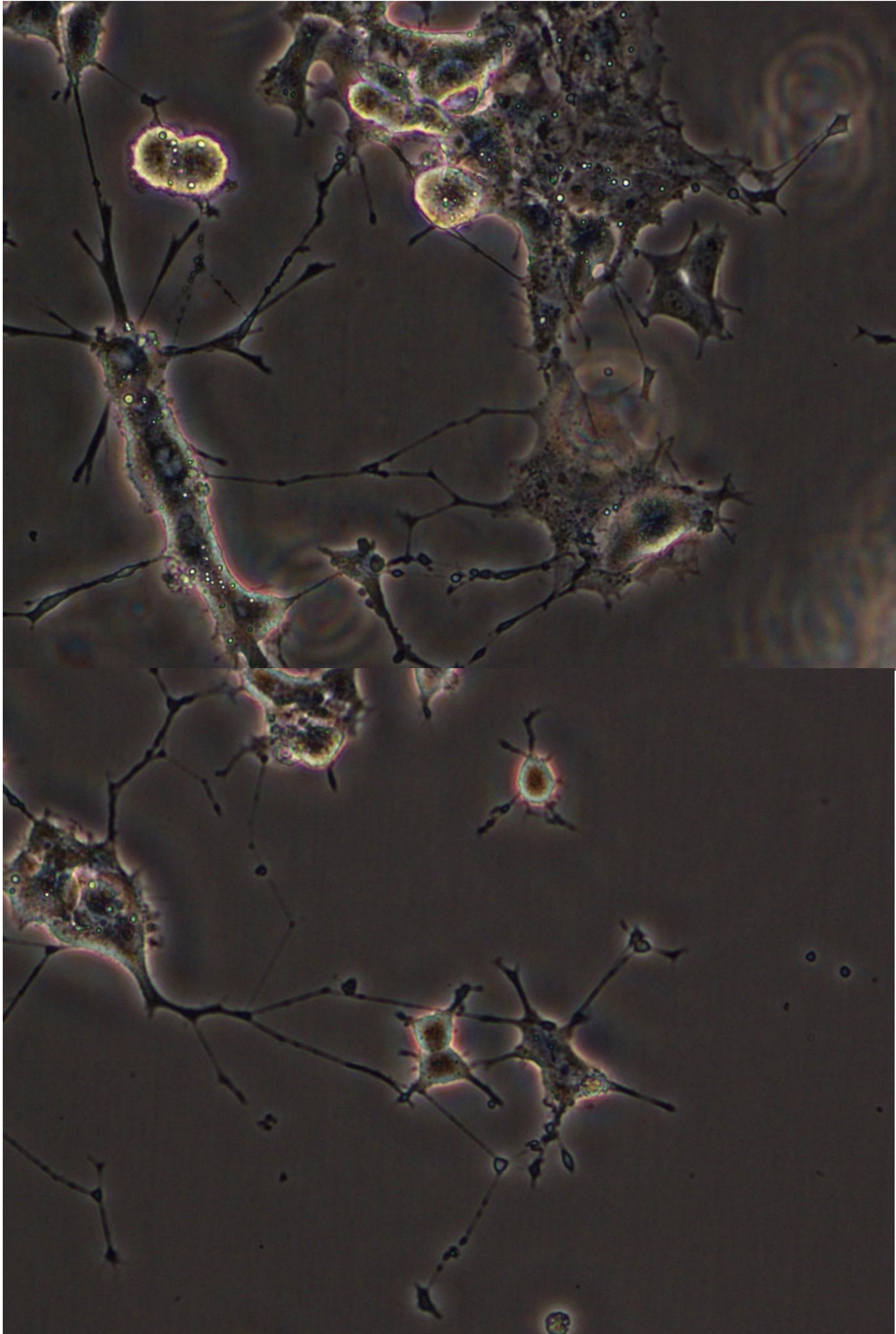

Figure 5. Two images of PC12 cells grown with NGF for two days *in vitro*

A too high concentration of KCl added will give an osmotic gradient capable of inducing lysis in the cell (*i.e*. the cells burst from the pressure). This limit was reached, and since registrations still were not made, a stricter reevaluation of the basic approach would perhaps have been beneficial. However, a lowering of the NaCl (changing growth medium to a "measuring medium") could have been one way forward; yet the extra procedure was never fully investigated, but was considered a needless extra feature as the cells were not required to survive anyway, and since they did not burst immediately. Alternatively, electrical stimulation rather than chemical could have been tested.

Throughout the recordings from the PC12 cells, only one recording stood out. At that time, I had entered a particularly large cluster of cells, to the extent and effect that recording and ground electrode did *not* measure from the same environment. Remember however that the clusters were of seemingly undifferentiated PC12 cells, that I had at this point not yet grounded all the equipment properly, and the lab table did not have gas suspension. Movement outside the cage could be registered, and subsequent registrations make this most likely. The random nature of the event increases the likelihood it was an outside disturbance.

As I mentioned earlier, however, theses clusters illustrated the possibility of an improved setup using 3D cell cultures. A 3D structure would be much more influential, and possibly required, when using the intended electrodes. Electrodes that the setup is intended for are meant for chronic implantation in a real, 3D brain, and thus are not ideal for measuring from a 2D surface with, and different designs might be affected by this to different degrees, which could be decisive.

Would electrical stimulation have been preferable? Will KCl only have the potential to induce exocytosis, and will then such an event be “loud” enough to be registered? Quite likely there is a reason this is not a cell type often used to study APs.

3.2. The Cortical Stem Cell Line

The cells looked good from the beginning, differentiating well, yet proliferation was later improved and confluency went from 60-70% to 80-90% (rough estimate). A 90-100% confluent monolayer is depictured in *Figure 6* (image from supplier of the cryopreserved cells).

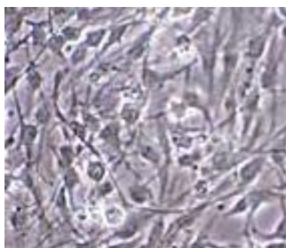

Figure 6. Mouse cortical neural stem cells [Figure 1a, Catalog No. SCR029, Chemicon® International]

From the shape, duration, and lack of similar peaks at the baseline, it is reasonable to assume the cortical cells are responsible for the peak in *Figure 7*. Based on shape and amplitude (reasonable for an extracellular recording), such a peak is considered an AP. The peaks also reoccur with increasing time-spans in-between (refractory times), and with slightly decreasing amplitudes. This too is reasonable for APs.

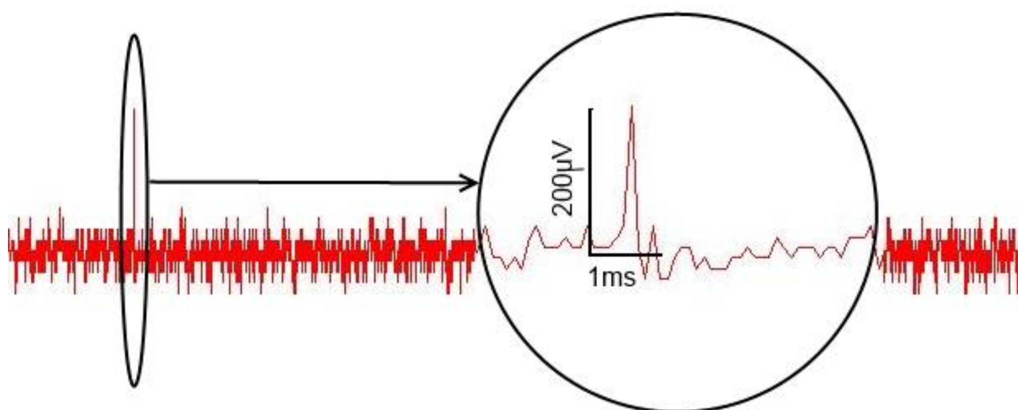

Figure 7. Recording of possible AP

In *Figure 8,* the peaks reoccur in a pattern which suggests that two cells might be firing. The time-span between the two peaks in *Figure 8* is approximately 20ms, and the two peaks reoccur several times at 15-20s intervals. This interval was consistent whether for one or two peaks (12-20s, varying more between than within samples).

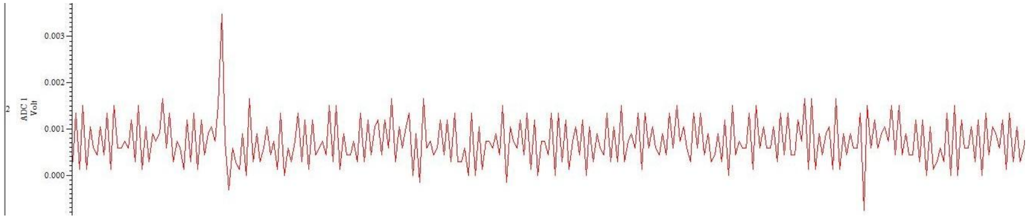

Figure 8. Possible recording of APs (signal amplified x10)

The amount of KCl needed to achieve AP-like responses was consistently 36µl, added in three batches (see *2.3.2. KCl for cortical neural stem cells*). The time it took for the cells to respond after the third KCl-addition varied, between 10 and 90s.

3.3. Possible Improvements of the Setup

Due to the slim angle of access allowed between the microscope and the Petri dish's rim along with the slim nature of the single, confluent layer of cells obtained with the cortical cells (and late with the PC12 cells), a part of the electrode will always be atop the cells, measuring against the background, lowering the signal-to-noise ratio.

Is the way to counter this problem the use of 3D cellular assays? These would further mimic the intended environment – a living brain – which is certain to be a good thing, is it not? Well, while 3D assays are full of potential, they are also less well studied, and less simple to produce. A good and consistent implementation could certainly have the potential to further the *in vitro*-system's merit, however.

One aspect which would have to be assessed is whether or not a (chemical) stimulating agent (*e.g.* KCl) would be able to spread – (somewhat) evenly – throughout such an assay. If only the outer/upper layer(s) is affected, little or nothing has improved – unless perhaps one uses the recording electrode to stimulate the cells.

With the open top – for easy access – solution, stability in the cell cultures system will only be maintained for a short time; as I have already argued, this accessibility is a significant part of the setup.

A closed system and a heated, CO2-profused sample holder – necessarily in connection to an *inverted* microscope – would be a huge investment for dubious gain. Even with a closed system, the contamination risks are high, and sample

might have to be discarded. It is also questionable whether one could get more from a sample than – like now – two or three opportunities. The cells will be exhausted, and the medium would have to be changed after tests, at the least.

3.4. Evaluation of the Project

During the PC12 experiments, I used levels of KCl high enough to potentially induce exocytosis. I arrived at those concentrations in steps. The osmotic gradient produced was damaging enough to the cells that some burst, limiting time and amount of cells from which to register. A medium with adjusted ionic levels (lowered Na^+, heightened K^+) is normally used in exocytosis experiments, but the change was deemed problematic here. This was about to be reevaluated, but instead the cell type was changed. This should be considered again, should PC12 cells be approached anew.

Possibly, there was a problem with cell adhesion, which too early on tainted the flexibility of the process. Once the cells were grown properly, exchanging the growth medium for phosphate buffered saline might well have been plausible. If so, greater pH-buffering ability would have been obtained, and life expectancy for the exposed cells prolonged.

The ideal time for measurements on a cell batch was not (empirically) quantified, but from assessing the PC12 cells based on appearance, I defined the window of opportunity as being between three days and six days differentiated with NGF. Shorter and they are still differentiating, longer and the terminally differentiated cells might even have started breaking down (possibly reducing responsiveness in cells yet alive).

When working with PC12 cells at least, the choice to continue using KCl as a stimulating agent for long might have been a mistake. At least *attempting* electrical stimulation would have been beneficial. Also, the choice of KCl level based on the levels required for exocytosis (and then *raising* the amount when no registrations appeared) might also have been a less favorable choice. Although the cells lasted long enough (before the osmotic pressure caused them to swell and sometimes burst) that they ought to have had time to respond to the treatment, were they at all able to begin with. However, I cannot, based on my project, see a reason one would choose PC12 cells for a similar setup.

Whether or not the cells were responsive ought to have been investigated in some way other than electronic registration. Fluorescent markers for displaying calcium influx might have been one such approach.

When working with the cortical stem cells registrations of apparent APs were at least achieved. Given more time, one could have brought this setup further, for example assessing more exactly the necessary KCl-level. The problem with narrowing down the stimulating agents volume span is the (presumed) variability between individual cells (we measure from just one, maybe two or a few cells at any time). To frequently reach above the cells' individual thresholds, an excess amount is readily used. Maybe the prolonged use of each sample demands too much? One can prolong the effective lifespan by introducing new equipment (*e.g.* CO_2-profusion). Yet the potential economical gain is offset by the investment in new equipment, and the scientific gain (heightened performance reliability) might be insignificant if appropriate considerations (particularly concerning the timing aspect) are taken; all preparations made and the procedure well practiced. Also, remember that the basic idea for this setup was for it to be quick and simple to use.

There was a lack of structure throughout the project, and more trial and error than ought be. This led not only to some very basic mistakes (mainly early on; *e.g.* the use of an almost definitely too large electrode), but also to an ineffective use of time. With more forethought, and more early expert input to catch basic flaws, the project might have had a lot better flow. My inexperience in the lab also limited my ability to perform as consistently as one would like, yet as I felt progress, a more experienced lab worker would likely find the procedures basic. This might be enough to make the setup viable.

4. Conclusions

I can *not* say – based on this project – that the setup can be considered an effective quick-assessment station for comparing fresh electrodes (intended for implantation). If an *in vitro* test is to be useful, higher level of consistency in response from the cells is a minimum requirement.

With the open top – for easy access – solution, stability in the cell cultures system will only be maintained for a short time; as I have already argued, this accessibility is a significant part of the setup. A closed system and a heated, CO_2-profused sample holder – necessarily in connection to an *inverted* microscope – would be a huge investment for dubious gain.

If the system is to be pursued in an as simple form as possible – yet with some merit; unlike the current version – the transition to 3D-assays along with a more narrowly specified amount of stimulating agent are priorities. If a good use of the cells can be maintained, a subsequent step might be to add an open-top heated sample holder, as to more accurately and for longer maintain the cells' responsiveness. However, all "improvements" are meaningless unless the cells can be made to consistently give off AP-like signals. During the project, I've seen hints that this could be achieved – for mouse neural cortical stem cells – but I cannot say that it as of yet has.

References

1. Kita J.M & Wightman R.M. Microelectrodes for studying neurobiology. Curr Opin Chem Biol. 2008; 12(5): 491–496.

2. Thelin J, Jörntell H, Psouni E, Garwicz M, Schouenborg J, Danielsen N, Linsmeier C.E. Implant size and fixation mode strongly influence tissue reactions in the CNS. PLoS One. 2011; 6(1): e16267.

3. Lind G, Linsmeier C.E, Thelin J, Schouenborg J. Gelatine-embedded electrodes—a novel biocompatible vehicle allowing implantation of highly flexible microelectrodes. *J Neural Eng*. 2010; 7(4): 046005.

4. http://www.med.lu.se/nrc/research/biocompatibility (2012 Jan 08)

5. Linsmeier C.E, Prinz C.N, Pettersson L.M, Caroff P, Samuelson L, Schouenborg J, Montelius L, Danielsen N. Nanowire biocompatibility in the brain – looking for a needle in a 3D stack. Nano Lett. 2009; 9(12): 4184-90.

6. Zhong Y, Bellamkonda R.V. Biomaterials for the central nervous system. J R Soc Interface. 2008; 5(26): 957–75.

7. Kohler P, Linsmeier C.E, Thelin J, Bengtsson M, Jorntell H, Garwicz M, Schouenborg J, Wallman L. Flexible multi electrode brain-machine interface for recording in the cerebellum. Conf Proc IEEE Eng Med Biol Soc. 2009; 2009: 536-8.

8. Abgrall P, Conedera V, Camon H, Gue A.M, Nguyen N.T. SU-8 as a structuralmaterial for labs-on-chips and microelectromechanical systems. Electrophoresis. 2007; 28(24): 4539–51.

9. Shah R.S, Chang S.Y, Min H.K, Cho Z.H, Blaha C.D, Lee K.H. Deep brain stimulation: technology at the cutting edge. J Clin Neurol. 2010; 6(4): 167-82

10. Lee K.H *et al*. Evolution of Deep Brain Stimulation: Human Electrometer and Smart Devices Supporting the Next Generation of Therapy. Neuromodulation. 2009; 12(2): 85–103.

11. Schiefer T.K, Matsumoto J.Y, Lee K.H. Moving Forward: Advances in the Treatment of Movement Disorders with Deep Brain Stimulation. Front Integr Neurosci. 2011; 5: 69.

12. Lebedev M.A, Nicolelis M.A. Brain-machine interfaces; past, present and future. Trends Neurosci. 2006; 29(9): 536-46.

13. Carmena J.M *et al.* Learning to Control a Brain-Machine Interface for Reaching and Grasping by Primates. PLoS Biol. 2003; 1(2): e42.

14. Parkinson's Disease: National clinical guideline for diagnosis and management in primary and secondary care. London: Royal Collage of Phsicians (UK); 2006.

15. Benabid A.L, Pollak P, Louveau A, Henry S, de Rougemont J. Combined (thalamotomy and stimulation) stereotactic surgery of the VIM thalamic nucleus for bilateral Parkinson disease. Appl Neurophysiol. 1987; 50(1-6): 344-6.

16. Johansson V, Garwicz M, Kanje M, Schouenborg J, Tingström A, Görman U. Authenticity, depression, and deep brain stimulation. Front Integr Neurosci. 2011; 5: 21.

17. Human Enhancement? Ethical Reflections on Emerging Nanobio-technologies. Edinburgh: Edinethics; 2007.

18. Greene L.A, Tischler A.S. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci U S A. 1976; 73(7): 2424-28.

19. Boulpaep E.L, Boron W.F. Medical physiology: a cellular and molecular approach. Philadelphia: Saunders; 2003.

20. van Grunsven L.A *et al.* Effect of nerve growth factor on the expression of cell cycle regulatory proteins in PC12 cells: dissection of the neurotrophic response from the anti-mitogenic response. Oncogene. 1996; 12(6): 1347-56.

21. Gilabert J.A, Montalvo G.B, Artalejo A.R. Rat Chromaffin cells primary cultures: Standardization and quality assessment for single-cell assays. Protocol Exchange. 2006; doi:10.1038/nprot.2006.294

22. Fujita T. Concept of paraneurons. Arch Histol Jpn. 1977; 40 Suppl:1-12.

23. Zhang B, Heien M.L, Santillo M.F, Mellander L, Ewing A.G. Temporal resolution in electrochemical imaging on single PC12 cells using amperometry and voltammetry at microelectrode arrays. Anal Chem. 2011; 83(2): 571-7.

24. Koizumi S *et al.* Characterization of Elementary Ca Release Signals in NGF-Differentiated PC12 Cells and Hippocampal Neurons. Neuron. 1999; 22(1): 125-37.

25. Mandel G, Cooperman S.S, Maue R.A, Goodman R.H, Brehm P. Selective induction of brain type II Na+ channels by nerve growth factor. Proc Natl Acad Sci U S A. 1988; 85(3): 924–28.

26. Fujita K, Lazarovici P, Guroff G. Regulation of the Diffrentiation of PC12 Pheochromocytoma Cells. Environ Health Perspect. 1989; 80: 127-42.

27. Rudkin B.B *et al.* Cell cycle-specific action of nerve growth factor in PC12 cells: differentiation without proliferation. EMBO J. 1989; 8(11): 3319-25.

Appendix I

I.I: Preparing PC12 cell medium

Materials

1. F12-K medium (Gibco)
2. Fetal calf serum (Gibco)
3. Normal Horse serum (Gibco)
4. Pen strep (Gibco)

Procedure

1. Preparation of PC 12 medium (Preparation of 100 mL)
    a. 86,5 mL F12-K Medium
    b. 10 mL (10%) Normal Horse Serum
    c. 2,5 mL (2.5%) Fetal Calf Serum
    d. 1 mL (1%) Pen/strep - antibiotics

I.II: Thawing of PC 12 cells

Materials

1. Frozen Cells
2. PC12 medium
3. T75 flask
4. 15 mL centrifuge tube

Procedure

1. Prepare a 15 mL vial with 10 mL medium (10 mL medium/vial).
2. Thaw the cells rapidly in a 37°C waterbath by swirling the vial in the water until only a small ump of ice remains.
3. Centrifuge at 300 g for 3 min.
4. Discard supernatant.
5. Resuspend the pellet in 10 mL medium
    a. After this step you may take a sample for cell counting and viability check.
6. Add the cell suspension to the T75 flask.
7. Determine the cell attachment after 24 h post-thaw.

I.III: Splitting PC 12 cells 3 to 1 in T75 flasks.

Materials

5. PC 12 cells in a T75 Flask
6. Accutase, (Millipore)
7. 15 mL centrifuge tube.
8. F12
9. Fetal calf serum
10. Normal Horse serum
11. Pen strep

Procedure

1. Prepare 50 mL PC12 medium accordning to protocol. Keep at room temperature (RT).
2. Remove all media from flask.
3. Add 3 mL 37°C Accutase to the flask and place it in the incubator at 37°C for approx 3-5 minutes.
4. Pipet the Accutase solution approx 5 times inside the flask and place the cell suspension in a 15 mL conical centrifuge tube. Add approx 7 mL PC12 media.
5. Wash the cells by centrifugation at 400 rcf for 5 min. at 4°C.
6. Decant the supernatant and resuspend the cell pellet in 30 mL PC 12 media.
7. Add 10 mL of cell suspension to each flasks.
8. Place the flasks in an incubator at 37°C, 5% CO_2 .
9. Change media every 3-4 days.

I.IV: Cryopreservation of PC 12 cells

Materials

12. PC 12 cells in a T75 Flask
13. Accutase, (Millipore)
14. Recovery™ Cell Culture Freezing Medium
15. Cool Cell
16. Dry ice
17. Dewar
18. 1 mL cryotubes

Procedure

1. Remove all media from the flask.

2. Add 3 mL 37°C Accutase to the flask and place it in the incubator at 37°C for approx 3-5 minutes.

3. Pipet the Accutase solution approx 5 times in the flask and place the cell suspension in a 15 mL conical centrifuge tube. Add approx 7 mL PC12 media.

4. Wash the cells by centrifugation at 300 rcf for 3 min. at 4°C.

5. Decant the supernatant.

6. Resuspend the cells in 3 mL Recovery freezing medium. Keep on ice.

7. Add 1 mL of cell suspension to each cryotube.

8. Place the cryotubes with cells in Cool Cell kept on dry ice and then place the Cool Cell at -80ºC in a freezer over night.

9. Store the cryotubes at -80°C.

Appendix II

EMD Millipore:

NEURAL STEM CELL BASAL MEDIUM

CATALOG NUMBER: SCM003
http://www.millipore.com/publications.nsf/a73664f9f981af8c852569b9005b4eee/a65321c3f0d3b54c852576dd0081e1cc/$FILE/SCM003.pdf

EMD Millipore:

CRYOPRESERVED MOUSE CORTICAL NEURSL STEM CELLS

CATALOG NUMBER: SCR029

http://www.millipore.com/userguides.nsf/a73664f9f981af8c852569b9005b4eee/67e27f0a0f6b56cd8525791e00686fb7/$FILE/SCR029.pdf