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Development of a test environment for the study of infrared neuromodulation

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Abstract

Multimodal microelectromechanical system (MEMS) implants, developed by the Research Group of Implantable Microsystems at the Faculty, enable infrared neuromodulation (INM) and electrophysiological recording in freely moving rodents for in vivo experiments.

This work provides a brief introduction to INM and its potential applications. Based on the literature, this paper describes various mechanisms involved in infrared neural modulation, neural interfaces for INM, brain implant fixtures, commercially available micro-drives, as well as analytical tools employed for processing extracellular recordings and videos of freely moving rodents.

To provide an interface for a given optical electrode (optrode) and to protect it from external impact, a micro-drive and a head stage have been developed. This optrode interface is designed for in vivo experiments on freely moving rats. The accuracy and durability of the micro-drives, alongside the physical and biological properties of the materials used, and the smoothness of the longitudinal displacement mechanism, have been tested.

The infrared optrode, featuring a micromirror needle tip, has been characterized based on in vivo experiments. A total of 8 hours of extracellular recording was used for data analysis across 4 days, from 4 rats. Neural activity recordings lasting one hour each were taken from the somatosensory cortex of each rat. The author has developed a Matlab graphical user interface (GUI) to investigate the variations in neural firing rate using an infrared optrode equipped with a micromirror needle tip. The experiments were carried out in a sequence of on and off periods of IR irradiation. The signal-to-noise ratio (SNR) of the optrode is also computed from the collected recordings.

Spike detection and sorting were carried out utilizing Kilosort2 while manual curation of the spike sorting was done in Phy. An algorithm for estimating SNR using root-mean-square (RMS) calculation, implemented in Matlab, is presented. Additionally, an amplitude-based SNR estimator and GUI for visualizing firing rates, both developed in Matlab, are demonstrated.

From the recorded signals, the effect of INM on the local field potential (LFP) was determined through wavelet and one-sided amplitude spectrum analysis. A Matlab GUI was also created to display the one-sided amplitude spectrum per channel for different frequency ranges.

In addition, a Python script was written to discern the position, velocity, and selected activities (climbing, moving or resting) of the rodent while undergoing INM in the video recordings. Such data provide valuable insight for our research team in assessing electrophysiological signals across diverse behavioural contexts.

The work comprises of the following sections. Firstly, the theoretical backgrounds are presented. Then, a detailed description of the fabrication process of the optrode interface, the experiment and the data analysis methods employed are provided. Finally, the outcomes of the various sections are summarised.

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1 Introduction

1.1 Infrared neuromodulation

Infrared Neuromodulation (INM) is a photomodulation technique that allows direct or indirect control of cellular activity by raising the temperature in a defined region of the targeted tissue. Numerous studies have demonstrated safe and replicable excitation or inhibition of neural firing in both in vitro and in vivo environments [1]. Nevertheless, the mechanism of neural infrared stimulation is still not fully understood.

Infrared neuromodulation (INM) utilises continuous-wave or pulsed infrared light in the wavelength range of (1400-2100 nm) to precisely modulate temperature changes in neurons, inducing or inhibiting action potentials [2], [3], [4], [5], [6]. Such modulation can evoke excitation or inhibition of neurons, distinguishing between infrared neural stimulation (INS) and infrared neural inhibition (INI).

There are several advantages of using infrared neuromodulation:

- it does not require any genetic modification of the neural tissue, unlike optogenetic stimulation, where a light-sensitive channel, such as rhodopsin, is necessary,
- there is no current spread, as with electrical stimulation,
- with INM the spot size of the stimulus matches the size of an optical fibre, which is typically a few tens of microns in diameter,
- if a well-controlled temperature change is established, INM can be applied repeatedly without causing any tissue damage,
- as INM is insensitive to external magnetic fields, this method is compatible with MRI and can be used in conjunction with various electrophysiological techniques [7].

Infrared neuromodulation finds application in multiple fields. One of its applications is the restoration of auditory perception. Cochlear implants are bionic devices that facilitate hearing restoration in patients, with several solutions relying on an intracochlear electrode array. These interfaces directly stimulate the auditory nerve by transmitting electrical signals. The primary drawback of conventional stimulation methods is their low spatial selectivity, which causes activation of neighboring spiral ganglion neurons due to the spread of current. Several studies have investigated the use of INM to restore lost hearing [8], [9].

Infrared neuromodulation is also used to study brain disorders, such as Parkinson's disease, which has a number of motor symptoms, including postural abnormalities, hypokinesia, rigidity, and tremor. It is well-known that the root of these symptoms is the degeneration of dopamine neurons in the substantia nigra pars compacta (SNc). Peoples et al (2012) demonstrated that near-infrared light exposure prevented SNc dopaminergic cell degeneration in an MPTP-induced mouse model of Parkinson's disease. [10]. Near-infrared stimulation can also be used to alleviate cognitive impairment resulting from traumatic brain injury [11], mitigate Alzheimer-related pathology in the cerebral cortex [12], and effectively reduce firing rates during seizure-like activity [13], potentially offering a treatment for epileptic patients.

2 Methodological overview

2.1 Mechanism of infrared neural stimulation

2.1.1 Heat-sensitive ion channels

Caterina et al described first a heat-sensitive ion channel, the first member of the TRPV (transient receptor potential vanilloid) channel family [14]. The TRPV1 channel, also known as the capsaicin receptor, is affected by the chemical compound found in hot chili peppers. The TRPV family comprises non-selective cation channels that are permeable to Ca^{2+} , K^+ , and Na^+ . These channels can be located in both the peripheral and central nervous systems.

Activation of the various heat-sensitive ion channels within the TRPV family depends on the absolute temperature and it does not depend on the heat change rate. TRPV1 channels typically begin to open at temperatures around 40°C. Its homologs TRPV2, TRPV3, and TRPV4 open at distinct threshold temperatures. TRPV2 activates at over 50°C, whereas TRPV3 and TRPV4 channels open at lower temperatures around 30°C [15].

2.1.2 Electrostatic mechanism

Initially, Shapiro et al hypothesized the connection between the thermal gradient resulting from pulsed infrared light absorption in neural tissue and the increase of the cell membrane's electrical capacitance. This reversible change in capacitance generates capacitive membrane currents which are independent of any specific ion channels [16]. This observation provides an explanation for the mechanism behind infrared neural stimulation (INS). Plaskin et al highlighted a mathematical error in the original paper of Shapiro et al. The corrected model predicts a reduction in membrane capacitance instead of an increase with the thermal gradient [17]. In a subsequent study, Plaskin and his team developed a new theory, named MechanoElectrical Thermal Activation, which predicts a $\sim 0.3/^{\circ}\text{C}$ increase in thermal capacitance. This increase arises from the deformation of the phospholipid bilayer, which causes ionic displacement currents [18].

Another possible explanation for INS is the formation of nanopores through the plasma membrane due to temperature changes. Beier et al. hypothesised that IR short waves destabilise the phospholipid bilayer within the plasma membrane by inducing a large temperature gradient. Slight changes in the plasma membrane may create transient, small-diameter nanopores that allow the influx of extracellular ions. The influx of these ions can depolarise the cell, leading to action potential generation or downstream activation of secondary messenger pathways to cause, for example, channel activation/inhibition [19].

2.1.3 Intracellular calcium waves

One possible explanation for the mechanism of infrared neurostimulation is the generation of transient intracellular Ca^{2+} waves. Iwanaga et al. irradiated HeLa cells with a femtosecond laser. They observed that IR stimulation caused sufficient Ca^{2+} release from the endoplasmic reticulum (ER) of the cell to trigger a Ca^{2+} wave [20].

Generation of Ca^{2+} waves by infrared stimulation was later observed in cardiomyocytes [21], in both astrocytes and neurons [22], in mouse hippocampal neurons and human glioblastoma cell lines [23], and in spiral ganglion neurons [24].

2.2 Mechanism of infrared neural inhibition

Cayce et al. were the first to describe that pulsed infrared neurostimulation resulted in a reduction in firing rate in rat somatosensory cortex in vivo. They hypothesised that the laser-induced inhibition was due to the effect of stimulating inhibitory neurons in superficial layers of the cortex [3].

Ganguly et al, using Hodgkin-Huxley based computational models, hypothesised that infrared neural inhibition results from thermally driven activation of voltage-gated potassium ion channels [25]. They tested this hypothesis by blocking voltage-gated ion channels during IR irradiation of unmyelinated Aplysia nerves. Their results also suggest that sodium channel blockers have no significant effect on IR inhibition.

2.3 Neural interfaces for INM

Infrared neuromodulation can directly or indirectly modulate neural activity. In the case of direct modulation, the IR light is absorbed by the tissue, resulting in neural excitation or inhibition through increased temperature. On the other hand, indirect modulation uses mediating components that convert incident infrared light into heat or high-energy visible photons.

2.3.1 Direct photomodulation

Fibre-based method

The IR light can be guided through an optical fibre positioned above the intact tissue, similar to optogenetic methods [22], [26]. This fibre-based approach is robust and applicable to depths of a few hundred microns. However, the optical fibre is too bulky for a precisely targeted stimulation and requires further recording implants in order to obtain any information about the evoked cellular response.[13].

MEMS-based devices

One possible solution to the problems mentioned above is the use of MEMS-based devices. These micro-electro-mechanical systems (MEMS) combine mechanical and electrical components to achieve more advantageous properties. They are manufactured with micron precision using technology from the semiconductor industry, in particular photolithography. MEMS technology makes it possible to mass produce these optrodes with reliable reproducibility and spatial precision [27]. The MEMS-based devices can have multiple functionalities in addition to providing IR light. They may have additional integrated sensors such as electrophysiological recording sites, temperature or neurochemical sensors [28].

Depending on the position of the optical actuators in the optrodes, passive and active probes can be distinguished. In the case of passive optrodes, the light is directed into the deep tissue from an external light source. In this setup, laser LEDs or diodes are integrated with waveguides. The main disadvantage of passive optrodes is the propagation loss of the light due to the large distance between the light source and the target [27]. The loss is due to Fresnel reflection: propagation loss at the interface between the waveguide material and the air, and at the end facet of the waveguide. Another source of propagation loss is the imperfections in total internal reflection through the waveguide.

In the case of active optrodes, the light source (e.g.: μ LEDs, VCSEL) is placed close to the target tissue, allowing direct stimulation of cells [29]. The use of active light-emitting components on the implantable part of the optrode eliminates the loss of light propagation through the waveguide and enables high power density stimulation. However, this arrangement can cause excessive thermal (parasitic) heating of the surrounding tissue [30]. If the optrode has electrophysiological recording sites, crosstalk between these sites and the supply current of IR stimulation may occur.

2.3.2 Indirect Photomodulation

Compared to direct photomodulation, where IR light is delivered directly to the tissue, indirect photomodulation uses micro- and nanoscale transducers to stimulate or inhibit neural activity. Indirect photomodulation techniques are based on near-infrared (NIR) radiation, which allows deeper penetration of light into tissue. This penetrating NIR light acts on the transducers to induce neural activity.

Polymer-mediated approaches

Conjugated polymers (CPs) can be used for neuromodulation [31]. Conjugated polymers are organic macromolecules that have a molecular backbone chain of alternating single and double bonds that create delocalised π -electrons. Some CPs exhibit strong absorption in the near infrared (NIR) wavelength range, which can act as effective photothermal agents by converting the absorbed light into heat.

Martino et al. showed that prolonged NIR illumination of a poly(3-hexylthiophene) (P3HT) thin film coated on glass substrates causes hyperpolarisation of human embryonic kidney (HEK293) cells grown on its surface [32]. Feyen et al. succeeded in optically controlling neuronal activity at the interface between conjugated polymers (P3HT, PCPDTBT) and mouse hippocampal slices using prolonged near-infrared illumination [31].

Upconversion nanoparticle mediated approaches

Photon upconversion (UC) is a nonlinear optical process characterised by the successive absorption of two or more photons followed by the emission of a photon at an energy level higher than the energy level of the individual absorbed photons. Nanoparticles with this ability are called upconverting nanoparticles (UCNPs) [33].

Lanthanide-doped upconverting nanoparticles have the advantage of absorbing NIR light and emitting light in the visible spectrum. UCNPs are currently used for in vivo drug delivery [34], deep tissue imaging [35], photodynamic therapy [36] and immunotherapy [37].

Upconverting nanoparticle-mediated optogenetics is now a popular line of research. Chen et al. used UCNPs that can upconvert near-infrared light into blue light emission [38]. The nanoparticles injected into the mouse brain excited channelrhodopsin-expressing dopaminergic neurons. In this setup, the near-infrared light was generated outside the skull at a distance of several millimetres.

Thermoplasmonic neurostimulation

The phenomenon of surface plasmon resonance is widely used in biosensors to determine the specificity, affinity and kinetic parameters of binding of different macromolecules [39]. Surface plasmon resonance (SPR) occurs when a photon of incident light strikes a metal surface. At a certain angle of incidence, part of the light energy couples with the electrons in the metal surface layer. The electrons begin to travel parallel to the metal surface. This oscillating electron motion is called a plasmon. The SPR angle of incidence at which resonance occurs depends on the refractive index of the sensing medium near the metal surface. In biosensors, detection is achieved by measuring the changes in reflected light.

Using the phenomenon of surface plasmon resonance, metal nanostructures can control temperature at the nanoscale. These metal nanostructures have the advantage of absorbing light at their plasmonic resonance and converting the energy of the absorbed light into an ideal nano-heat source. This absorption can be remotely controlled by light. This photothermal phenomenon is known as thermoplasmonics. Typical applications of these gold nanoparticles are in the field of photothermal cancer therapy, photothermal imaging, protein tracking, nanosurgery, drug delivery, nanochemistry and optofluidics [40]. In addition, Eom et al. showed that irradiation of gold nanorods with pulsed NIR light induces increased local heat to modulate neural activity [41].

2.4 Commercial micro-drives and brain implant devices in the literature

Electrophysiological recording of brain activity is an essential technique for understanding the neural basis of behaviour in healthy or in an ill subject. To understand how the brain represents information about the internal and external world, recordings of electrical neural activity in rodents are used [42], [43] to find the neural correlation between sensory and perceptual processing and decision making [44]. In general, electrophysiological methods can be divided into extracellular and intracellular recordings. In contrast to intracellular recordings, extracellular recordings are widely used in freely moving animals, which allows for stable recordings from several minutes to even hours [45]. To record extracellular activity, cortical probes like ECoG can be utilised to capture electrical signals from the surface of the cortex, without the need to penetrate brain tissue. Alternatively, penetrating probes may be deployed

to capture neural activity from deeper regions of the brain. For this study, penetrating probes have been utilised.

For extracellular recordings, a variety of penetrating neural probes based on microwire electrodes [46], carbon fibre electrodes [47] and micromachined electrodes [48] have been developed and applied in the last decades. The most common characteristics of these silicon electrodes in chronic recording are that they are easy to break, expensive, and typically implanted for a single use per animal. In many cases, the most commonly used moveable electrodes are permanently implanted and cemented to the skull, which precludes reimplantation in the same subject and results in the need for an increased number of experimental animals [49].

Several designs have been proposed for the construction of micro-drives. In the case of a micro-drive, a probe is attached to a mechanism that allows the probe to move in a vertical direction with high precision. The manually operated micro-drives introduced in the last decades were not so successful [50], [51]. Several automatic micropositioning systems have also been developed using brushless DC micromotors [52], [53]. Another type of micropositioning system consists of a piezo motor and a Hall effect sensor [45]. Unlike electromagnetic devices, piezoelectric systems are not affected by electromagnetic noise. Another solution is a hydraulic micro-drive, which can move the electrodes precisely, but it is bulky and too heavy [54]. Among the many micro-drives developed to date, the power screw mechanism is the most widely used solution for electrode positioning, whether it is a manual or automated mechanism.

The most important requirements for micro-drives are ease of implantation and the precise, seamless movement of the electrodes. As a means of prolonging chronic recordings in freely moving animals for long periods of time, Polo-Castillo et al. have developed a low-cost, reusable and reimplantable device design [55]. Numerous components of their design are produced using 3D printing. The micro-drive comprises a head-plate that is permanently affixed to the skull of rodents (mice or rats). Dental cement, a biocompatible material, was used for the fixation. Furthermore, the micro-drive's design ensures reusability and replaceability, allowing for easy attachment and detachment from the baseplate, enabling efficient implementation and reimplantation. Additionally, to prevent lateral deviation of the electrode shaft during vertical displacement, an electrode shuttle system was developed. The electrode shuttle system comprises a piston element (see Figure 1 (a)), which moves through a bushing (b) to limit displacement. A compression spring (c) provides a preload between the piston and the nut-screw system or piezoelectric actuator, ensuring zero backlash and granting the mechanism high stiffness, load capacity and resistance to shock and vibration. The piston functions as a shuttle, with a stainless steel guide cannula (e) fixed concentrically around a polyimide tube (d). Electrodes are inserted through the polyimide tube. One setback of this design is that it cannot accommodate probes of any size. If the probe is bulky and lacks a long, thin shaft, the shuttle system cannot be utilised since the electrodes are positioned in the polyimide tube. The overall displacement length of this micro-drive is 2.5 mm, which is relatively short.

Daal et al provided a comprehensive protocol for detailing the chronic implantation and reimplantation of Neuropixels probes in rats and mice. They incorporated 3D-printed elements in their micro-drive design, which allows for stable and long-term in vivo recordings of freely moving rodents [56].

Chung et al developed a micro-drive system that accommodates silicon probes of any shape [57]. The 3D-printed micro-drive comprises three components: a shell, a body, and a slider (refer to Figure 2 right). The slider is bonded with the silicon probe and moves linearly concerning the body as the screw turns. The body has a trapezoidal structure that accommodates the slider's shape, and screws are used to move the slider, secure the body to the shell, and anchor the micro-drive to the micro-drive handle. The shell envelops the body and is the sole segment of the micro-drive fixed to the animal's skull. This facilitates the retrieval of the electrode with the slider and body through unscrewing the shell. The micro-drive handle establishes a link between the body and the stereotaxic post, enabling effortless implantation and reimplantation. To protect the micro-drive system, it is covered with a cap. The headgear consists of a head-plate, a probe connector holder (known as a C-holder), and a cap (refer to Figure 2 on the left). The head-plate is horizontally fixed to the skull of the mice and has a big opening for accessing the skull. The connector is affixed to the holder, situated at the rear of the

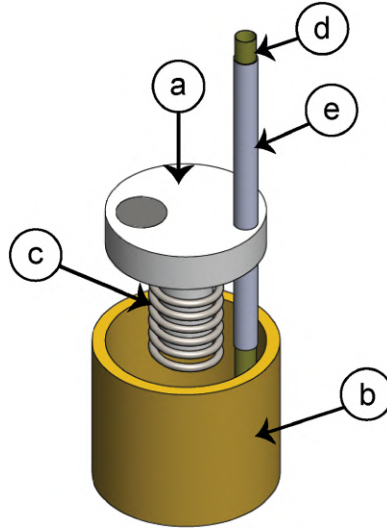


Figure 1: Electrode shuttle system. (a) Piston, (b) bushing, (c) compression spring, (d) polyimide tube, (e) cannula guide [55].

head-plate, and secured with screws. The cap covers the C-holder and the front part of the head-plate.

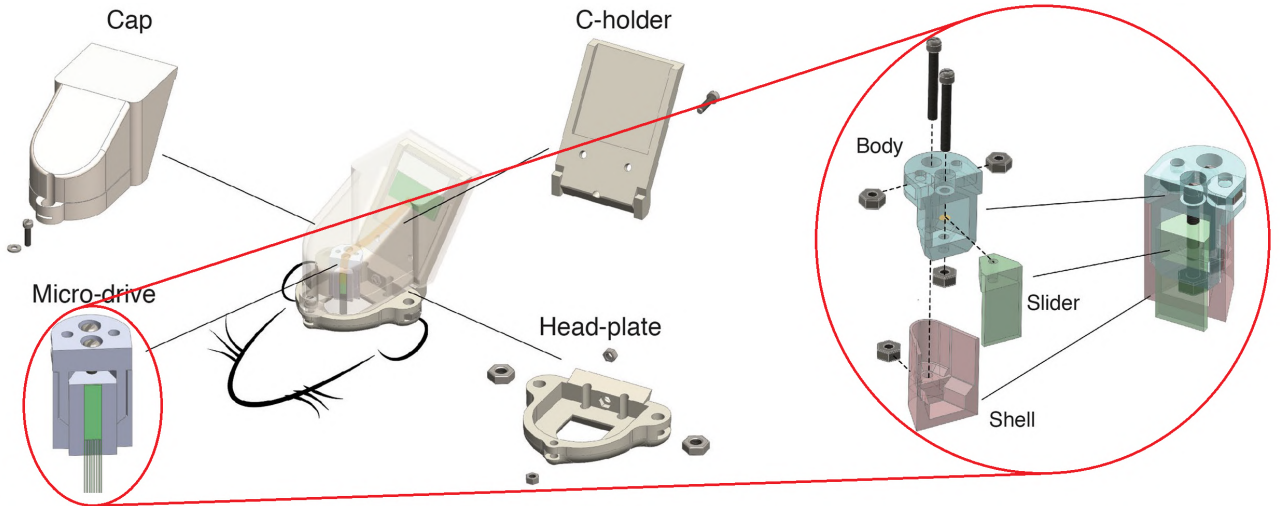


Figure 2: On the left, the hat enclosure comprises a micro-drive (lower left), a cap (upper left), C-holder (upper right), and a head-plate (lower right). [57]. On the right, the slider (green) is embedded in the body (blue) and moves vertically upon turning the front screw. The back screw holds the body embedded in the shell (red).

The commercially available nano-drives developed by Cambridge NeuroTech company have advantageous properties [58]. The small size of the nano-drives allows for fixing two nano-drives on the skull of a rodent, with a vertical displacement of 7.5 mm - a significant advantage compared to other options [55]. This mechanism moves the probe smoothly without any lateral movement. The primary disadvantages of this nano-drive are twofold: it is intended for single use and is relatively expensive.

2.5 Analysis of extracellular recordings

Extracellular recordings represent a fundamental and widely employed electrophysiological method in neuroscience research, serving to understand how neurons encode and process information, as well as in clinical applications (e.g., brain-machine interface). Recent advancements in microelectrode array fabrication have allowed for the simultaneous recording of neural signals from a large number of neurons at the same time [59].

2.5.1 Local Field Potential (LFP), Single (SUA) and Multi-Unit Activity (MUA)

The local field potential (LFP) and neural spikes are the two primary components of recorded neural signals. The local field potentials (LFPs) can be acquired by applying a low-pass filter (e.g. <300 Hz) to the raw neural signals that were recorded. LFPs are believed to serve as input for local neuronal networks [60]. The local field potential represents a summed synaptic activity of numerous neurons within the proximity of the recording electrode (a few hundred micrometres radius) [61]. Conversely, by high-pass filtering (i.e. >300 Hz), neural spikes can be derived from the raw recorded neural signal, representing the output of the local neuronal networks [60].

Neural spikes can be categorized into two main parts: single-unit activity (SUA) and multiunit activity (MUA). SUA refers to the timing of individual neuronal spikes, or action potentials. This activity is typically identified through the process of spike sorting, which involves thresholding and classifying the spikes. MUA, on the other hand, refers to the collective spikes recorded within a radius of $140\text{--}300\text{ }\mu\text{m}$ around the electrode [42]. However, obtaining single-unit activity (SUA) and multi-unit activity (MUA) from extracellular recordings is a challenging process. Several algorithms are available to solve this problem.

2.5.2 Basic steps for spike sorting

Spike sorting is the computational process of extracting the action potentials of single neurons from extracellular recordings. However, it is a complex process owing to the constant variability in extracellular recordings and the presence of overlapping electrical fields from neighboring neurons.

The traditional method of spike sorting involves four stages: preprocessing, spike detection, clustering and postprocessing (see Figure 3). In the preprocessing phase, the raw signal acquired through recording is subjected to band-pass filtering, typically between 300 Hz and 3000 Hz, to isolate the activity of a limited number of neurons in proximity to the electrode. Nevertheless, the filtered signal may also contain background activity - resulting from the firing of more distant neurons. The band-pass filtered signal is the superimposed activity of different neurons.

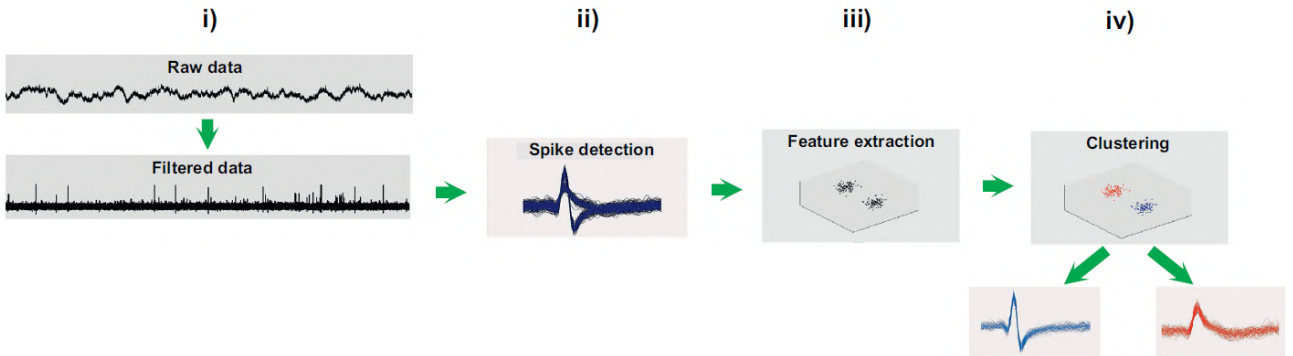


Figure 3: Basic steps for spike sorting. Firstly (i), the recorded raw data undergoes band-pass filtering, typically between 300 Hz and 3000 Hz, to capture the activity of select neurons near the electrode, excluding background noise. The language will remain impartial and we will try to avoid non-technical terms where possible. Secondly (ii), spikes are detected through amplitude-based thresholding on the band-pass-filtered data. Next (iii), discriminant attributes of spike shapes are extracted. Finally (iv), the extracted characteristics serve as the inputs to the clustering algorithm which clusters the waveforms and links each cluster to a unit (neuron) [62].

During the spike detection process, it is necessary to separate the spikes of each neuron from one another. By using thresholding based on amplitude, individual spikes can be distinguished from the background. However, determining the threshold value can pose problems, whether done manually or automatically, if the recorded signal has low signal-to-noise ratio (SNR) or high variation over time. This can arise in chronic recordings where the amplitude of spikes gradually decreases due to tissue responses (microglial sheath formation around the electrode) and/or micromotion of the electrodes

[63]. Furthermore, a threshold-based technique may detect large neurons, such as pyramidal neurons, preferentially, and disregard the spiking activity of small neurons [64].

Following spike detection, putative spike waveforms (which are typically 2 to 3 ms in length) are stored as input for the feature extraction step. Sorting of spikes by amplitude is not viable as neighboring neurons can have the same peak amplitude. Each neuron possesses a distinct action potential shape, largely dictated by the morphology of its dendritic tree, relative distance, and orientation to the recording electrode. Different characteristics of detected spikes can be utilized as input for the clustering process. Examples of these features include peak amplitude and spike width. Improved performance of the clustering algorithm is often observed with a greater number of discriminative features.

After capturing the relevant features of the spikes, neurons are arranged into distinct clusters. Some spikes cannot be separated due to a low signal-to-noise ratio. Therefore, they're clustered into multi-unit activity. Multi-unit clusters form via the superposition of unclustered spikes, which usually have relatively low amplitudes and lack a typical single neuronal refractory period. Post-processing steps involve merging or splitting clusters, depending on whether two clusters belong to the same neuron or one cluster contains multiple distinct neurons.

2.5.3 Kilosort for Spike Sorting

Kilosort is a widely used and accurate algorithm for automatic spike sorting of recorded data [65]. Kilosort has multiple versions, including the latest Kilosort4, which offers enhanced features that outperform other spike sorting algorithms. This section provides a detailed introduction to the Kilosort2 spike sorting algorithm used in our electrophysiological data analysis experiments.

Early spike sorting algorithms necessitated human curation due to imperfect clustering. Therefore, Kilosort clustering has a bias towards "over-splitting," resulting in more clusters than the actual units. To simplify human curation, cluster merging is preferable to cluster splitting. A software tool named Phy was created to assist with manual validation of clustering results.

As extracellular recordings exhibit non-stationarity, manual inspection is necessary. The non-stationary attributes of the data stem from the varying waveform of spikes on an hourly or accelerated basis. Vertical movement or "drift" of the probe is the key contributor to this variation. Drift is primarily due to tissue relaxation after the insertion of the probe and the animal's movement [66]. To compensate for this non-stationarity, Kilosort2 employs drift tracking, which, unlike later versions of Kilosort (from Kilosort 2.5), does not require a geometric model of the probe.

While all versions of Kilosort were primarily developed for Neuropixels probes, they can also be used for other types of probes. Kilosort2 appears to be the optimal selection for analysing data recorded by various types of probes as it conducts drift tracking without necessitating the channel geometry of the probe to be exact.

2.5.4 Spike sorting process

The detailed implementation of the different versions of the Kilosort spike-sorting algorithm is presented by Pachitariu et al. [65], [67]. All versions of Kilosort operate on a GPU to process significant amounts of recorded electrophysiological data. The input file is binary, and the output produces ".npy" files employed for data visualization.

The preprocessing step in every version of Kilosort comprises temporal filtering and channel whitening, which together execute high-pass filtering at 300 Hz channel-wise and minimize the cross-channel correlations, respectively. These two linear operations diminish the strong spatiotemporal correlations of the background electrical activity in the brain. From Kilosort2.5, the preprocessing step has been upgraded with drift correction.

In Kilosort, the procedures for spike detection and feature extraction are grouped together as "template deconvolution." The templates in this step represent the typical spatiotemporal waveforms of neurons. These templates are utilised to identify spikes and extract their characteristics, even if multiple spikes overlap on the same channels simultaneously [67]. The Kilosort version used determines

the clustering algorithm employed when establishing the templates, which are created from recorded data. Kilosort1/2/2.5 make use of scaled k-means, whilst Kilosort3 utilises recursive pursuit, and Kilosort4 is based on graph clustering.

Kilosort1/2/2.5 do not necessitate further clustering, as the templates must correspond to the final units in a one-to-one fashion. However, in Kilosort2/2.5, this correspondence is less stringent due to the use of splitting (bimodality pursuit algorithm) and merging in the post-processing step. Consequently, compared to Kilosort1, less manual intervention is necessary.

One-to-one matching between the learnt templates and final units is irrelevant for Kilosort3/4, thus the templates are discarded following. That is why the templates are discarded following the template deconvolution stage in subsequent versions. Subsequently, only the extracted features are employed to cluster the spikes. In Kilosort3, recursive pursuit is employed, while Kilosort4 utilises graph clustering as the clustering algorithm. Additionally, splitting and merging are utilised in the postprocessing stage.

2.5.5 Phy: Graphical User Interface for data visualization and curation

The spike sorting algorithm results can be visualised in Python with the assistance of the *phy* library. Phy is an open-source Python library offering a graphical user interface to manually curate and visualize an enormous amount of electrophysiological data [68]. It is intended for use on multi-electrode arrays with hundreds of recording sites, such as Neuropixels probes.

Views

The phy GUI interface comprises diverse views (Figure 4) that represent data from distinct aspects to facilitate manual curation and visualisation of the results of spike sorting.

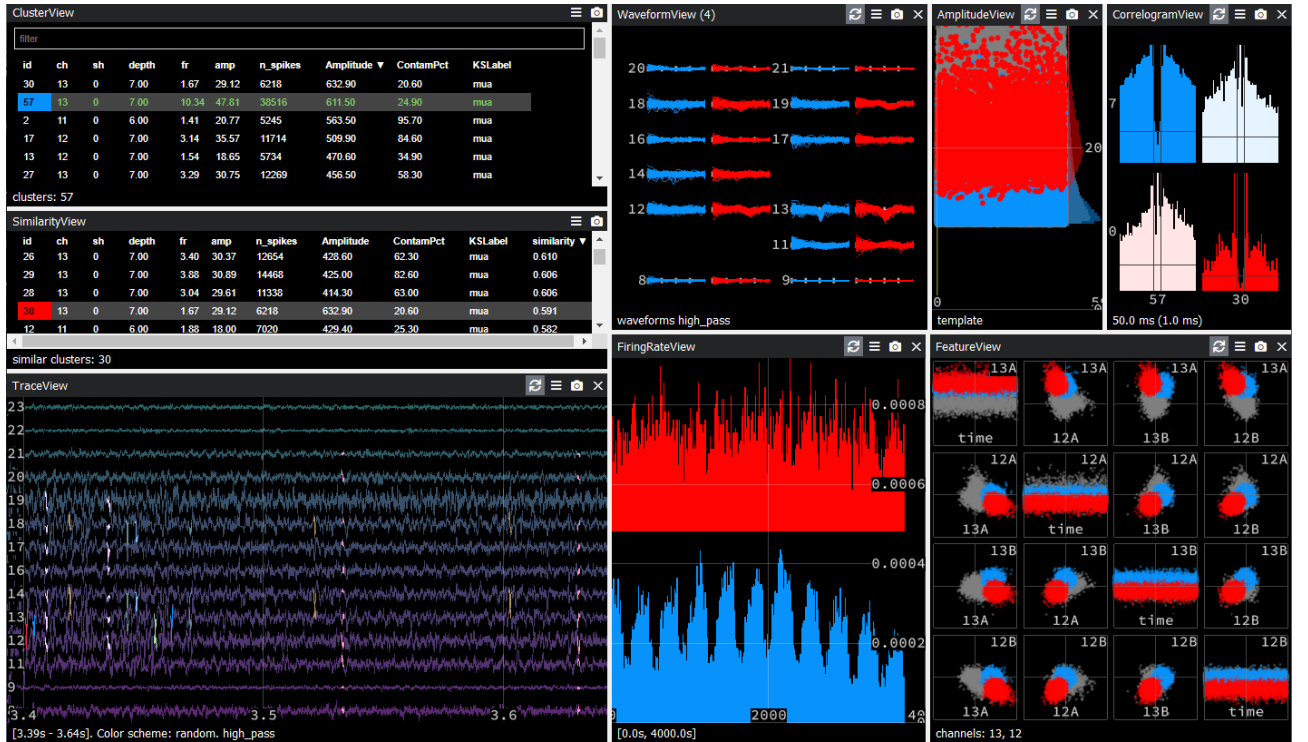


Figure 4: The Phy GUI interface presents several views that depict the spike sorting algorithm’s output data. On the top left is the Cluster view, on the middle left is the Similarity view, on the bottom left is the Trace view, on the top middle is the Waveform view, on the bottom middle is the Firing rate view, on the top right is the Amplitude and Correlogram view, and on the bottom right is the Feature view.

The Cluster view displays the clusters determined by the spike sorting algorithm. Each cluster is assigned a unique identifier, a channel number which denotes the nearest probe site, and a depth value that is calculated using the geometry of the probe and the channel number associated with the cluster.

The spike's average *amplitude*; *n_spikes*, which represents the number of spikes in a cluster during the recording; and *KSlabel*, indicating the quality of the cluster: good (individual neuron, SUA), multiunit activity (MUA) or noise, are all important markers. The clusters can be classified manually into good (green), mua (light grey), and noise (dark grey) during curation.

The Similarity view displays the similarity between the selected cluster in the Cluster view and the other clusters. This view features identical columns as the Cluster view, with an extra column containing similarity scores that the spike sorting algorithm computes. It aids in the manual curation process, whereby a higher similarity score signifies a higher probability that the two clusters belong to the same neuron.

The Waveform view displays the waveform of chosen clusters on various channels. The waveforms may be presented in spike waveforms, where each spike is visible, mean spike waveforms (average of the individual spikes), or template waveforms.

The Feature view exhibits the principal component features of the selected clusters on the appropriate channel (where the spike is best detected).

The Correlogram view displays the auto-correlograms in the diagonal and cross-correlograms in the off-diagonal between all selected clusters. The default 2 ms refractory period indicated by the two vertical lines and the baseline firing rate represented by the horizontal line.

Moving on to the Trace view, raw data is illustrated across all channels, where clusters are assigned distinct colours. It is possible to show all the clusters or only those selected.

The Amplitude View displays the amplitude of spikes over time for the chosen clusters, along with vertical histograms on the right. The grey spikes represent background spikes detected on the selected cluster's channel, but originating from other channels.

The Firing Rate View illustrates the number of spikes in the function of time for the selected clusters, allowing for observation of their firing patterns.

Manual clustering

Manual clustering aims to improve the result of the automatic spike sorting algorithm by human judgement [69]. The outcome can be enhanced in certain cases: if the automated algorithm identified two distinct clusters that belong to the same neuron (which requires merging), or if the automated algorithm assumed that the spikes belonged to the same group but originated from multiple neurons (which requires splitting), or if the automated algorithm identified clusters that are not well-isolated neurons and the reviewer must determine whether these clusters represent multi-unit activities or noise.

During manual curation, every cluster is reviewed individually in the Cluster view. Firstly, potential splits are looked for. If there is a distinguishable group of points in the Feature view of the cluster that can be separated, it is possible that the cluster includes spikes from two or more neurons. In this instance, creating new clusters can be achieved by drawing a polygon around a set of the defined groups of points during this splitting step.

The next step is to classify (label) the cluster as either MUA, noise, or SUA. A cluster qualifies as MUA or noise on these grounds: if the selected cluster's waveform amplitude compared to the baseline is minimal, if the waveform's shape is noisy, or if no clear refractory period is present in the auto-correlation graph (Correlogram view). In other words, the auto-correlation graph's values aren't low between the two vertical lines.

Once the cluster is deemed SUA, potential merges must be verified. Select the most similar cluster from the Similarity view to this selected cluster. If the waveforms of the two clusters are very similar, the auto-correlograms and cross-correlograms in the Correlogram view have similar shapes and good refractory periods, and both clusters remain stable over time (as shown in the Firing rate and/or Amplitude view) or they continuously drift into one another, and the group of points of the two clusters overlap in the Feature view, then these two clusters belong to the same neuron and should be merged. Select the next most similar cluster in the Similarity view and repeat this step until all possible merges have been checked (or the similarity score remains high). After merging, classify the cluster as "good", i.e. SUA, and repeat these steps for all unclassified clusters in the Cluster view.

The GUI stores the label for each cluster in a log file. The results of manual clustering can be further analyzed in Matlab or Python with custom codes.

2.6 Wavelet analysis

The wavelet transform of a time-evolving signal depends on two variables: scale (or frequency) and time [70]. Wavelets offer a means to represent the frequency components of a signal as a function of time. Whilst the standard Fourier transform also represents the frequency content of a signal, it does not provide easily accessible information concerning time-localization, such as that of high frequency bursts.

Time-localisation can be achieved by using the windowing technique to cut off a specific slice of the signal, which is well-localised, and then performing its Fourier transform. This produces the windowed Fourier transform, a commonly used method for achieving time-frequency localization. Using the described method, the LFP of the recorded brain signals was determined by calculating their one-sided amplitude spectrum over time, as described in Section 5.5.1.

The wavelet transform also offers a similar time-frequency description, with a few noteworthy differences. Both wavelet and windowed Fourier transforms involve taking the inner products of the signal with a set of functions. However, the key difference between the two methods lies in the shapes of these analyzing functions. In windowed Fourier transforms, the functions all consist of the same envelope function, translated to the appropriate time location, and filled with higher frequency oscillations. The functions have the same width irrespective of their frequency. In contrast, the functions in the wavelet transform have time-widths that suit their frequency. At high frequency, they are narrow, while at low frequency, they are broader. Consequently, the wavelet transform has better capability than the windowed Fourier transform to magnify very short-lived high frequency phenomena. This is particularly useful when examining signal transients.

The functions utilized in the wavelet transformation are commonly referred to as "wavelets". The mother wavelet is what they are produced from. Examples of typical mother wavelets include Haar, Morlet, and Mexican hat wavelet. The wavelets (or daughter wavelets) are formed by scaling a and translating b the mother wavelet ψ in time:

$$\psi^{a,b}(x) = \frac{1}{\sqrt{a}}\psi\left(\frac{x-b}{a}\right).$$

The wavelet transform is:

$$W_\psi(f)(a,b) = \frac{1}{\sqrt{a}} \int_{-\infty}^{\infty} f(t)\psi\left(\frac{t-b}{a}\right)dt.$$

2.7 Video tracking

Behavioural studies often utilise video recording to capture animal movement during experiments. Efficient research requires software and algorithms capable of tracking and analysing the position of an animal during experiments. It is therefore essential to use video tracking applications. 28 open source software have been comprehensively compared in an excellent review by Panadeiro et al [71]. The tracking programs follow a standard process that involves image acquisition, calibration, detection, trajectory generation and data analysis. However, certain programs might omit calibration and trajectory generation steps.

The method of image acquisition determines how video images are produced. There are two primary methodologies: offline, involving the processing of a sequence of images from a video file, or online, which utilises a live camera to continuously stream each image. One benefit of using the offline mode is that intricate algorithms can perform intensive computations and access past and future frames to process a given image. The online approach utilises real-time computations, reducing storage capacity requirements, yet elevating computational demands due to the need to evaluate each frame at the same pace as the camera frame rate to prevent data loss. Currently, real-time tracking is feasible solely through basic algorithms that are unable to cope with complex backgrounds or multiple targets.

The calibration step establishes the mapping between the camera's natural units (pixels) and the real world units. A calibration algorithm capable of correcting for image distortion and perspective errors is used when capturing images of a 2D surface with a fixed camera. It is crucial to note that

the distance from the centre to the edges does not increase linearly. Calibration techniques often rely on the pinhole mathematical camera model [72] to solve the equations that describe how an object in the real world appears projected onto the image plane by an ideal camera lens. Various calibration techniques use a simple scale transformation to convert image coordinates to world coordinates. This is done by multiplying the image coordinates by a constant.

The initial stage of detection involves determining the location of animals within each image of the recorded video. Various detection algorithms comprise steps, including background subtraction, for eliminating stationary features from the environment, segmentation, for separating objects that indicate potential animals, and filtering, for eliminating erroneous detections.

Background subtraction algorithms seek to eliminate environmental features that could potentially distract from the detection of animals. Animals in a natural environment experience dynamic lighting conditions that can alter their appearance through shadows, reflections, and other natural phenomena. The two most prevalent types of background subtraction techniques include static image-based techniques, which are implemented when background objects and illumination remain unchanged during the experiment, and dynamic model-based techniques, which are employed when there are changes in illumination or other gradual variations in the background. Static methods typically utilise either a reference frame or video of the background without animals. Dynamic methods, however, utilise moving or decaying average models or Gaussian mixture models. It should be noted, however, that such methods may not reliably detect animals that remain static for a significant portion of the experiment. Novel approaches employ deep learning algorithms to distinguish pixels from the background and foreground, which proves to be more robust than alternative techniques. However, this method presents complexity and demands an enormous volume of training data, consequently it is highly computationally intensive approach.

Segmentation is carried out after performing background subtraction. Its aim is to distinguish potential animals in the image and to apply a filtering step to eradicate false positives. The most widely used segmentation method is known as 'thresholding.' This simple approach employs a reference value to isolate pixel regions of varying brightness within the image. Thresholding is a computationally efficient technique that relies on contrast and demands that animals are visible as bright objects against a dark background or dark objects against a bright background. Nevertheless, it is very sensitive to false-positives and false-negatives in non-uniform images. As a result, the majority of analyzed software utilizes a variation of this technique. To enhance sensitivity and robustness of detection, certain techniques identify particular animal features in the image. For example, the ellipse fitting strategy can be applied. Another viable approach to animal segmentation is the implementation of machine learning techniques. To implement this approach, users have to indicate the precise location of each animal in several video frames prior to the automated detection phase. Thereafter, the animal is identified in the remaining frames using machine learning techniques.

During the trajectory generation step, a group of trajectories are generated based on an animal's positions in subsequent video images. The primary challenge of trajectory generation is to link potential targets with previous trajectories if multiple animals are present. The Kalman filter, a prediction-correction method [73], is the most widely used technique for dealing with this problem. Kalman filtering and similar techniques may be unreliable when tracking animals in scenarios with occlusions or multiple interacting animals. This limitation is observable in situations where two animals cross paths and change direction after a collision.

Data analysis entails extracting data from the output of the trajectory generation phase to acquire the necessary behavioural data for the experiment. This data comprises statistics concerning moving velocity, distance, animal orientation, exploration rate or activity rate. Data analysis is typically a post-processing procedure performed after completing all the previously mentioned steps.

3 Micro-drive and head-stage fabrication

3.1 Rapid prototyping

To fixate the optrode, created by the research group, to a rodent's skull, the author has developed a micro-drive. When the rodent is in motion, the micro-drive is susceptible to different physical effects, thus requiring protection for both the micro-drive and the optrode. Therefore, a head stage has also been devised.

3.1.1 Micro-drive

To meet the criteria of being affordable, lightweight, quickly reproducible, allowing for easy vertical displacement tuning of the micro-drive and implantable, a model was devised based on Chung et al's design [57]. This model will hereinafter be referred to as a *simple type* micro-drive.

Its design comprises of three modular components: a shell, a body, and a slider, with the optrode affixed to the slider. The slider consists of two components. The first component is connected to the body, while the second component is where the optrode is glued. The optrode-holding second component fits into the first component and can be easily replaced. The slider moves up and down vertically relative to the body, enabling precise adjustments using an M2 (2 mm x 20 mm) stainless steel screw with a 0.4 mm thread pitch (see Figure 5). The maximum vertical movement of the slider is 7 mm. The body's trapezoidal shape was replaced with a square to prevent the slider from detaching from the surrounding body of trapezoidal shape. The body envelops the slider, and the shell encloses the body. Only the shell is attached to the animal skull in the micro-drive. Consequently, the optrode along with the slider and body can be retrieved by unscrewing the body with the slider from the shell.

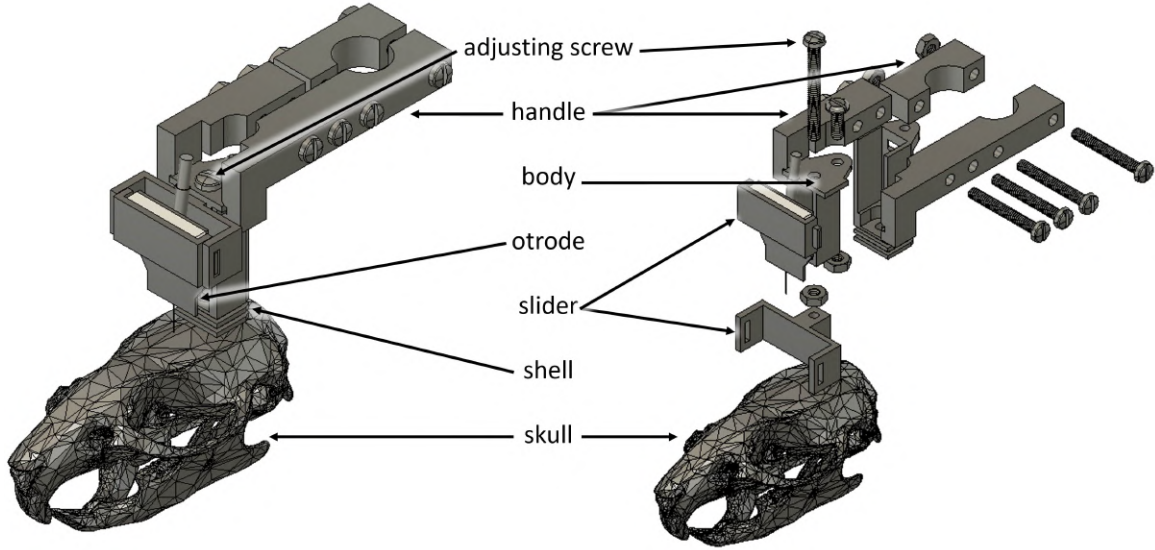


Figure 5: On the left-hand side, the *simple type* micro-drive system comprises the slider embedded in the body, which is in turn embedded in the shell. The shell is then cemented to the skull, while the micro-drive handle holds the body in place during both implantation and reimplantation. Using the adjusting screw, the vertical movement of the slider can be finely tuned. On the right-hand side, there is an exploded view of the *simple type* micro-drive system.

During the process of implanting and reimplanting the micro-drive, a handle is employed to securely fasten it to the stereotaxic post, which has a diameter of 7.9 mm. The body is laterally compressed by the handle to ensure stable fixation, as shown in Figure 5. The stereotaxic post is wrapped by the micro-drive handle, and screws are used for further fixation.

To achieve a smoother vertical movement of the micro-drive, a new design was created with inspiration from Cambridge NeuroTech's nano-drive [58]. This version will be referred to as the *complex type* micro-drive. Unlike the *simple type* micro-drive, its body has two distinct components, an inner and

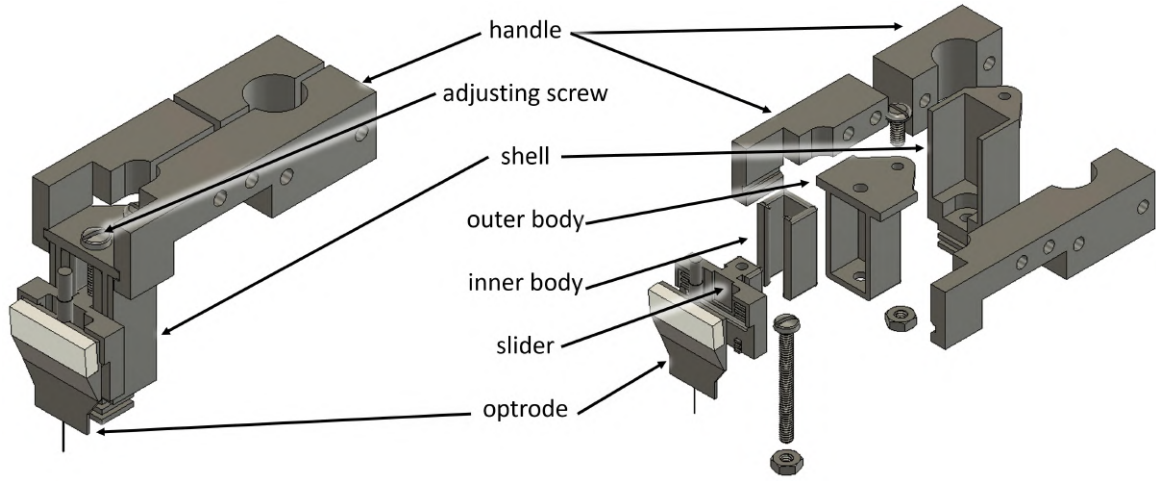


Figure 6: On the left-hand side, the *complex type* micro-drive system comprises the slider firmly embedded in the inner body that is wrapped by the outer body, which is further held embedded in the shell. The shell is then cemented to the skull, while the micro-drive handle holds the body in place during both implantation and reimplantation. Using the adjusting screw, the vertical movement of the slider can be finely tuned. On the right-hand side, the view of the *complex type* micro-drive system is illustrated in an exploded view.

an outer body. The inner body is C-shaped when viewed from above. The inner body surrounds the slider, which is controlled by an adjusting screw, and the slider moves vertically within it (see figure 6). This design holds the slider more securely and prevents any undesired lateral movement. The outer body encompasses the inner body, which in turn contains the slider and the shell. Compared to the *simple type* micro-drive, this design has larger dimensions as a consequence of the extra components provided by the inner and outer body. One additional difference is that the slider consists of a single component. The optrode is glued directly to that component of the slider. The micro-drive handle is designed in a similar fashion to that of the *simple type* model, though the size is different.

During the development of the micro-drive, an improved version of the *simple type* micro-drive system was created, as illustrated in Figure 7. According to the results of the vertical displacement mechanism's smoothness (refer to section 3.2.2), there is no significant difference between the simple and complex micro-drive systems. However, on the basis of the simpler structure of the *simple type* microdrive, this version has been upgraded. The key difference between this version and the previous version lies in the circular shape of its components, shell, body and slider, which makes this version suitable for both additive and subtractive manufacturing techniques. Another distinction can be observed regarding the procedure of connecting the body to the handle, allowing the two handle screws to slide into the body's grooves prior to being firmly secured in position. Formerly, the handle compressed the body sideways to secure it, however it has been discovered that this method appears to be unstable, as discussed in section 3.2.3.

3.1.2 Head Stage

Freely moving rodents expose the implanted optrode to various physical effects, such as hits caused by the animal's movement or scraping. To prevent damage to the optrode fitted with the micro-drive system, a head stage was developed, as shown in Figure 8.

The main elements of the head stage include the base, cover and top. The base is cemented and screwed onto the animal's skull for fixation. The grooves on the base enhance the attachment. It should be noted that the micro-drive is not affixed to the base, but instead attached directly to the skull. This design prevents any impact on the head-stage from directly affecting the micro-drive. A cover is positioned on the base to conceal the micro-drive along with the optrode. The cover comprises two parts, with a larger frontal part and a smaller rear part, facilitating easier implantation.

The optrode has two connectors, one for optical stimulation via the optical cable and another

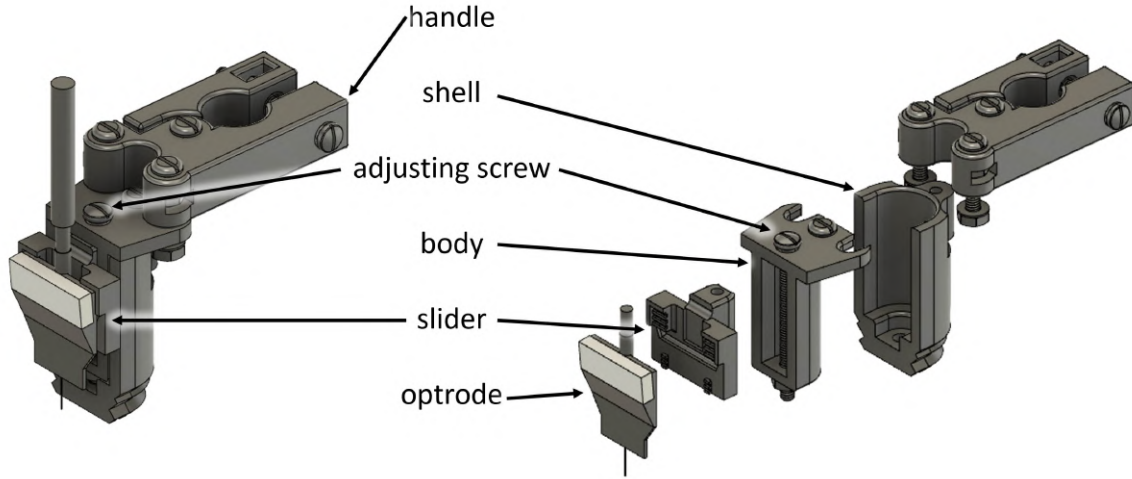


Figure 7: On the left-hand side, the upgraded version of the *simple type* micro-drive system is depicted. On the right-hand side, an exploded view of the micro-drive system is presented.

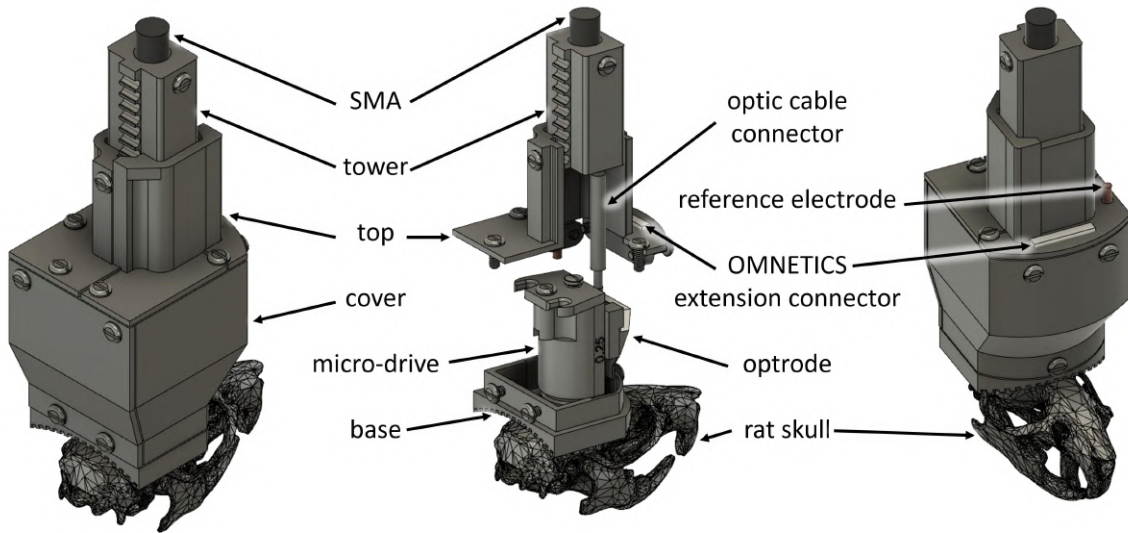


Figure 8: Components of the head stage and micro-drive system from various angles. The base and the micro-drive are separately affixed to the rodent's skull using cement. A cover is then placed on top of the base to conceal the micro-drive with the optrode. The cover is comprised of two parts, a larger front part and a smaller rear part to ease the implantation procedure. The top part features various connectors, including SMA for the optic cable, OMNETICS for transmitting the recorded electrophysiological data from the optrode, as well as a connector for the reference electrode. This top part is also made up of two components. When adjusting the penetration depth of the optrode, one can reach the adjusting screw by removing the smaller part of it.

for electrophysiological recording via the PCB. The top part is designed in a way that the external optical cable connects to the optrode via the SMA connector, whereas the recorded brain signals are transmitted to a computer through the OMNETICS connector. There is also a reference connector for the reference electrode. The upper part encircles the tower, which holds the SMA connector in place. The tower's vertical position can be adjusted relatively to the upper part. This adjustment is necessary when the penetration depth of the optrode changes as the optical cable is stiff with a fix length within the head stage. The top part consists of two components. During the surgery, as the optical cable

within the head stage connects to the optrode, the larger upper part secures the tower. If it is necessary to adjust the penetration depth of the optrode, simply removing the smaller top component will allow access to the screw responsible for adjusting the vertical movement of the micro-drive.

3.1.3 Design

Fusion 360, a cloud-enabled 3D CAD, CAM, CAE, and PCB design software developed and sold by Autodesk [74], was utilised for the design. This software encompasses all the necessary tools required for creating prototypes starting from sketch until fabrication.

This software provides various benefits, such as swift file transfer and computation, streamlined workflows that facilitate effortless design modifications and parameter adjustments in comparison to AutoCAD. Additionally, cloud-based remote access enables users to work on multiple computers. Its user-friendly interface and comprehensive documentation of functions enhance its usability. Numerous tutorial videos are also available online. Students can take advantage of the free educational licence. In addition, the construction and adjustment of individual components within the assemblies can be easily executed, while joints between them can be made to replicate the model's behaviour. Furthermore, an animation can be employed to showcase the model and its characteristics.

3.1.4 Fabrication

The models were fabricated using 3D printing technology. However, due to their fine details and small dimensions, Fused Filament Fabrication (FFF) 3D printing cannot be utilized for their production. FFF is a method of additive manufacturing that involves pushing a continuous filament of thermoplastic material through a heated nozzle to create objects layer by layer. The nozzle diameter and layer height are the determining factors in printing's XY and Z resolutions. The nozzle diameter can range from 0.1-1 mm, while the layer height typically measures around 0.2 mm [75].

Digital Light Processing (DLP) 3D printers provide a better resolution compared to Fused Filament Fabrication (FFF) printers. The XY resolution for DLP printers varies from 35-100 μm , with the Z resolution ranging between 25-300 μm [76]. DLP technology utilises a digital projector screen to project the design layer over the whole platform, curing all points concurrently. The material used is a liquid resin that hardens to plastic using UV light. This method provides greater speed and precision in comparison to FFF technology.

Stereolithography (SLA) 3D printing machines bear resemblances to DLP printers. However, unlike DLP printers, they cure individual cross-sections of a layer using a circular point, rather than the whole layer at once. This procedure, done through a UV light source (a laser), makes SLA printers slower than their DLP counterparts. Another contrasting feature is the shape of their basic unit - in SLA, it is circular, thus creating a smoother surface, while in DLP, it is square-shaped pixels.

The models were fabricated through DLP printing in the Robotics Laboratory at the University, using the Phrozen Sonic Mini 8K printer [77]. The resolution applied was 50 μm , while the Phrozen Nylon-Green Tough 3D Printer Resin [78] was utilised. As the slicer program accepts STL files, the models designed in Fusion 360 were converted into this format. In the slicing software, parameters of the 3D printing process are defined. The software then converts the STL file into instructions that can be executed by a 3D printer. We utilized the ChiTuBox slicer program, which is compatible with the prevailing resin-based 3D printing technology.

Fabrication of the models was also carried out at the HUN-REN Research Centre for Natural Sciences using a Form 2 SLA printer by Formlabs [76]. The models were printed using Surgical Guide Resin due to its biocompatibility and stiffness [79]. PreForm, Formlabs' own slicer for its 3D printers, was utilized.

Subsequently, numerous prints with varying parameters were conducted to ascertain the optimum distance between the movable parts. For the micro-drive of the *simple type*, in the case of using a DLP printer, the distance between the slider and the body is 0 mm, whereas the gap between the body and the shell is 50 μm . Concerning the micro-drive of the *complex type*, the distance between the slider and the inner body is 0 μm , whereas the gap between the inner and outer body is 25 μm , and the

gap between the outer body and the shell is $50\text{ }\mu\text{m}$. In the instance of the improved version of the *simple type* micro-drive, utilizing SLA printing technology, the distance between the slider and body is $25\text{ }\mu\text{m}$, and $30\text{ }\mu\text{m}$ between the shell and body.

3.2 Description of tests

3.2.1 Reproducibility

The dimensions of the 3D printed and designed models differ, as a result of the limited resolution of the 3D printer and the characteristics of the resin employed. The accurate dimensions are essential at the connections of the model parts where they slide against one another, particularly at the slider, which moves in a vertical direction towards the body. These dimensions were measured.

Another vital determinant of successful printing is suitable support. 3D printing constructs parts by layering them, thus necessitating a preceding layer to build upon. Depending on the model's complexity, an appropriate support structure is necessary. If an element overhangs more than 45° , support is required. This is because, as shown in figure 9, if the support is not dense enough, the overhanging parts can fall down, causing the print to fail. Conversely, if the support is too dense, the surface of the parts that connect to the support will be rough. The correct and optimal support is demonstrated in figure 10, which enables successful printing.

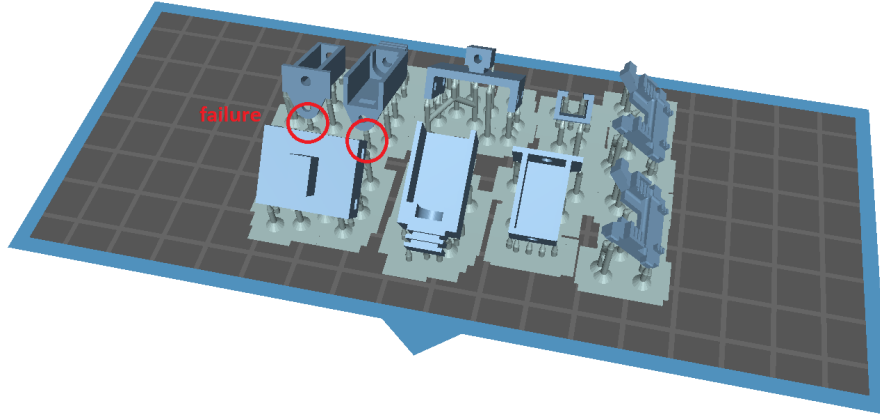


Figure 9: Arrangement of the parts of the *complex type* micro-drive in the ChiTuBox slicer resulted in failed 3D printing due to inadequate support.

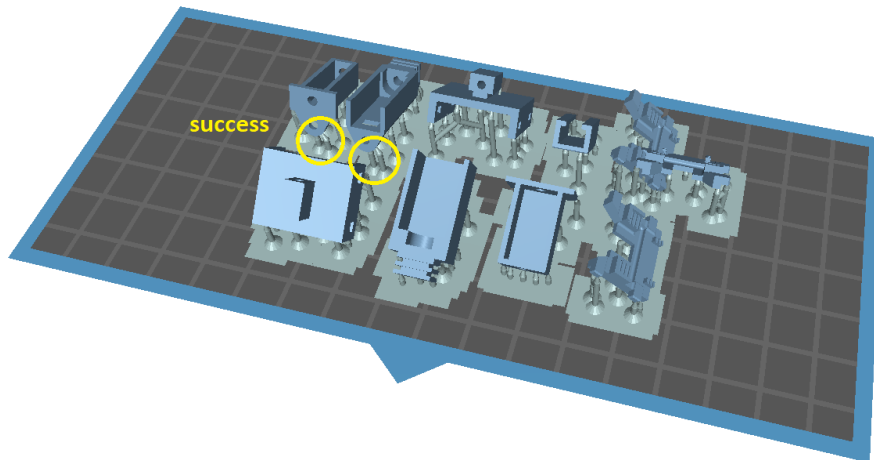


Figure 10: Arrangement of the components of the *complex type* micro-drive in the ChiTuBox slicer resulted in a successful print owing to the adequate support.

3.2.2 Wobbling

For effective infrared neuromodulation and adequate electrophysiological recordings, smooth vertical movement of the optrode coupled to the micro-drive is essential. In order to prevent damage to neurons during the adjustment of optrode penetration depth, it is essential to avoid any lateral movement (wobbling) of the optrode shaft.

This lateral, peak-to-peak displacement was assessed under a light microscope (refer to Figure 1 in the Appendix). A needle was attached to the slider for each micro-drive design and the lateral movement of the needle tip was examined (see Figure 2 in the Appendix). A brief video was recorded for every test. The scale bar measures 1 mm in length. In the case of the nano-drive commercially available from Cambridge NeuroTech (refer to Figure 11), no lateral displacement was observed. In our study, we observed a lateral displacement of approximately $100\text{ }\mu\text{m}$ in both the *simple type* (Figure 12) and *complex type* micro-drives (Figure 13), which we consider to be acceptable.

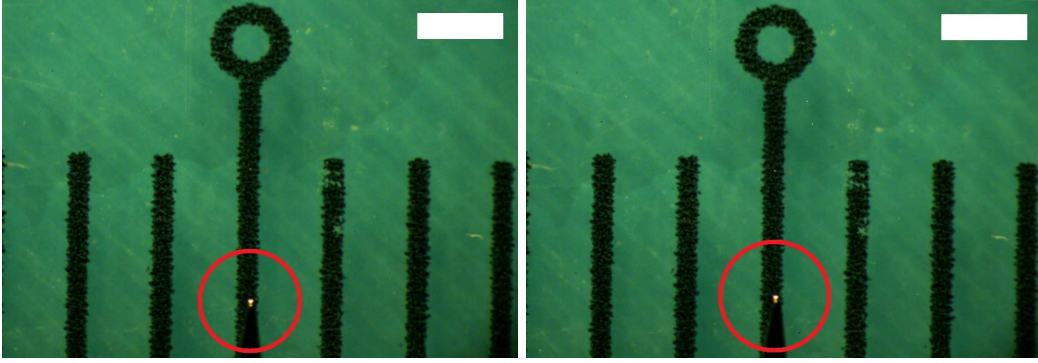


Figure 11: Wobbling examination of the nano-drive from Cambridge NeuroTech reveals no lateral movement. The length of the scale bar measures 1 mm.

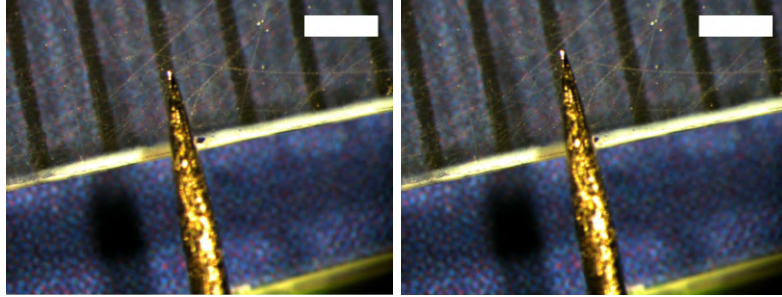


Figure 12: Wobbling test of the *simple type* micro-drive, indicating a slight horizontal shift. The scale bar measures 1 mm in length.

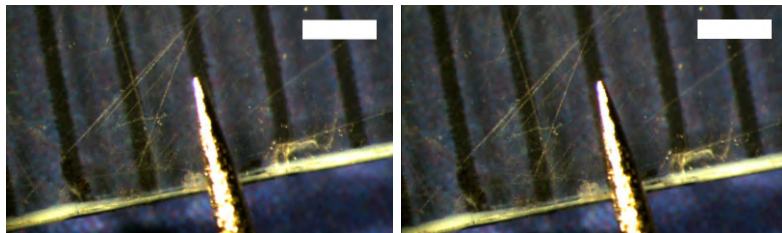


Figure 13: Wobbling test of the *complex type* micro-drive. A slight horizontal shift is apparent. The scale bar measures 1 mm in length.

3.2.3 In vivo performance

The *complex type* micro-drive was tested in vivo on a rat at the HUN-REN Research Centre for Natural Sciences. In an acute experiment, an anaesthetised rat was secured to a stereotaxic frame (refer to Figure 5 in the Appendix) to replicate a previous test that utilised an infrared neuromodulation protocol. The purpose of the experiment was to examine the micro-drive and the infrared neuromodulation with an optrode that features a shaft with an asymmetric tip. The optrode was affixed to the micro-drive, which in turn was attached to the stereotaxic post. In this experiment, the micro-drive was not cemented to the rat’s skull.

Vertical displacement tests were not conducted in this acute experiment, only in subsequent ones. However, during the current experiment, the micro-drive handle failed to fix the apparatus securely, causing some wobbling. Lateral squeezing is not an appropriate fixation method. Consequently, the handle configuration was modified in the improved version of the *simple type* micro-drive (see Figure 5 and Figure 7). To attach the body to the handle, the two handle screws are slipped into the grooves on the body before firmly securing them in place. For the time being, the micro-drive was fixed to the micro-drive handle temporarily using a screw.

3.2.4 Water test

One day prior to the in vivo experiment, the micro-drive and optrode were placed in a container filled with distilled water to prevent the optrode shaft from drying up. The tested micro-drive was made from Phrozen Nylon-Green Tough 3D Printer Resin. Prior to the in vivo experiment, it was observed that the *complex type* micro-drive had swollen and had become soft. The slider had become too elastic to firmly secure the optrode, causing the vertical displacement mechanism unusable. In addition, the swollen body couldn’t be attached to the micro-drive handle. In order to conduct the in vivo experiment, the optrode was fixed to another micro-drive that was not exposed to water.

Due to the observation that parts printed with Phrozen Nylon-Green Tough 3D Printer Resin become soft and swollen, a water test was conducted on both this resin and the Surgical Guide Resin. The purpose of the test was to simulate the wet environment of living tissue to which the micro-drive will be exposed. Furthermore, the suitable printer resin could be selected for the long term experiments with freely moving rodents.

Various parts of the micro-drive, printed with two different types of resins, were tested during this water test. Figure 14 illustrates the components used for the water test. The components with the label *N* were printed using Phrozen Nylon-Green Tough 3D Printer Resin, while label *T* refers to Surgical Guide Resin prints. Components labelled *N1* and *N2* originate from *simple type* micro-drives, where *N1* is the body with a slider and *N2* is the shell. The remaining components are derived from *complex type* micro-drives. *T1* serves as the inner body, *T2* as the slider, and *N3* and *T3* as the outer bodies.

The experimental setup is depicted in Figure 15. The components were immersed in tap water; specifically, the *N2* and *T1* components were submerged in the water, while the other components were suspended above the water and not in contact with it. The glass was tightly sealed to create a wet environment for the components. After half a day, the parts were removed from the glass.

After removing the components from the glass container, their dimensions were carefully measured. The dimensions of the components were measured before and after the water test to determine whether they maintained their shape. Additionally, the stiffness of the components was assessed before and after the water test. Ensuring the stiffness is crucial for the micro-drives used in chronic implantation, as the components need to maintain their shape and positioning within a wet environment.



Figure 14: Micro-drive components for the water test. Components labelled with N are printed with Phrozen Nylon-Green Tough 3D Printer Resin, while those labelled T are made from Surgical Guide Resin prints.

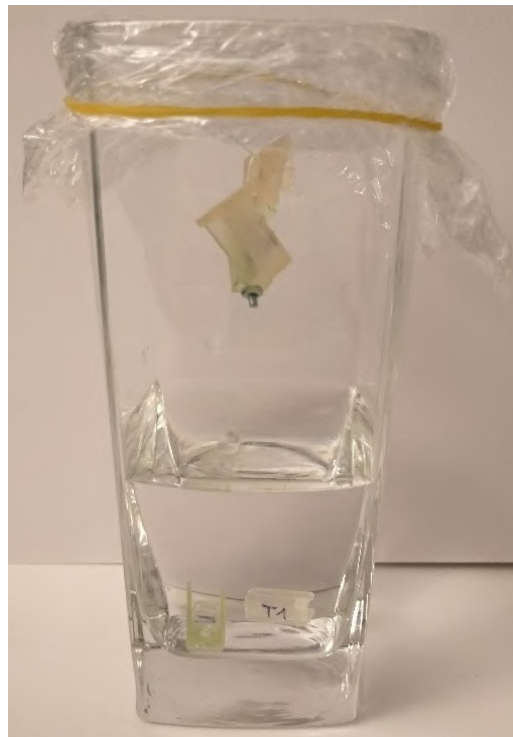


Figure 15: The water test's setting entailed the placement of several components in a glass of tap water, with some being submerged while the others were suspended above the water, without contact. The glass container was sealed to create a wet environment for the components.

4 Experiment

4.1 IR optrode

A single-shank, multisite silicon-based optrode with a micromirror needle tip was used for infrared neuromodulation and extracellular recordings [80]. The optrode was designed to maximize optical efficiency, reduce optical loss, and minimize tissue damage during device insertion.

Figure 16 displays the geometrical properties of the optrode shaft. The optrode has a 5mm long implantable shank possessing a cross-section of $170\ \mu\text{m} \times 200\ \mu\text{m}$ (width $\times$ thickness) with a parabolic-shaped tip, which is defined by the equation $y = 0.0025x^2 + 0.5766x$. At a distance of $145\ \mu\text{m}$ from the tip, 16 linear channels are located on the shank, with inter-site distances of $100\ \mu\text{m}$; these channels serve as extracellular recording sites that detect neural activity upon intracortical IR illumination. The recording sites are square-shaped with side lengths of $30\ \mu\text{m}$, resulting in a site area of $900\ \mu\text{m}^2$.

Planar and bulk MEMS technology was used to fabricate the optrode chip from p-type monocrystalline silicon. The electrophysiological recording sites were fabricated with sputtered platinum patterned by standard photolithography and lift-off technology. The recording sites are coated with porous Pt, also known as platinum black, to minimise their impedance. In addition to creating wire bonding pads to establish an electrical connection with an external connector, a groove was carved out for optical fibre alignment using the technique of deep reactive ion etching (DRIE). To smooth the uneven edges of the silicon chip following DRIE, the rough sidewalls were polished.

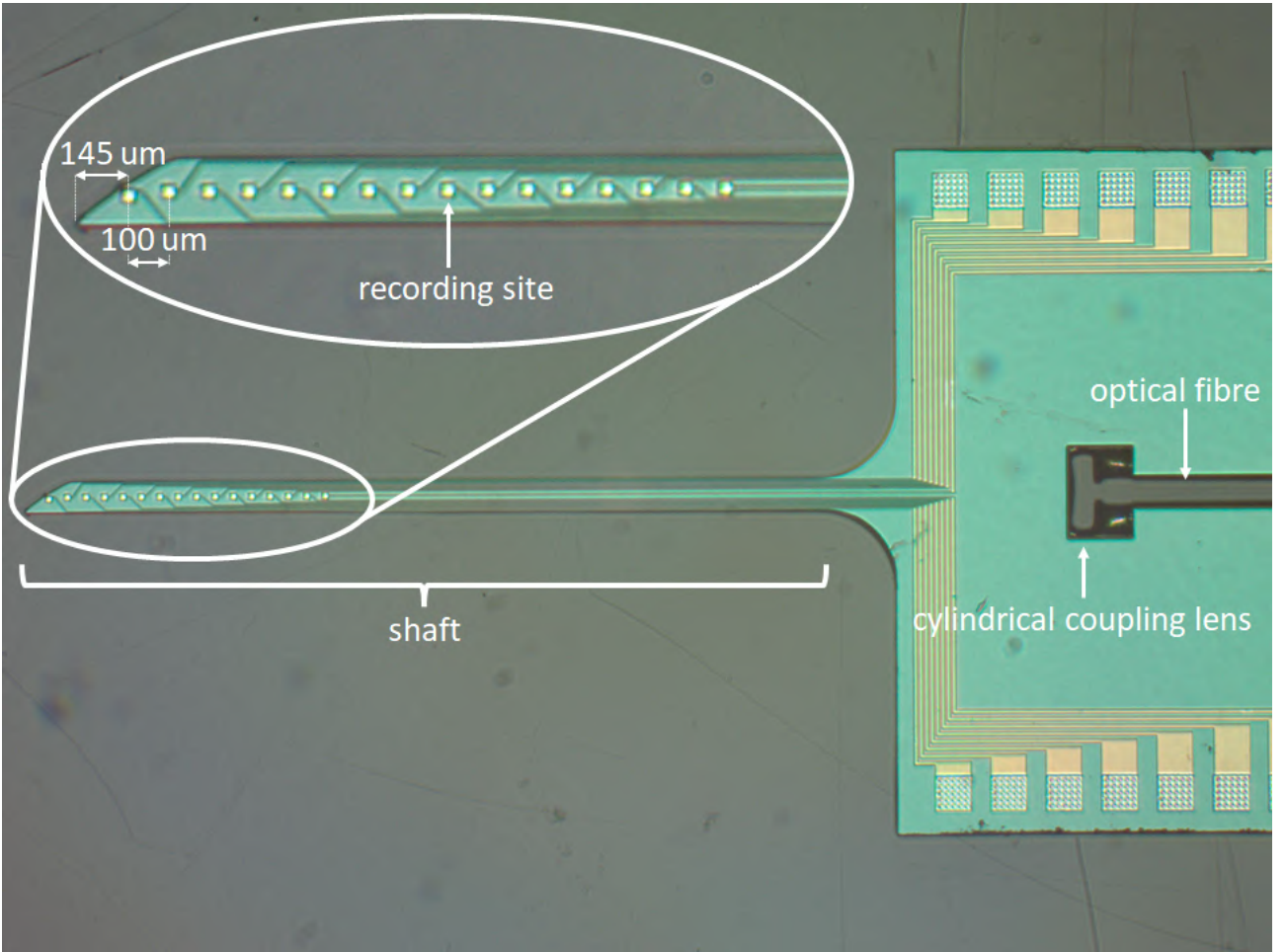


Figure 16: The optrode chip with a parabolic-shaped (micromirror) needle tip. The shaft is 5 mm in length and contains 16 linearly placed recording sites. The distance between the centre of the first site and the tip is $145\ \mu\text{m}$. The spacing between the centre of the sites is $100\ \mu\text{m}$.

The optrode chip is affixed to a custom-printed circuit board (PCB), specifically made for implantation in rodents. The PCB allows for electrical connections and guarantees correct alignment and

fixation of the optical fibre. The optical fibre itself, having a $105\text{ }\mu\text{m}$ core diameter, transmits IR light received from an external source (LPSC-1550-FG105LCA-SMA laser diode, Thorlabs Inc., USA).

4.2 Animal surgery

Four rats (weight, $327.5 \pm 52.5\text{ g}$; 1 female and 3 males) were anaesthetised with an intramuscular injection of a combination of ketamine and xylazine . A homeothermic heating pad was connected to a temperature controller in order to maintain their physiological body temperature. The deeply anaesthetised rats were positioned within a stereotaxic frame. After that small craniotomies and durotomies were performed over the somatosensory cortex. An optrode with the previously mentioned parameters was then carefully inserted into the brain at a depth of 2 mm to avoid rupturing blood vessels on the brain surface, and with slow insertion (a few $\mu\text{m/s}$) to prevent dimpling. A further screw electrode was inserted above the cerebellum as a reference electrode. Throughout the experiment, physiological saline solution at room temperature was administered into the cavity of the craniotomy to prevent the dehydration of the neocortex.

4.3 Optical stimulation protocol

To evaluate the effect of IR stimulation provided by the optrode with a micromirror needle tip, the given protocol was employed. Preceding the recordings, a 10-minute-long spontaneous activity was recorded separately. During the 1-hour long recordings, a 2-minute-long recordings without IR irradiation was performed initially. Later, a sequence of 2-minute-long infrared radiation exposure (laser is switched on, referred to as ON period) followed by a 4-minute resting period (laser is switched off, OFF period) was repeated ten times. The objective of the 4-minute OFF period during IR illumination is to allow sufficient time for the tissue to return to its initial temperature. A laser diode with an IR centre wavelength of $\lambda = 1550\text{ nm}$ (LPSC-1550-FG105LCA-SMA, Thorlabs Inc., USA), driven by a Keithley 2611B SYSTEM SourceMeter (Keithley Instruments Inc, OH, USA), was employed. Each onset of the laser diode was synchronized with trigger signals also generated by the Keithley 2611B SYSTEM SourceMeter.

5 Data analysis

In this section, we analyse the recorded and pre-processed electrophysiological data from various perspectives, including signal-to-noise ratio, firing rate patterns, neuron type, and spectral analysis. Our algorithms are implemented in Matlab. Lastly, a Python script for rodent tracking is presented, which enables the location of the freely moving rat to be determined from recorded videos.

5.1 Electrophysiological recording and spike detection

Extracellular recordings were conducted using Intan RHD2132 amplifiers with 32 channels, which were connected to an RHD-2000 Evaluation Board. Heating was initiated by a square pulse generated by the Keithley 2611B SYSTEM SourceMeter. The signals were synchronised via the analogue input of the Intan system, which was connected to a computer. The sampling rate was 20 kHz.

The individual neuron spikes must be extracted for analysis of the recordings. The automated spike sorting software Kilosort2 was utilized for spike extraction. The software's details are presented in Section 2.5.3. The manual review of the detected single-unit clusters was executed in Phy. The procedures of the manual curation are detailed in Section 2.5.5.

5.2 Signal-to-noise ratio estimation

5.2.1 RMS-based SNR estimation

In order to validate the effectiveness of optrode in conducting in vivo extracellular recordings, an RMS-based algorithm was utilized to calculate the signal-to-noise ratio (SNR) of the optrode according to Fiáth et al's research [81]. This algorithm considers the fact that during the down-states of ketamine-xylazine-induced slow-wave activity, most cortical neurons stop firing for a few hundred milliseconds [82]. These brief time windows allow for an estimation of the recording's noise level. During up-states, neurons display high spiking activity, which can be utilised to estimate the signal level. The ratio of up-states (high spiking activity) to down-states (low spiking activity) provides the SNR of the recording.

The algorithm was implemented using mostly built-in Matlab functions. The modified algorithm's steps are illustrated in Figure 17. The initial step involved applying a band-pass filter (500-5000 Hz) and rectification to the raw signal (Figure 17a) to extract the multiunit activity (MUA) from the local field potential (LFP) (Figure 17b). The band-pass filtered signal underwent smoothing with a moving average (Figure 17c) using a window size of 10 ms.

In the subsequent step, the up and down states are ascertained via thresholding. The average of the smoothed signal is set as the threshold. If the signal points surpass the threshold, they are classified as the up-state. Thresholding leads to minor gaps in the "true" up-states. To "bridge" these gaps, a data point is identified as an up-state if an up-state point lies within its assigned 50 ms radius. Figure 17c displays the output of the thresholding and "gap filling" processes. The black data points represent the up-state.

In the last step, up- and down-states exceeding 200 ms in duration are chosen. To determine the RMS value of actual up- and down-states, which are typically several hundred milliseconds long, and to eliminate falsely detected states identified by the algorithm, the minimum duration criterion is required. To prevent the use of the up-and down-states' transition zones, a 150 ms interval at the center of these chosen states is taken for RMS calculation. Both the up- and down-states are calculated using the formula $\text{RMS} = \sqrt{\frac{1}{n} \sum_{i=1}^n x_i^2}$. The signal-to-noise ratio (SNR) is computed by dividing the average root mean square (RMS) value of the up-states by the average RMS value of the down-states. Figure 17d illustrates the boundaries of the states, indicated by vertical black lines. The central intervals, lasting 150 ms, for the states which are at least 200 ms long, are displayed in green and red for up-states and down-states respectively.

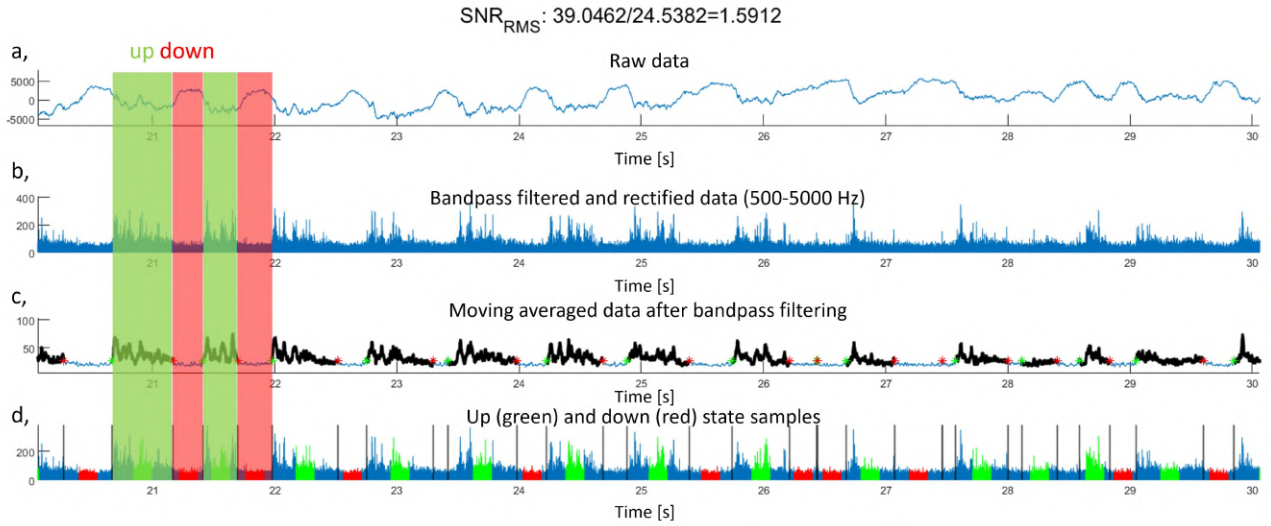


Figure 17: Steps for estimating SNR based on RMS. Two up and down states are highlighted with green and red shades respectively. Subplot (a) shows the raw data, subplot (b) shows the data after it has been band-pass filtered (500-5000 Hz) and rectified (absolute value), subplot (c) shows the result of the thresholding and "gap filling", where up-state points are black, and subplot (d) shows the points used for RMS calculation, with up-state highlighted in green, and down-state highlighted in red.

5.2.2 Amplitude-based SNR estimation

The amplitude ratio of the signal and noise offers an alternative method for calculating the signal-to-noise ratio. The algorithm presented here combines the previous SNR calculation method with the outcomes of the spike sorting algorithm (e.g. Kilosort). The aforementioned algorithm is applied to obtain down-states, thereby enabling the calculation of noise amplitude. The spikes detected by Kilosort supply the information necessary for computing signal amplitude.

Firstly, the raw signal undergoes high-pass filtering (at 300 Hz) to isolate the spikes. Following this, the amplitude of the noise is determined. The down-states are then divided into 5 ms intervals, with the difference between their maximum and minimum values calculated to obtain their amplitude. The noise level is obtained by averaging these amplitude values.

The spike sorting algorithm (Kilosort, in this case) output provides the signal's amplitude. Kilosort establishes the spikes' centres, and to determine their signal amplitude, 1 ms intervals (since the action potential is roughly 1 ms long) were extracted from the high-pass filtered data surrounding the aforementioned centres. The signal level was calculated based on the average amplitude of these extracted intervals.

False spikes may occasionally be detected by the spike-sorting algorithm. To mitigate the impact of erroneously identified spikes, the algorithm involves only those spikes with amplitudes exceeding 1.5 times the previously measured noise level when calculating the signal level. Consequently, the SNR is determined by dividing the signal level by the noise level. However, the algorithm has a drawback in that it can either yield no SNR value or no value smaller than 1.5.

Figure 18 depicts the stages of the amplitude-based signal-to-noise ratio (SNR) calculation. The up- and down-states are highlighted by green and red shading, respectively. In Figure 18b, spikes are illustrated by green lines and red dots indicate the detected spike centers. The amplitude of the spikes is represented in magenta and black dots, which denote their maximum and minimum values. In the up-states the low-amplitude spikes are excluded, highlighted in red in Figure 18c. The signal and noise levels are marked by a red and a black horizontal line, respectively, as depicted in Figure 18d.

5.3 Firing rate visualization

The experiment aimed to establish how infrared neuromodulation impacts neuronal spiking rates. To achieve this, a graphical user interface (GUI) was developed by the author that illustrates the firing

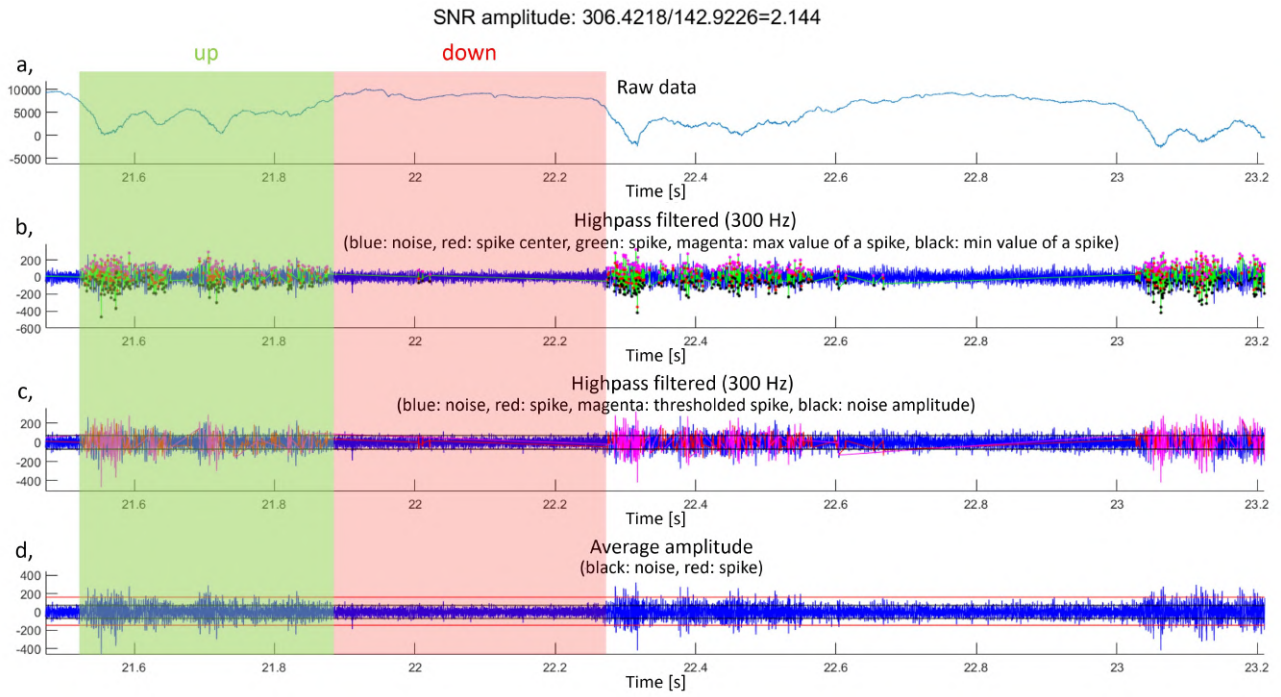


Figure 18: Steps for estimating amplitude-based SNR. Green and red shades highlight up and down states respectively. Subplot (a) shows the raw data, subplot (b) depicts data filtered for high-pass (300 Hz) with detected spikes, subplot (c) illustrates data filtered for high-pass (300 Hz) with thresholded spikes, and subplot (d) plots the amplitude level of the signal (red) and the noise (black horizontal line).

rate of neurons.

The GUI needs two inputs: the timing of the infrared stimulation and the result of Kilosort following manual curation in Phy. Firing rates for the clusters, which are classified as SUA or MUA during curation, were obtained from this input, representing the number of spikes over time. These firing rates were visualised using the Matlab GUI, shown in Figure 19.

The diagram in the upper section displays the timing of IR stimulation, with yellow indicating the ON period of IR irradiation, and blue indicating the OFF period. The timing is represented with red vertical lines on the other plots. The time interval can be set in the GUI, determining the size of time intervals for which the number of spikes is counted.

The *Spike rate over time* plot quantifies the total number of spikes as a function of time for each of the various clusters. The brighter (yellow) colour indicates a higher firing rate than the darker (blue) regions. The plot portrays whether cluster X has a similar colour to cluster Y, at different times t_1 and t_2 , indicating similar firing rates. The plot also identifies which clusters have higher firing rates compared to others (inter-cluster firing rate), as well as which clusters require further analysis.

The *Normalised spike rate over time* plot qualitatively represents how the firing rate changes over time within a cluster, (intra-cluster firing rate). The brighter yellow regions reflect a higher firing rate, while darker blue regions indicate a lower firing rate. This plot summarises how the clusters respond to IR irradiation, indicating whether the optical stimulation protocol affects all clusters or only a smaller group of them. However, it is not suitable for quantitative comparison of firing rates between different clusters.

The *Spike rate of cluster X* graph displays the number of spikes over time for the chosen cluster. This allows for an individual analysis of various clusters, and quantitative observation of changes in firing rates based on infrared irradiation.

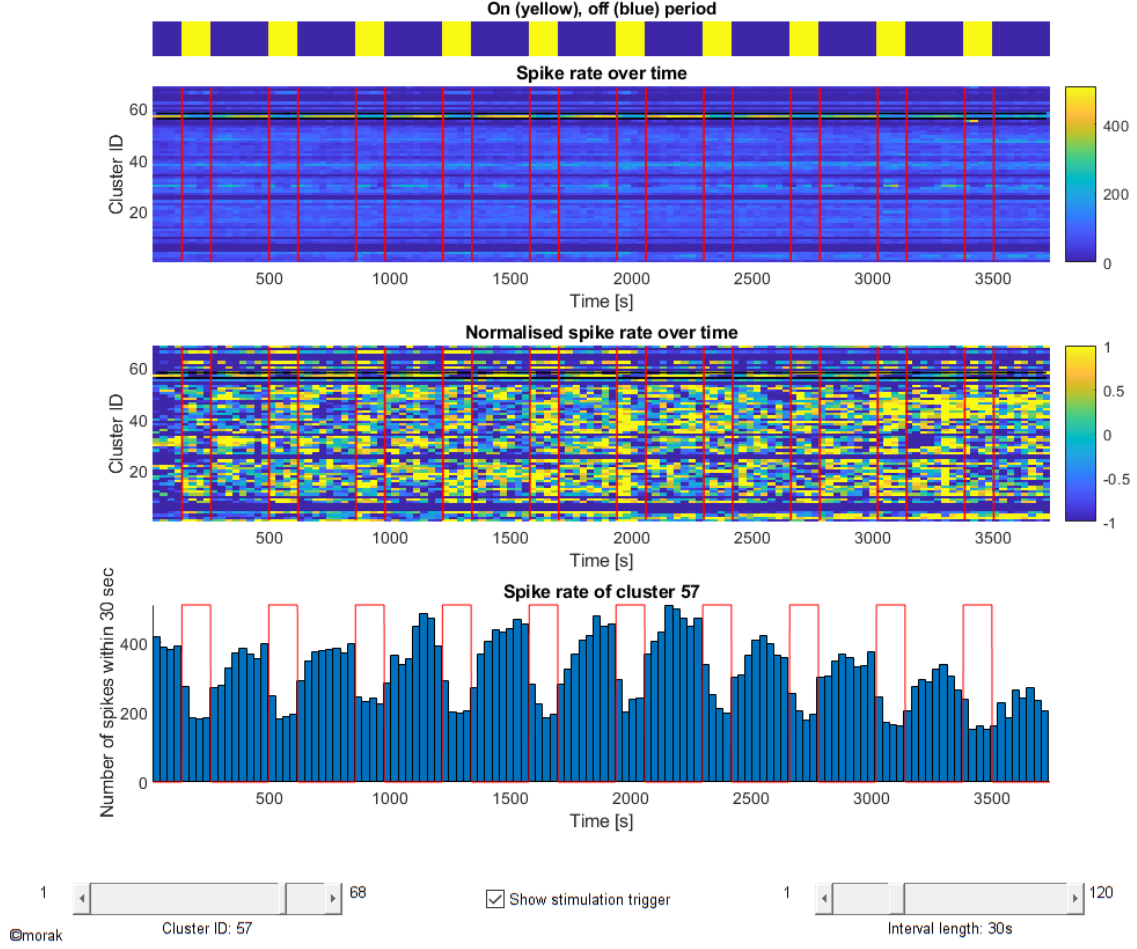


Figure 19: GUI for visualising firing rates. The *Spike rate over time* plot displays the inter-cluster firing rates, while the *Normalised spike rate over time* plot shows the intra-cluster firing rate. The *Spike rate of cluster X* plot illustrates the firing rate within a cluster.

5.4 Pyramidal vs interneuron detection

During the experiment, our curiosity extends beyond the change in firing. We aim to explore the reaction of various neurons to IR irradiation. In the cerebral cortex, two main types of neurons can be distinguished - the pyramidal neuron and the interneuron [83]. While pyramidal neurons are regular-spiking and excitatory, interneurons are fast-spiking and inhibitory neurons.

The selection of neuron group is determined based on the spike waveform. If the duration of the action potential at half amplitude is greater than 0.6 ms, the neuron is classified as a pyramidal neuron; otherwise, it is classified as an interneuron [84].

Figure 20 illustrates the average waveform and type of the chosen cluster across different channels. Utilising the optrode geometry and insertion depth, the channel depth can be calculated. If the waveform exhibits a typical action potential shape, the selection cluster's type (pyramidal or interneuron) is determined using half amplitude-based thresholding. In this scenario, a waveform is categorized as an action potential if the horizontal line drawn at half amplitude intersects it twice, and the time points of intersection are not the first or last points of the window. In Figure 20, the red dashed line illustrates the interval between the two intersection points.

5.5 Spectral analysis

The analysis of the recorded brain signal until now has been limited to the higher frequency domain. However, we are also interested in the effect of infrared irradiation on the lower frequency range, namely

Mean waveforms of cluster 57

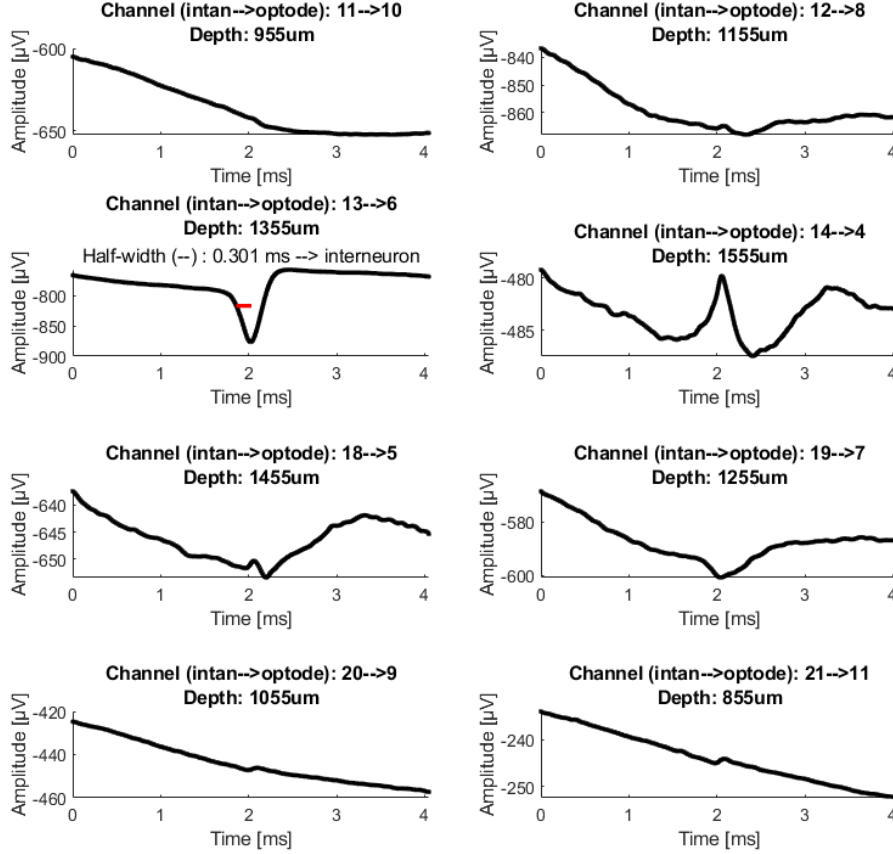


Figure 20: Mean waveforms of the chosen cluster on various channels are examined to determine the type of neuron based on the duration of the action potential at half amplitude (red dashed line).

the local field potential (LFP). Firstly, we introduce the one-sided amplitude spectrum, followed by the wavelet analysis to conduct spectral analysis on the lower frequency range.

5.5.1 One-sided amplitude spectrum

The one-sided amplitude spectrum represents the magnitude of a signal's frequency components in the frequency domain. By only considering positive frequencies, it simplifies the interpretation of frequency content. This way, it is easier to identify and analyse dominant frequency components and spectral characteristics.

To calculate the one-sided spectrum, the initial step involves pre-processing the raw data recorded from the brain activity of rodents. A notch filter is employed to attenuate the interfering frequency of power-line hum, which is essential since the analysis is carried out in the lower frequency range. Then, the signal undergoes low-pass filtering at 200 Hz and is down-sampled from a sampling frequency of 20 kHz to 2 kHz.

The next step involves calculating the single-sided amplitude spectrum using the method outlined in [85]. To do so, the Discrete Fourier Transform (DFT) of the pre-processed signal is calculated using an algorithm like the Fast Fourier Transform (FFT). This leads to a spectrum that is complex-valued, including both magnitude and phase information. As the FFT involves a scaling factor L , which represents the length of the original signal, it is important to adjust the complex-valued spectrum by dividing it by L . Additionally, it is necessary to calculate the complex magnitude of the FFT spectrum. Consider the double-sided amplitude spectrum, where the positive frequency spectrum is the complex conjugate of the negative frequency spectrum. Note that the peak amplitudes of the time domain

signal are halved in this case. To obtain the single-sided spectrum from the two-sided spectrum, take the non-negative component of the two-sided spectrum and multiply it by 2.

The single-sided spectrum was calculated for each optrode channel after pre-processing the signal. The frequency ranges observed for each channel were delta (0.5-4 Hz), theta (4-7 Hz), spindle (8-15 Hz), low beta (15-20 Hz), high beta (20-30 Hz), low gamma (30-50 Hz), and high gamma (50-80 Hz).

To obtain a normalised amplitude value for each frequency range, the amplitude values within the range are added together and then divided by the sum of the amplitude values for the entire spectrum. These normalised amplitude values at different frequency ranges are calculated for the ON, OFF, and control (Ctrl) intervals. The duration of the ON interval indicates the period during which the IR irradiation was active. The first half of the period during which the IR irradiation was switched off is referred to as the OFF interval, whereas the second half of this period is known as the control interval. The tissues around the tip of the optrode are heated during IR irradiation. In contrast, the tissue's temperature returns to normal during the OFF interval. Consequently, the control interval serves as a reference period for comparison with the ON period.

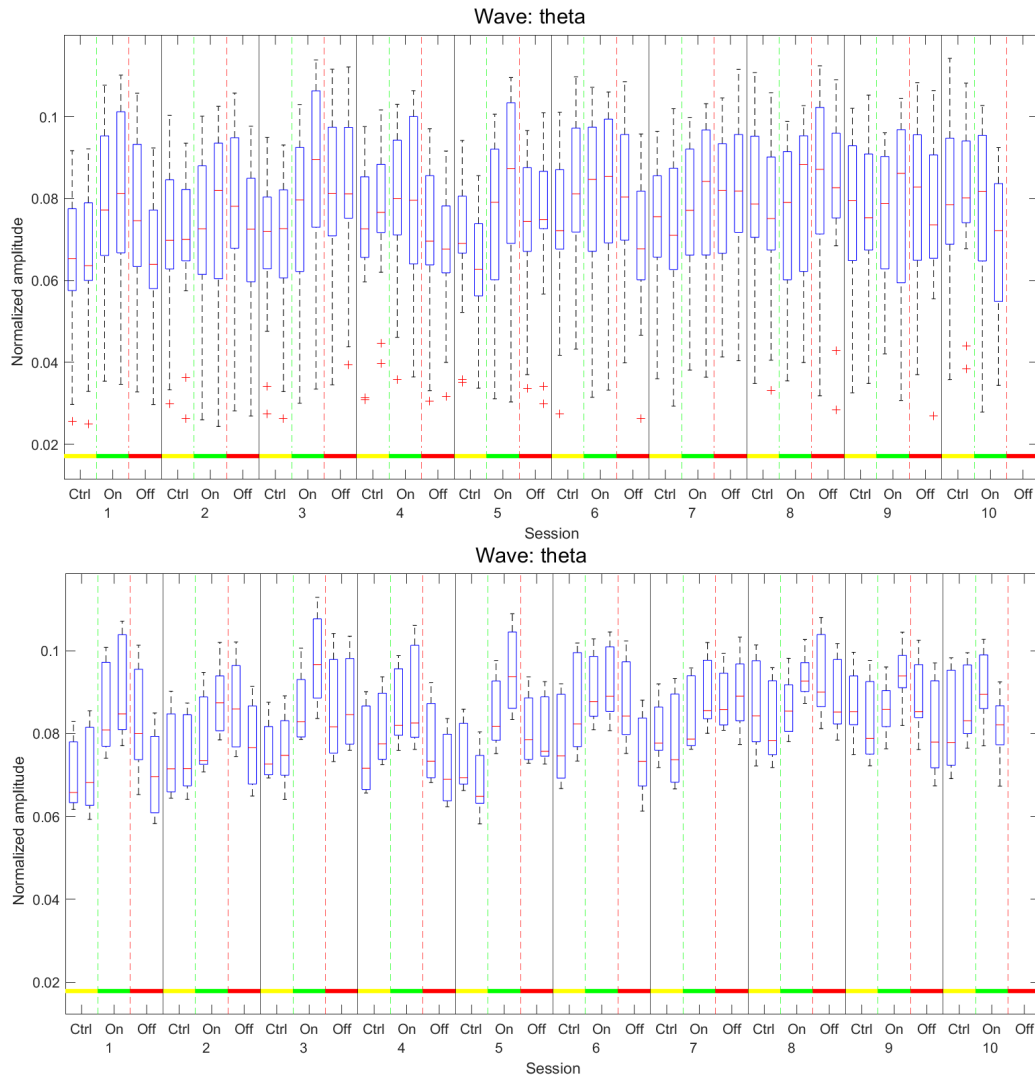


Figure 21: The box plot displays the normalised amplitude of intervals in the theta frequency range. It contains two plots, one presenting statistical data for all channels and the other for the selected channels. The central mark indicates the median, while the bottom and top edges of the box correspond to the 25th and 75th percentiles, respectively. The most extreme data points that were not classified as outliers are shown by the whiskers, while outliers are represented by a '+' marker symbol. At the bottom, yellow, green, and red lines demonstrate the control, ON, and OFF periods, respectively. The trials (sessions), which consist of 1 control, 1 ON, and 1 OFF interval, are separated by vertical black lines.

The periods of ON, OFF and control are subdivided into intervals lasting 30 or 60 seconds. During these intervals, the channels' normalised amplitude for various frequency components is computed. Figure 21 displays the box plot presenting the normalised amplitude of intervals within the theta frequency range. Each box presents the statistical data for all channels (upper plot) or the chosen channels (lower plot). The box plot shows the median through its central mark and the 25th and 75th percentiles through the bottom and top edges of the box, respectively. The whiskers represent the range of the most extreme data points not classified as outliers, and the outliers are displayed individually with a '+' marker symbol. The control, ON, and OFF periods are graphed at the bottom with yellow, green and red lines, respectively. The sessions (trials) comprising of 1 control, 1 ON and 1 OFF intervals are divided by black vertical lines.

As depicted in Figure 21, the top plot of the box plot representation for all channels does not reveal any pattern during the IR stimulation protocol when compared to the bottom plot, which shows only a group of channels. To identify the channels that follow the same pattern during the IR stimulation protocol, a GUI was developed to display the individual channels' normalized amplitude values.

Figure 22 displays the developed graphical user interface for representing the normalised amplitude of each channel within a specified frequency range. The control, ON, and OFF intervals are divided into 30 or 60-second segments, as previously mentioned in the box plot representation. In this particular case, the intervals last for 60 seconds. The active channels can be chosen by selecting the checkboxes on the right-hand side of the GUI. The curves of the active channels are plotted in different colours and contribute to the thick blue curve representing the mean normalised amplitude value. The inactive channels are shown in grey. On the right-hand side, we can select the frequency range from the drop-down menu. By choosing a curve shown as a dashed line, we can examine its pattern and the relevant channel number displayed at the bottom of the legend. The plot containing all the channels can be saved by clicking on the "Save plot" button.

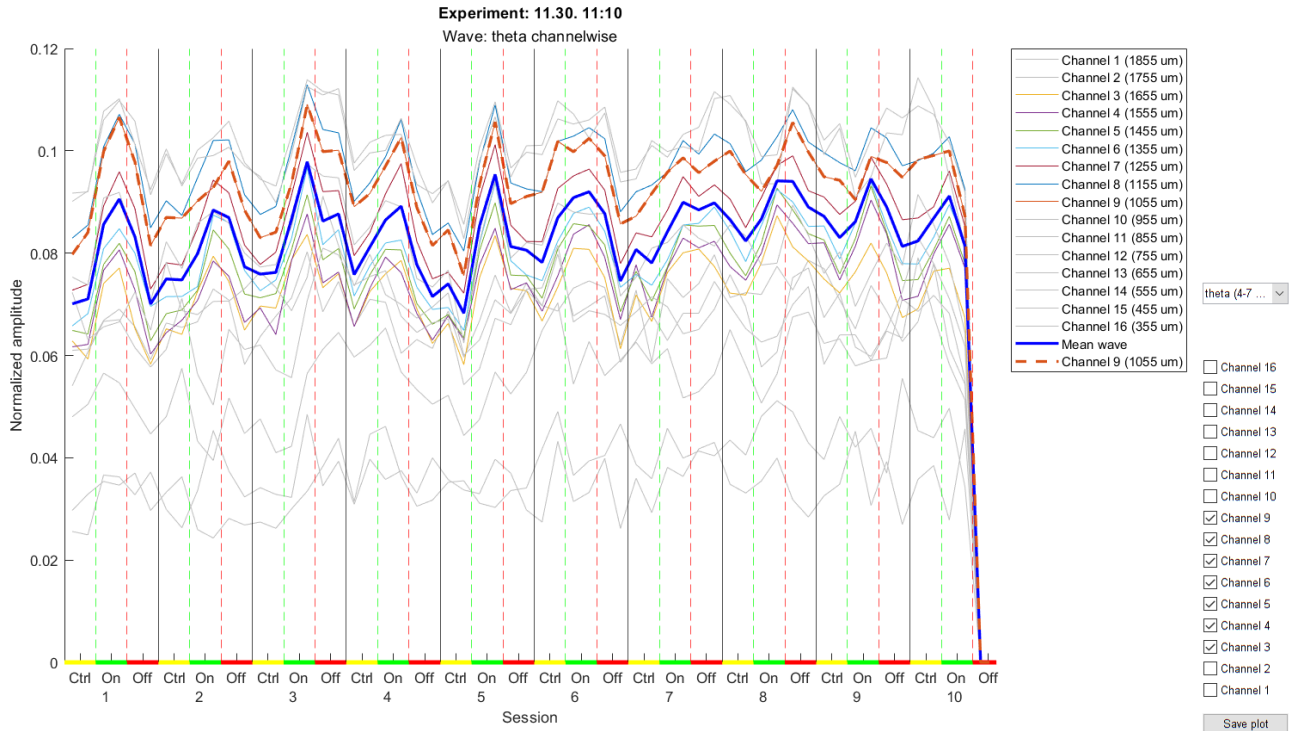


Figure 22: The visual display of the developed graphical user interface, which represents the normalized amplitude of every channel. The curves of the active channels, which have been selected by checking the right-hand box, are shown in different colours while inactive channels appear grey. The frequency range can be adjusted using the drop-down menu on the right-hand side. A selected channel is marked with a dashed line, whereas the thick blue curve indicates the average value of the active channels. The entire graph, comprising all the channels, can be saved by clicking the "Save plot" button.

5.5.2 Wavelet analysis

The signals recorded for analysis lasted approximately one hour. Considering the high computational requirements, the wavelet transformation is particularly time-consuming for signals of this length. Thus, the signal undergoes downsampling from a 20 kHz sampling frequency to 1 kHz in the initial stage.

The subsequent stage of the wavelet analysis employs the Morlet wavelet transformation [86], which is particularly valuable in the analysis of signals displaying a clear oscillatory component. This proves especially useful when analysing oscillatory brain signals.

The frequency range of the transformation was computed between 0-30 Hz, with a resolution of 2 Hz. After applying the wavelet transformation to the signal, normalization was carried out at each frequency, followed by a moving average operation with a window size of 30 seconds to accentuate the wavelet amplitude alterations.

Figure 23 depicts the complex amplitude of the wavelet transform of the signal on the left and the result of the moving average operation on the right. Higher complex amplitude values are represented by yellowish colours, while lower values are represented by blueish colours. The green horizontal lines at the bottom of the plots represent the time when the infrared irradiation was on.

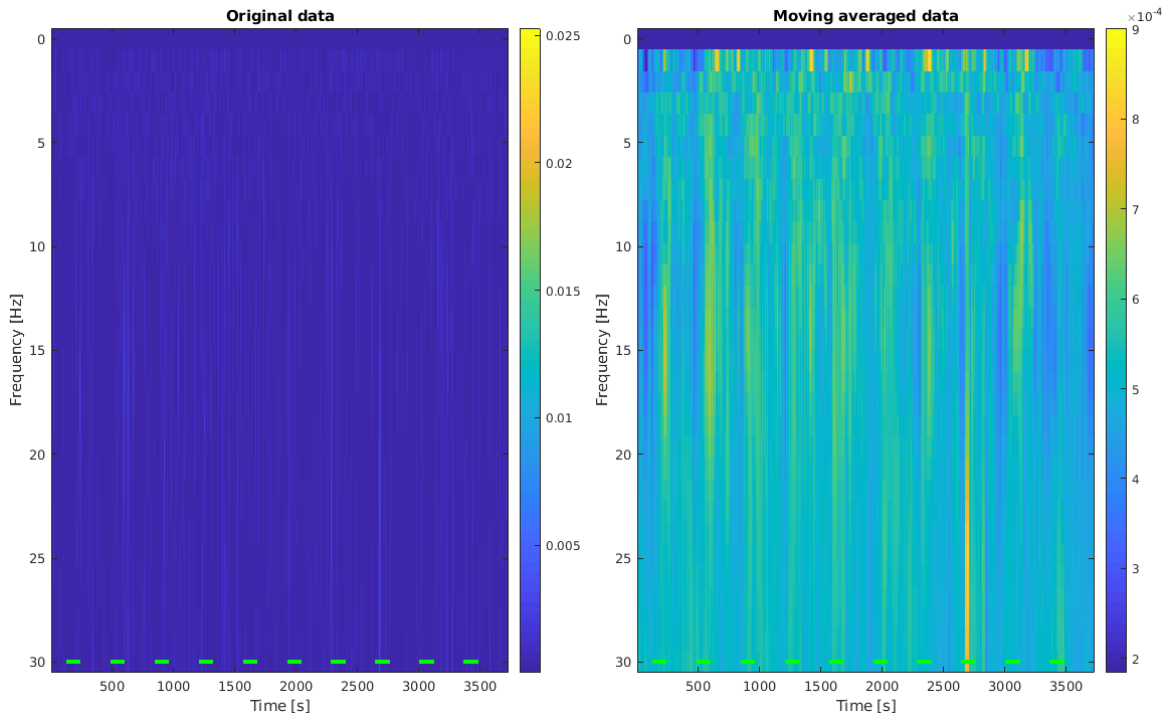


Figure 23: Application of moving average operation on the wavelet-transformed signal with a window size of 30 seconds to emphasize amplitude changes of the wavelet at specific frequencies over time. Higher complex amplitude values are represented by yellowish colours, while lower values are represented by blueish colours. The left plot shows the original wavelet transform, while the right plot displays the moving averaged wavelet transform. The ON period of the infrared irradiation is marked by green horizontal lines at the bottom of each plot.

5.6 Video tracking

The research group has initiated experiments on freely moving rodents using infrared neuromodulation. They conduct electrophysiological recordings of the brain activity alongside video recordings to discern any behavioural changes. These videos can also be utilized to analyze the electrophysiological data within a behavioral context. Based on this finding, the research group can analyse the recorded brain activity, including the rate of spikes and local field potentials (LFPs) during periods of rest or

when the rat is free to move around its cage. Electrophysiological data has been recorded solely from anaesthetised rodents up until this point.

Many open-source freely moving animal-tracking software, rely on thresholding. However, in our case, detecting rodents is challenging due to the bright wood chips background and the white rat. Simply applying thresholding is not a viable option. Additionally, the rat, in our study, wears a head stage with two wires that extend through the ceiling of the experimental environment, which can overlap with the rat’s body in the recorded video. To overcome these challenges and customize video tracking software for our particular setting, a Python script was written.

The video is analyzed on a frame-by-frame basis. The algorithm responsible for identifying the rodent is executed on each frame. The steps of the Python video tracking script are illustrated in Figure 24. Firstly, darker pixels are excluded from the frame. To accomplish this, the frame is transformed into a grayscale image and then a binary threshold is applied to generate a mask. Applying the mask to the original image leads to Figure 24b. The threshold was determined experimentally. In the following step, our objective is to eliminate the wood chips in the background. Considering the non-uniform illumination conditions, contrasting variability, and uneven backgrounds within the working environment, Gaussian adaptive thresholding has been utilised on the greyscaled and thresholded frame. The adaptive thresholding technique assumes that local regions of an image possess more uniform illumination and lighting than the image in its whole. The application of adaptive thresholding caused the wood chips to appear darker, as illustrated in Figure 24c. The wood chips were then filtered out by utilizing Gaussian blur and binary thresholding (Figure 24d), with the resulting binary image serving as a mask on the original image (Figure 24e). The next step is a binary thresholding based on the HSV colour space of the rat. Morphological operations (erosions and dilations) were then applied to remove noise, as depicted in Figure 24f. Subsequently, the edges were smoothed using a Gaussian blur, as shown in Figure 24g, after performing the opening morphological operation. The Otsu threshold was used in the next step (Figure 24h) to obtain a binary image. If the animal’s body is separated into two parts by the wires in the frame, it can be reconnected into a coherent object by combining the Gaussian blur and the Otsu threshold operations. Finally, a contour detection function determines the largest contour of the binary image, which is projected onto the original image depicted in Figure 24i. The resulting green contour represents the detected rat, while the white dot indicates the centre of the contour, showing the position of the rodent.

The onset of the ON period during infrared neuromodulation is indicated by a blue LED flash in the video, aiding data analysis such as correlating behaviour with stimulation or assessing electrophysiological data during different phases of the stimulation. Although the LED’s position remains stable during a recording, it could alter across experiments. Prior to beginning video tracking, the location of the blue LED must be established manually by formulating a rectangle around it. By selecting the four corners of the LED, we can determine the area of the blue flashing light demonstrated by the light green rectangle located on the left in Figure 25.

Based on the recorded video, it is possible to discern various behaviours exhibited by the rodent, including resting, moving, and climbing. In our experimental design, we consider that the rat is climbing when its position is detected outside the bottom of the cage which is covered with wood chips. Owing to the fact that the position of the cage may differ across various recordings, the cage floor has to be manually selected by designating the four corners of the cage, akin to selecting the LED. The chosen bottom area is presented as a light brown shade on the right side of Figure 25. The rat detection procedure starts after selecting both the LED and the cage floor.

The video tracking output is saved in .csv format. The data file comprises the rodent’s x and y coordinates. Using consecutive coordinates, the Euclidean distance is calculated. The position and distance are measured in pixels. Knowing the distance and the time difference, the speed of the rat is calculated by dividing the distance by the time difference. The resulting file also includes information on whether the blue LED flashes at a specific time. Additionally, the file indicates whether the animal is climbing or not.

The data provided by the .csv file is presented in Figure 26. The figure has six subplots, of which the top-left displays the x-y position of the rodent. It should be noted that the origin point in the subplot is located at the bottom left, whereas in the video it is located at the top left. By examining the

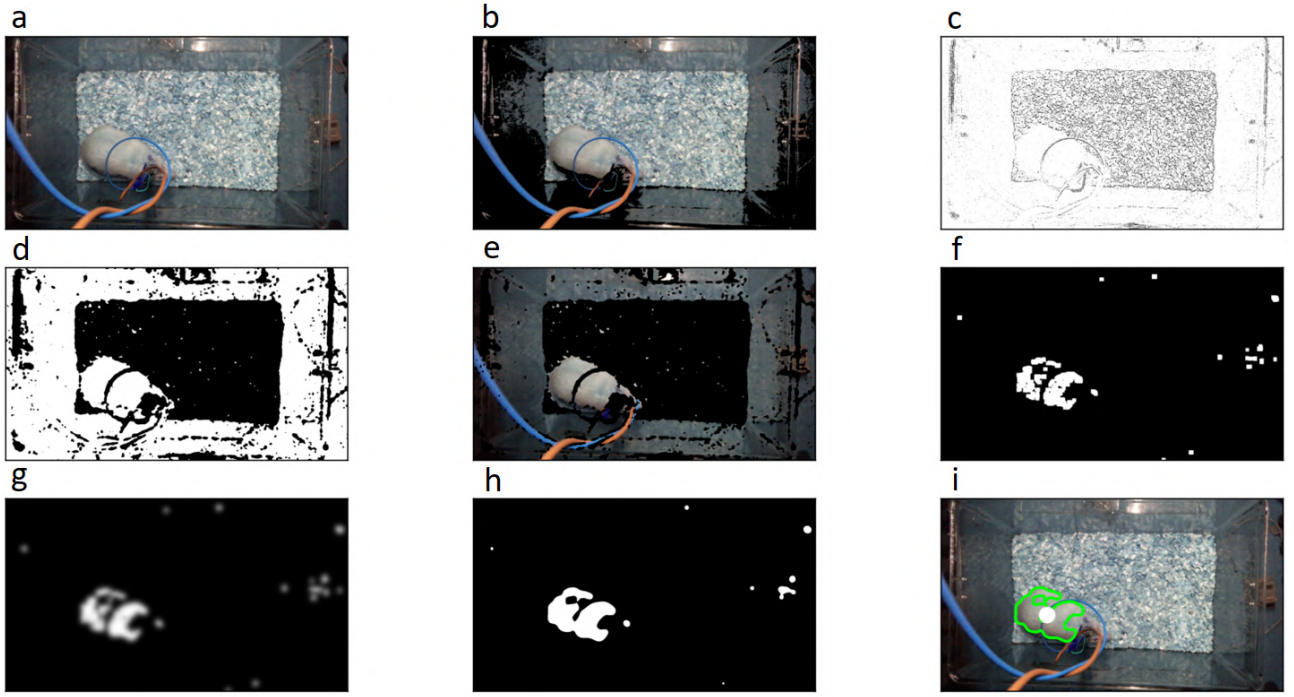


Figure 24: Steps in the video tracking Python script. a, Original frame. b, Masking the darker regions by converting the frame into greyscale and applying binary thresholding. c, Implementing Gaussian adaptive thresholding on the greyscale frame. d, Generating the mask to filter out the wood chips by using Gaussian blur and binary thresholding. e, Filtering the wood chips from the coloured frame. f, The application of binary thresholding based on the HSV colour space of the rodent, alongside the implementation of morphological operations to reduce noise was performed. g, A Gaussian blur was applied. h, The Otsu thresholding technique was used. i, The largest contour detected in the previous step was projected onto the original frame in green colour, with the white dot marking the contour's centre and hence, the rodent's position.

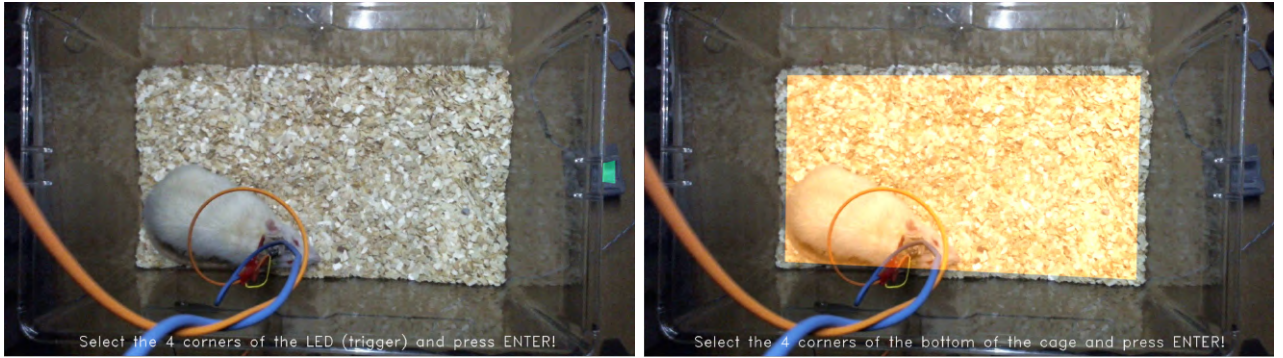


Figure 25: The floor of the cage and a blue LED are chosen prior to initiating rodent detection. The manually selected area of the LED is denoted by a rectangle in light green on the left, while the selected area of the cage floor is marked by a light brown rectangle on the right.

animal's x-y position, users can determine the duration of time the animal spent in a specific region, as depicted in the bottom-right subplot. By selecting two points on the plot that define the diagonal of a rectangular shape, the area can be chosen for analysis. In this particular example, the animal spent 90% of its time within the designated rectangular area highlighted by the dashed red line.

The top middle subplot illustrates the speed of the animal as a function of time. This graph can help in determining if the animal is in motion or at rest. The upper right subplot displays whether the animal is standing (climbing) at a specific time point. The lower left and middle subplots exhibit the x and y positions of the animal with respect to time, respectively. These two subplots are useful for

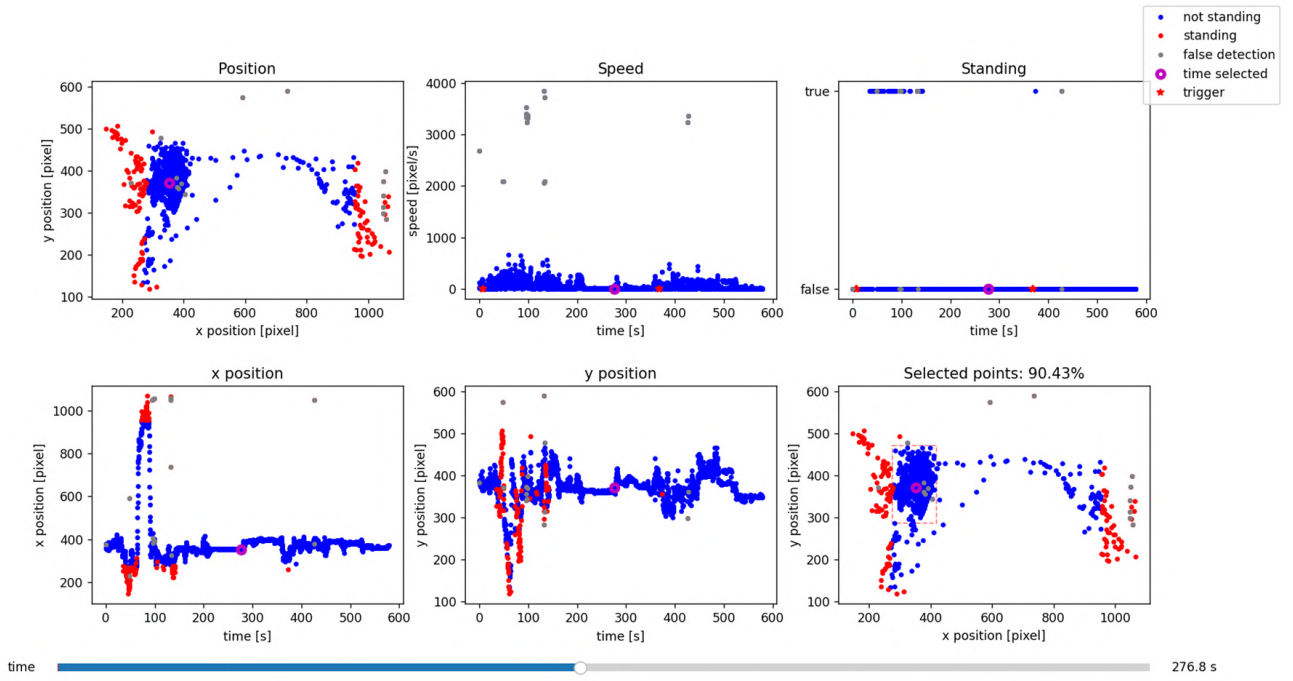


Figure 26: Visualization of the video tracking output. The subplots display the rodent’s x-y position, velocity, standing (climbing) status, x and y coordinates over time, and the duration spent in a designated area. When the animal is standing at a certain time instance, the data is represented by a red dot, otherwise it is blue. Grey dots are used to indicate erroneous detections. The red stars mark the start of the ON period during stimulation. The value corresponding to a specific time point selected using the bottom slider is indicated by a purple circle on the subplots.

identifying when the animal has, for example, moved from one side of the cage to the other.

In Figure 26, red dots indicate when the animal is climbing, while blue dots indicate when it is not. If a detection is incorrect, the corresponding point is represented by a grey dot. If the animal’s speed exceeds 1000 pixels per second, the corresponding detection is deemed erroneous. The red stars in the top-middle and top-right subplots mark the instances when the blue LED flashed, hence serving as the onset of the ON period. A slider located at the bottom of the figure allows users to select a specific time point, and the values associated with this point are visualised as purple circles in the subplots.

6 Results

6.1 Micro-drive and head-stage fabrication

To implant the optrode in the rat’s brain, the author developed two types of micro-drives. The micro-drive holds the optrode in place and allows adjustment of the penetration depth of the optrode. Furthermore, in order to protect the micro-drive equipped with optrode, a head-stage was devised and securely fixed onto the rat’s skull surrounding the optrode. Prior to implantation, it is crucial to examine the 3D printed components for functionality and durability.

6.1.1 Assessment of the precision of 3D fabrication

Due to the limited resolution of 3D printing and the inherent properties of the printing resin, the dimensions of the CAD model invariably differ from those of the printed version. Consequently, there may be discrepancies in the fitting of the printed components of the model. To ensure a smooth displacement of the slider, the ideal offset between the CAD design and the fabricated model must be determined through experimentation.

To determine the necessary offset, the printed model’s dimensions must be measured. Table 1 summarises the measurement results. The labels correspond to the parts discussed in 3.2.4. The measured dimensions of the components are displayed in Appendix Figures 3 and 4.

Dimensions [mm]			
resin type	label	CAD	fabrication
Phrozen Nylon-Green Tough 3D Printer Resin	N1	7x6.5	6.97x6.54
	N2	8x6.5	8.01x6.57
	N3	9x6.5	9.03x6.54
Surgical Guide Resin (Formlabs)	T1	7x5.5	7.18x5.68
	T2	15x10	15.25x10.17
		4.998x7	5.22x7.14
	T3	9x6.5	9.22x6.89

Table 1: The dimensional measurement results. Parts produced through Phrozen Sonic Mini 8K DLP printer, with Phrozen Nylon-Green Tough 3D Printer Resin, are precise. However, parts fabricated through the Form 2 SLA printer, using Surgical Guide Resin, exhibit an absolute error of 0.1-0.25 mm compared to the CAD design.

When using the Phrozen Sonic Mini 8K DLP printer with Phrozen Nylon-Green Tough 3D Printer Resin, the accuracy achieved is 50 μm , the maximum resolution of the 3D printer. For the *simple type* micro-drive, the optimal offset values are as follows: 0 mm for the distance between the slider and the body, and 50 μm for the distance between the shell and the body. For the *complex type* micro-drive, distances are 0 mm between the slider and the inner body, 25 μm between the inner and outer body, and 50 μm between the shell and the outer body.

The precision of printing with the Form 2 SLA printer using Surgical Guide Resin is reduced, resulting in an absolute error range of 0.1-0.25 mm as compared to the CAD design. Consequently, in the case of the improved version of the *simple type* micro-drive, numerous attempts were made to ascertain the necessary offset values. Following multiple test prints, the optimum distances between the slider and body and between the shell and body were found to be 25 μm and 30 μm , respectively.

6.1.2 Wobbling

To implant the micro-drives, it is necessary to test the vertical displacement of the optrode. Ensuring a smooth movement of the optrode shaft without lateral deviation is crucial for successful infrared neuromodulation and appropriate electrophysiological recordings.

The optrode shaft’s lateral movement was tested under a light microscope. To avoid using the expensive and fragile optrode, a needle was attached to the micro-drive slider instead. The nano-drive, available commercially from Cambridge NeuroTech, served as a benchmark for the micro-drive developed. As expected, no lateral needle displacement was noticed in the case of the nano-drive. The micro-drives, both the designed *simple* and *complex types*, exhibited a small lateral deviation within the 100 μm range while the needle was subjected to vertical displacement. This lateral deviation is considered to be acceptable for us.

6.1.3 In vivo performance

In an in vivo experiment, the complex type micro-drive underwent testing on a rat at the HUN-REN Research Centre for Natural Sciences. The acute test replicated a prior experiment that utilized an infrared neuromodulation protocol. To secure the micro-drive, it was not cemented onto the rat’s skull but attached to a stereotaxic post by its handle instead. The experiment did not test the micro-drive’s integrated vertical displacement mechanism. During the acute implantation procedure, the optrode’s positioning was performed using computer control.

The micro-drive design appeared suitable for infrared neuromodulation with electrophysiological recording. Various tests were conducted to confirm whether the micro-drive adequately stabilizes the optrode, whether connectors are attachable to the optrode while the micro-drive is connected to the stereotaxic post via the micro-drive handle, and whether electrophysiological recording can be performed in this arrangement.

During the experiment, the optrode required the optical cable and OMNETICS connector to be connected. This design enabled successful connection of the required components. However, it was discovered that the micro-drive handle did not securely fasten the micro-drive’s body. Squeezing the body laterally with the handle proved insufficient. As a temporary solution, the micro-drive was fixed to the handle with a screw. The micro-drive’s handle underwent modifications in a subsequent version, resolving the issue. Based on the brain activity observed during the experiment, the micro-drive showcases considerable potential in electrophysiological recording. This claim is supported by later sections, which include detailed analysis of the recorded brain activity.

6.1.4 Water test

An experiment was conducted to replicate the impact of a wet environment on the components of the micro-drive. The objective was to choose the suitable printer resin for the long term experiments with freely moving rodents. Two types of resins were analysed: the Phrozen Nylon-Green Tough 3D Printer Resin and Surgical Guide Resin. Certain components of the micro-drive produced utilizing these resins were immersed in a glass of water or suspended above the water. The container was sealed to maintain a humid environment for the components. Following a half-day interval, the components were removed from the water.

The results of the water test are presented in table 2. It was observed that parts printed using Phrozen Nylon-Green Tough 3D Printer Resin displayed increased dimensions and loss of structural integrity, becoming soft and swollen (Figure 27). After drying, the parts did not revert to their original shape. However, those printed with Surgical Guide Resin demonstrated no alteration in their physical properties or dimensions.

According to the results of the water test, it is apparent that the Phrozen Nylon-Green Tough 3D Printer Resin is not suitable for the production of micro-drives. In a wet environment, the components of the micro-drive become swollen and soft. Unlike the previously mentioned resin, parts manufactured with Surgical Guide Resin preserve their dimensions and physical properties. Therefore, this particular type of resin appears to be a suitable material for creating micro-drives.

In summary, the improved version of the *simple type* micro-drive, printed with Surgical Guide Resin and protected by the head stage, is suitable for long-term in vivo experiments with freely moving rodents. This is confirmed by the fabrication outcome, water test, and in vivo performance of the micro-drive.

Dimensions [mm]				
resin type	label	before water (22.11.2022. 17:20)	after water (23.11.2022. 10:42)	experience
Phrozen Nylon-Green Tough 3D Printer Resin	N1	6.97x6.54	7.21x6.68	swollen soft
	N2	8.01x6.57	8.67x7.06	
	N3	9.03x6.54	no test	
Surgical Guide Resin (Formlabs)	T1	7.18x5.68	7.17x5.68	no change
	T2	15.25x10.17 5.22x7.14	15.24x10.14 5.22x7.14	
	T3	9.22x6.89	9.24x6.87	

Table 2: The result of the water test. The properties of parts printed with Surgical Guide Resin remained unchanged, while those printed with Phrozen Nylon-Green Tough 3D Printer Resin became enlarged, swollen, and soft.

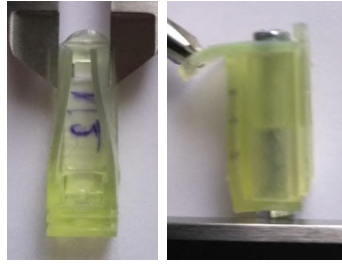


Figure 27: Components 3D printed with Phrozen Nylon-Green Tough Resin become swollen and soft.

6.2 Data analysis

The main aim of the experiments was to examine how infrared irradiation affects neural activity. Besides the neural effect of IR stimulation, the SNR of the optrode was calculated to evaluate the performance of the optrode on the recorded data.

Extracellular neural activities were recorded from four anaesthetised rats (one female and three males) on four different days. Two approximately one-hour-long recordings were obtained from the somatosensory cortex of each rat per day. Altogether, eight roughly 1 hour recordings were used for analysis. The days of the experiments can be categorized into two parts. The initial two experiments were conducted over a span of three days, while the last two were carried out within a period of two days, one month after the initial experiments.

Within each approximately 1 hour recording, the following optical stimulation protocol was repeated 10 times after the 2 minute recording without IR exposure: a 2 minute IR exposure (ON period) was followed by a 4 minute resting period (OFF period) in which the IR source was switched off.

6.2.1 RMS-based signal-to-noise ratio

RMS-based SNR estimation is obtained using the observable up- and down-states of neural activity in rats anaesthetised with ketamine-xylazine, which induces slow-wave activity in the cortical neurons. During the up-states, the neurons exhibit high activity, while they remain silent during the down-states. Signal levels are derived from calculating the RMS value of the up-states, and the RMS value of the down-states reveals the noise level. The ratio of the two provides the SNR.

The RMS-based SNR calculator algorithm described above used more than 500 up- and down-states in the 1 hour recordings to determine the SNR value. Figure 7 in the appendix displays the SNR measurements for the eight recordings. The initial two plots in each recording display the SNR value of nine intervals when the IR irradiation was ON and OFF, respectively. The third plot illustrates the SNR on the whole ON (in green) and OFF (in red) periods in each channel.

The signal-to-noise ratio (SNR) value remains consistent for a given rat within a single experimental

day, but the recordings from the first and last two days reveal significant variations. On the initial two days, the SNR value on the second channel was excessively low for the algorithm to recognize the up- and down-states. Conversely, during the final two days, the algorithm struggled to detect the up- and down-states on numerous channels, particularly those located closer to the skull.

Figure 8 in the appendix presents a quantitative summary of the mean and standard deviation of the SNR values calculated channel-wise and interval-wise for both the ON and OFF periods. The mean channel-wise SNR value is 1.77 ± 0.08 during the ON period and 1.74 ± 0.06 (excluding the outlier third recording) during the OFF period (refer to Figure 7 in the appendix, the first two plots of every recording). The interval-wise SNR values of the 8 recordings show 1.83 ± 0.42 for the ON period and 1.82 ± 0.40 for the OFF period, as displayed in the third plot of each recording in Figure 7 in the appendix. The mean signal-to-noise ratio values demonstrate similarity for both channel-wise and interval-wise recordings. Nonetheless, the standard deviation of the interval-wise SNR values was at least 4 times greater compared to that of channel-wise SNR values. The increased standard deviation is to be anticipated, as all channels are employed in the interval-wise calculations. There is no observable difference in the SNR values between the ON and OFF periods.

6.2.2 Amplitude-based signal-to-noise ratio

In the calculation of SNR based on amplitude, the noise level is determined by the amplitude of the down-states, whereas the signal level is based on the amplitude of the detected spikes by Kilosort. The ratio of the signal amplitude to the noise amplitude determines the amplitude-based SNR.

The SNR values in the eight approximately one-hour-long recordings are displayed in Figure 9 in the appendix. In each recording, the first two graphs display the SNR value of the 9 intervals when the IR irradiation was on and off, respectively, similar to the case of the RMS-based SNR calculation. The third graph illustrates the SNR on the whole ON (green) and OFF (red) periods channels-wise.

The amplitude-based SNR estimation's result is qualitatively analogous to the RMS-based estimation. Likewise, within the same day and in the same rat, the SNR value does not differ qualitatively. There is a significant variation in the number of channels for which the SNR could be calculated between the first two days and the last two days of the experiment. In the last two days, the SNR could be estimated on fewer channels compared to the first two days, but on more channels compared to the case of RMS-based SNR estimation. Based on the channel-wise comparison of the ON and OFF periods (Figure 9 in the appendix), no significant difference exists in the SNR value of the ON and OFF intervals, except for the third experiment. In the third experiment, there may be errors because of the unlikely large SNR values in the OFF periods.

Figure 10 presents a quantitative summary of the mean and standard deviation of the SNR values calculated for each channel and interval during both the ON and OFF periods. The mean channel-wise SNR value for the 8 recordings is 1.92 ± 0.07 during the ON period, and 1.77 ± 0.09 (excluding the outlier standard deviation of the third recording) during the OFF period, as shown in Figure 9 in the appendix (the first two plots of each recording). The interval-based signal-to-noise ratio (SNR) is 1.96 ± 0.20 for the ON period and 2.07 ± 0.30 for the OFF period across the 8 recordings (refer to Figure 7 in the appendix, the third plot of each recording). Compared to the channel-based recordings, the SNR means are slightly higher for interval-based recordings; however, the interval-based SNR standard deviation is about three times larger than that of the channel-based SNR standard deviation. This increased standard deviation is expected as all channels are used in the interval-based calculation. Compared to the RMS-based SNR estimation, the mean amplitude-based SNR estimation value has increased, and the standard deviation has decreased. The higher signal-to-noise ratio and reduced variability observed in the amplitude-based estimation are attributed to the constraint in the algorithm that the spike amplitude must be 1.5 times greater than the noise level. Nevertheless, the difference in SNR between the ON and OFF periods increased.

6.2.3 Spike rate over time

The study aimed not only to determine the SNR value of the optrode but also to observe changes in neural activity during the periods of the IR irradiation being turned on and off. This involved observing not only the firing rate of the neuron, but also the type of neuron and its depth in the brain. The author developed a GUI for this purpose (refer to Section 5.3).

In some cases, during IR irradiation at the same depth from the skull, the firing rate activity of different neurons is found to be opposite. The neural firing activity of an experiment is demonstrated in Figure 28, conducted on the 26.10.2022. at 12:00, where neurons globally followed the ON and OFF intervals of IR irradiation. The *Spike rate of cluster 28* depicts that cluster 28 showed a reduced firing rate during the ON intervals.

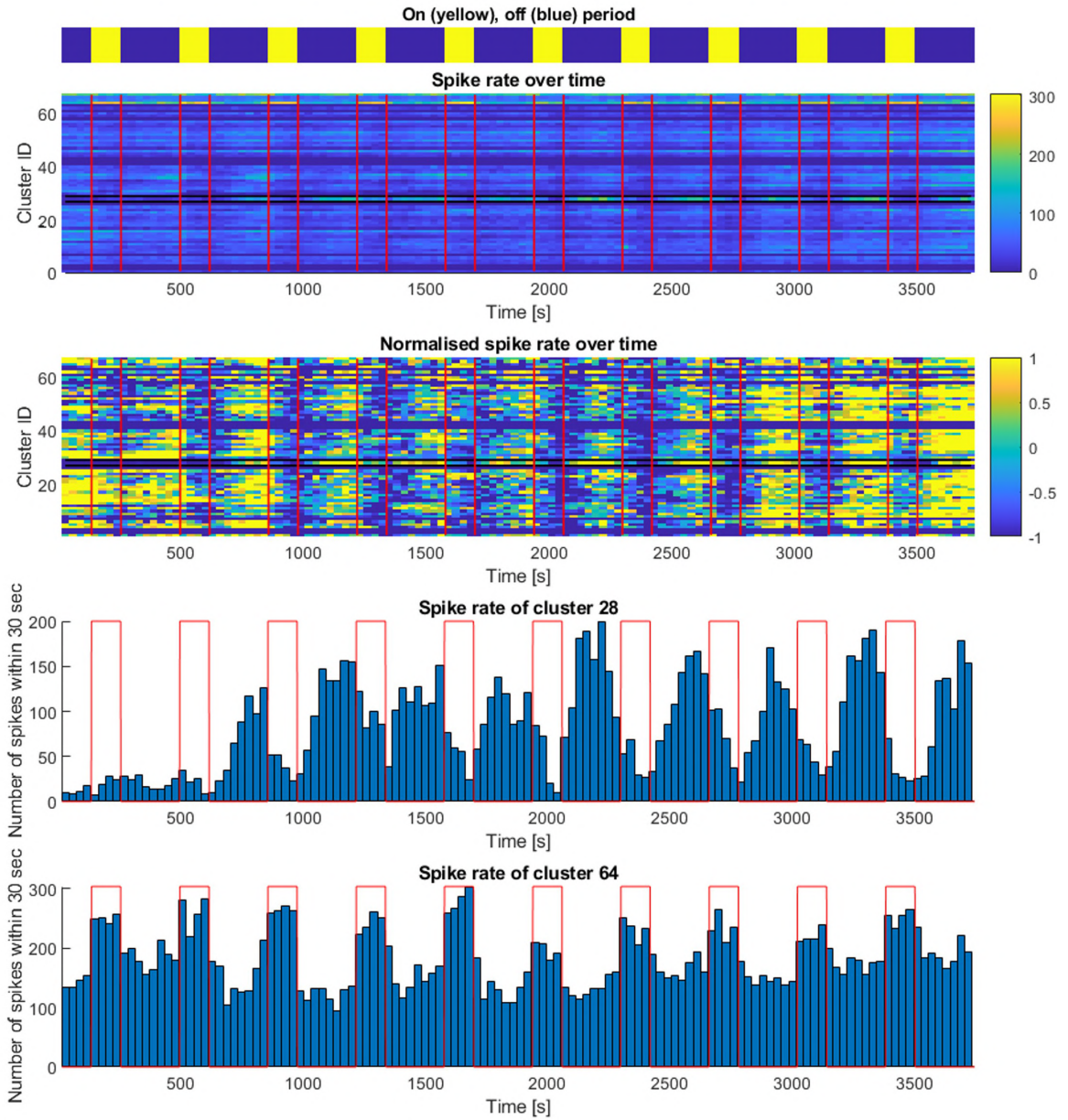


Figure 28: Spike rate during the experiment conducted on the 26.10.2022. at 12:00. In the *Normalized spike rate over time* plot, it is apparent that the clusters (neurons) demonstrate activity in synchronization with the IR stimulation, on a global scale. The spike rate of cluster 28 indicates a decrease in neural activity while cluster 64 exhibits higher neural activity during the ON period of the IR stimulation.

Based on the waveform of cluster 28 at a depth of 1555 μm from the skull (on the left side of Figure 29), it can be concluded that the neuron is a pyramidal neuron (as its action potential's half amplitude width is greater than 0.6 ms). However, during the same experiment, cluster 64 displayed an increased firing rate activity during the ON interval (as shown at the bottom of Figure 28). Based on its half amplitude width (on the right side of Figure 29), cluster 64 is an interneuron at the same depth with an opposite firing rate pattern compared to cluster 28, which is a pyramidal neuron.

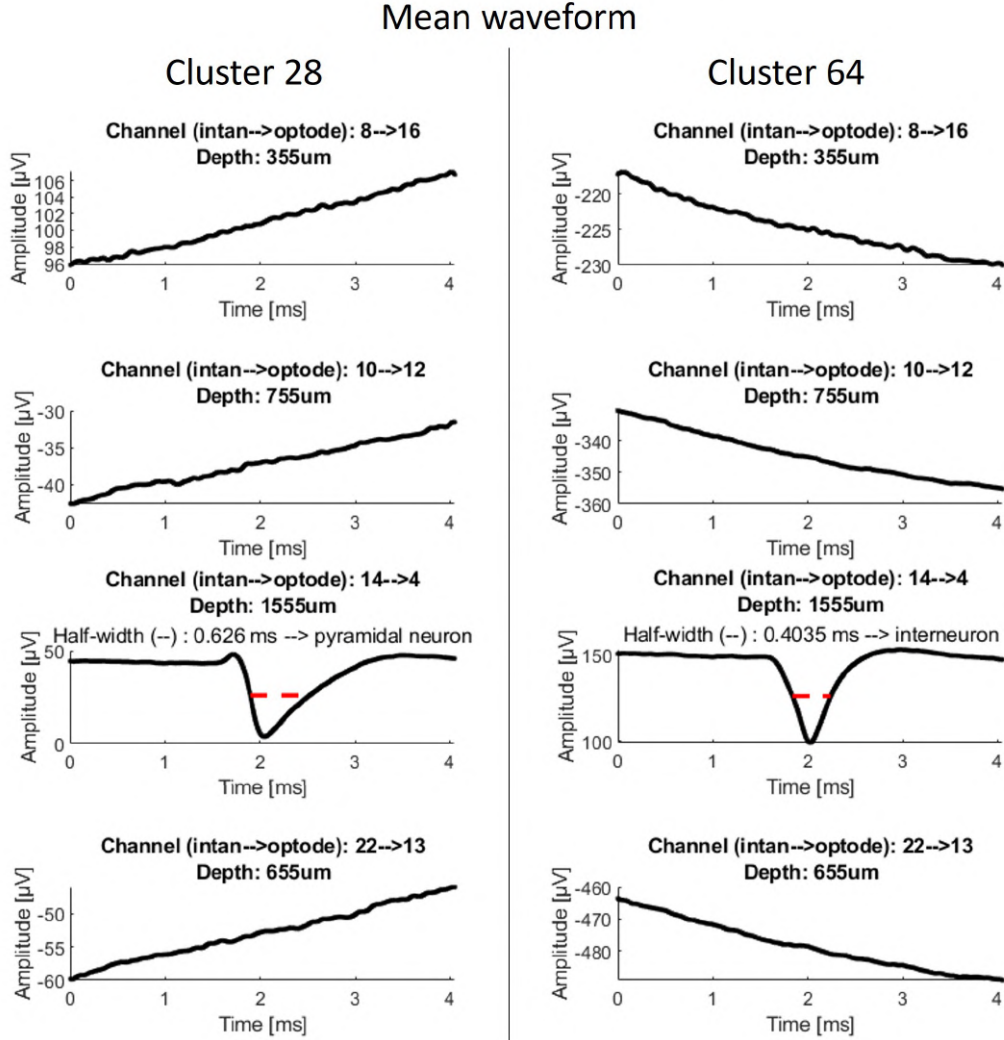


Figure 29: Mean waveforms of cluster 28 and cluster 64 were recorded from various channels during the experiment conducted on the 26.10.2022 at 12:00. On the left, cluster 28 represents the mean waveform of a pyramidal neuron, while on the right, cluster 64 represents the mean waveform of an interneuron. These two clusters are positioned at the same depth, precisely 1555 μm deep from the surface of the skull.

In another case, the firing rate activity of various neurons exhibits an opposite pattern during IR irradiation at varying depths. Figure 30 depicts the neural firing activity of an experiment conducted on the 28.10.2022. at 11:54, wherein cluster 30 portrayed a decreased firing rate during the ON intervals. Based on the corresponding waveform at a depth of 1455 μm (on the left side of Figure 31), the neuron appears to be an interneuron. However, in the aforementioned experiment, cluster 45 exhibits higher firing rate during the ON interval with a diminishing firing rate pattern over time (as shown at the bottom of Figure 30). The neural activity of this cluster was recorded at a depth of 1055 μm . According to its waveform (on the right side of Figure 31), it can be identified as a pyramidal neuron. To summarise, cluster 45 represents a pyramidal neuron, located at a distinct depth, with an opposite firing rate pattern to that of cluster 30, which represents an interneuron.

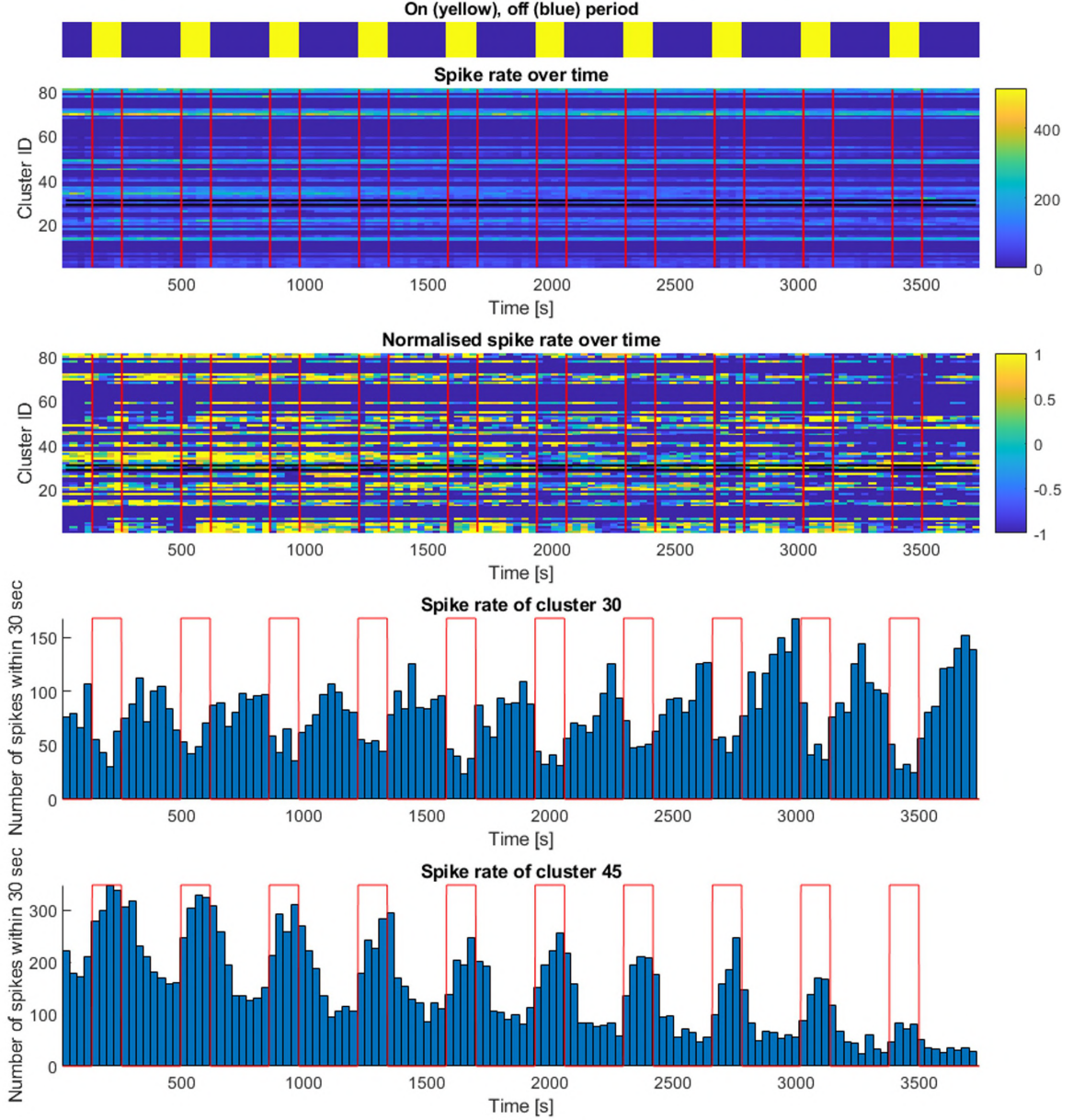


Figure 30: Spike rate during the experiment conducted on the 28.10.2022. at 11:54. The spike rate of cluster 30 indicates a decrease in neural activity while cluster 45 exhibits higher neural activity with a decreasing trend during the ON period of the IR stimulation.

Based on the above results, we can say that the firing activity of some neurons follows the IR stimulation protocol. It can be concluded that both pyramidal neurons and interneurons can show either increased or decreased firing rate activity during IR irradiation. Furthermore, one experiment showed that at the same depth, certain neurons exhibited increased spiking rates during ON intervals, while others had decreased rates. In view of these findings, further research is needed to understand the evoked responses by also considering the initial brain states and network topology.

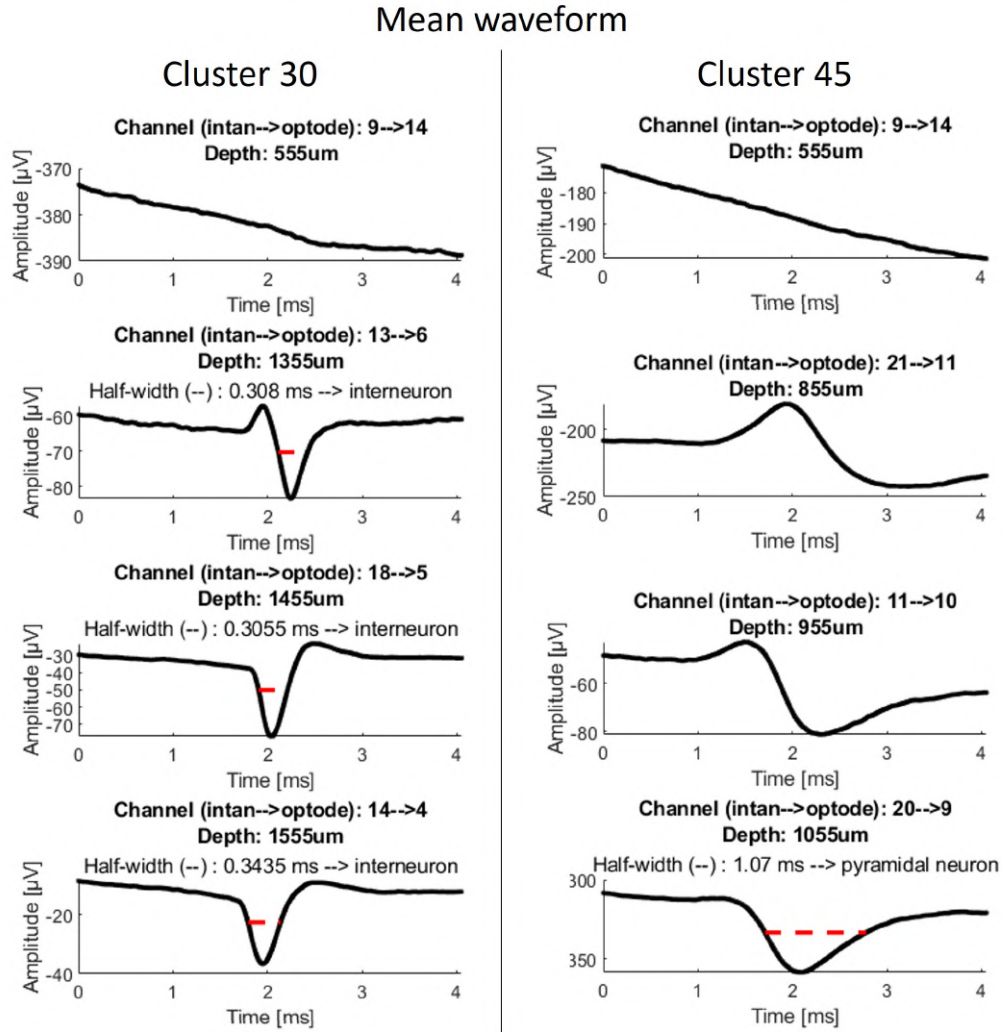


Figure 31: Mean waveforms from cluster 30 and cluster 45 were recorded from multiple channels during the experiment conducted on the 28.10.2022. at 11:54. The mean waveform on the left indicates an interneuron, while the mean waveform on the right represents a pyramidal neuron. These two clusters are situated at varying depths: cluster 30 at a depth of 1455 μm and cluster 45 at a depth of 1055 μm from the surface of the skull.

6.2.4 One-sided amplitude spectrum

The author developed a graphical user interface (GUI) to objectively analyse the local field potential (LFP) recorded from electrophysiological signals. This GUI calculates the one-sided amplitude spectrum for the ON, OFF, and control periods of the stimulation in different frequency ranges. The periods are further partitioned into 30 or 60-second time intervals. As illustrated in Figure 32, the graph appears smoother in the 60-second segments (lower plot), in contrast to the noisier graph observed in the 30-second intervals (upper plot).

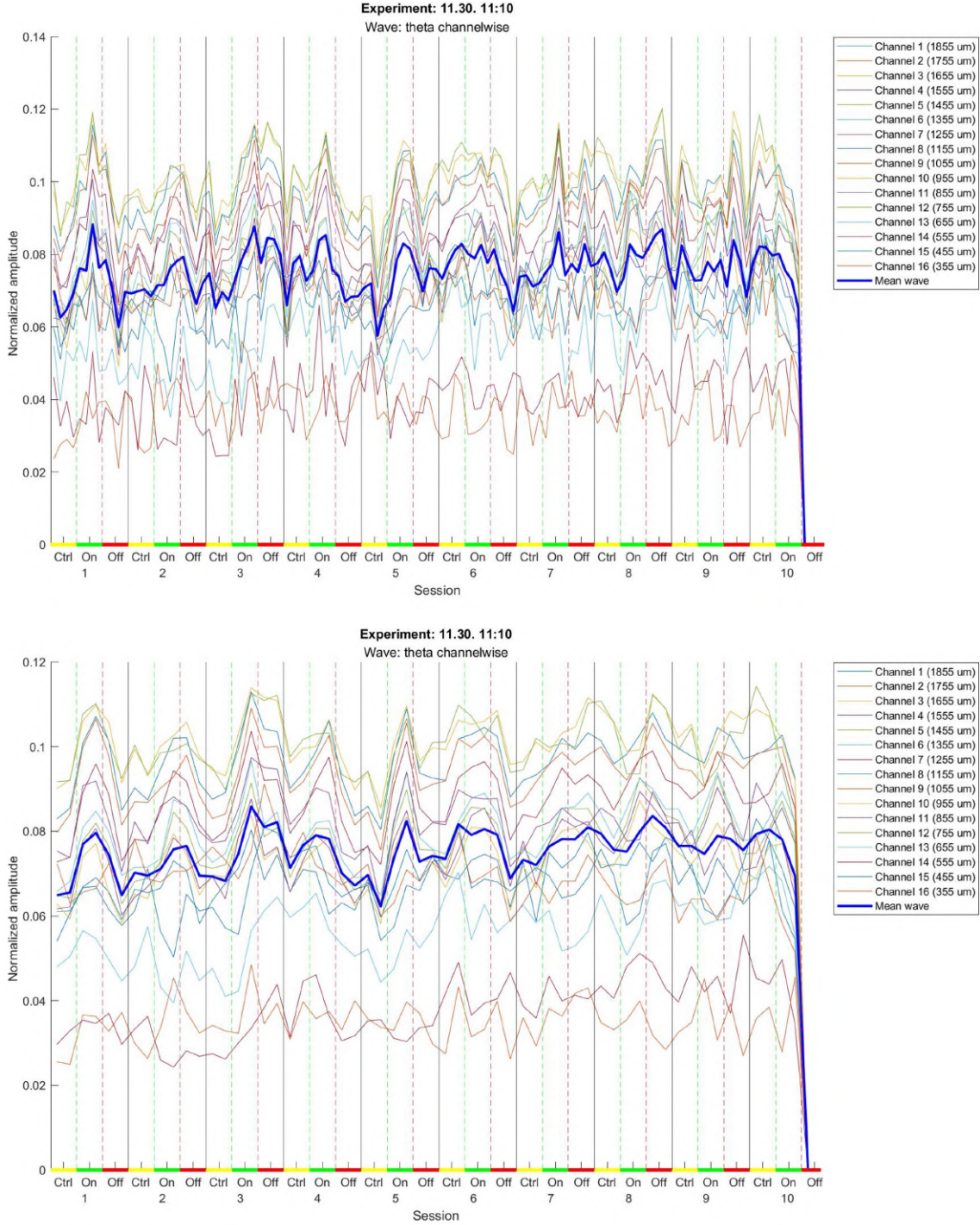


Figure 32: Comparison of the one-sided amplitude spectrum calculation graph using 30 or 60-second segments. It illustrates that the curve at the top, resulting from 30-second segments, is noisier compared to the curve at the bottom which is an outcome of 60-second segments.

The results indicate that brain signals primarily correspond to the stimulation protocol in the spindle (8-15 Hz), low beta (15-20 Hz) and high beta (20-30 Hz) frequency ranges. Within the ON interval, one-sided amplitude spectrum values are higher compared to control and OFF interval values. It is apparent that not all channels follow the same pattern during stimulation. A selected subset of channels more clearly highlights the effect of IR irradiation, as demonstrated in Figure 33. The above-mentioned pattern is observed in the selected 6-12 channels in the spindle frequency range during stimulation (lower plot), specifically the higher values in the ON intervals compared to both the control and OFF intervals.

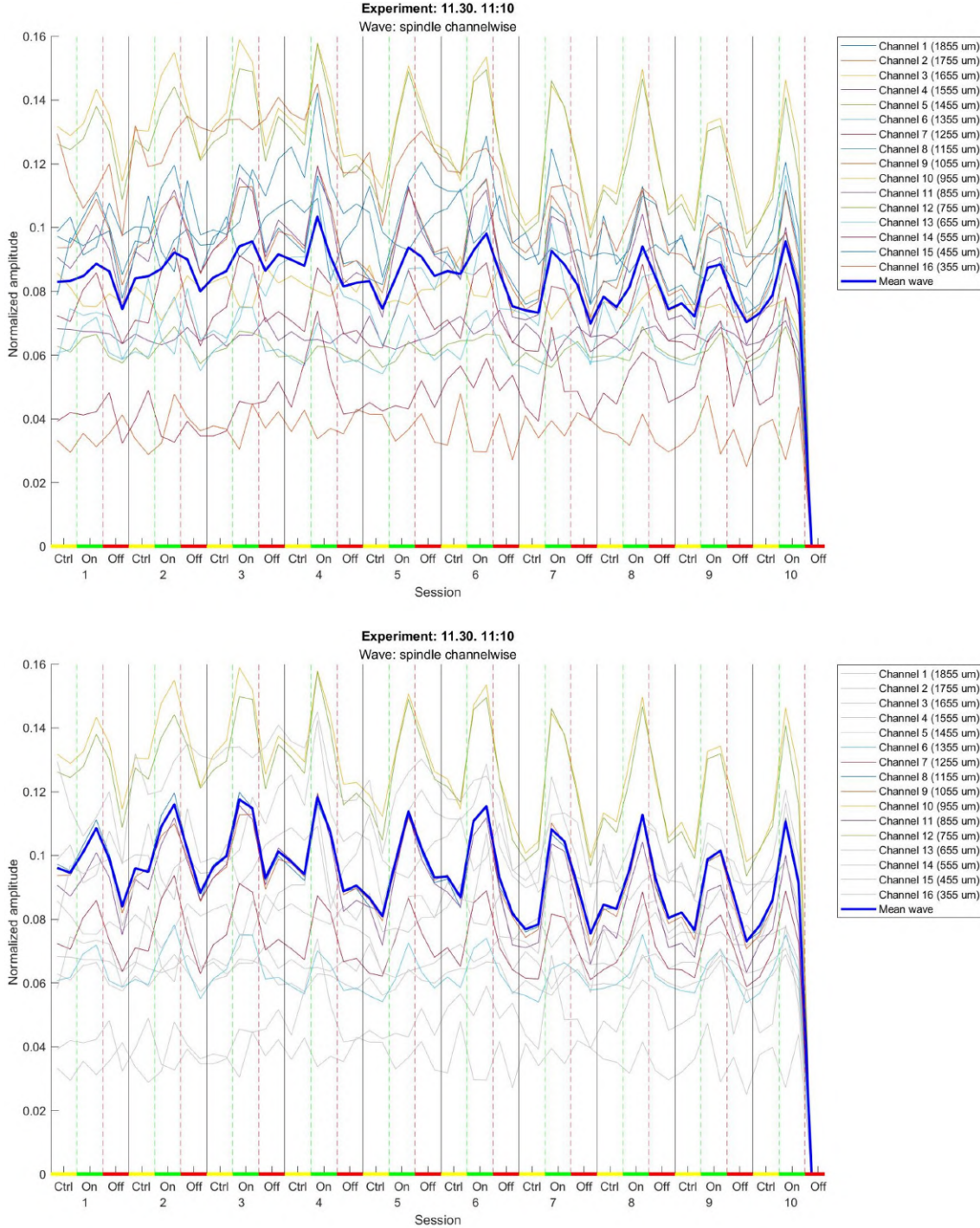


Figure 33: The effect of IR stimulation is more evident on a subset of the channels. The upper plot shows that all channels are active, while at the lower plot, channels 6-12 are active. The mean value of the active channels, as shown by the blue curve, indicates that a subset of the channels exhibit higher values during the ON intervals relative to the control and OFF intervals in the course of stimulation.

6.2.5 Wavelet analysis

Morlet wavelet transformation was applied to the recorded electrophysiological signal for analysing oscillatory brain signals. The complex amplitude values of the wavelet transform was frequency-wise normalized and moving-averaged on each channel to determine if the signals follow the pattern of the infrared stimulation.

Few channels did not exhibit any alterations during the stimulation, as illustrated in Figure 34. Yellowish colour indicates higher complex amplitude values while bluish colour indicates lower values. The green horizontal lines at the bottom illustrate the ON period of the stimulation. In this specific instance, as depicted in Figure 34, channels 2 and 4 exhibit no variation throughout the IR irradiation, while the remaining channels indicate heightened levels during the ON intervals. On the basis of the 8 recordings, there is a significant difference in value between the ON and OFF periods in the frequency range of 15-30 Hz.

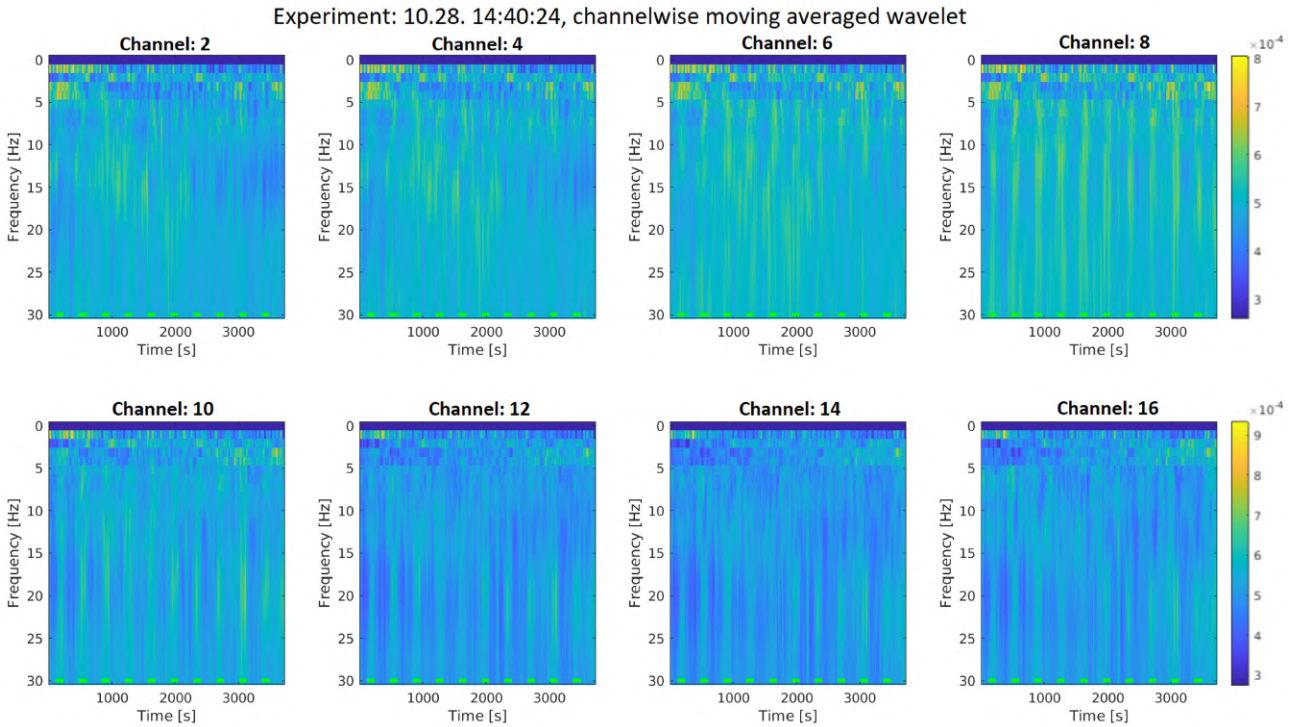
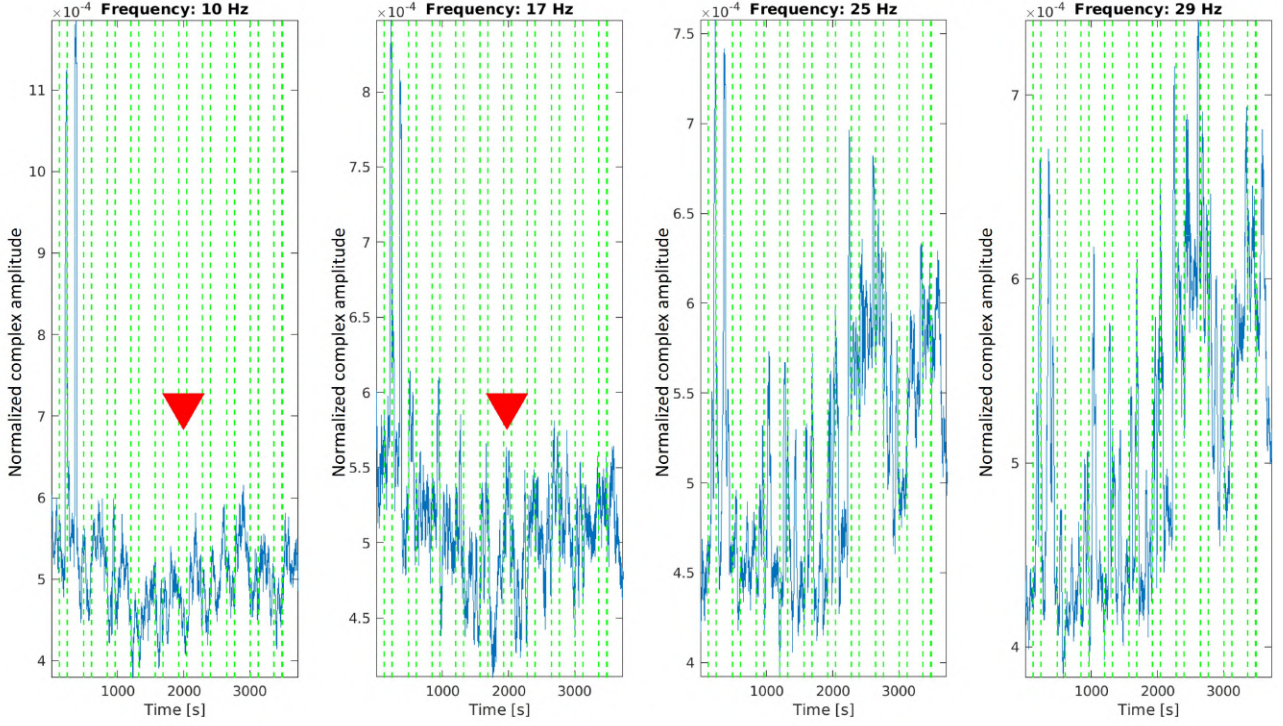


Figure 34: Channelwise wavelet transformation was conducted during an experiment on the 10.28. at 14:40:24. Higher values of complex amplitude are indicated by yellow shades while lower values are indicated by bluish shades. The green horizontal lines positioned at the bottom illustrate the ON periods of the stimulation. No variation was observed throughout the IR irradiation in channels 2 and 4, while the remaining channels indicated increased levels during the ON intervals within the frequency range of 15-30 Hz.

Complex amplitude value patterns can differ between channels within the same recording and at the same frequency. Additionally, the pattern of complex amplitude values may be opposite at one frequency on a channel during an experiment compared to another frequency on the same channel. An example of these observations is illustrated in Figure 35. At a frequency of 10 Hz, channel 4 exhibits an increase in complex amplitude values during OFF periods in this instance. In contrast, channel 12 demonstrates higher values during ON periods for the same frequency. Moreover, on channel 4, values are higher during OFF periods at 10 Hz, and lower during OFF periods at 17 Hz. At a frequency of 17 Hz, channel 12 exhibits a significant amplitude change between the ON and OFF periods.

Experiment: 10.26. 12:00:13, channel 4, moving averaged wavelet at different frequencies



Experiment: 10.26. 12:00:13, channel 12, moving averaged wavelet at different frequencies

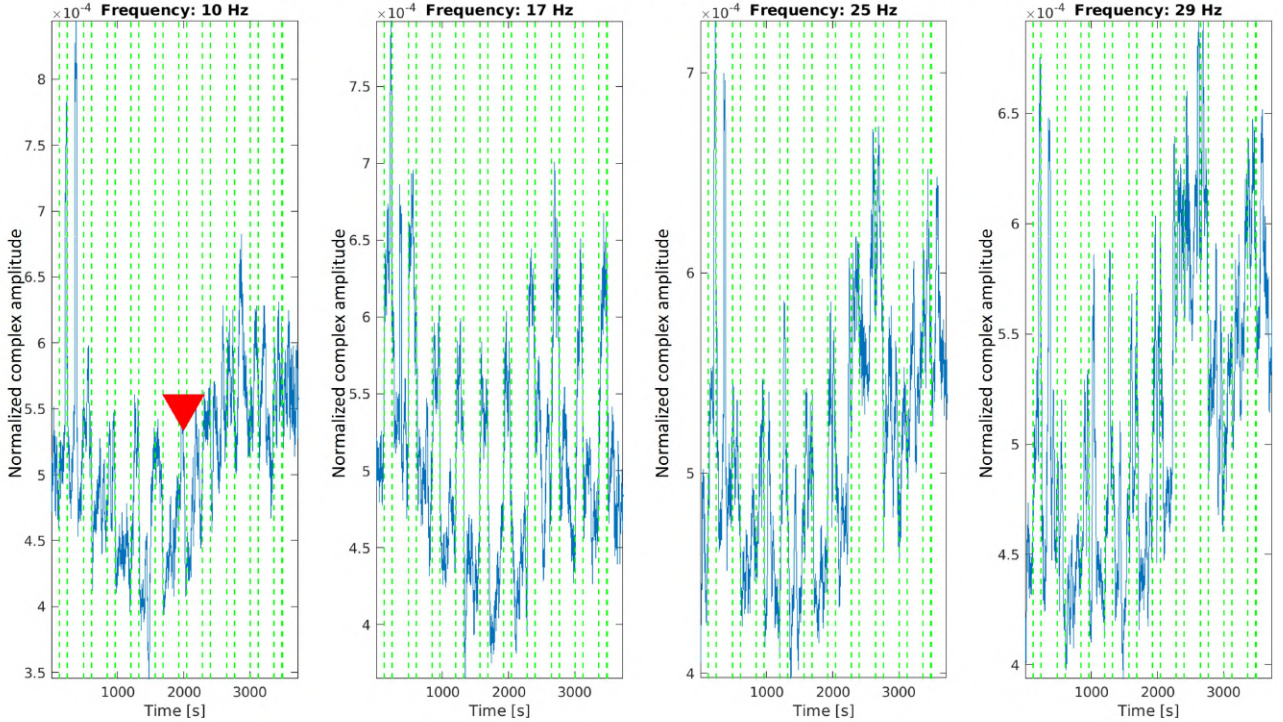


Figure 35: Moving averaged wavelet transformation was performed on channels 4 (upper plot) and 12 (lower plot) of the experiment conducted on the 26. 10. at 12:00:13, with frequencies of 10, 17, 25 and 29 Hz. The green dashed vertical lines show the applied stimulation pattern, where 2-minute ON and 4-minute OFF periods alternate, with this sequence repeating ten times. During the OFF periods at 10 Hz, channel 4 shows increased complex amplitude values, while during the ON periods at the same frequency, channel 12 shows higher values. Moreover, the values in channel 4 demonstrate higher levels during OFF periods at 10 Hz and during ON periods at 17 Hz. Notably, the red triangles accentuate one ON period that highlights the earlier observations.

6.2.6 Video tracking

The research team has commenced experiments using infrared neuromodulation on rodents that are able to move freely. They are performing electrophysiological recordings of brain activity along with video recordings to detect any behavioral changes. I created a Python script for video tracking to assist the team in analyzing the recorded electrophysiological data within a behavioral context.

The author has developed a video tracker script that can detect white rodents in backgrounds of wood chips, as illustrated in Figure 36, located on the left-hand side. The green contour acts as a representation of the identified animal, whilst the white dot presents the center of the detected area, indicating the rat's position. Another desirable objective was to identify rats when their body was overlapped with a wire. This goal was successful in most cases, as indicated on the right-hand side of Figure 36.

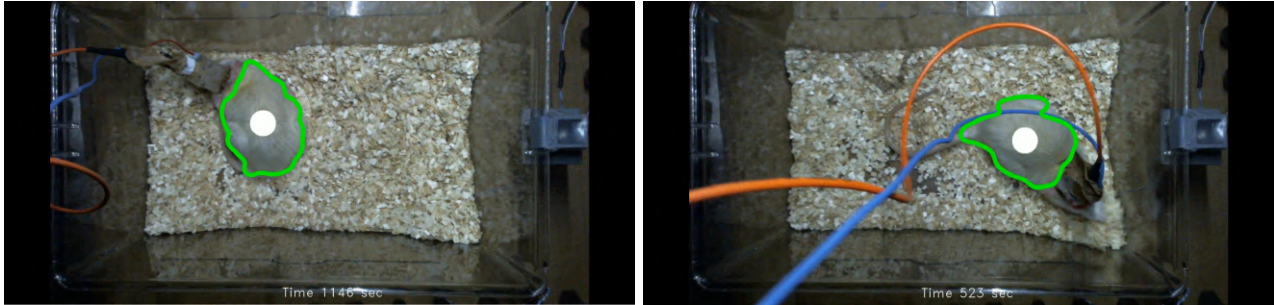


Figure 36: The Python script developed detects rats in recorded videos. On the left is a successful detection of the rodent, while on the right the appropriate detection is made regardless of wires. The green contour indicates the detected animal, and the white dot represents the center of the detected area, which is the rat's position.

The detection exhibits inaccuracies, which stem from the cage's changing position across experiments, reflections caused by non-diffuse light sources, or incorrect camera resolution settings. The main reason for detection errors arises from the reflections on the wall, which share a similar colour and brightness to that of the rat, as shown in Figure 37 on the left-hand side. Misidentification can be detected when the animal's speed exceeds 1000 pixels per second. Based on this criterion, the highest detection error encountered was below 6%.

However, there was a scenario where changing the camera's resolution led to a 17% detection error. This is due to the usage of predetermined thresholds by the algorithm. In this particular case, the video recording was brighter than usual.

The wire that may overlap with the animal's body can impede the algorithm, resulting in the detection of only the larger part of the rodent. This phenomenon is illustrated on the right-hand side of Figure 37.

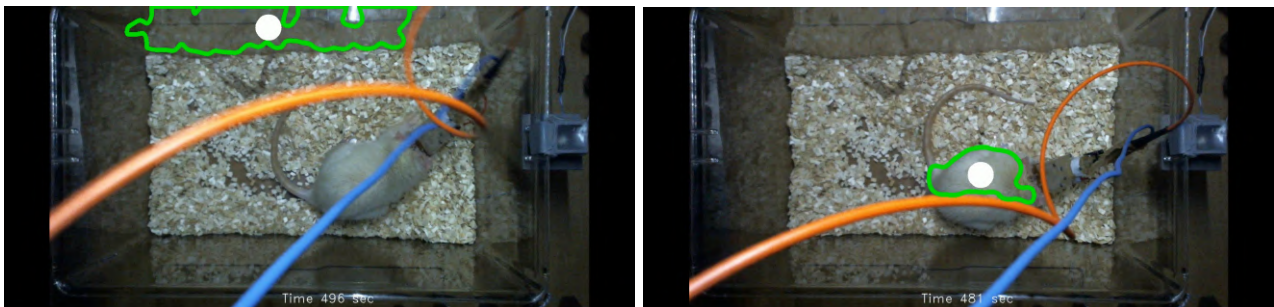


Figure 37: Detection error caused by reflections on the wall (left) and cable (right).

Improving detection accuracy can be accomplished in several ways. Using a diffuse light source and applying a coloured material to the cage wall can reduce reflections from the wall, which are the main source of detection errors. Additionally, increasing the darkness of the wood chips' colour would improve the algorithm's robustness by increasing the contrast between the animal and the background.

Other potential methods to improve accuracy include the use of machine learning techniques instead of thresholding. Such techniques necessitate the use of feature extraction methods or deep neural networks, which require numerous manually annotated images since no existing database is available.

7 Conclusion

This work describes the procedure used for infrared neuromodulation based on literature review, the potential neural interfaces for INM, and the analytical tools employed for processing extracellular recordings and video acquisition in freely moving conditions.

Additionally, a micro-drive and a head-stage were developed to fix the optrode to the skull of a rat whilst providing protection. The design, fabrication process, material selection, durability and functionality testing were described in detail.

In vivo performance of the infrared optrode with a micromirror needle tip was characterised using eight one-hour-long recordings in four rats. Spikes were detected and sorted from the recordings using Kilosort2, followed by manual curation in Phy. Further analysis and visualization of the results were conducted in Matlab. The RMS-derived signal-to-noise ratio (SNR) of the optrode was evaluated using a recommended algorithm from literature. Furthermore, an alternative technique, based on amplitudes, was proposed for estimating SNR by the author of this report. SNR was computed in terms of channel-wise and interval-wise values, and the mean SNR of at least 1.7 on a channel-wise basis and 1.8 on an interval-wise basis was achieved using both methods. The standard deviation was less than 0.1 for channel-wise and a maximum of 0.4 for interval-wise calculations.

A Matlab graphical user interface was developed to display the firing rate of neurons in response to infrared irradiation. Additionally, the type of neuron was determined from the waveform. The results indicate that neurons changed their firing rate during the IR stimulation protocol. Examples showed that both pyramidal and interneurons can exhibit an increase or decrease in firing rate activity during IR irradiation. Moreover, within the experiment, certain neurons exhibited an increase in spiking rates, whilst others displayed a decrease in spiking rates at the same depths.

The local field potentials (LFPs) of the recorded brain signals were analysed using one-sided amplitude spectrum calculation and wavelet transformation. A Matlab GUI was developed to visualize the one-sided amplitude spectrum per channel, across various frequency ranges. It was found, that at certain frequency range of the LFP, a consistent pattern with the infrared stimulation emerged, displaying a significant difference in amplitude between the ON and OFF periods of the stimulation, primarily in the frequency range of 15-30 Hz.

The author has developed a Python script that identifies the rodent's location during IR stimulation in the recorded video. The detection allows for identification of the position, speed and some behaviours such as climbing, moving or resting. These data provide valuable information for the research group to analyse electrophysiological signals in various behavioural contexts.

In the subsequent phase, further neural activity will be recorded and analyzed in freely moving rodents over a longer period of time. Our goal is to observe the variability of the SNR of the optrode and the effects of infrared neuromodulation on spike rates and LFP in the long run. Employing the developed tools, different behavioural contexts will be analyzed to study the mentioned aspects.

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