Comparison of tactile, electrical, and magnetic neurostimulation methods on an earthworm model

by

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Contents

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Abstract

Micro-magnetic stimulation is a promising technique for the treatment of neurological conditions, allowing long-term implant stability and effective neuronal regulation. Despite this potential, further research is essential to fully understand its mechanisms. The earthworm, with its primitive nervous system comprising two linear giant fibers, the medial giant fiber (MGF) and the lateral giant fiber (LGF), is proposed as a suitable model for investigating magnetic stimulation. The goal of this thesis is to compare tactile, electrical, and magnetic stimulation in an earthworm model. Electrical stimulation triggered both giant fibers a single time and also a third longer spike of unknown origin. In contrast, tactile stimulation selectively triggered one type of fiber depending on stimulation site. The fiber was usually triggered multiple times and the third spike was absent. Several action potentials from the MGF were recorded after magnetic stimulation. The stimulus artifact blocked the first 10 ms of recording and therefore a direct relation between stimulus and stimulation could not be established. The stimulus was 1.4 µs long and reached 2.45 kV/m. The peak electric field required to achieve stimulation for a pulse of that length was estimated at 22.6 ± 10.5 kV/m. Magnetic stimulation did not reach this value and therefore it is unclear whether this action potentials were triggered by the magnetic pulse. In tactile stimulation, crosstalk is negligible and in electrical stimulation, it becomes a concern only when the distance is less than 2 cm. In contrast, magnetic stimulation presents significant crosstalk issues, which can be effectively mitigated by increasing the distance between the stimulation and recording sites. These findings suggest that the earthworm is a suitable model for future research exploring the phenomenon of magnetic stimulation.

I. Introduction

Electric fields, whether originated internally from neurons or applied externally, affect the transmembrane potential of neurons, thereby affecting the likelihood of action potentials [1, 2]. This influence has driven the extensive use of electric stimulation in investigating the structure and functionality of the brain [3, 4, 5]. Furthermore, ongoing assessments are being conducted to explore the potential of electric stimulation in addressing compromised or lost function in a wide range of neurological conditions including Parkinson's disease [6, 7], blindness [8], epilepsy [9] and depression [10]. Despite these promising results, there are several concerns regarding the long-term viability of the implantable electrodes required to deliver the stimulus. The injuries induced by the introduction of electrodes into the brain initiates a diverse array of biological responses. These include inflammatory reactions that have the potential to result in glial scarring and the encapsulation of the electrode. Such responses have the capacity to modify the spatial distribution of the electric fields produced by the stimulation, potentially leading to a reduction in effectiveness [11]. Another important issue is that electrodes usually activate all nearby neurons in certain brain region [12, 13]. This results in the dispersion of activation beyond the immediate vicinity of the stimulating electrode, potentially compromising the efficacy of the stimulation [12].

Recent research indicates that the use of implantable micro-coils to induce magnetic stimulation might provide a feasible method for regulating neuronal activation [14, 15]. Electric fields induced by magnetic stimula-

tion present spatial asymmetry, which results in stronger activation forces in specific orientations. Activation is therefore constrained spatially when utilizing coils, potentially enabling precise and effective stimulation [14, 16, 15]. Micro-coils also offer the advantage of magnetic fields that can easily penetrate biological tissues, making them less affected by variations in the impedance of the surrounding tissue. Consequently, the activation patterns are likely to exhibit greater stability over time when using coils [17]. Furthermore, these magnetic implants do not require direct galvanic contact with biological tissues, and therefore an electrochemical interface with the brain is not necessary. This mitigates the risk of an intense immune reaction and inflammatory response upon implantation, reducing biofouling concerns. Consequently, implantable magnetic stimulation alleviates numerous safety limitations associated with the waveforms applicable for driving these devices [18].

Despite the potential of micro magnetic stimulation, the technology is still in its initial phases of development and therefore there are still safety and effectiveness concerns that must be addressed before experimenting on human subjects [19]. In this context, lower animals such as invertebrates serve as excellent experimental models for early-stage research due to their lower ethical concerns, simpler physiology and lower cost [20, 21]. A literature review was conducted with the aim of identifying the most adequate model to study the magnetic stimulation phenomena. Experiments involving the stimulation of cockroaches, earthworms, sea slugs, and Venus flytraps were analyzed based on the specific requirements of magnetic stimulation.

It was concluded that no organism clearly outperforms the others as all of them were well suited for specific contexts and limited in others. Choosing the right subject for study depends on the focus of the research. Earthworms might be preferable when orientation is a key factor, provided that the issue of their restraint is managed. On the other hand, if spatial effects are not a priority, the Venus flytrap offers a reliable option for working with non-neural excitatory cells. Insect legs can be beneficial for initial visual assessments of stimulation efficacy. For advanced studies, the sea slug's large neurons make it ideal for conducting intracellular recordings (Appendix F).

The earthworm was ultimately selected due to its primitive and linear nervous system [22], which facilitates the development of models to describe the effects of the stimulation. An additional advantage of the earthworm model is that the same neural pathway can be selectively activated by both electrical and tactile means [23, 24, 25]. This allows direct comparison of stimulation methods, which combined with the relative simplicity of the earthworm anatomy could help characterizing some of the fundamental principles of magnetic stimulation. Such an approach has already been successfully implemented with ultrasound neurostimulation [26].

With this in mind, this thesis presents a comparison between tactile, electrical, and magnetic stimulation in an earthworm model with the aim of highlighting differences between these three stimulation methods to better understand their interaction with the nervous system. The theoretical framework is presented in section II. The methodology employed to record the neurological signals in the worm and extract the data necessary for analysis is described in section III. The experimental results are presented in section IV. Finally, section V discusses the main challenges encountered in this work and possible ways to address them for future research. and the main challenges encountered during this project.

II. Theory

The theory section begins with Maxwell's equations, which were applied to optimize coil design. Following this, the earthworm nervous system is presented to describe the neural pathways that are stimulated. This is accompanied by a key principle of electrical stimulation, linking stimulation parameters to neural excitation. Finally, a model of the stimulation process is provided to estimate delay and conduction velocity.

i. Electromagnetism

The peak electric field is a critical variable in triggering neural stimulation [27]. It is therefore advantageous to design the coil so that the induced field is as high as possible. Maxwell's equations were used to compute an approximation of the induced electric field around the coil. For this purpose, the coil was defined as an infinitely long solenoid. This calculation can be found in Appendix B and led to the following equation:

$$E(t=0) = -\frac{V_0}{8rN} \tag{1}$$

Where V_0 is the voltage used to charge the capacitor, r is the distance from the center of the coil and N is the number of turns. This expression suggests that the optimal parameters to maximize the induced field include

increasing the voltage and reducing the number of turns and size of the coil.

ii. Earthworm nervous system

The nervous system of the common earthworm (*Lumbricus terrestris*) can be divided into three parts: central, peripheral, and sympathetic nervous system. The central nervous system includes the brain ring, which consists of a series of ganglia arranged as a collar around the pharynx, and the ventral nerve cord, which extends from the sub-pharyngeal ganglia to the posterior end of the body. The peripheral nervous system comprises nerve branches that emanate from the central nervous system and innervate the body of the animal. It includes afferent sensory fibers and efferent motor fibers. Finally, the sympathetic nervous system is composed of a nerve plexus located within the gut and other internal organs [28].

In neuroscience, the ventral nerve cord is of special interest due to its facilitation of direct action potential (AP) measurement. Extracellular recordings can be acquired without requiring any dissection and its role in the escape reflex, which is activated by mechanical stimulation, ensures that action potentials can be selectively triggered. The ventral nerve cord includes three giant fibers: the medial giant fiber (MGF) and two lateral giant fibers (LGF), which due to their segmental connections can be considered a single functional fiber. These axons are larger than any other nerve cell in the earthworm (0.07 and 0.05 mm in diameter, respectively) and present myelin-like glial cell wrapping, especially in MGF. As such, conduction velocities in these nerves are unusually high for invertebrates [29, 30].

The giant fibers are involved in the escape reflex by transmitting electrical signals from sensory neurons in the skin to giant motor neurons, which in turn activate muscles causing the animal to twitch. This response can be amplified by interneurons that trigger additional action potentials in the giant fibers. In the MGF, the escape reflex pathway is known and is depicted in Figure 1. Sensory neuron signals from the anterior region are directed to the MGF, while those from the posterior region are routed to the LGF. Further documented differences between both fibers include conduction velocity, which is faster in MGF, and stimulation threshold, which is higher in LGF for all stimulus durations [23, 24].

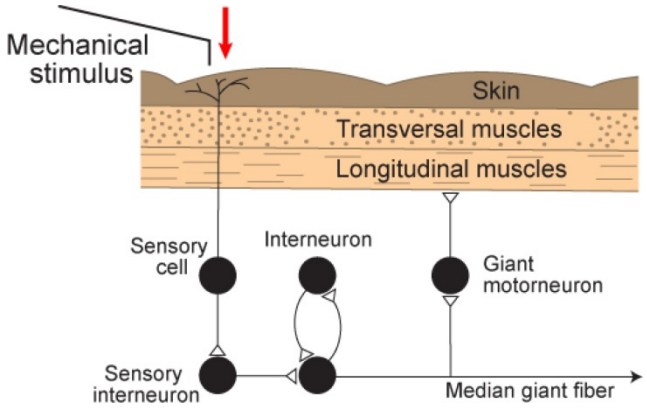

Figure 1: *Elements of the escape response mediated by the MGF pathway [23].*

iii. Strength - duration curve

Excitability of nerve tissue is dependent on the strength and the duration of the applied stimulus [31, 32]. This is because depolarization of an excitable membrane requires flow of electrical charge across the membrane. The charge transferred is proportional to the current and the time [33], and therefore shorter pulses require stronger stimulus to successfully initiate an action potential. There is a minimum stimulus amplitude under which stimulation is impossible, even for an infinite pulse, called the rheobase [34]. The specific relation between stimulus strength and pulse duration was characterized by Louis Lapicque and is given by the following expression [35]:

$$a = r + \frac{rc}{t} \tag{2}$$

Where a is the stimulus amplitude r is the already mentioned rheobase, t is the pulse length and c is the chronaxie, which is the shortest pulse that will elicit action potentials with an stimulus amplitude of twice the rheobase [36]. A typical strength - duration curve can be found in Figure 2. Although rheobase and chronaxie are usually determined for galvanic contact electrodes, Lapicque's equation has also been applied to external electric fields [37].

iv. Propagation model

In order to describe the propagation of the measured action potentials, a simple model describing the mechanical and electrical stimulation process was made. This model was based on the description of the MGF escape reflex

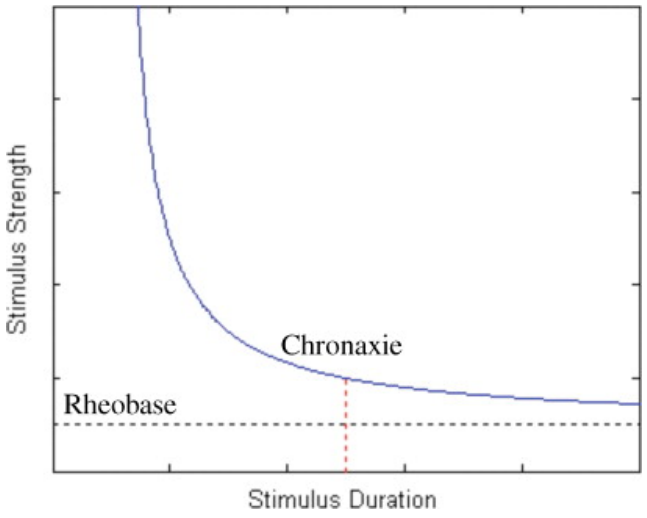

Figure 2: *Strength - Duration curve displaying the chornaxie and rheobase [38].*

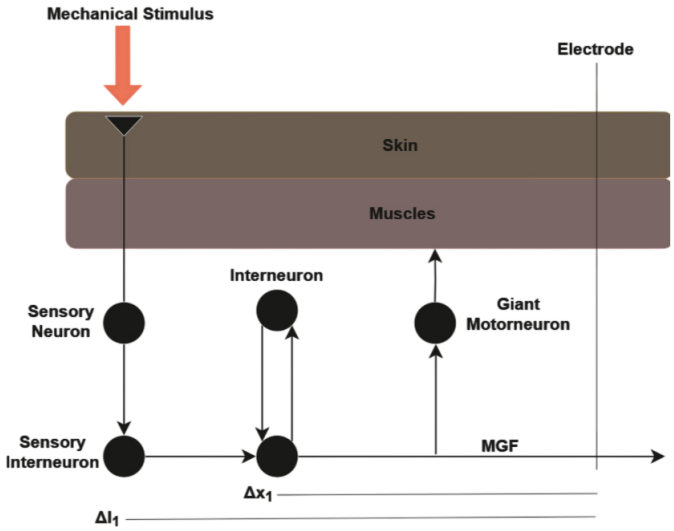

Figure 3: *Tactile stimulation model.*

pathway illustrated by Kladt et al. [23]. The time between stimulation onset and recording of the action potential, the propagation time, was defined as the sum of the generation time and the conduction time. The generation time includes all events from stimulus onset until the generation of the action potential in the giant fibers. In tactile stimulation these events include the transduction of the mechanical signal and synapses between sensory neurons and interneurons. In electrical stimulation, generation time only includes the depolarization of the giant fibers. Conduction time consists of the transmission of the action potential through the giant fiber. This was assumed to remain the same for all stimulation methods.

The equations describing the tactile propagation model (Figure 3) are the following:

$$T_{\mathrm{t}} = t_{\mathrm{gt}} + t_{\mathrm{ct}} \tag{3}$$

$$t_{\mathrm{ct}} = \frac{\Delta x_1}{v_{\mathrm{ct}}} \tag{4}$$

T_{t}: Propagation time in tactile stimulation

t_{gt}: Generation time in tactile stimulation

t_{ct}: Conduction time in tactile stimulation

Δx_1: Distance between giant fiber and recording electrode

v_{ct}: Conduction velocity in tactile stimulation

The equations describing the electrical propagation model (Figure 4) are the following:

$$T_{\mathrm{e}} = t_{\mathrm{ge}} + t_{\mathrm{ce}} \tag{5}$$

$$t_{\mathrm{ce}} = \frac{\Delta l_2}{v_{\mathrm{ce}}} \tag{6}$$

T_{e}: Propagation time in electrical stimulation

t_{ge}: Generation time in electrical stimulation

t_{ct}: Conduction time in electrical stimulation

Δl_2: Distance between giant fiber and recording electrode

v_{ce}: Conduction velocity in electrical stimulation

Assuming that conduction velocity does not change between stimulation methods ($v_{ct} = v_{ce} = v_c$), that in tactile stimulation the distance between the sensory interneuron and the giant fiber is 0 ($\Delta x_1 = \Delta l_1$) then the generation time can be calculated as follows:

$$t_{\mathrm{gt}} = T_{\mathrm{t}} - \frac{\Delta l_1}{v_{\mathrm{c}}} \tag{7}$$

$$t_{\mathrm{ge}} = T_{\mathrm{e}} - \frac{\Delta l_2}{v_{\mathrm{c}}} \tag{8}$$

Ideally, conduction velocity could be measured accurately

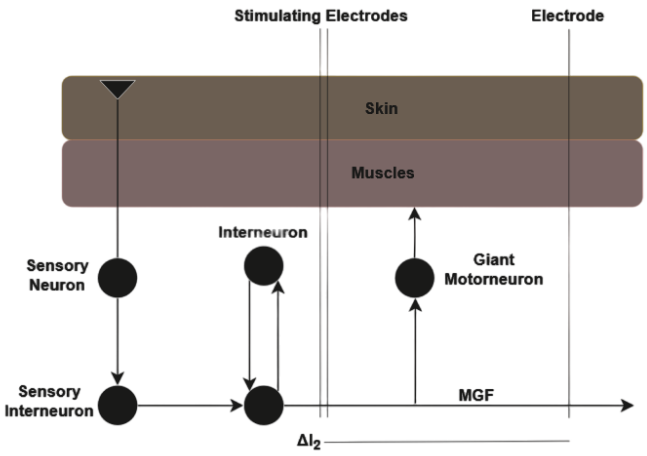

Figure 4: *Electrical stimulation model.*

by using two pairs of recording electrodes and two amplifiers. This was impossible in our case due to limitations in equipment. As electrical stimulation directly depolarizes the axon where action potentials are recorded, rather than the previous neurons in the pathway, generation times for this stimulation method were assumed to be zero. Therefore Equation 5 and Equation 6 now become:

$$T_e = t_{ce} \tag{9}$$

$$v_c = \frac{\Delta l_2}{T_e} \tag{10}$$

Which in combination with Equation 7 gives the generation times for tactile stimulation following our model assumptions:

$$t_{gt} = T_t - \frac{\Delta l_1}{\Delta l_2}\, T_e \tag{11}$$

III. Methods

i. Animals

Earthworms of the species *Lumbricus Terrestris* were purchased from a worm farm (Wormenkwekerij Wasse) and kept at 5 - 8 °C in a small refrigerator. Water was added in small quantities every two weeks to keep the soil moisturized. Before every experiment, the animals were anesthetized by immersion in a 10 % ethanol solution for eight minutes (subsection i). The worms were released after the experiment.

ii. Experimental set up

Figure 5 shows a picture of the set up, and additional pictures can be found in Appendix E. A Hammond 1411S aluminum enclosure with dimensions 76x102x203 cm was used as a Faraday cage. Four perforations were made to accommodate BNC connectors soldered to the recording and stimulation electrodes, which consisted of stainless steel insect pins (Ento Sphinxs No. 000). A ruler was placed in the border of the Faraday cage as a reference to calculate distances.

The two recording electrodes were connected to a SR560 differential pre-amplifier (Stanford Research Systems) with a gain of 2000 and band-pass filter with cut-off frequencies from 300 to 1000 Hz. The visualization equipment consisted of a TEK TBS2074 digital oscilloscope (Tektronix). Signals were acquired with a sample rate of 312500 samples per second. The stimulating electrodes were connected to a EDU33212A waveform generator (KEYSIGHT) which was used to deliver voltage pulses.

Magnetic stimulation was delivered by charging a 400 V 220 µF capacitor (Rubycon) with a 0-300 V power supply (Delta Elektronika), which then was discharged into a single turn coil. This coil was made up of two 15 mm parallel wires with a separation of 7 mm between them (Figure 23). A TN3015H-6G thyristor (STMicroelectronics) was used as a switch. The thyristor was activated using a BC547 bipolar junction transistor (NXP Semiconductors). An schematic of the stimulation circuit is depicted in Figure 24. The transistor was operated with a EDU36311A DC power supply (KEYSIGHT) and another EDU33212A waveform generator. The circuit was placed into a flexible holder, facilitating the manipulation of the coil so that it could be placed and fixed at the desired distance.

iii. Recording of action potentials

Two recording electrodes were inserted into the animal at positions corresponding to either the anterior or posterior thirds of its longitudinal axis, maintaining a separation of 1 cm between them. A ground electrode was inserted in the center through a wrapping of aluminum foil [23], which was used to reduce stimulation artifact (subsection ii). Tactile stimuli were applied to both the anterior (head) and posterior (tail) regions of the specimen to

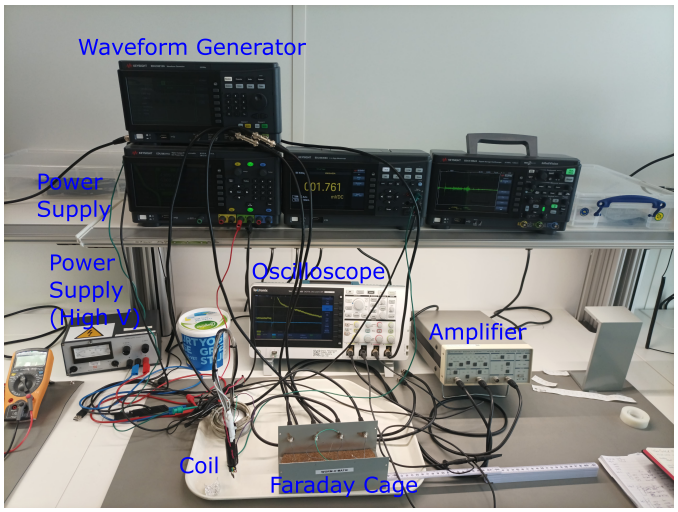

Figure 5: *Experimental set up and equipment.*

assess neural responsiveness. Animals that showed no response to tactile stimulation were excluded from the study.

iv. Mechanical stimulation

Mechanical stimulation was performed by pressing the animal with an oscilloscope probe on its skin surface. The voltage between the probe and a reference electrode was displayed in a dedicated channel in the oscilloscope so an artifact was created when the probe was in contact with the worm. The peak of the artifact was used as a time referent for the stimulus onset.

v. Electrical stimulation

Two additional electrodes were inserted mirroring the recording electrodes along the short axis. Electrical pulses of 100 µs duration and 5 V peak-to-peak amplitude were delivered by synchronizing two 2.5 V voltage pulses with opposite polarity. The pulses were set to a frequency of 0.1 Hz so that there was enough time to examine the response and store it before the next pulse was delivered. The electrical stimulus generated an artifact that was used as a time referent for the stimulus onset.

vi. Generation times and conduction velocities

The distances between stimulus site and recording electrodes (l_1 and l_2) were calculated with ImageJ (National Institutes of Health). The software was used to calculate the pixel to length ratio in pictures where the ruler and the electrode placement were visible. The distance in pixels from the stimulus site to the recording electrode was then measured and the ratio was used to convert it to centimeters. For electrical stimulation, the distance was taken from the cathode, as depolarization occurs in that region [39, 40]. To account for uncertainties, 12 distances were generated based on a normal distribution, with the mean equal to the value obtained from the software and a standard deviation of 0.1 cm. The propagation times (T_t and T_e) were obtained by calculating the differences between time of stimulation onset and the first peak of the recorded action potential. Finally, the average generation time and conduction velocity for each fiber was calculated by performing a Monte Carlo simulation on Equation 10 and Equation 11, respectively, iterating over all values for T_t , T_e , Δl_1 , and Δl_2. More information about the Monte Carlo method can be found here [41].

vii. Strength-duration curves

Strength duration curves were computed by following the standard protocol for electrical stimulation but adjusting the input voltage and pulse duration to achieve a success rate close to 50 %, which was defined as the threshold. Then pulse duration was reduced, and the input voltage increased until the success rate was again at threshold levels. This process was repeated until the maximum input voltage was reached. The resulting set of pulse amplitude and duration data points were then fitted to the Lapicque equation, yielding the values for the rheobase and chronaxie that best described the response.

The input voltage was converted to electric field by modeling the stimulating electrodes in an electrostatic simulation using Falstad's Circuit Simulator. It was assumed that the potential is equally distributed across the surface of the electrode. Consequently, a coronal (top-down) plane was used, allowing the electrodes to be modeled as circles since the voltage is uniform at every cross-section. The ventral nerve cord was assumed to run parallel to the two electrodes, with distance (d) between 0.2 and 1.25 mm. A schematic of the model can be found in Figure 6. In Falstad, distances were introduced in meters rather than millimeters to better accommodate to the size of the canvas (12 x 12 m). The electrodes were defined as conducting circles of 0.3 m in diameter with a separation of 10 m between them. Each circle was set to a potential of 0.5 V, equivalent to a 1 V pulse. Only the field parallel to the axon was considered, as it is the primary contributor in neurostimulation [42]. The results of the simulation

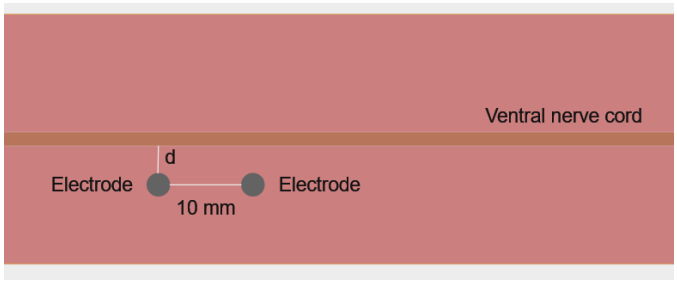

Figure 6: *Model for estimation of electric field during electric stimulation.*

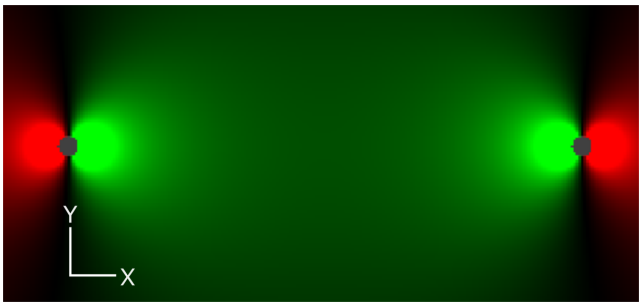

Figure 7: *Electric field parallel to the ventral nerve cord (E_x) from a Falstad simulation. Each electrode was set to 0.5 V. Green indicates left to right direction and red the opposite.*

are presented in Figure 7. Finally, the electric field for a 1 V peak-to-'peak pulse was estimated by taking the maximum field at distances d = 0.2 m to d = 1.25 m using steps of 0.05 m and calculating the average, which after re-scaling back to millimeters, yielded a result of 140 V/m.

The estimation of the induced electric field pulse length and duration was based on the work of Johan Meyer, who generously shared his calculations of the induced field from the coil at a 5mm distance from a 30 V discharge. His results are described in Appendix D.

viii. Magnetic stimulation

The coil was covered with some tape in order to isolate it in case it came into contact with the worm. The coil was placed 1 millimeter over the posterior end of the worm, as that is where the animal is at its thinnest and therefore the neuron is closer. The voltage in the collector of the transistor was set to 5 V and the capacitor was charged to 160 V. The stimulation was delivered by applying a 10 µs 5 V peak-to-peak pulse to the base of the transistor to activate the thyristor, closing the circuit and allowing the capacitor to discharge into the coil. The magnetic pulse generated a stimulus artifact that was used as a time referent for the stimulus onset. In order to maximize the induced electric field, charging the capacitor up to 300 V (the limit of the power supply) would have been desirable. However, it was found that the thyristor used in the circuit immediately failed after a 200 V discharge and could only withstand 170 V discharges for 5 - 10 repetitions. Consequently, the capacitor was charged at 160 V, which allowed the delivery of more than 10 pulses before failure. An approximate curve describing the relation between the discharge voltage and the failure of the thyristor can be found in Figure 16.

IV. Results

i. Electrical and tactile stimulation

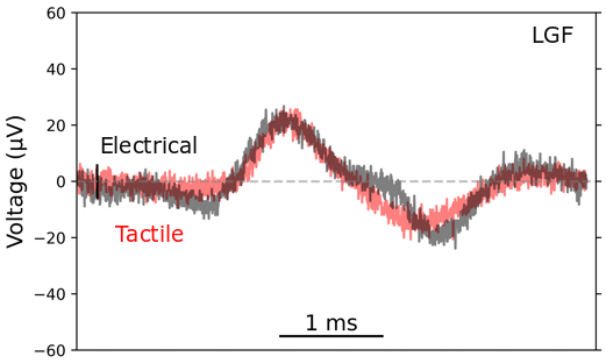

Figure 8: *Average of action potentials measured from the LGF. Red denotes the average AP triggered by tactile stimulation (n = 6) while the average pulse triggered by electrical stimulation is shown in black (n = 6). The standard deviation is shown by the black bar at the beginning of the plot. The differences between the Action potentials traces (5.1 µV) are within the boundaries of measurement error (5.7 µV) and therefore we conclude that the two stimulation methods trigger the same action potentials.*

The action potentials triggered by tactile and electrical stimulation are compared in Figure 8 (LGF) and Figure 9 (MGF).

The maximum difference between the action potential traces from the LGF is 5.1 µV , which falls within the boundaries of the standard deviation 5.7 µV and therefore

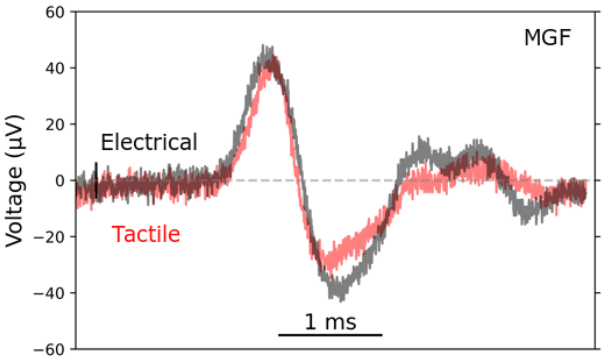

Figure 9: *Average of action potentials measured from the MGF. Red denotes the average AP triggered by tactile stimulation (n = 6) while the average pulse triggered by electrical stimulation is shown in black (n = 6). The standard deviation is shown by the black bar at the beginning of the plot. Although there is a small difference in amplitude between the two action potential traces (11.8 µV), duration and shape indicates that the signal comes from the same fiber.*

it is safe to conclude that the two stimulation methods trigger the same action potentials.

In the MGF however, there is an observable difference between the average traces from the two stimulation methods. Specifically, the amplitude of the action potentials triggered by tactile means is 11.8 µV lower than that of those elicited by electrical stimulation. Nevertheless, this difference is not large and considering that duration and overall shape are mostly equal it is unlikely that the signals come from different fibers. There are some indications that this small difference in amplitude was caused by the stimulating electrodes altering the conductivity of the worm. This possibility is explored in Appendix C but additional experiments are necessary to confirm it.

Electrical activity in the ventral nerve cord was recorded following electrical stimulation, tactile stimulation in the tail and tactile stimulation in the head (Figure 10). Electrical stimulation triggered both the MGF (M) and the LGF (L) while tactile stimulation triggered only one giant fiber depending on the stimulation region. When the stimulus was delivered in the posterior section of the animal (Figure 10D-F) the LGF was triggered and when the stimulus was delivered in the anterior region (Figure 10G-I) the MGF was activated. During tactile stimulation the MGF and LGF action potentials were propagated in opposite directions, but in electrical stimulation both APs were conducted in the same direction. This can be appreciated by the polarity of the pulses, which is the same for the MGF and the LGF in electrical stimulation but opposite in tactile. These discrepancies most likely stem from differences in how each stimulation method depolarizes the giant fibers. Electrical stimulation generates an external electric field that, if sufficiently strong, directly depolarizes the MGF and LGF and propagates the action potential in both directions [43]. Conversely, tactile stimulation activates the giant fibers only after the integration of the stimulus into the escape reflex pathway. The MGF pathway receives input from sensory cells in the anterior region and the LGF from sensory cells in the posterior region [29, 30]. This guarantees that only one fiber is activated depending on the stimulation region and that the action potential is propagated in the natural direction only.

Electrical stimulation triggered the giant fibers only once, while after tactile stimulation several action potentials following the stimulus were often observed. This is perhaps a consequence of the differences in the stimulus duration, which was 0.1 ms in electrical stimulation but over 50 ms in tactile stimulation.

Electrical stimulation also triggered a third, longer spike (3) which is absent in tactile stimulation. This spike could be a result of muscle twitches triggered by the external current generated by the stimulus [44]. In tactile stimulation, muscle contraction is impaired after sufficient anesthetization [24]. A possible explanation could be that the anesthesia blocks the natural neural pathway that leads to muscle contraction while still allowing the direct stimulation of the muscle by external currents. In any case, more research is needed to determine the origin of this spike.

In electrical stimulation, the action potentials arrive just a few milliseconds after the stimulus is delivered. In tactile stimulation, however, no action potential arrived within the first 20 milliseconds following stimulation onset. This again can be explained by how the two stimulation methods trigger the giant fibers. The delay observed in tactile stimulation is a consequence of the time it takes to integrate the signal into the escape reflex pathway, while in electrical stimulation this pathway is bypassed and conduction along the fiber can begin much earlier. The action potential also arrived with more temporal variation in tactile stimulation than in electrical stimulation.

Five recordings of the ventral nerve cord like the one

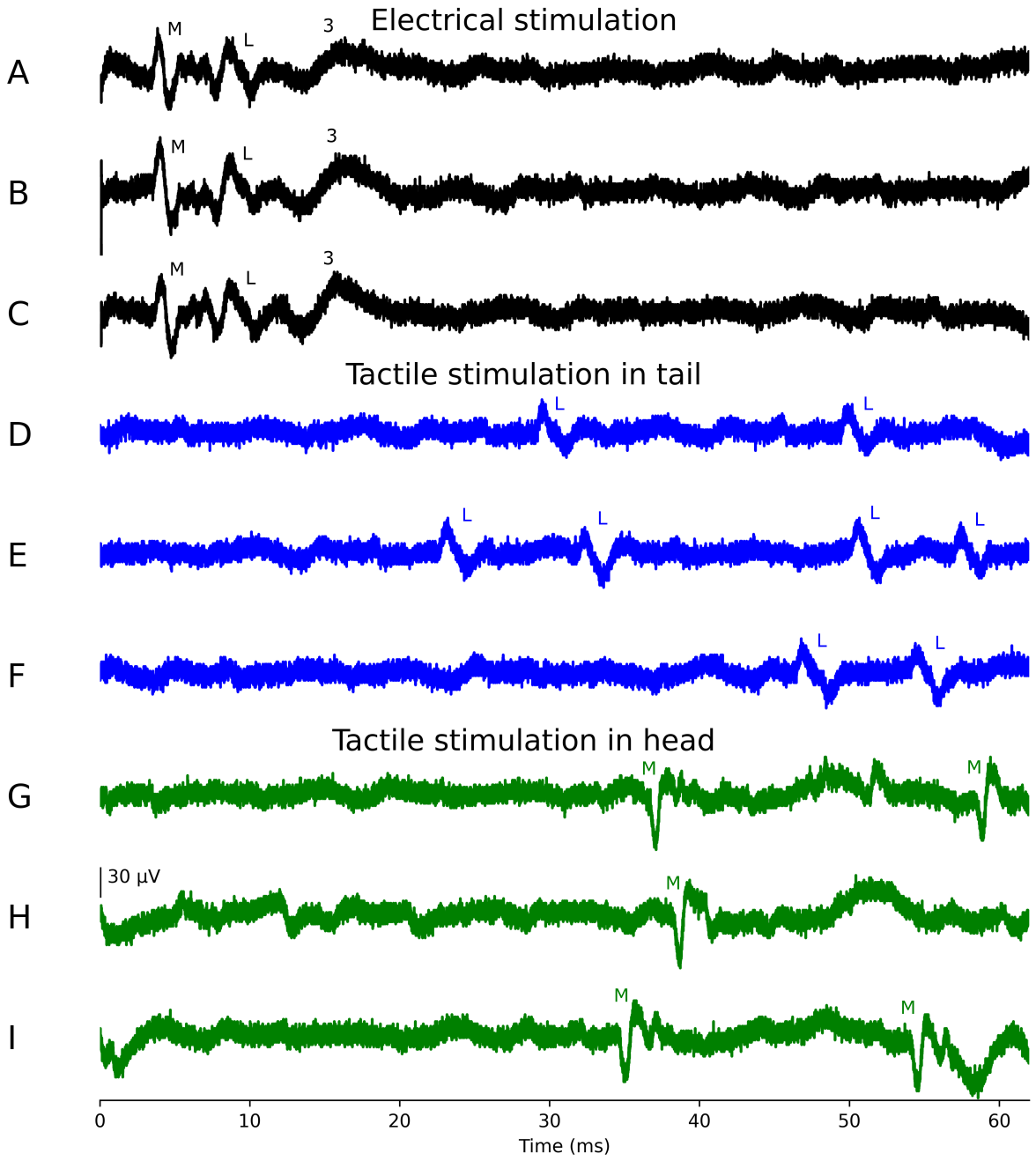

Figure 10: *Response signals measured in the ventral nerve cord following electrical stimulation (A-C), tactile stimulation in the tail (D-F) and tactile stimulation in the head (G-I). Axis scale is indicated in chart H. Electrical stimulation concurrently triggers both MGF (M) and LGF (L) with very short, repeatable, arrival times. Both fibers are triggered only once, with MGF arriving before LGF to the recording site. Electrical stimulation also triggers a third longer spike (3) possibly of muscular origin, which is absent in tactile stimulation. Tactile stimulation triggers the LGF pathway when the stimulus is delivered in the posterior section and the MGF pathway when the stimulus is delivered in the anterior part. This opposite directionality is appreciated by the inverted polarity of the LGF and MGF Action Potentials. Unlike electrical stimulation, tactile stimulation can trigger several action potentials in the same fiber following a single stimulus. The action potential arrives later and with more temporal variation in tactile stimulation.*

Fiber	Velocity (m/s)	Generation time (ms)
LGF	8.0 ± 0.3	21.8 ± 4.3
MGF	15.0 ± 0.3	35.6 ± 2.8

Table 1: *Average velocity of each fiber and the generation time of their associated flight reflex pathway. These values were estimated with a model that calculates the generation time and conduction velocity from the total time and distance from stimulus and recording. The MGF conducts the AP 7.0 ± 0.4 m/s faster than the LGF, but its pathway takes 13.8 ± 5.1 ms longer to reach and depolarize the giant axon. The table shows average and standard error from data from five different worms.*

displayed in Figure 10 were used to estimate the conduction velocities and generation times of the LGF and the MGF with a model that describes the mechanical and electrical stimulation process (subsection iv). The resulting values can be found in Table 1. The velocities of the MGF and the LGF were estimated at $(15.0 \pm 0.3)\,\mathrm{m/s}$ and $(8.0 \pm 0.3)\,\mathrm{m/s}$, respectively. The generation times for the MGF and the LGF escape reflex pathways were estimated at $(35.6 \pm 2.8)\,\mathrm{m/s}$ and $(21.8 \pm 4.3)\,\mathrm{m/s}$. This means that although the MGF is $(7.0 \pm 0.4)\,\mathrm{m/s}$ faster than the LGF, its associated escape reflex pathway takes $(13.8 \pm 5.1)\,\mathrm{m/s}$ longer to reach and depolarize the giant fiber. These values are consistent with the action potential arrival times observed in Figure 10. In electrical stimulation, where generation times were assumed to be zero, the faster MGF fiber conducts the action potential to the recording electrodes earlier than the LGF. In tactile stimulation, generation times are not negligible and the faster pathway of the LGF can sometimes compensate the slower velocity of the giant fiber and conduct the action potential earlier than the MGF pathway.

The model used to estimate these values can be validated with the literature. The generation times for electrical stimulation were calculated by Jérémy Vion-Bailly et al [26] at 1.3 ms for MGF and 2.4 ms for LGF. These times are significantly shorter than those obtained for tactile stimulation, making the assumption of zero generation times for electrical stimulation acceptable. This confirms that generation times in tactile stimulation are longer than in electrical stimulation. Furthermore, the estimated velocity values align closely with the literature. Vion-Bailly et al. [26] reported velocities of 16.6 m/s for MGF and 9.0 m/s for LGF in ethanol-anesthetized worms following electrical stimulation. Similarly, Nikolay Kladt et al. [23] reported velocities of 16.1 m/s and 6.9 m/s for MGF and LGF, respectively, in chlorobutanol-anesthetized worms. Since the velocity is implicitly included in the equation used to calculate generation times (Equation 11), this consistency with the literature further validates the model and the obtained time values.

ii. Magnetic stimulation

Electrical activity in the ventral nerve cord was measured following tactile stimulation in the head (Figure 11A) and magnetic stimulation (Figure 11B). In the latter figure, a large stimulation artifact can be observed, which indicates that high electric fields were delivered. The average of the action potentials present in Figure 11A-B was taken and compared in Figure 11C. The difference between the traces are within the boundaries of measurement error, confirming that action potentials from the MGF fiber were recorded after magnetic stimulation.

It is, however, unclear whether these APs were actually triggered by the induced electric field. The response of the worm closely resembled that of intense tactile stimulation, with a rapid succession of APs, rather than that of electrical stimulation, which triggers a single action potential. Since both magnetic and electrical stimulation rely on generating an external field that depolarizes the neuron, it would be expected for both methods to result in similar responses. Therefore, magnetic stimulation is also expected to bypass the escape reflex pathway and generate action potentials shortly after the delivery of the pulse. Unfortunately, the stimulus artifact blocked the first 10 milliseconds of signal and therefore it was impossible to know if the first action potential was triggered in the expected time frame. Information before the stimulus is unavailable and consequently the possibility of non-stimulation factors inducing a panic state in the worm cannot be dismissed even though the giant fibers are usually silent unless specifically triggered by tactile stimulation in the head or tail[24, 45].

iii. Strength-duration curves

Strength-duration curves for the MGF for electrical stimulation of four different worms were estimated, as seen in Figure 12. Experimental data were fitted to the Lapicque equation (Equation 2), and the chronaxie and rheobase values that best described the response were obtained. These curves estimate the input voltage and pulse duration necessary to trigger action potentials 50 % of the times. Some of the variability in these curves could be a consequence of differences in distances between electrodes and the ventral nerve cord in each experiment. The

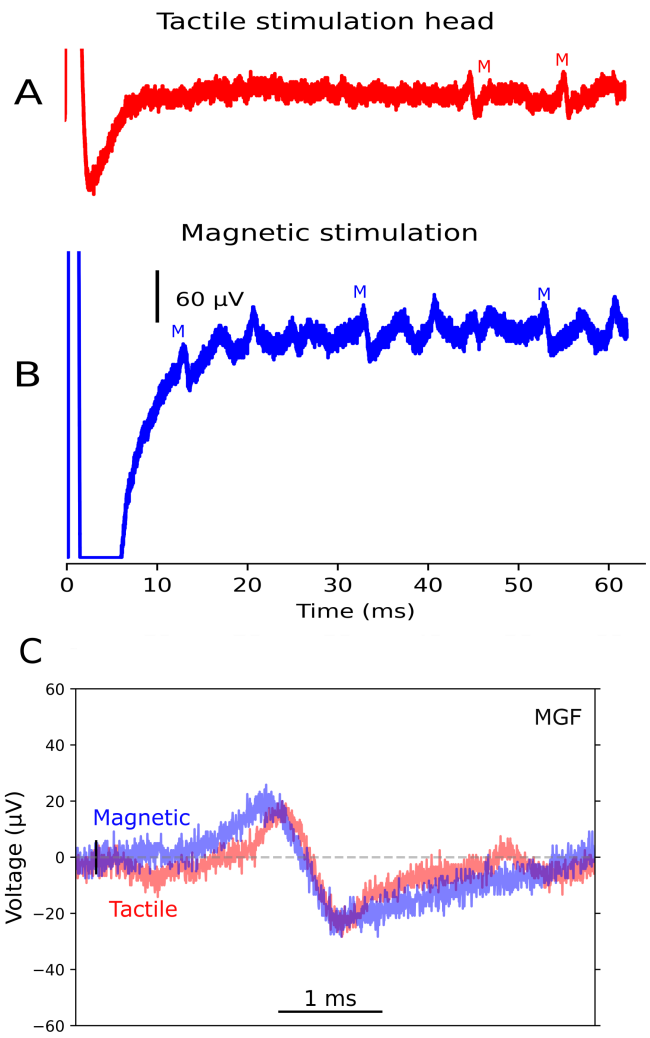

Figure 11: *Electrical signals measured in the ventral nerve cord following tactile (A), and magnetic (B) stimulation at t = 0. Axis scale is shown in chart B. The average of the action potentials measured following the stimulation is shown in (C), with the standard deviation shown at the beginning of the plot. Notably, action potentials from the MGF fiber (M) were recorded after magnetic stimulation. The response consisted of a train of action potentials following the stimulus, a phenomenon sometimes observed in tactile stimulation but not in electrical. The stimulus artifact blocked the first 10 ms and information before the stimulus is not available. Therefore, it is unclear whether these APs were actually triggered by the induced electric field.*

average values for the four worms were $r = (1.4 \pm 0.4)$ V and $c = (0.16 \pm 0.06)$ ms, which gives an approximation of the strength duration curve of the MGF that can be expected following this protocol and set up. A rough equivalence between the input voltage and the electric field parallel to the giant axon, see right axis of Figure 12, was established by performing an electrostatic simulation in Falstad. This allowed a direct comparison between electrical and magnetic stimulation.

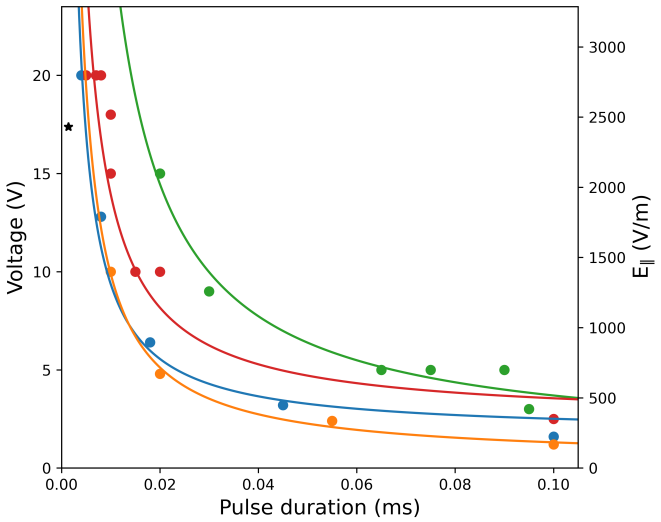

Figure 12: *Estimation of the strength - duration curves for electrical stimulation for the MGF of four different worms. The curves were generated by fitting experimental data to the Lapicque equation. The average rheobase and chronaxie are r = 1.4 ± 0.4 V and c = 0.16 ± 0.06 ms respectively. The right axis represents a rough estimate of the electric field generated by the voltage pulses during electrical stimulation. The asterisk indicates the estimation for magnetic stimulation at E = 2450 V/m and t = 0.0014 ms. Magnetic stimulation did not reach the threshold values delimited by the strength-duration curves.*

According Figure 20, the induced electric field from magnetic stimulation was modeled as a pulse of 2450 V/m amplitude and 1.4 µs in duration, shown in Figure 12 as an asterisk. Based on the estimated strength duration curve, a pulse with this length would require an amplitude of (22.6 ± 10.5) kV/m for upper threshold stimulation. The induced electric field from magnetic stimulation falls below this value, making it unlikely that above-threshold intensities were delivered, although stimulation could still be possible if enough pulses are applied.

iv. Stimulus artifacts

Electrical activity in the ventral nerve cord was recorded after tactile, electrical, and magnetic stimulation at two different distances between stimulation and recording sites Figure 13. A stimulus artifact is present in all graphs where the stimulus is delivered at t = 2 ms, meaning that crosstalk affects the three stimulation methods. In electrical and magnetic stimulation, the artifact is probably caused by spread of the electric field generated by these methods. For tactile stimulation, crosstalk is maybe a consequence of movement artifacts when pressing the worm.

In tactile stimulation (Figure 13A-B), separating the recording electrodes from the stimulation site from 2 cm to 5 cm led to a reduction of the stimulus amplitude from 440 µV to 65 µV and a reduction in stimulus duration from 10 ms to 7 ms. Generation times for tactile stimulation are (35.6 ± 2.8) ms for the MGF and (21.8 ± 4.3) ms for the LGF (Table 1) and therefore the stimulation artifact will not interfere with the recording of action potentials. This makes crosstalk negligible in tactile stimulation, even at very short distances.

In magnetic stimulation (Figure 13E-F), action potentials are expected a few milliseconds after the pulse. Consequently, large artifacts like the one observed in Figure 13E are a concern since they could completely block the triggered action potentials. Separating the recording electrodes from the stimulation site from 2 cm to 4 cm led to a reduction of the stimulus amplitude from 635 µV to 296 µV and a reduction in stimulus duration from 18 ms to 3 ms. The reduction in artifact duration is specifically significant and it demonstrates that increasing the distance between stimulation and recording by just a few centimeters can considerably decrease the impact of the artifact.

In electrical stimulation (Figure 13C-D), increasing the distance between the recording electrodes and the stimulation site from 3 cm to 5.5 cm reduced the stimulus amplitude from 715 µV to 120 µV and the stimulus duration from 1 ms to 0.2 ms. Due to the very short generation times in electrical stimulation, the time between the stimulus and the action potential largely depends on the distance between the stimulation and recording sites. With a conduction speed of 15 m/s (Table 1), action potentials follow the stimulus by 0.67 ms for each centimeter of separation. Therefore, at distances below 3 cm, crosstalk may interfere with the acquisition of action potentials, but its impact is minimal at longer distances.

V. Discussion

One of the main limitations encountered during the experiments was the inconsistency of the anesthetic effects. Despite using the same exposure time, some worms recovered after just five minutes, while others became completely unresponsive. Furthermore, the effects of the anesthesia were short-lived, making it challenging to conduct experiments lasting longer than 15 minutes. Given the variability among individual worms, it was essential to compare the stimulation methods within the same specimen, which extended the duration of the experiments. The awakening of the worms often led to the premature termination of many experiments due to increased motion artifacts before the second stimulation method could be adequately tested. Additionally, even when noise levels were low, the movements of the worm altered the distances between the electrodes and the stimulation site, invalidating some experiments where distance was a key value.

For future experiments, it is recommended to improve the anesthesia protocol so that experimental conditions remain stable for longer time. This could be achieved, for instance, by employing neuromuscular blockers such as rocuronium, that was shown to inhibit muscular contractions in worms for longer than 2 hours, or by performing the experiments in an airtight box, which prevented evaporation and prolonged the effect of ethanol up to 3 hours [46]. Alternatively, other immobilization methods can be explored, such as confining the worm in a tight chamber [25].

Although the model used to estimate conduction velocities and generation times proved to be robust, it is recommended that future research directly measures the velocities of the giant fibers. This approach will result in more accurate values for the generation times during tactile stimulation and also enable the estimation of generation times during electrical and magnetic stimulation. Furthermore, it would allow the verification of the assumption that the stimulation method does not affect conduction velocity.

Additionally, to facilitate more accurate comparisons between electrical and magnetic stimulation, the applied electric field stimulus should be as similar as possible in the two stimulation methods. One way to achieve this is by employing sinusoidal waveforms to deliver the voltage in electrical stimulation, rather than using pulses. This approach would result in a stimulus shape more comparable to that of magnetic stimulation, thereby facilitating

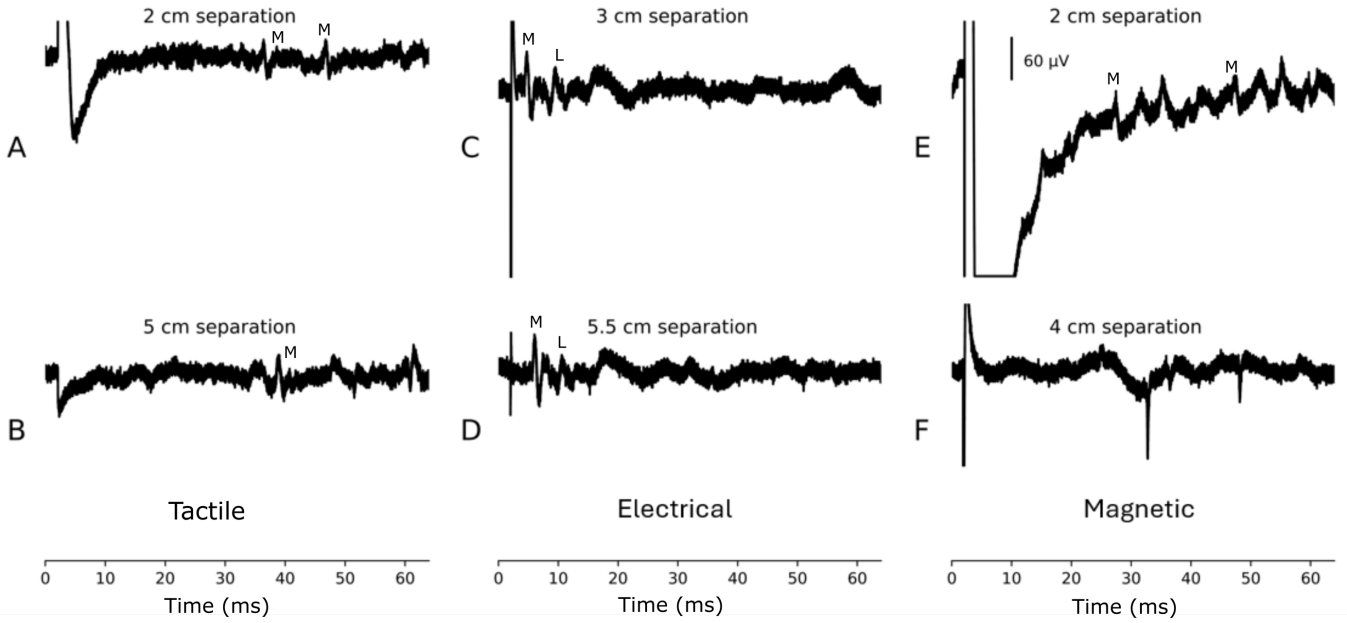

Figure 13: *Signal recorded from the ventral nerve cord following tactile (A –B), electrical (C-D), and magnetic stimulation (E-F) at two different separations between stimulation and recording. Axis scale is shown in chart E. The signals are clipped so that the action potentials form the MGF (M) and LGF (L) could be distinguished. All stimulation methods are affected by crosstalk, which generates an artifact following the delivery of the stimulus at t = 2 ms. Separating the recording electrodes from the site of stimulation reduces the amplitude and duration of the artifact in the three methods.*

the comparison of both the amplitude and duration of the applied fields. If during electrical stimulation the rheobases and chronaxies were calculated with sinusoidal waveforms, the magnetic pulse could be more reliably compared to the resultant strength-duration curve.

Finally, according to the estimated strength-duration curves (Figure 12), it is recommended to increase the amplitude and/or duration of the induced electric pulse to improve the chances of successful stimulation. The electric filed strength can be increased by charging the capacitor at a higher voltage. Following the Lapicque equation, there is no limit in how much you can increase the amplitude of the stimulus. However, very high voltages raise safety concerns and might pose challenges in the design and operation of the circuit. In this work, the circuit used to deliver magnetic stimulation was limited to 160 V discharges because the thyristor used as a switch started failing at higher voltages, breaking immediately at 200 V (Figure 16). This effect was likely caused due to the high currents flowing through the switch. Therefore, to increase the induced field the circuit should be modified to allow higher voltage discharges. It is important to account for the possible increase in resistance in the circuit, which could result in less effective stimulation despite higher voltages due to a reduced current passing through the coil. Alternatively, increasing the pulse duration will reduce the field amplitude necessary for stimulation. However, for pulses longer than the chronaxie ($0.16 \pm 0.06\,ms$), the gains diminish as the strength-duration curve converges towards the rheobase. Pulse duration can be extended by raising the inductance of the coil, although this would also decrease the magnitude of the induced field [27]. If safety concerns can be effectively addressed and high-voltage power supplies along with high-current rated components are available, it is recommended to increase the amplitude of the induced field. Alternatively, if these conditions cannot be met, prolonging the duration of the pulse up to the chronaxie may be more advantageous to improve the likelihood of stimulation.

VI. Conclusions

The goal of this thesis is to compare tactile, electrical, and magnetic stimulation in an earthworm model. Electrical stimulation concurrently triggers both the LGF and the MGF fibers while tactile stimulation selectively triggers one type of fiber depending on stimulation site: the LGF in the posterior and the MGF in the anterior end of the worm. Tactile stimulation can trigger several spikes in the MGF or in the LGF after a single stimulus, while electrical stimulation triggers only one spike in each fiber. Electrical stimulation also triggers a third longer spike which is absent in tactile stimulation. The MGF conducts the action potentials $(7.0 \pm 0.4)\,\mathrm{m/s}$ faster than the LGF, but its pathway takes $(13.8 \pm 5.1)\,\mathrm{s}$ longer to transmit the stimulus to the giant fiber. As electrical stimulation bypasses the reflex network, delay between stimulus and action potential firing is longer in tactile stimulation.

Action potentials from the MGF were recorded following magnetic stimulation. The recorded signal consisted of a train of action potentials following the stimulus, a type of response observed only in tactile stimulation. The generation time was unknown because the stimulus artifact blocked the first 10 ms of signal and therefore a direct relation between the stimulus and AP triggering could not be established. Electrical stimulation follows a typical strength duration response with a minimum stimulus amplitude $r = (1.4 \pm 0.4)\,\mathrm{V}$ and a time constant $c = (0.16 \pm 0.06)\,\mathrm{ms}$. Based on this curve, the induced field at the magnetic pulse width of 1.4 µs should be $(22.6 \pm 10.5)\,\mathrm{kV/m}$ The electric field generated by the magnetic field pulse was estimated at $2.45\,\mathrm{kV/m}$ and consequently magnetic stimulation did not reach the critical field value. Therefore, it cannot be concluded that the recorded action potentials were triggered by the magnetic pulse.

Crosstalk is present in the three stimulation methods, but the impact of the artifact varies. In tactile stimulation, generation times are $(35.6 \pm 2.8)\,\mathrm{ms}$ and $(21.8 \pm 4.3)\,\mathrm{ms}$ for the MGF and the LGF, respectively. The artifact duration is 10 ms at a 2 cm distance, making crosstalk negligible. In Magnetic stimulation, however, action potentials are expected just a few milliseconds after the pulse, so large artifacts are a concern. Increasing the distance between stimulation and recording from 2 cm to 4 cm reduced the peak-to-peak amplitude and duration of this artifact from 635 to 296 V and from 20 to 3 ms. In electrical stimulation, crosstalk has minimal impact at distances greater than 3 cm. However, at closer distances, even an artifact lasting 1 ms can interfere with the recording due to the very short generation times.

This study showed that the earthworm's nervous system can be used to highlight fundamental mechanisms of various neurostimulation methods. This indicates that the earthworm is a suitable animal model for future research in the field of micro-magnetic stimulation.

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A. Experimental protocol

i. Anaesthesia protocol

Earthworms were immersed in concentrations of 5 % and 10 % ethanol for periods of time ranging from 3 to 10 minutes. The objective was to establish an anaesthesia protocol that relaxed the worm during 7 - 15 minutes, suppressing their escape reflex, while making sure that the worm could recover afterwards. Deep anaesthesia was unwanted because it could have effects in neuron excitability and conduction velocity, making AP detection difficult or even impossible [24].

The worms were submerged in a specific anaesthetic solution (control, 5%, 10 %) for a certain time (3, 5, 7.5, 10 min). Afterwards the worms were briefly rinsed and cleaned, and a timer was set. Every minute the head or tail of the worm was tapped with a brush with the intention of triggering the escape response. The time at which the worm started moving or showing the reflex was written down. The whole process was recorded with a camera.

All worms reacted violently when encountering the ethanol solution and moved vigorously during the first two minutes of exposure. Five percent ethanol was largely ineffective in relaxing the worm while 10 % concentrations were insufficient after 5 minutes and too effective after 10 minutes.

An exposure time of 7.5 minutes with 10 % ethanol was found as the optimal anaesthetic protocol for our purposes. The results of the experiment can be found on Table 2.

Exposure time	Control (tap water)	Ethanol 5%	Ethanol 10%
3 min	-	No effect	-
5 min	-	No effect	Excitatory effect
7.5 min	-	-	8 min effect
10 min	No effect	3 min effect	Full immobilization (no recovery)

Table 2: *Effects of ethanol concentration on worm immobilization. Every concentration and exposure time was tested on a different worm. A concentration of 10 % ethanol with a exposure time of 7.5 min was the only protocol that relaxed the worm for longer than 7 minutes.*

ii. Electrical stimulation artifact

The aim of this experiment was to evaluate the stimulation artifact generated by electrical stimulation in a worm mock-up and test different ways to reduce it. The main idea was to use a double input configuration to balance the charge injected [47] into the mock-up, and to wrap a piece of aluminium foil between stimulation and recording electrodes to provide a low resistance way out for the current and reduce crosstalk [23].

The mock-up consisted of a wrapped piece of wet cloth 19 cm long and 0.5 cm in diameter. The magnitude of the stimulation artifact was assessed while changing the stimulation set up. Electrode distance, input voltage, amplifier gain, number of stimulation electrodes, and presence or not of aluminium foil were changed during the experiment.

Using a double input configuration and wrapping the mock-up with aluminum foil significantly reduced the amplitude of the stimulation artifact (Figure 14). Adding aluminum foil to single and double input configurations decreased the artifact amplitude by factors of 3.3 and 1.47, respectively. Without aluminum foil, the double input configuration reduced the artifact amplitude by a factor of 3.4, and with foil, by a factor of 1.53. The effectiveness of the aluminum foil largely depended on its contact with the cloth. Regarding the input electrodes, the most effective configuration was placing the two stimulating electrodes parallel to each other and achieving charge balance by adjusting the input voltage of each channel until the artifact was removed. This would however greatly increase the stimulation threshold as the electric field generated would be perpendicular to the axon [42]. Consequently, this method was not considered for stimulation experiments. In terms of duration, the double input configuration had no significant influence, whereas the aluminum foil reduced the duration by a factor of 4.

Low input voltages and low amplifier gain also diminished stimulation artifacts (Figure 15). Lowering the peak-to-peak amplitude of the input voltage from 5 V to 1.5 V reduced the artifact amplitude by a factor of 2.5. Keeping the voltage the same but decreasing the amplifier gain from 5000 to 1000 reduced the amplitude by a factor of 3.3. High gains also extended the artifact duration, likely due to the amplifier needing more time to reset after overloading. Separating the stimulation and recording electrodes also reduced artifact amplitude. However, since in our protocol we stimulate both the head and the tail of the animal, it is necessary to place the electrodes at least a couple of centimeters away from these regions, which reduces the potential separation between the two pairs of electrodes.

Placing aluminum foil between stimulation and recording electrodes was an effective measure of reducing both stimulus artifact amplitude and duration. Delivering the pulse with two stimulation electrodes rather than one also diminished the amplitude of the artifact, but the effect on its duration was not significant. Finally, lowering the input voltage and amplifier gain also led to smaller artifacts.

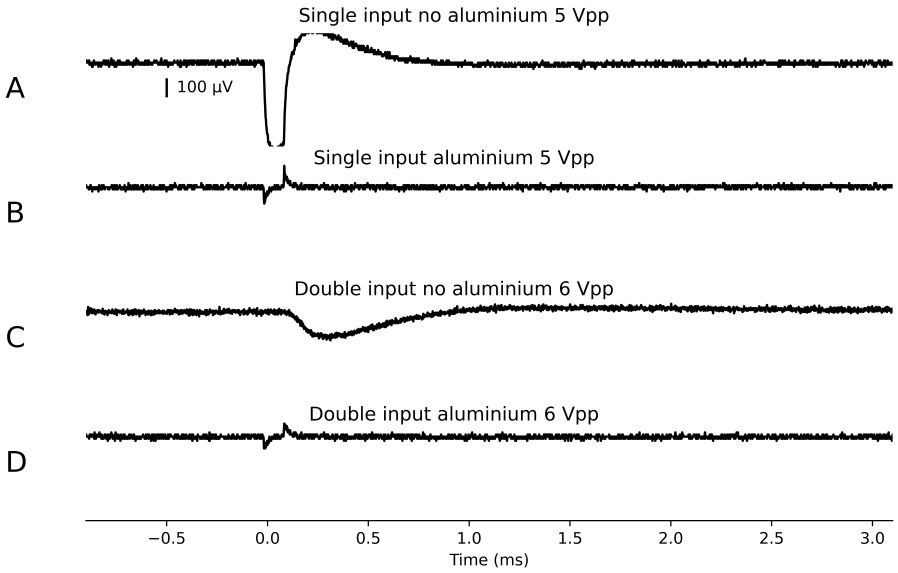


Figure 14: *Effect of input configuration and aluminium foil in stimulation artifact from electrical stimulation measured in a cloth mock-up. The stimulus was delivered at t = 0 ms. Axis scale is indicated in chart A. The measurements were made with a gain of 1000. Using aluminium foil reduces both the duration and the amplitude of the stimulation artifact, while employing a double input configuration reduces the amplitude only.*

iii. Discharge Voltage

The voltage used to charge the capacitor is a crucial parameter in magnetic stimulation, as it directly affects the current flowing through the coil and, consequently, the induced electric field [27]. To increase the amplitude of the induced field and maximize the likelihood of successful stimulation, it is advisable to raise the discharge voltage as high as reasonably possible. However, For safety reasons, it is recommended to limit the energy of high voltage capacitors to 10 J [48]. Based on this guideline, the maximum voltage considered to charge the $220\mu F$ capacitor was 300 V ($E = \frac{1}{2}CV^2$).

During experimentation, it was discovered that the circuit could not withstand discharges at 300 V. Specifically, the thyristor failed after a single discharge at 200 V. This is probably a consequence of the high current flowing through the thyrisor. By incrementally increasing the voltage, it was determined that the thyristor could endure between 5 to 10 discharges at 170 V. An approximate curve describing the relation between the discharge voltage and the number of discharges necessary for failure was calculated (Figure 16). From this data, the discharge voltage was set at 160 V as a compromise to balance the high induced fields and the durability of the circuit.

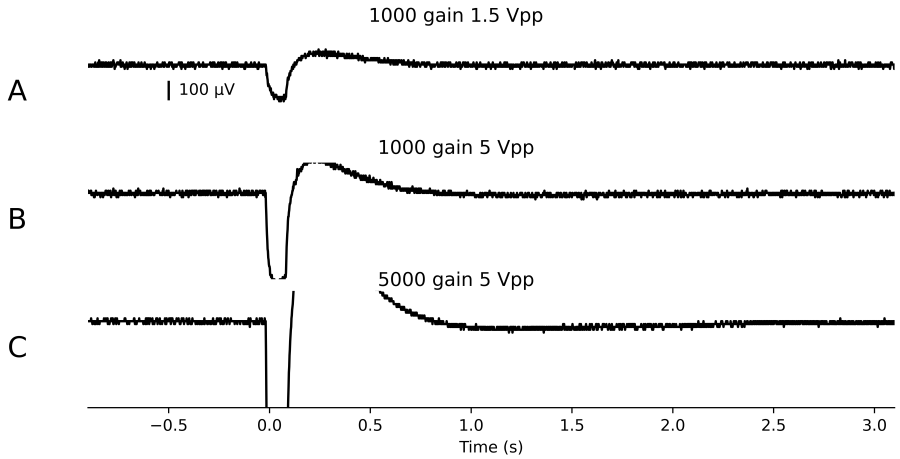


Figure 15: *Effect of input voltage and amplifier gain in stimulation artifact from electrical stimulation measured in a cloth mock-up. The stimulus was delivered at t = 0 ms. Axis scale is indicated in chart A. The measurements were made with no aluminium and single input. Increasing the amplification and input voltage led to larger stimulation artifacts. Amplification also increased the duration of the artifact, probably because the amplifier needed more time to reset after overloading. The signals in B and C are clipped in order to keep the same axis as in Figure 14.*

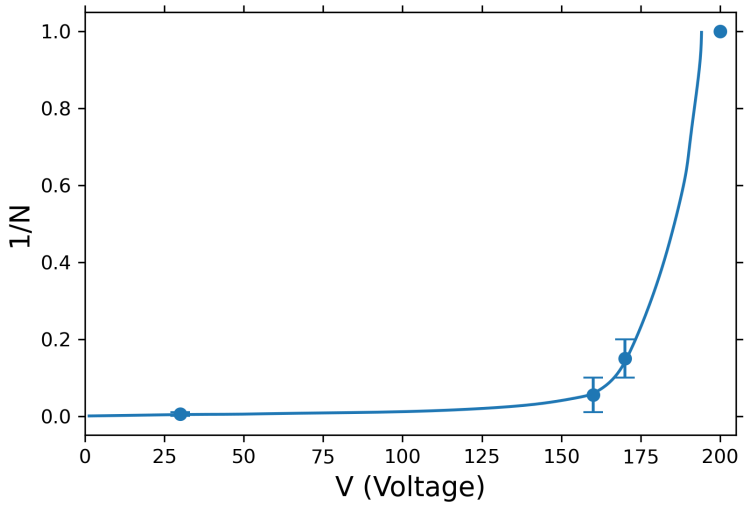
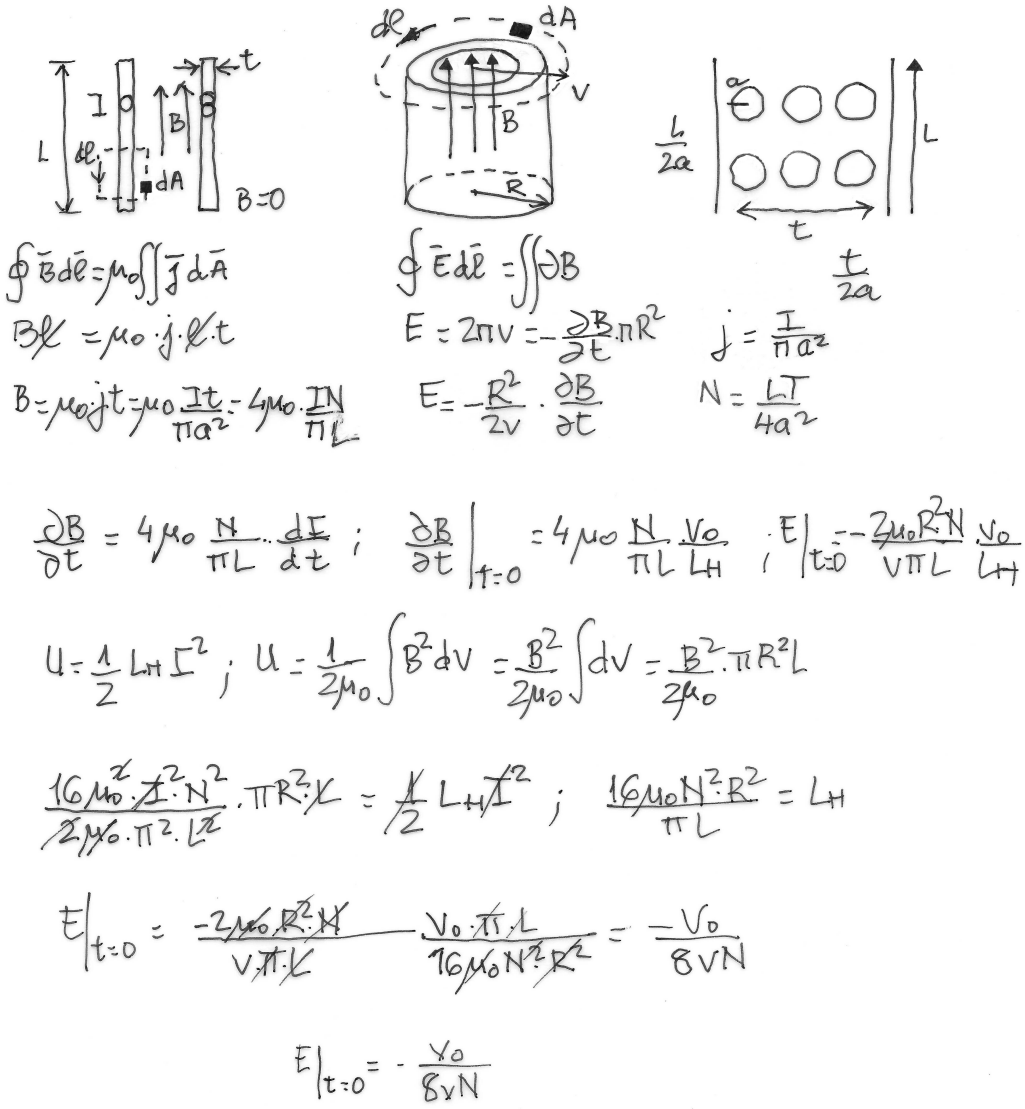


Figure 16: *Relation between discharge voltage and number of discharges necessary before failure. Voltages of 200 V caused immediate failure, and the number of repetitions required for failure significantly decreased at voltages above 160 V.*

B. Electromagnetic calculations for coil design



$$\oint \bar{B}\,d\bar{l} = \mu_0 \iint \bar{j}\,d\bar{A} \qquad \oint \bar{E}\,d\bar{l} = \iint \dot{B} \qquad j = \frac{I}{\pi a^2}$$

$$Bl = \mu_0 \cdot j \cdot l \cdot t \qquad E = 2\pi v = -\frac{\partial B}{\partial t}\pi R^2 \qquad N = \frac{LT}{4a^2}$$

$$B = \mu_0 j t = \mu_0 \frac{It}{\pi a^2} = 4\mu_0 \frac{IN}{\pi L} \qquad E = -\frac{R^2}{2v}\cdot\frac{\partial B}{\partial t}$$

$$\frac{\partial B}{\partial t} = 4\mu_0 \frac{N}{\pi L}\cdot\frac{dI}{dt}\,; \quad \left.\frac{\partial B}{\partial t}\right|_{t=0} = 4\mu_0 \frac{N}{\pi L}\frac{V_0}{L_H}\,; \quad \left.E\right|_{t=0} = -\frac{2\mu_0 R^2 N}{v\pi L}\frac{V_0}{L_H}$$

$$U = \frac{1}{2}L_H I^2\,; \quad U = \frac{1}{2\mu_0}\int B^2 dV = \frac{B^2}{2\mu_0}\int dV = \frac{B^2}{2\mu_0}\cdot\pi R^2 L$$

$$\frac{16\mu_0^2 \cdot I^2 \cdot N^2}{2\mu_0 \cdot \pi^2 \cdot L^2}\cdot\pi R^2 L = \frac{1}{2}L_H I^2\,; \quad \frac{16\mu_0 N^2 R^2}{\pi L} = L_H$$

$$\left.E\right|_{t=0} = \frac{-2\mu_0 R^2 N}{v\pi L}\cdot\frac{V_0\cdot\pi\cdot L}{16\mu_0 N^2 R^2} = -\frac{V_0}{8vN}$$

$$\left.E\right|_{t=0} = -\frac{V_0}{8vN}$$

Figure 17

C. Additional results

i. Effect of stimulation electrodes on AP amplitude

It was theorized that the observed difference in the MGF action potential amplitude for tactile and electrical stimulation might be caused by the insertion of the stimulating electrodes into the worm. These electrodes could potentially alter the conductivity of the inside of the worm, which directly affects the shape of the extracellular action potential [24]. Additional experimental data were analyzed to directly compare tactile stimulation with and without the placement of the stimulating electrodes. The peak-to-peak AP voltages can be found in Table 3. Based on these data, there is some indication that the presence of the stimulating electrodes increases the amplitude of the action potential in the LGF. The MGF amplitude, however, remained constant, perhaps due to the stimulation electrodes being placed between the tail and the recording electrodes, and therefore not interfering with the propagation of the MGF AP. Unfortunately, the experiment was terminated due to the increased levels of noise and movement artifacts before the stimulation electrodes could be placed between the head and the recording electrodes. As such, it is difficult to definitely conclude that the stimulation electrodes increase action potential amplitude. Further experiments need to be made to verify this hypothesis.

Fiber	Electrical (µV)	Tactile no electrodes (µV)	Tactile electrodes (µV)
LGF	66.9 ± 6.7 (13)	60.6 ± 7.1 (13)	69.4 ± 6.2 (8)
MGF	100.8 ± 7.3 (13)	88.0 ± 6.4 (10)	86.3 ± 2.5 (4)

Table 3: *Peak-to-peak AP voltages measured during electrical stimulation, tactile stimulation with no stimulating electrodes, and tactile stimulation with stimulating electrodes. Amplitude increases with stimulating electrodes in LGF but not in MGF.*

ii. Intensity threshold

The stimulation threshold at pulse duration of 100 µs was estimated by delivering ten pulses and keeping record of whether each pulse triggered an action potential so the probability of success could be calculated. The amplitude of the pulses was then changed, and the process repeated. The purpose of this experiment was to fix the duration of the stimulus so that the effect of the amplitude could be assessed. The results were fitted into a two parameter log logistic function with upper limit 1 and lower limit 0 [49], which was used to estimate the threshold voltage. This model is depicted in Figure 18, with an estimated threshold voltage of 0.96 ± 0.03 V. Minor variations in the input voltage led to significant changes in the likelihood of triggering an action potential. This highlights the importance of achieving sufficiently high stimulus strengths to successfully stimulate the neuron. From the electrostatic simulation of the electrode configuration (Figure 7), this voltage would result in an electric field of 134 ± 4 V/m, which is consistent with the 30 - 300 V/m range commonly reported in the literature [50, 51]. In the future, once magnetic stimulation can be reliably achieved, this approach can be used to study the relationship between stimulation intensity and probability. Comparing the electrical and magnetic thresholds in the same worm could provide new insights into the mechanisms of magnetic stimulation.

iii. Magnetic stimulation

Action potentials following magnetic stimulation were recorded in two instances, both in the same worm and with 30 seconds of difference between them. These two recordings are displayed in Figure 19.

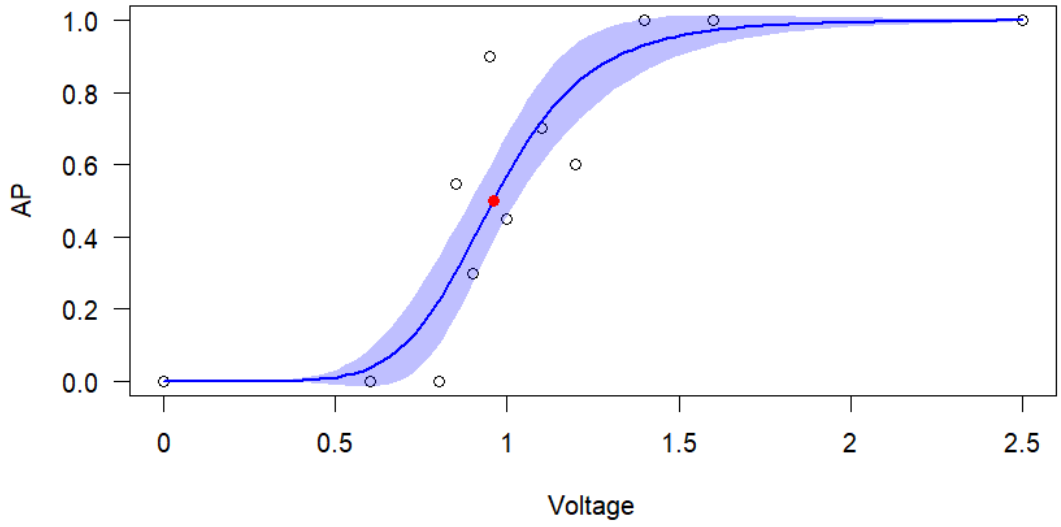


Figure 18: *Probability of triggering an action potential as a function of applied voltage fitted into a Log-logistic model and showing the 95 % confidence interval. The red dot indicates the estimated threshold (50 % chance of triggering an AP) at V = 0.96 V. Minor changes in input voltage lead to large variations in the probability of triggering an AP.*

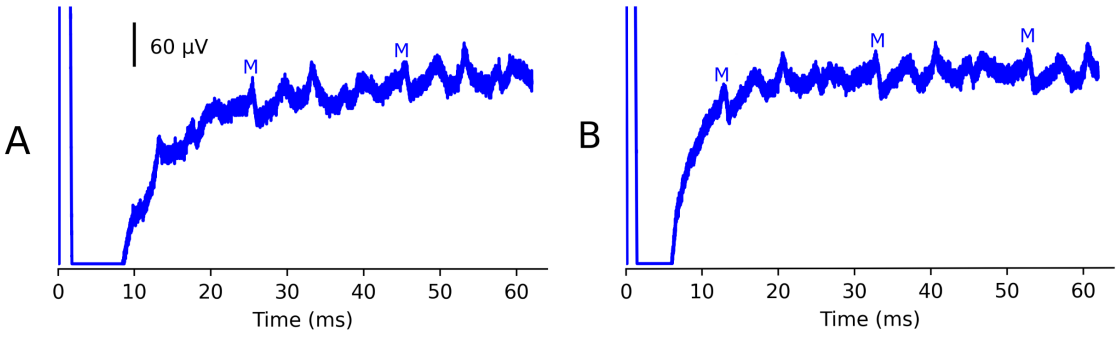


Figure 19: *Recordings of the ventral nerve cord following magnetic stimulation. M denotes action potentials from the MGF. Scale is shown in chart A.*

D. Magnetic stimulus

The estimation of the field generated parallel to the axon by a 30 V discharge in the stimulation circuit provided by Johan Meyer was used to determine the strength and duration of magnetic stimulation. The electric field was estimated from the measurement of the voltage across the capacitor over time. The original signal (Figure 20 orange trace) shows many peaks due to the finite difference method used to approximate the derivatives necessary to calculate the electric field. Consequently, the signal was smoothed with a second-order low-pass Butterworth filter with a cutoff frequency of 3 MHz. Since the same circuit was used to discharge the capacitor in both cases, the time constant of the capacitor remains the same. Therefore, the voltage over time in the high voltage case can be expressed as:

$$V_{\text{high}}(t) = \frac{160}{30} V_{\text{low}}(t) \tag{12}$$

Since the electric field was estimated using linear operators, the electric field for a 160 V discharge (Figure 20 blue trace) can be obtained by multiplying the electric field from a 30 V discharge by the quotient of 160 and 30. The resultant field can be divided in two pulses: a negative pulse with an amplitude of 2450 V/m and a duration of 1.4 µs, and a positive pulse with an amplitude of 875 V/m and a duration of 2.6 µs. In order to incorporate this stimulus into the strength-relation curve defining the stimulation threshold, the stimulus should be modeled so that it closely matches the square voltages pulses delivered during electrical stimulation, which were used to estimate the curve. Therefore, only the first pulse, where the peak field was reached, was considered when defining the magnetic stimulus. Although the second pulse could also play a minor role in stimulation, this possible loss is compensated by the fact that the first pulse is assumed to have an amplitude of 2450 V/m for 1.4 µs, overestimating its actual strength. This approach is also consistent with how magnetic stimulus length is typically defined in the literature [52, 53].

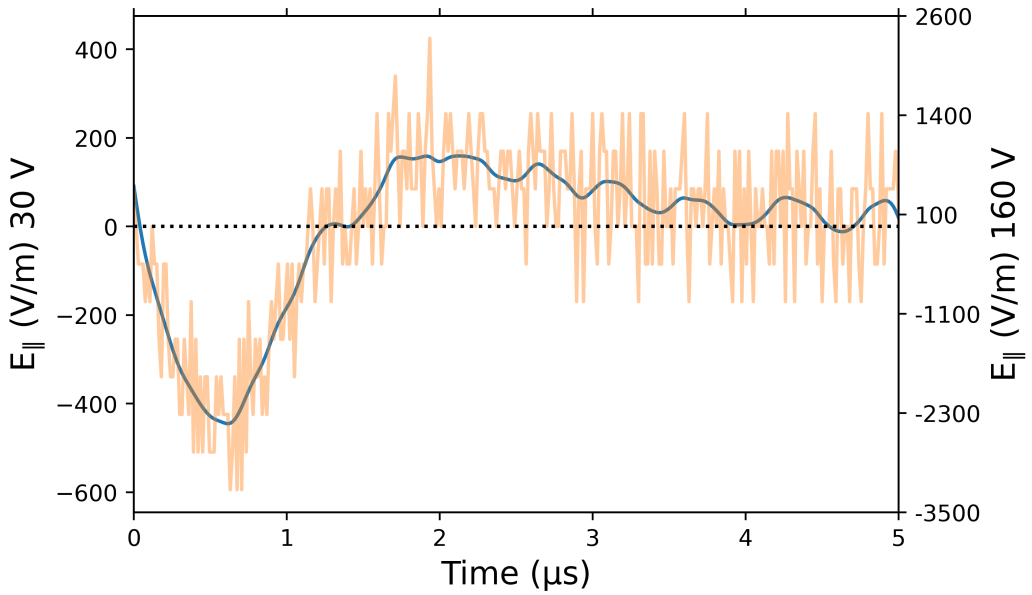


Figure 20: *Estimated induced electric field from magnetic stimulation in the direction parallel to the axon. The orange curve represents the original estimation, while the blue curve represents the filtered signal ($f_c = 3MHz$). The field was calculated following a 30 V discharge (left axis) and the right axis represents the re-scaling for a 160 V discharge, which was the voltage used in stimulation attempts.*

E. Set up

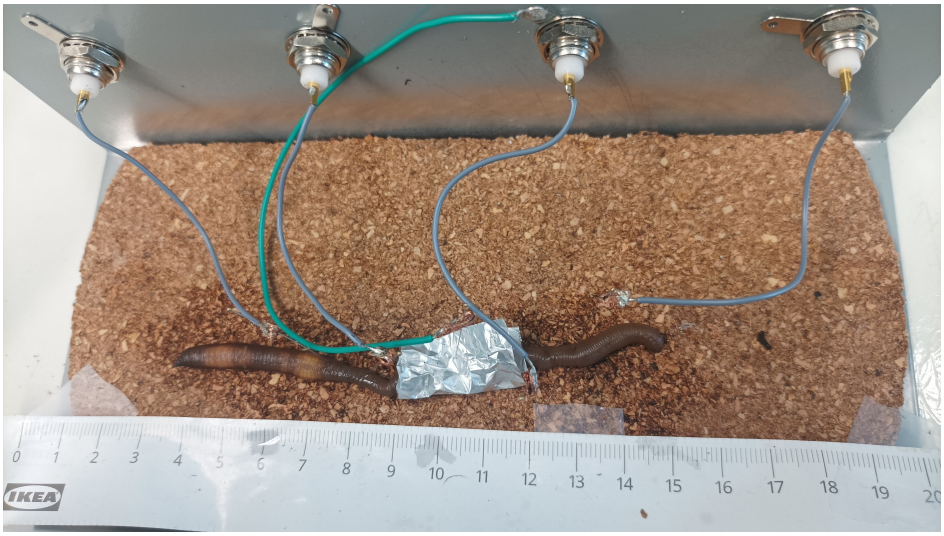


Figure 21: *Close up view of the worm in the Faraday cage before an experiment involving electrical and tactile stimulation.*

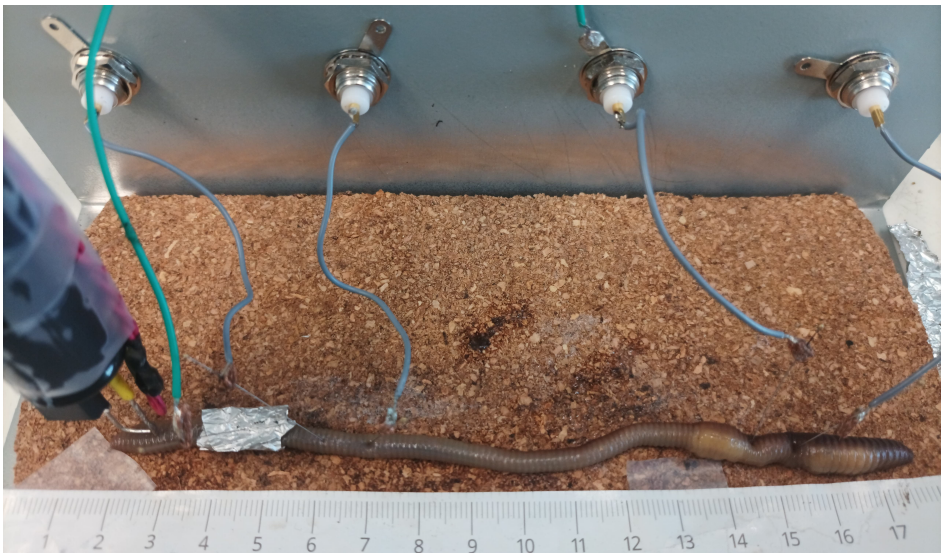


Figure 22: *Close up view of the worm in the Faraday cage before an experiment involving magnetic and tactile stimulation.*

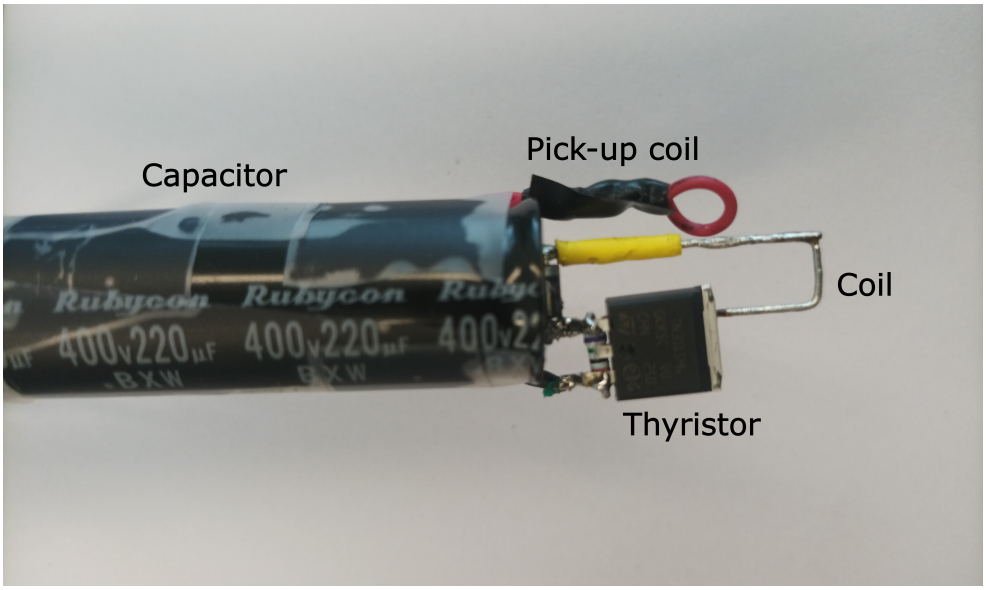


Figure 23: *Close up view of the coil and some parts of the circuit used to deliver magnetic stimulation. The pick up coil was not part of the stimulation circuit but was used for estimations of the electic field.*

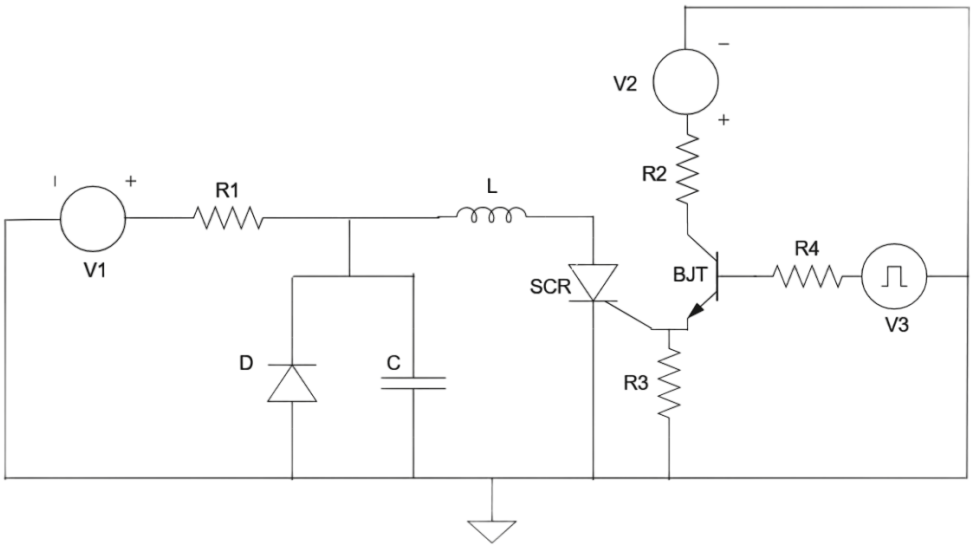


Figure 24: *Schematic of the circuit used to deliver magnetic stimulation.*

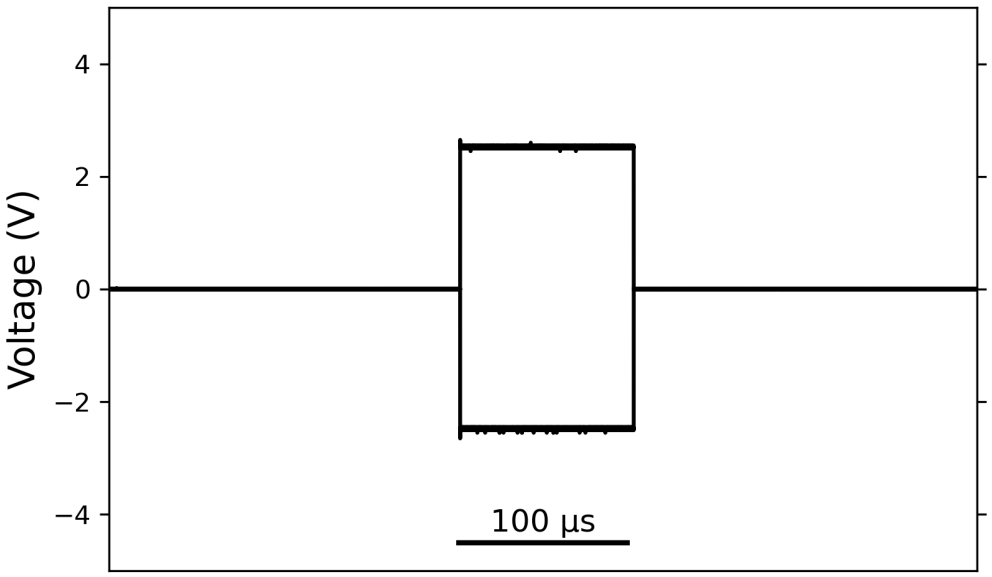


Figure 25: *Voltage pulse delivered during electrical stimulation.*

F. Literature review

The paragraphs describing the relevance of micro magnetic stimulation have been summarized, as they were already explained in the introduction of the thesis. No additional modifications were made.

Contents

Invertebrate animal models for magnetic stimulation research

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Delft University of Technology
July 14, 2024

I. Introduction

Micro magnetic stimulation has been proposed as a neuromodulation technique that could enhance the control of neuronal activation [1, 2], and improve the stability and biocompatibility of implants [3, 4]. Despite its potential, the development of micro magnetic stimulation is still in its early stages, and significant concerns about its safety and effectiveness need to be addressed before it can be tested on humans [3]. Furthermore, the underlying mechanisms by which magnetic stimulation affects neurons are not yet fully understood [5]. In this context, invertebrates and other lower animals are particularly valuable for early-stage research because they pose fewer ethical issues, have simpler physiological systems, and are more cost-effective to study [6, 7]. Mollusks such as Aplysia or Lymnaea have been extensively used in neurological research due to the large size of their neurons, well-defined behavioral responses, and characterized neuronal circuit [8, 9]. Furthermore, their relatively long lifespan makes them valuable for investigations in chronic disease research [10]. Likewise, insects are also attractive as model organisms for neuroscientific studies, given their small and simpler brains, notable stereotypy across individuals, and manifestation of a wide array of behaviors [11]. Leeches and other annelids have also undergone extensive examination as models in neuroscience research. Their rudimentary nervous systems, sparse coding, and simple behaviors render them appealing for investigating behavioral patterns in the context of neuronal activity [12]. Finally, while plants lack nervous systems, they utilize ionic mechanisms to generate and propagate action potentials in a manner highly reminiscent of neuronal processes [13]. These action potentials play a crucial role in triggering rapid plant movements, such as in Dionaea muscipula, and have been utilized in pioneering experiments testing the functionality of the first artificial organic neurons [14].

Invertebrates are therefore especially appealing for preliminary research as they serve as a low-cost, more ethical, experimental platforms in which the fundamental principles of physiology can be better understood. Every species offers its own benefits and limitations and is better suited for different contexts. Determining the most appropriate model for addressing the specific requirements of the research question plays a crucial role in the success of any investigation.

Therefore, the goal of this literature review is to identify the most adequate animal model for studying the principles underlying magnetic stimulation of neurons. This is done by reviewing existing literature on neural stimulation in annelids, insects, mollusks and plants with rapid movements. Criteria for what constitutes an ideal model are defined to serve as a framework for analysis of the literature.

II. Methods

i. Requirements

Our goal is to learn the fundamental biophysical principles of the magnetic stimulation of neurons. Accordingly, the animal model should provide a rudimentary nervous system in which local effects can be analyzed, providing strong and distinctive signals after stimulation. The impact of the orientation of the field is of particular interest as in future applications it would be difficult to guarantee optimal positioning of the coil after implantation [15]. Large nerve fibers in 1D arrangements are therefore preferred so this effect can be better studied. The neurons should also remain sensitive over an extended period to facilitate the study of multiple effects and their combinations within the same animal. Additionally, it is important to consider that we lack experience with dissection, voltage clamping, and other techniques essential for intracellular measurements. Therefore, to ensure that several subjects are used to obtain strong statistical significance, preparation for stimulation and recording should not require advanced skills or specialized equipment. Finally, the organism should require minimal maintenance as we lack infrastructure or expertise for animal care. Taking everything into account, the following requirements were established:

-The being should be maintained in care intervals longer than a week and without requiring specialized care knowledge or infrastructure.

-Preparation for stimulation and recording should only require standard electrical equipment and ordinary skills.

-Stimulation should trigger a response that can be measured clearly and distinctively.

-Nerves should have a sufficient size and a linear arrangement so the impact of orientation can be assessed.

-Experiment should be able to run for at least 2 hours after preparation.

The literature will serve as the basis for evaluating the degree of adherence of the four organisms to each criterion. A scoring system ranging from one to five was employed based on available evidence, with one indicating poor adherence and five signifying excellent adherence. Considering the aforementioned importance of spatial effects in the mechanisms of magnetic stimulation, this criterion was given a weight of 1.5. Maintenance has been assigned a weight of 0.5, while the remaining criteria have been kept at a weight of 1.

ii. Research methods

An extensive literature search was conducted on neural stimulation annelids, insects and mollusks. In the case of plants, the search was limited to the action potential of the Venus flytrap and the Shameplant, as their electrical networks are known to resemble animal nervous systems [16, 17]. The literature was retrieved using the web of science platform, which provides access to 8 different databases (Web of science Core Collection, Current Contents Connect, Derwen Innovations Index, Grants Index, KCI-Korean Journal Database, MEDLINE, ProQuest Dissertations & Theses Citation Index, and SciELO Citation Index). Four search queries were designed for each category as the characteristics of the literature for each one was diverse. For the animals, the choice of keywords was to cover research about large and common species in neuroscience research belonging to a certain taxon (“Annelid OR Earthworm OR Leech”) regarding neuronal stimulation (“Neuron” AND “Stimulation”). Considering that plants do not have nervous system, the term “neuron” was replaced by “Action Potential” in their search query. As the goal of the study was to identify the most suitable models for studying the fundamental neural effects of magnetic stimulation, the search was adapted to retrieve papers published during the early stages of neurological research in invertebrates. The timeframe was based on the citation report associated to each species, which delineate the annual count of publications and citations. For insects and mollusks, only articles published before or during 1980 were considered while for annelids the cutoff year was set in 1990. This decision was informed by the comparatively slower development of neurological research in annelids compared to the other two taxa. Further filters were applied to enhance the relevance and quality of the retrieved articles. Specifically, language was limited to English, the topic was confined to "Neurosciences Neurology", only affiliations with a minimum of 5 publications were considered, and papers with fewer than 10 citations were excluded from the analysis. No filters were applied for plants as the total literature available was very limited.

Table 1: *Filters applied to each search in Web of Science*

Search	Language	Topic	Publication years	Affiliations	Citations	Hits
Insect	English	Neuroscience Neurology	until 1980	>4 publications	>9	50
mollusk	English	Neuroscience Neurology	until 1980	>4 publications	>9	50
Annelid	English	Neuroscience Neurology	until 1990	>4 publications	>9	57
Plant	-	-	-	-	-	61
Educational	-	-	-	-	-	45

An additional query for retrieving literature regarding neurophysiology education with invertebrates was implemented, as experiments described in such papers often focus on the basics of neurology. In order to identify educational articles, the following keywords were used: ("Student" OR "Undergraduate" OR "Classroom") AND ("Neuroscience" OR "Neurophysiology" OR "Physiology") AND "Action Potential" in combination with the keywords used for the species in the previous 4 queries. No filters were used in this query. Details of the five searches can be found in Table 1.

Finally, more detailed information about the caring requirements of cockroaches and Venus flytraps was found performing a simple search in google.

The exclusion criteria for screening were established based on their capacity to provide information relevant to the requirements, which aim to provide a framework for addressing the research question. Articles unrelated or with incomplete information were excluded. Intricate experiments involving a wide array of interventions and equipment were also excluded, as their complexity is not ideal for studying the fundamental principles underlying phenomena like magnetic stimulation. Lastly, a preliminary evaluation of the retrieved literature during the screening determined that the species best meeting these requirements within each taxonomic group were cockroaches, sea slugs, earthworms, and Venus flytraps for insects, mollusks, annelids, and plants, respectively. With the purpose of keeping the review focused, only articles featuring these species were selected for analysis. Figure 1 displays a flowchart describing this process.

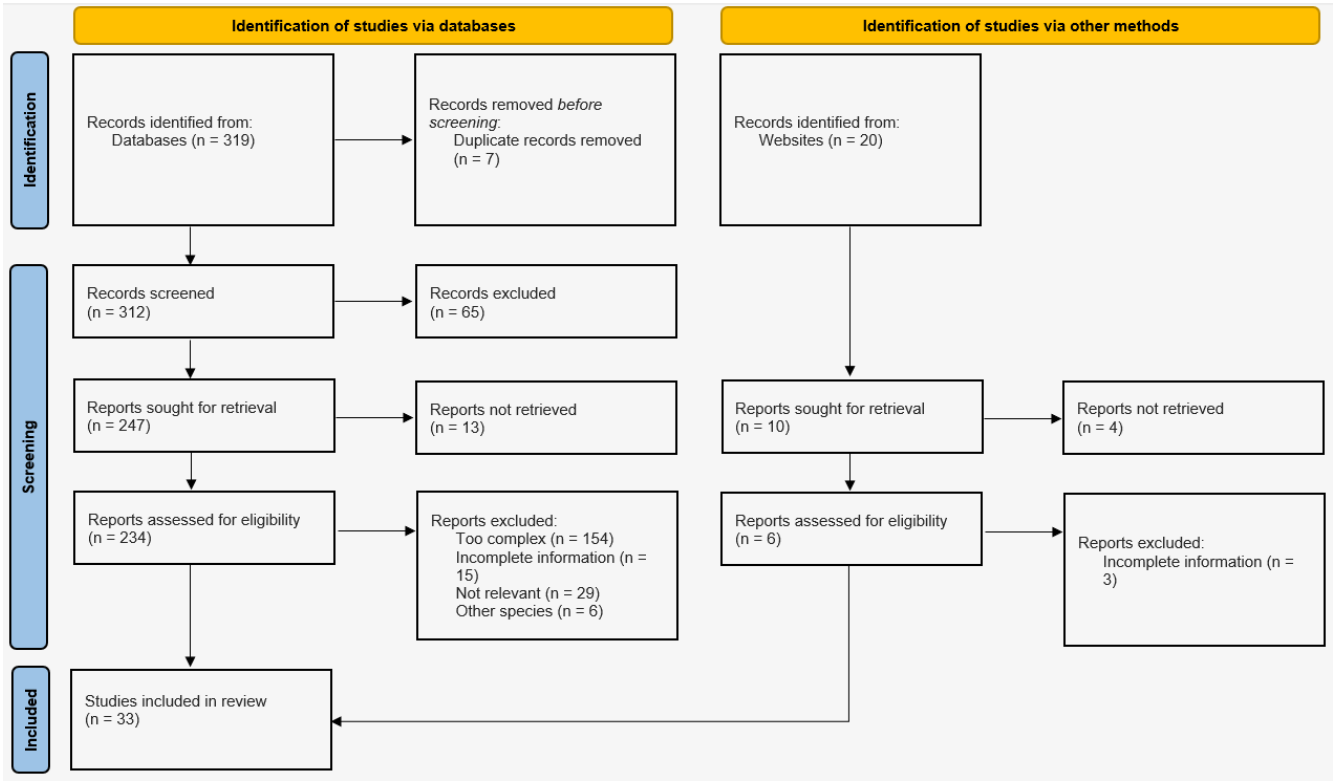

Figure 1: *Flowchart describing the methodology for article retrieval*

III. Evaluation of the literature

i. Maintenance

There are several considerations that should be evaluated when assessing the maintenance requirements of a living being. These are housing, up-keeping of the housing, and required food. "Housing" refers to the space and environmental conditions that the organism needs to survive and thrive. "Upkeep" denotes the interventions necessary to sustain optimal living conditions and the frequency at which they should be undertaken. Lastly, "required food" encompasses the type and frequency of feeding regimen required by the organism. Through these considerations, the complexity and interval of care was delineated, providing a framework for ranking organisms in terms of their maintenance needs.

i.1 Cockroach

Cockroaches can be housed in plastic or glass containers with access to water and food, typically comprising rodent chow, pet food, or fruit [18, 19, 20]. While fresh food isn't required daily, insufficient food availability may trigger cannibalistic behaviours among cockroaches [20]. It's also important to remove spoiled food and dirty water to prevent the proliferation of flies and the accumulation of toxic fermentation gases [21]. Supervision intervals of the order of one or two weeks would therefore be recommended.

i.2 Sea slug

Regarding sea slugs, they require large tanks filled with artificial sea water under controlled conditions to replicate their natural habitat [22, 23]. Therefore, the typical maintenance demands of a saltwater aquarium can be expected. Concerning alimentation, some researchers fed slugs of the species *Pleurobranchaea californica* on raw squid in a weekly basis [23, 24]. Conversely, species of the genus *Aplysia* were fed on dried seaweed in various experiments [25, 26], but the frequency of feeding was not stated. In any case, regular aquarium maintenance, including cleaning and monitoring, should be conducted at least weekly to ensure optimal conditions for sea slug health and habitat stability.

i.3 Earthworm

Earthworm colonies can be sustained in containers with soil bedding in temperature and moisture controlled environments [27, 28, 29]. Feeding involves enriching the soil with manure or debris. Notably, earthworms of the species *Lumbricus terrestris* can survive in these containers for over a month at low temperatures without maintenance [30, 31]. If maintaining a long-term colony is desirable, regular maintenance should occur approximately weekly. However, if resupplying is not a concern, these animals can be maintained with caring intervals on the order of a month.

i.4 Venus Flytrap

Venus flytraps require prolonged exposure to sunlight and contact with distilled water in order to grow and thrive [32, 33, 34]. The international carnivorous Plant society (ICPS) recommends that the plant should always be in contact with distilled water [35]. The ICPS also states that the Venus flytrap needs to consume live prey regularly for optimal health, but some research groups grow these plants without any feeding [36, 37]. As the velocity at which the soil dries out depends on several factors, and no specific watering intervals were found in the literature, determining the precise frequency of care proves challenging. Nevertheless, it is presumed to range approximately every one to two weeks for optimal care.

ii. Set-up

This criterion was evaluated based on two pivotal aspects. Firstly, the complexity of organism preparation was subjected to scrutiny, encompassing considerations such as the temporal demands, requisite expertise levels, and the array of materials and equipment essential for execution. This examination provided insights into the logistical challenges and

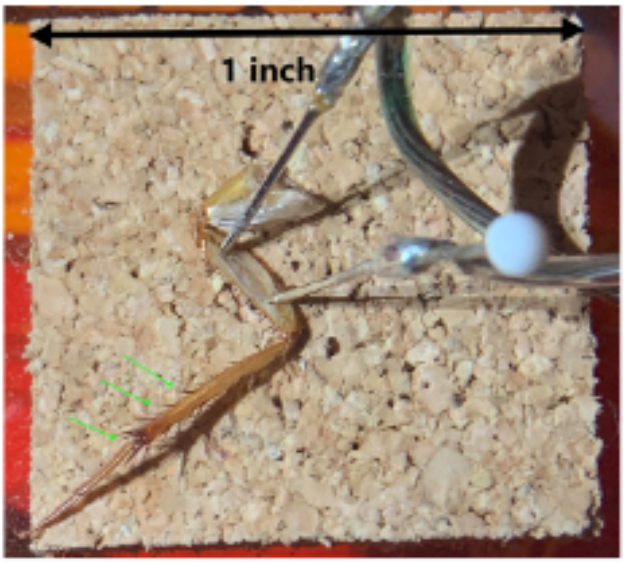

Figure 2: *Cockroach leg preparation for recording [40]*

resource requirements associated with the preparatory phase. Secondly, attention was directed towards the recording instruments, where several parameters were assessed. These included the total number of instruments required, their accessibility, and the degree of technical intricacy involved in their operation. This emphasis on instrument selection underscores the significance of reliability and ease of use in facilitating data acquisition.

ii.1 Cockroach

The methods required to experiment in cockroach preparations are complicated, often involving dissections even for extracellular recordings [38]. N. Orida and R. K. Josephson [39], however, report an intact preparation wherein fine copper wires mounted in a polyethylene cuff are used to record extracellular signals from the ventral nerve cord of freely walking insects. The nerve was exposed by making an incision in the abdominal sternites applying CO_2 anesthesia. The nerve cord segment situated amidst the fifth and sixth abdominal ganglia was then encased within the cuff, securely fastened around the nerve cord by melding the cuff edges using a heated probe. Subsequently, the sternites were repositioned, and the abdomen was sealed closed using wax. Signals were then acquired with an amplifier, oscilloscope, and threshold discriminator. Even though the surgical intervention is limited in time, executing it without previous experience would be challenging. Furthermore, this preparation demands the use of cuff electrodes, which are not standard electronic equipment.

Experimenting in the isolated leg of the cockroach offers an alternative to address these issues. The leg can be amputated with small scissors and cooling in a refrigerator or CO_2 can be used as an anesthetic [18, 19, 40]. Preferably, the severed limb should be the metathoracic leg as it is the largest and therefore easier to handle [18]. The limb is then placed on top of a cork and action potentials can be acquired by inserting two electrodes in the femur [40] or in the coxa and the femur [18], see Figure 2. The necessary equipment consists of an oscilloscope, amplifiers, and filters. The use of a Faraday cage is recommended to further reduce electromagnetic noise [18, 40]. Neural signals can therefore be recorded with a simple preparation and using only standard electrical instruments, although it is important to note that the electrodes should be both thin and stiff to effectively penetrate the limb.

ii.2 Sea Slug

Preparation for recording action potentials in sea slugs is not straightforward as it requires long invasive interventions. Preparations described in literature can be classified in three groups: Dissection, electrodes implantation, and live brain recording.

Dissections allow the study of concrete neural pathways in isolation and involve very complex procedures. For example J. Byrne et al. studied the properties of the neurons innervating the siphon skin and mantle shelf in *Aplysia Californica* [41]. The sea slug dissection for this experiment involved securing the relaxed animal to a wax tray with pins, making incisions along its left side to expose internal organs like the gill, purple gland, and mantle shelf. These were carefully dissected, with the skin section containing the abdominal ganglion transferred to a Lucite chamber for experimentation. The ganglion was pinned for visibility, and the sheath covering mechanoreceptor neurons was removed. Finally the chamber was perfused with Instant Ocean solution, maintaining a constant temperature for experimentation. Regarding equipment, single-barreled microelectrodes filled with potassium citrate were used for measuring, and film and FM tape with a four channel recorder for recording. This method therefore necessitates the use of non-standard instruments.

The nervous system of intact sea slugs can be studied by implanting electrodes in the giant nerves of these animals after anesthetizing them by the injection of isotonic $MgCl_2$. The surgery involves longitudinal incisions that expose the ganglia, allowing the implantation of double cuff electrodes in the area of interest. The incision is then sutured and the animals are left 24 hours to recover. Necessary electronic equipment includes the already mentioned double cuff electrodes, filters, amplifiers, and FM tape recorder. [25, 26]. J. S. Cobbs and H. M. Pinkser [26] reported that 84 % of specimens had usable records after implantation, which implies that experience with the surgical procedure is crucial for the success of the experiment.

The relation between behaviors and neural signals can be analyzed by performing in vivo brain recordings [22, 23, 42]. The required procedure is described by M. P. Kovac and W. J. Davis [23]: A dorsal incision, 1 cm to 2 cm long, is made over the cerebropleural ganglion, the sea slug's brain. Fishhooks and silk threads are used to keep the incision open, revealing the brain and immobilizing the specimen for the experimentation. The brain is then pinned to a wax-covered platform, exposing its nerve roots for electrode attachment. Suction electrodes can be used to stimulate and record extracellularly [23], but intracellular recordings with KCl filled glass capillary electrodes are more prominent in these preparations [22, 42]. Nevertheless, none of these electrodes can be defined as standard electronic instruments. Additional necessary equipment included amplifiers and oscilloscopes [22, 23].

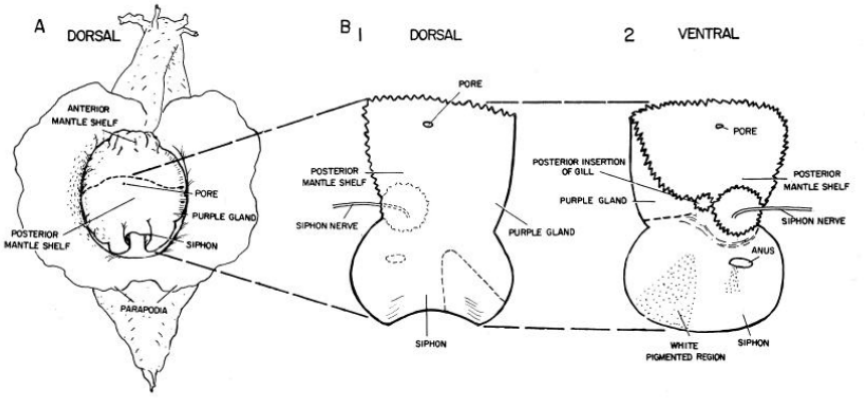

Figure 3: *Sea slug dissection [41]*

ii.3 Earthworm

Recording extracellular signals in earthworms, however, can be done with minimal preparation. Researchers from Iowa state university measured the conduction velocity of the axons of freely moving earthworms placed over an electrode grid [28, 29, 43]. Following tactile stimulation, the transmitted action potential was detected by amplifying and filtering the acquired signal. The distance of the electrode pairs on the grids varied according to the size of the worm. These same researchers proposed an alternative set up, consisting of two concentric vinyl rings with 40 pairs of silver wire recording electrodes [44]. Although these preparations do not require any surgery or dissection, it is necessary to design and assembly specific recording equipment. More recently, Kyle M. Shannon et al. [31] described an experimental set up that allows the measurement of axonal conduction velocity with standard instruments. Earthworms were anesthetized by a 5 minute immersion in 10 % ethanol and placed over a Styrofoam board inside a Faraday cage. Two recording electrodes and a ground electrode were inserted into the worm and connected to a custom 880 gain amplifier with a band-pass filter from 300 to 1300 Hz. Finally, after tactile stimulation, the signals were measured with the audio program Audacity in a laptop. This set up offers the possibility of recording action potentials with more typical instruments while maintaining a straightforward animal preparation. Robert Bäring and Christiane K. Bauer [30] describe a similar experiment wherein the earthworm is immobilized in a custom restraining chamber rather than with anesthesia. The chamber limits the type of stimulation that can be delivered, as the animal becomes less accessible, and needs to be assembled. However, it offers a more consistent way of restraining the animal compared to anesthesia.

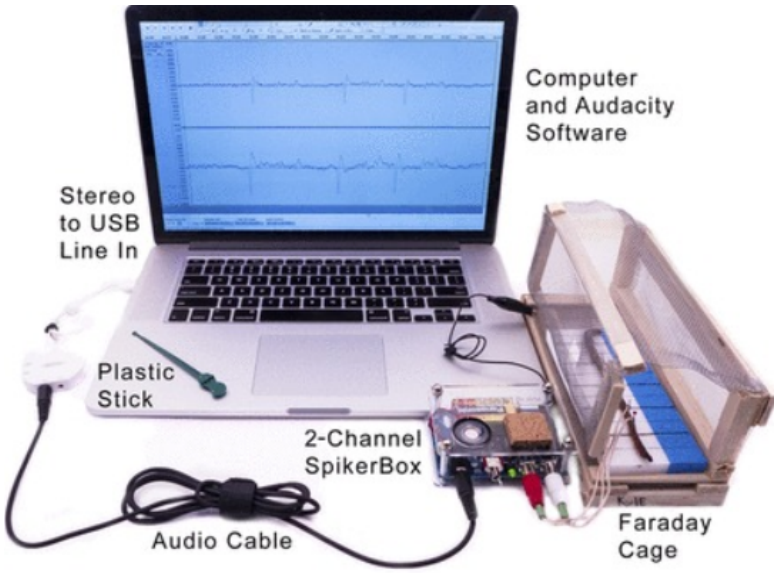

Figure 4: *Earthworm experiment set up [31]*

ii.4 Venus Flytrap

Actions potentials can be measured in Venus flytraps following a procedure similar to that of electrocardiograms. The static nature of plants facilitates the manipulation of the organism when preparing the experiment, and signals can be detected by fixing surface electrodes soaked in a conductive solution in contact with the leaf [32, 34, 45]. A supporting platform may be necessary to guarantee the stability of the electrodes [34, 45], especially if the trap is to be closed during the experiment. Alternatively, recording from a lower leaf near the soil could mitigate this concern. Signal acquisition methods vary, but some authors have reported favorable results employing only an amplifier and an analog-to-digital converter [45, 34]. Additionally, employing a Faraday cage is common practice across experiments to mitigate electromagnetic interference [32, 33, 34, 46, 47].

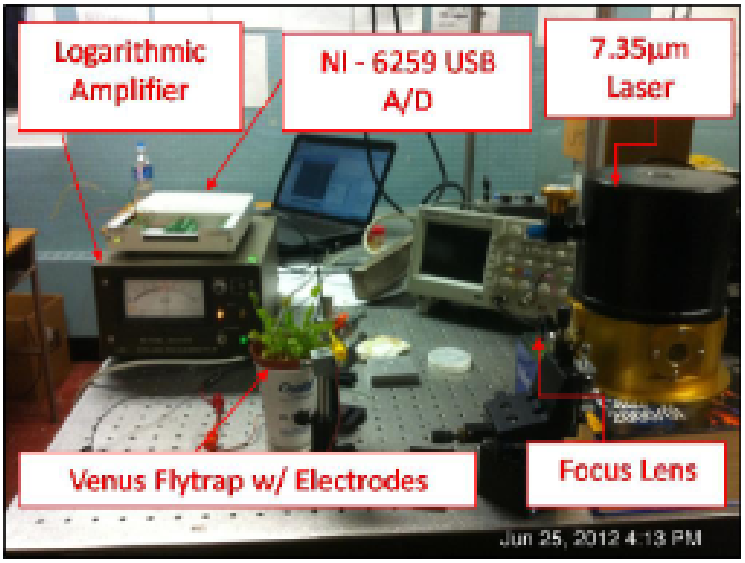

Figure 5: *Venus flytrap experiment set up [45]*

iii. Signal

This criterion was evaluated in terms of how easy was to distinguish individual, triggered, action potentials in the recordings. The assessment process involved an examination along two primary axes. Firstly, attention was dedicated to appraising the levels of noise present, with a focus on those potentially emanating from stimulation artifacts, that for magnetic stimulation are expected to be large. Secondly, the reliability of stimulus-response relationships was evaluated by assessing the robustness of the of the correlation observed between the stimulus and the subsequent generation of action potentials.

iii.1 Cockroach

The cockroach leg serves as an excellent platform for measuring extracellular action potentials, a fact supported by its extensive recommendation for high school and college students in experiments [18, 19, 40]. In these experiments, the primary stimulation method consist of mechanically triggering the spines in the leg of the insect, each of them associated with a single sensory axon [19, 40]. However, employing magnetic stimulation poses a potential challenge for this setup. The small size of the cockroach leg, around 2 cm as seen in Figure 2, would difficult the separation of the recording electrodes from the electric field induced by the coil. Action potentials in cockroach legs are of the order of 1 millisecond [18, 40] and therefore very susceptible to stimulation artifacts. While extending the leg may seem like a feasible solution, altering the angle between the leg and the tibia triggers action potentials [18] thereby complicating the establishment of a correlation between the magnetic pulse and the neural response. Remarkably, Raddy L. Ramos et al. [18], applied electrical stimulation to the leg and reported satisfactory results concerning noise levels and action potential measurement. Their stimulation protocol consisted of a single current pulse of 1 mA with a duration of 250 µs. As it can be seen in Figure 6, the magnitude of the stimulation artifact resembled that of the triggered action potentials, which in combination with the short duration of the pulse enabled the recording of the neural signal with minimal interference of noise. It is unclear, however, if the parameters necessary for magnetic stimulation can be adjusted to comply with these conditions. Raddy L. Ramos et al. did not provide recordings following stronger and longer pulses making it challenging to estimate the potential impact of larger stimulation artifacts. Another limitation of the cockroach model regarding magnetic stimulation lies in its abundant spontaneous activity in the leg [18]. While this activity is useful for oscilloscope calibration, it may hinder the establishment of a robust stimulus-response relationship, particularly under near-threshold conditions where spontaneous activity might be mistaken for evoked potentials. Finally, the high

neuronal density inside the leg may difficult the identification of single action potentials [40].

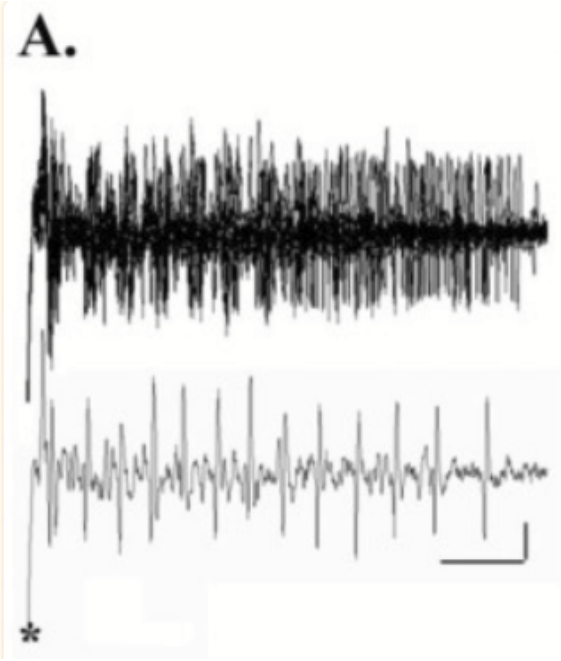

Figure 6: *Electrical stimulation of sensory axons in cockroach leg. Asterisk denotes offset of stimulation. Top is the overlay of 20 traces [18]*

iii.2 Sea slug

Sea slug giant neurons enable the recording of action potentials from specific neurons with a precision difficult to achieve in other species [26, 48, 49, 41]. This capability is demonstrated in Figure 7 which presents recordings from six distinct brain neurons following the elicitation of swimming behavior in the slug. It is important emphasize that these recordings are intracellular, necessitating more complex instruments and protocols, as discussed previously. Typically, the nervous system of these animals is stimulated through mechanical [26, 41] or electrical [25, 48] means. With regard to magnetic simulation, the substantial size of the animals allows for a significant distance between the recording electrodes and the stimulation coil. Figure 8 illustrates extracellular recordings in two neurons (R1 and R2) from the abdominal ganglion of *Aplysia Californica* following mechanical stimulation at different anatomical regions [26]. No stimulus artifacts were recorded at any of the stimulation sites; however, this does not guarantee that magnetic stimulation would not induce such artifacts. Unsurprisingly, when electrical stimulation is delivered close to the recording site, stimulus artifacts become apparent [48]. Nevertheless, it is noteworthy that signals could be registered from stimuli in widely different places, indicating that the coil could be placed very far from the electrode and still trigger signals in the target neurons. This capability may potentially reduce the noise from the stimulus. Spontaneous activity in the sea slug nervous system varies greatly depending on which neurons are being recorded. J. S. Cobbs and H. M. Pinkser [26]reported that spontaneous firing in R1, R2, and L1 was limited to when the animal crawled and was mostly silent otherwise. This finding would facilitate the establishment of a strong stimulus-response correlation. This quiescent neuronal activity can be appreciated in Figure 8. However, recordings in the circumesophageal ganglia [25], contained significantly more spontaneous activity making it more difficult to distinguish individual action potentials.

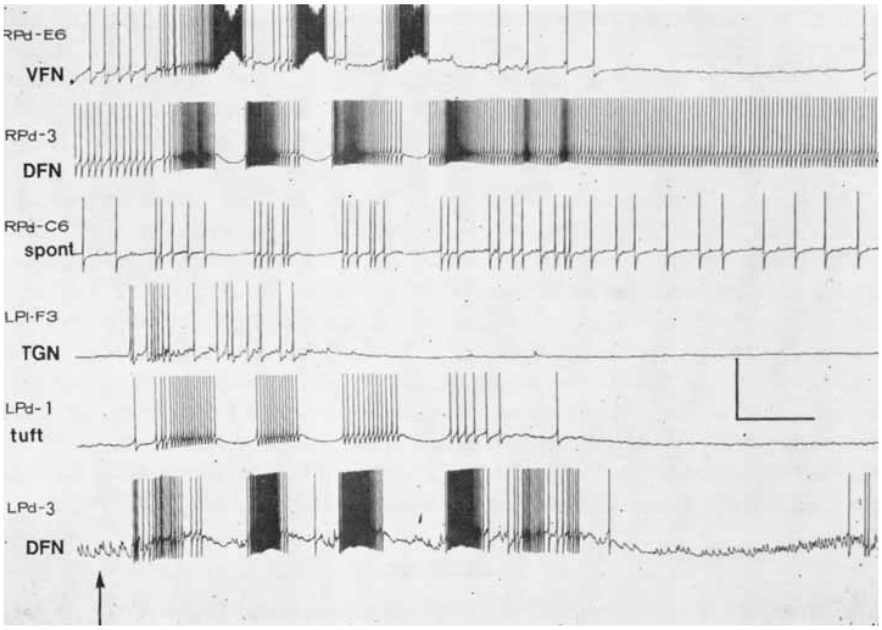

Figure 7: *Signal recordings from 6 cells in the sea slug brain following escape-swimming elicited by a drop of concentrated salt solution applied to the oral veil [49]*

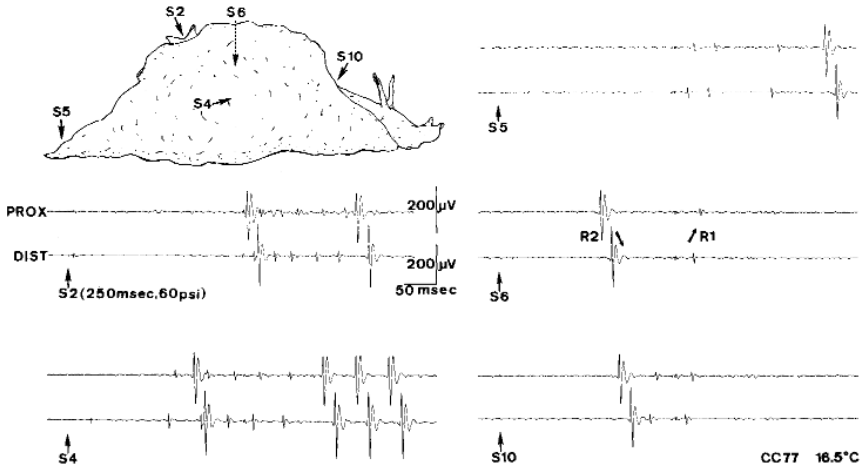

Figure 8: *In vivo responses in R1 and R2 neurons in* Aplysia*. Large spikes corresponded to signals from R2, which traveled from proximal to distal regions, while the small spikes were triggered by R1 and conducted in the opposite direction [26].*

iii.3 Earthworm

In earthworms extracellular neural signals can be acquired straightforwardly by simply placing the animal over electrodes. Tactile or electrical stimulation is usually delivered on the head or tail, while the recording electrodes can be positioned anywhere along the length of the earthworm [31, 29, 44]. In the context of magnetic stimulation there is therefore potential for separating the electromagnetic coil from the electrodes. Figure 9 displays signal recordings in awake earthworms following a 1.2 V stimulus pulse with a duration of 0.1 ms [30]. As can be observed, the noise

generated by the applied voltage does not interfere with the triggered action potential. As previously stated, whether magnetic stimulation would elicit stimulus artifacts of the same nature is unknown. However, in this setup, the stimulating electrodes were placed in the first third of the worm, while the first pair of recording electrodes were positioned in the middle. Thus, there is ample room for separation in the event that the noise produced by magnetic stimulation is too high. Finally, Kyle M. Shannon et al. reported that spontaneous activity in the worm was very low [31], which helps establishing a strong relation between stimulus and triggered signal. It is noteworthy that this may also complicate debugging, as it may be difficult to determine whether the absence of action potentials is caused by the worm or by the recording equipment.

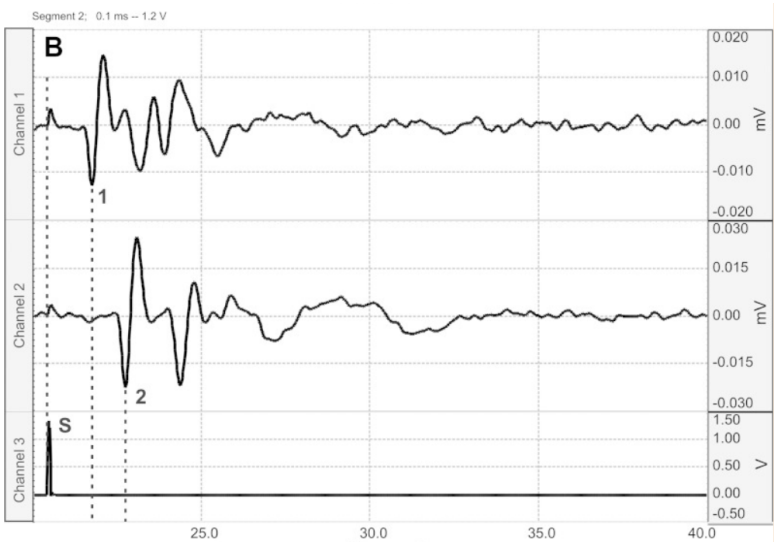

Figure 9: *Differential recordings in two location in an earthworm following a 1.2 V stimulus pulse [30]*

iii.4 Venus Flytrap

The physiology of the Venus flytrap offers excellent advantages for acquiring strong and distinct signals. Its action potential has a duration of the order of seconds [45, 36, 32, 34], although the precise duration appears to vary depending on electrode placement and sampling protocol, and conflicting literature does exists [47]. As depicted in figure Z, the large duration of this action potential greatly facilitates its identification. This characteristic proves highly beneficial for experiments involving magnetic stimulation, as the electromagnetic pulse generated would be orders of magnitude shorter than the action potential. Consequently, spatial separation between recording electrodes and the stimulation site would not be essential, affording greater flexibility in the setup. Additionally, high-frequency signals could be safely filtered out, thereby enhancing the recording quality. Moreover, spontaneous activity in the Venus flytrap is virtually non-existent, as the action potential is only triggered under specific conditions, primarily prey capture and wounding [34]. Consequently, every recorded action potential could be reliably correlated with the applied stimulus. An important consideration is that it is crucial to avoid closing the trap, as doing so would hinder the stimulation of the trigger hairs. Therefore, stimulation should be administered at intervals of at least 30 seconds to ensure that the trap remains open [36]. Lastly, it is important to note that the excitatory cells of the Venus flytrap are not neurons; hence, their electrochemical properties differ and may not accurately represent neuronal responses to magnetic stimulation.

iv. Orientation

In evaluating suitability for exploring the spatial effects of magnetic stimulation, the nervous system characteristics play a pivotal role. This criterion was consequently assessed based on three of these characteristics. Firstly, nerve size was considered, with a preference for larger nerves as they facilitate alignment of the electric field along the axons.

Secondly, the spatial disposition of nerves was examined, favoring simple linear arrays for optimal alignment of the electric field. Additionally, the complexity of the nervous system was evaluated, with a preference for low nerve density to ease neural identification and determine the nerves being stimulated accurately.

iv.1 Cockroach

The anatomy and physiology of the leg of the cockroach may present obstacles in the evaluation of the field orientation effects. Firstly, the leg's dimensions, approximately 2 cm in length as illustrated in Figure 2 would present challenges in aligning the induced field along the axons of the nerves. This would be further complicated by the fact that the leg follows a zig-zag structure and cannot be stretched without stimulating sensory neurons and thus altering the natural firing pattern [18]. Finally, the cockroach leg exhibits a notably high neuronal density, encompassing sensory neurons in the hair cells, sensory neurons encoding the angle between the tibia and femur, and motor neurons regulating locomotion [18, 19]. Consequently, discerning the specific cells affected by the electric field may become a difficult venture.

iv.2 Sea slug

Sea slugs, in contrast, offer a different scenario. The giant neurons contained in their nervous system would greatly facilitate the alignment of an electric field along a linear section of the axon. Moreover, their nerves follow a straightforward arrangement, as depicted in Figure 10, directly connecting the various ganglia. This organized neural structure further enhances the ease of alignment. Finally, these animals have a very low neuronal density, thanks to their relatively low number of neurons [41]. Identifying the targeted neuron would therefore be feasible, enabling the establishment of a direct relation between stimulation and field angle.

iv.3 Earthworm

The anatomy and physiology of the nervous system in earthworms offer opportunities similar to those observed in sea slugs. Although smaller in size, earthworms possess two large nerve systems running along their body: the median giant fiber (MGF) and the lateral giant fiber (LGF) [27, 30, 31]. The substantial size and linear orientation of these nerves render them excellent targets for investigating the spatial effects of magnetic orientation as the angle between axons and electric field could be easily estimated by the angle between the coil and the large axis of the animal (see Figure 11). Additionally, earthworms exhibit low neuronal density which combined with the myelination and large size of the MGF and LGF increases the likelihood of stimulating these two nerve networks and facilitates neural identification [31]. Differentiating between the LGF and the MGF could present challenges as they very close together and follow a similar arrangement. Fortunately, the excitation threshold of MGF is lower than that of LGF so at low intensities isolating MGF would be possible [30].

iv.4 Venus Flytrap

In the case of Venus flytraps, there are several notable considerations in the context of exploring the spatial effects of magnetic stimulation. The small size of its leaves would present a practical challenge, potentially impeding the precise alignment of the magnetic field with the excitatory structures. Furthermore, Venus flytraps do not have a specialized excitable tissue like animals [47] and therefore the stimulated cells may vary in shape and are probably anatomically dissimilar to neurons. This lack of anatomical clarity could hinder the establishment of a precise spatial relation between the applied magnetic field and the target cells, which is critical for the evaluation of the impact of orientation. Finally, the complexity of the excitatory network within the Venus flytrap remains largely unexplored [32], rendering the identification of specific stimulated cells a challenging endeavor.

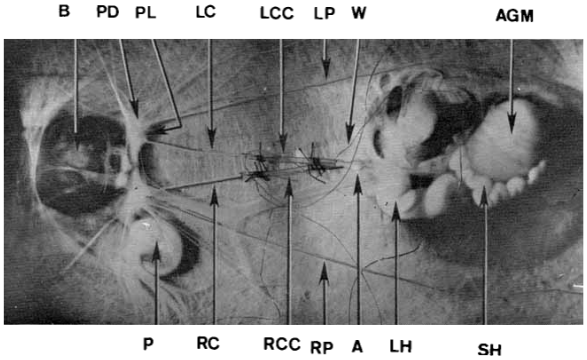

Figure 10: *Nervous system of* Aplysia Californica *A = Abdominal Ganglion; AGM = Accessory Genital Mass; B = Buccal Mass; LH = Large Hermaphroditic Duct; LP = Left Posterior Pedal Nerve; P = Penis; PD = Pedal Ganglion; PL = Pleural Ganglion; RP = Right Posterior Pedal Nerve; SH = Small Hermaphroditic Duct. [26]*

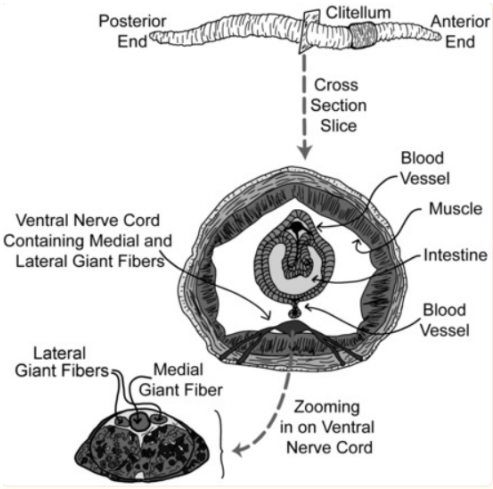

Figure 11: *Overview of earthworm anatomy with cross-section[31]*

v. Time

This criterion was evaluated based on two conditions. Firstly, the maximum viable experimental time, which is defined as the duration between the preparation of the experiment and the point at which action potentials cannot be reliably recorded. Secondly, the stability of the preparation, defined as the additional interventions required to maintain the experiment for as long as possible.

v.1 Cockroach

The cockroach leg provides a stable experimental platform in which neural activity can be recorded for over 12 hours after detachment [18]. Neurons survive in standard environmental conditions and since the leg does not have autonomous movement no restraint is required.

v.2 Sea slug

Sea slug experiments require the maintenance of sea water-like environmental conditions for the stability of the preparation, whether with the intact animal or dissected tissue. Typically, this involves continuous perfusion of the

chamber containing the sample while maintaining a constant temperature [41, 26, 42]. Depending on the experiment type, several additional considerations can be used to evaluate preparation stability. Live brain recordings require slug immobilization using hooks and threads [42]. Although specific durations were not reported, an animal with an open brain wound would likely be unstable and difficult to maintain for extended periods of time. Feasible experimental durations were also not reported in the experiment involving dissection [41], although they are unlikely to exceed the 12 hours reported by Raddy L. Ramos et al. [18] for the cockroach leg, given the larger scale of this dissection. Finally, in studies involving electrode implantation, action potentials could be measured for time periods of the order of days until the animal died or its health deteriorated so much that recordings become unusable [26]. Therefore, if recordings are made shortly after surgery, extended experiment durations are plausible.

v.3 Earthworm

Experimenting on intact earthworms for extended periods of time presents several challenges. The primary consideration is that the animal needs to be restrained in order to avoid artifacts from muscle contraction. Neuronal activity can be measured in intact, unrestrained earthworms [28, 29, 44]. However, the noise generated by muscle contraction has a larger amplitude than the action potentials and is originated just a few milliseconds later, as showed in Figure 12. Consequently, artifacts of muscle origin could easily override the action potentials. Anesthetizing the worms is a possible solution to address this issue. Earthworms under mild anesthesia do not exhibit muscle contractions while maintaining neural excitability. Nevertheless, the effects of the anesthesia only last for between 5 and 10 minutes, and although a longer exposure to the narcotic could prolong muscular immobilization, neural excitability would also decrease [31]. These concerns may be mitigated by restraining an awake worm in a chamber so that the animal is unable to move [30]. As it can be seen in Figure 9 neural activity can be observed with minimal noise interference by employing this set up. Such a preparation has its own disadvantages, as discussed previously, but could potentially extend the experimental time from minutes to an hour as the worm still requires frequent moisturizing [31].

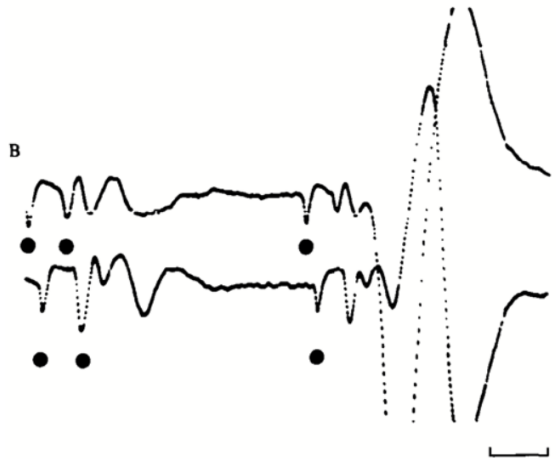

Figure 12: *Recording from freely moving earthworm. Dots are placed under action potentials while the larger wave is originated by muscle contractions. The time scale is 2ms.[44]*

v.4 Venus Flytrap

Action potentials can be reliably triggered in Venus Flytraps for hours [34, 50]. Restraint is no needed as the plant does not move other than to close its trap, which can be avoided by separating stimulation impulses 30 seconds between each other. As the intact organism is used, action potentials can be recorded under standard environmental conditions, although it is important to note that temperatures over 30 ºC increase trap sensitivity and could lead to unintended trap closure [36].

Table 2: *Table displaying the compliance scores of each organism with respect to individual requirements, with the weight of each requirement indicated in brackets. A score of 1 denotes poor compliance, while a score of 5 signifies excellent compliance.*

Weight	Maintenance (0.5)	Setup (1)	Signal (1)	Orientation (1.5)	Time (1)	Total
Earthworm	5	5	5	5	1	21
Cockroach	4	5	2	2	5	17
Sea slug	1	1	5	5	3	17
Venus flytrap	4	5	5	1	5	18.5

IV. Discussion

Following the evaluation of the literature, grades were assigned to each organism, reflecting their adherence to every requirement. Drawing upon the insights gleaned from our analysis and discussion of the retrieved literature, the extent to which each organism aligns with the requisite criteria was discerned. These grades, indicative of the organisms' proficiency in meeting predefined standards are displayed in Table 2.

i. Maintenance grading

Although the precise caring interval of the Venus flytrap, sea slugs, and cockroaches may vary; it is certainly shorter than the month that the earthworm can survive without intervention. This difference gives the earthworm an advantage, warranting its highest score in this criterion. Maintenance of cockroaches requires feeding and removing leftovers, while the Venus fly trap necessitates watering and ensuring adequate sunlight exposure These tasks, although essential, do not demand substantial time commitment or specialized knowledge, thus meriting these organisms a score of four points. In contrast, the maintenance of a large saltwater aquarium, essential for the sustenance of sea slugs, entails a significantly higher degree of effort and expertise. As a result, the sea slug receives a score of one point in this evaluation.

ii. Setup grading

The three sea slug preparations previously described necessitate surgical or dissection procedures, demanding proficiency and experience for satisfactory execution. Additionally, signal measurements employ non-standard instruments like cuff electrodes or capillary electrodes. While recording action potentials in sea slugs with conventional equipment might be feasible, no literature examples were found. Due to these shortcomings, sea slugs receive the minimum score in this criterion. Cockroach preparations, although potentially as intricate as sea slug setups, allow for the recording of neural activity with minimal intervention on the insect using its leg, requiring no previous expertise and common equipment. The same holds true for Venus flytraps. Therefore, these two organisms receive the highest score. Regarding earthworms, if designing and manufacturing recording equipment is to be avoided, the animals need to be anesthetized so they can be restrained during the experiment. However, widely available ethanol can serve as a sedative, inducing narcotic effects through simple short immersion, thus adding minimal complexity to the setup. Consequently, earthworms also receive the highest score.

iii. Signal grading

In earthworms and sea slugs, potential stimulus artifacts from induced electromagnetic pulses can be mitigated by simple separation between the coil and recording electrodes, facilitated by their anatomy. Moreover, both species exhibit low spontaneous activity, particularly in certain nerves of sea slugs, which allows for robust stimulus-response correlations. Consequently, they both satisfactorily meet the criteria for evaluation and are assigned the maximum score. The long action potentials observed in Venus flytraps enable the separation of the stimulus artifact in time rather than in space. Additionally, the absence of spontaneous activity greatly facilitates stimulus-signal correlations in this species.

Consequently, the Venus flytrap also receives a score of five points. Conversely, the small size of the cockroach leg greatly complicates the spatial separation of recording and stimulation processes. Although one experiment reported that electric noise did not significantly interfere with the action potential signals, the options to mitigate potential artifacts from magnetic stimulation would be limited if they were to be longer and stronger. Furthermore, the cockroach leg exhibits spontaneous activity and a high neuronal density, making correlations between stimulus and action potentials challenging to establish. Given these limitations, the cockroach is awarded 2 points in this criterion.

iv. Orientation grading

Both sea slugs and earthworms exhibit highly suitable anatomy and physiology for studying the effects of orientation in magnetic stimulation, owing to their large neurons and low neuronal density, alongside straightforwardly arranged nervous systems. Sea slugs have larger neurons, but their nervous system is slightly more complex as it can be seen when looking at Figure 10 and Figure 11. Earthworms may face challenges in distinguishing between the MGF and LGF. While additional information about the sea slug nervous system is necessary to assess potential similar issues, it would not be unexpected if afferent and efferent nerves are similarly arranged as in earthworms. With current data, both earthworms and sea slugs receive a score of 5 points in this criterion. Conversely, the small size of the cockroach leg hinders the exploration of spatial effects in magnetic stimulation. Despite a relatively uncomplicated neural network arrangement, the leg's zigzag disposition and high neuronal density difficult uniform alignment of the electric field with respect to the axons and neural identification. Considering these shortcomings, the cockroach receives a score of 2 points. Finally, Venus flytraps possess a complex and incompletely understood electrochemical network, featuring non specialized excitatory cells that may have diverse shapes and arrangements. Establishing a relation between field angle and excitation would be extremely challenging under these circumstances and therefore Venus flytraps get the minimum score.

v. Time grading

The Venus flytrap and the cockroach leg can be experimented on for hours without necessitating additional interventions to maintain preparation stability, resulting in the maximum score for both organisms. Experiments with sea slugs required the constant maintenance of specific environmental conditions. While the precise duration of viability varies and depends on the specific experiment, action potentials can be measured in sea slugs for longer than a day after electrode implantation surgery. Taking into account these considerations, the sea slug receives a score of three points in this criterion. Finally, earthworms need to be restrained in order to reduce muscular noise. Anesthesia is the simplest option but its effects only last between 5 and 10 minutes. Restraining earthworms within a custom chamber presents an alternative that may potentially extend the viable experimental time, albeit the design and construction of such a chamber significantly augment the workload associated with the experiment. Consequently, earthworms receive a score of 1 point in this criterion.

V. Conclusion

In conclusion, an assessment of the strengths and limitations inherent in insect, sea slug, earthworm and Venus flytrap models in relation to the investigation of magnetic stimulation of neurons was conducted. In terms of maintenance, the earthworm only requires care intervals of a month while for the other three the care intervals are of the order of a week. The complexity of the preparation for stimulation and recording is comparable for the insect leg, earthworm, and Venus flytrap, while for the sea slug it is notably more intricate as it requires surgery or dissection. The earthworm and sea slug both exhibit a low neuronal density, which simplifies neural identification, especially for the later. However, the earthworm's neural arrangement follows a linear pattern along its body length, which makes it more suitable for the exploration of spatial effects. This may be difficult in Venus flytraps due to their homogeneous excitatory network and in insect legs due to its size. Regarding signal acquisition, separation between stimulation and recording can be performed in time in the plant and in space in the earthworm and the sea slug. This is very challenging in the insect leg due to its small size. Finally, concerning experimental time, the earthworm and sea slug need to be restrained to guarantee stability

in long experimental sessions while the Venus flytrap and the insect leg do not require any adjustment. Considering all these factors, no single organism clearly outperforms the others. The final choice would therefore be determined by the specific experimental requirements. If the study of orientation is important, the earthworm may be a better candidate, but concerns regarding restraint need to be addressed. If spatial effects are not essential, the Venus flytrap can provide a stable platform if testing in non-neural excitatory cells is acceptable. Insect legs can be useful for preliminary experiments to assess stimulation effectiveness visually, while the complexity of the sea slug preparation makes them better suited for more advanced experiments in which their giant neurons facilitate intracellular recordings.

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