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Nociceptive Flexion Reflex (NFR) detection at a single Motor Unit (MU) level

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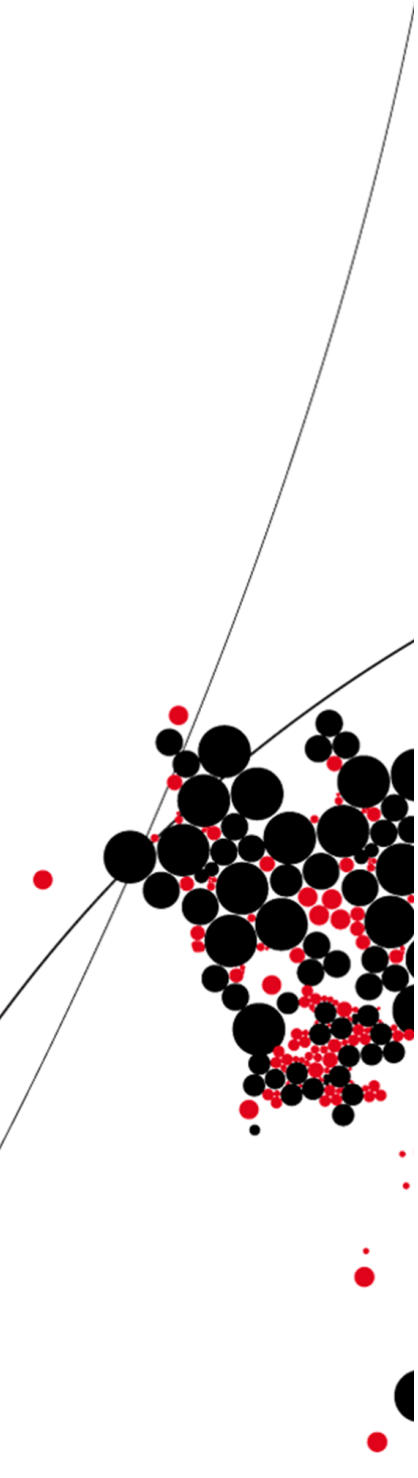

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ABSTRACT

Background: The Nociceptive Flexion Reflex (**NFR**) is a reliable tool for studying pain, and it can be considered as an objective index for pain threshold. However, despite the widespread necessity to study the mechanism of nociception in spinal circuitry, there is currently no valid paradigm to explore the NFR at a single motor unit level.

Objectives: This study will investigate the possibility of online NFR detection while the High-Density-Surface-Electromyography (**HDsEMG signal**) is recorded. Additionally, an offline method is implemented to examine whether it is feasible to identify a single **Motor Unit (MU)** activity from HDsEMG signals and to confirm the occurrence of NFR at the single MU level.

Methods: Ten healthy subjects without any history of chronic pain were invited to this study. After receiving the noxious electrical stimulation, those who showed reflexes and passed their NFR threshold were eligible for the rest of the analysis. The online NFR detection algorithm was developed using a combination of **MTT-NFR** method, to vary the stimulation intensities while the HDsEMG was recorded from the bicep femoris muscle during the 20% of **MVC**. Different numbers of MUs were identified from each subject during a particular batch, using the **Convolutive Blind Source Separation** batch decomposition algorithm. The SIL measure (similar to PNR (pulse to noise ratio)) indicated the accuracy of extracted MU. The similarities between the extracted MUs from two subsequent batches were evaluated by employing the merging algorithm. To limit the decomposition error, we implemented a method based on the Coefficient of Variation for the Inter-Spike Intervals (CV_{ISI}) that can ensure the reliability of the MU spikes train. Subsequently, the offline reflex responses were analyzed with the metrics called (**CUMSUM-PSTH**) and (**CUMSUM-PSF**). The stimulus-wise contribution for each MU was examined individually to find any relationship between the stimulation intensity, reflex prevalence, and reflex latency.

Results Among 10 people, only 2 subjects met the two analysing inclusion criteria. Applying logistic regression to the subject's demographic property indicates that height is a significant factor influencing the probability of inducing a reflex. A total of 171 spike trains were identified from batch decomposition, which was then limited to 30 MUs after employing the merging algorithm. 63.66% of the extracted MUs showed the reflex property in their CUMSUM-PSF. There was no linear correlation between the stimulation intensities and reflex prevalence, and reflex latency. However, the study indicated that MUs with higher SIL are more capable of showing excitation.

Conclusion In conclusion, despite having the non-comfortable stimulation procedure, the online NFR can be obtained non-invasively, recording the HDsEMG signals. Additionally, offline NFR assessment confirmed the occurrence of a reflex in the single MU level. However, comparing the excitatory MUs and non-excitatory MUs SIL values indicates that the decomposition algorithm may affect the offline NFR detection process.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

1.1 Motivation

Chronic pain is defined as an unusual sensation of the pain continuing beyond the normal healing phase. Generally, pain is considered chronic if it lasts more than three months [1]. In 2019, the US national health interview survey reported that 20.4% of adults had chronic pain and 7.4% of adults had chronic pain that frequently limited their life or their work activities (also called high-impact chronic pain) [2]. In European communities, chronic pain of moderate to severe intensity occurs in 19% of adults, which seriously affects the quality of their social and working lives. However, only a few percent of patients are served to treat the pain with the help of specialists and sufficient medicine, and most of them received inadequate treatment [3].

In recent decades, due to the high prevalence of chronic pain, exploring for a reliable and stable tool for pain assessment received more attention than before. However, the clinical evaluation for chronic pain is still complicated due to the considerable variation of the patients' sensations to pain. Among the methods for chronic pain evaluation, Nociceptive Flexion Reflex (NFR), also called R3 reflex, a stimulus-induced spinal reflex, is considered as a relatively stable tool in the study of pain [4]. Therefore, to tackle the aforementioned problem, this study explores a way to detect the nociceptive reflex as a metric for the pain threshold through both online and offline implementations which will be discussed later.

The previous study of pain demonstrated that the intensity of stimulation required to elicit an NFR might be used as an objective index of pain threshold [5]. Several studies have also confirmed that there is a linear correlation between the NFR threshold and the subjective perception of pain, and the magnitude of the reflex response is correlated with the intensity of the perceived

pain [5], [4]. Furthermore, the NFR threshold may be assessed using a Multi-Threshold-Tracking (MTT) algorithm to vary the intensity and other physical properties of stimulation. Therefore, to study the relationship between the pain perception and the stimulation threshold that induces NFR, an MTT-NFR experiment to generate different sequences of stimulation in combination with the NFR detection algorithm (online and offline) was established.

1.2 Goals

Previous studies confirmed that inducing the lateral malleolus in the location of the sural nerve pathway while the subject maintains a standardized angle of knee flexion will generate a nociceptive flexion reflex (NFR) in the bicep femoris muscle [6]. However, as a **primary objective**, this research aims to investigate whether it is possible to detect an NFR using the MTT algorithm and the HDsEMG (high-density-surface EMG) electrodes. To accomplish this goal, the HDsEMG signal will be recorded during the entire experiment, and the online MTT-NFR algorithm keeps exploring for a nociceptive reflex occurrence [7]. Furthermore, the **secondary objective** of this assignment is to examine whether it is feasible to identify a single Motor Unit (MU) action potential from the HDsEMG signal and to implement an offline method to confirm the occurrence and to calculate the prevalence of NFR at the single MU level. To tackle this conclusive objective, the HDsEMG signal of several participants will be recorded, and then the retrieved HDsEMG data will be decomposed into single MUs for each participant. Further analysis will be carried out to detect the NFR in offline and online manners.

1.3 Expected contribution

It is expected that the developed project may contribute to subsequent experiments and work. The main expected contributions are:

- The development of a suitable setup experiment to elicit the desired muscle activity.
- The implementation of the MTT algorithm in combination with an online nociceptive flexion reflex (NFR) detection in the HDsEMG signals.
- The decomposition of High-density surface EMG (HDsEMG) signals to single motor units (MU) using a sophisticated decomposition algorithm.
- The development of a method to detect the occurrence of an NFR for each individual motor neuron using offline analysis.
- The development of a method to elaborate on the relationship between the NFR occurrence and NFR prevalence with stimulation intensity using offline analysis.

CHAPTER 2

BACKGROUND

2.1 Pain

According to IASP (International Association For the Study of Pain), the recent definition of chronic pain was revised to “An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" that persists more than three months [8]. Various issues arise in the study of pain. Pain is relatively defined as a subjective tool that means the patient has learned how to express his level of pain sensation with the application of the word since his early life. Therefore, there is no absolute scale for the pain sensation. Besides, the nature of the pain is multidimensional which can be altered to another mechanism like anxiety [9]. These challenges in the study of pain will emphasize the essence of exploring a reliable and a stable tool for pain assessment.

The new ICD (International Classification of Disease) category of chronic pain comprises the most relevant clinical disorders which are classified in the following seven groups: (1) Chronic primary pain: this is a pain in one or more anatomic regions that persists for longer than three months and can not be expressed by other chronic conditions, (2) Chronic cancer pain includes pain caused by the cancer itself or the pain caused by the cancer treatment, (3) Chronic posttraumatic and postsurgical pain, this is defined as a pain that would develop after a surgical procedure, (4) Chronic neuropathic pain which is caused by any kind of lesion, injury or disease to the somatosensory nervous system itself, (5) Chronic headache and orofacial pain which is defined as headaches or orofacial pains, (6) Chronic visceral pain is a persistent or a recurrent pain that originates from the internal organs of the head and neck region and the thoracic, and the last category is (7) Chronic musculoskeletal pain which represents a persistent pain that arises as part of a disease process or the response to a noxious stimulation that directly affects bones, joints, muscles, or related soft tissues. The last category is limited to the nociceptive pain [1]. Our

study, therefore, concentrates only on the nociceptive pain, which is the normal response to the repetitive stimulus, initiated with the nociceptors at the neuronal level.

2.1.1 Neurophysiology of nociceptive pain

2.1.1.1 Peripheral processing

According to Willer studies [9], stimulation of the sural nerve (either on or through the skin) elicits two different reflex responses in the bicep femoris muscle: the first short latency and low threshold reflex which corresponds to a tactile reflex (also called RII) and the second longer latency and higher threshold reflex which corresponds to a nociceptive flexion reflex (NFR) (also called as RIII). The threshold of RIII is then considered as the threshold of a pain sensation. However, the pain sensation does not occur unless the pain transmission mechanism (also called nociception) evolved with the help of nociceptors. Nociceptors are free nerve endings in the skin that are relatively sensitive to a noxious stimulus. Once a nociception has occurred, the nociceptive action potentials are transmitted by the peripheral nervous system to the brain through the ascending pathway while the modulation of the pain will be transmitted back to the spinal cord through the descending pathway. During the nociception, the first-order afferent neurons travel from the periphery (skin, cornea, visceral organs) to the spinal cord via the dorsal horn. Nociception has occurred when two types of fibers are activated. Smaller, myelinated $A-\delta$ fibers transmit nociception rapidly, which produces the initial fast pain. Larger, unmyelinated C- fibers transmit what is called second pain which has aching qualities and lasts longer than the first pain. Then, the axon of the first-order neurons will synapse with the second-order neurons at the level of the brain stem and the third-order neurons will transmit the signal to the somatosensory cortex to perceive the intensity of the painful stimulus (see figure 2.1),[10].

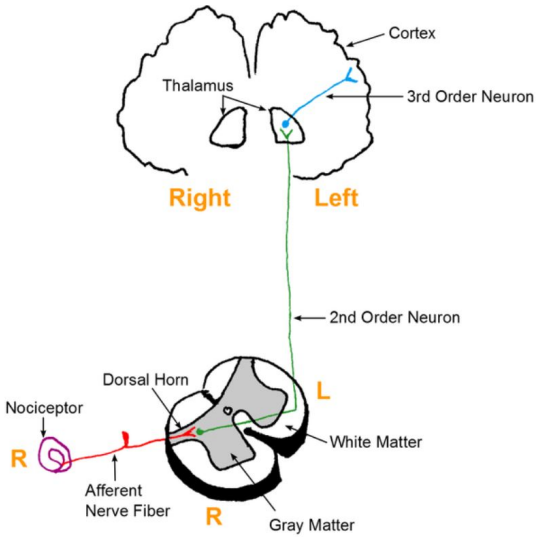


Figure 2.1: Nociception mechanism and It's relative pathways, retrieved from [11]

2.1.1.2 Central processing

After a stimulation has been received, nociception will be transmitted to the spinal cord via the $A-\delta$ and C fibers. The fibers terminate in the dorsal horn, which comprises various laminae where the afferent neurons establish synapses [10]. $A-\delta$ fibers project mainly in laminae I with some given branches in laminae V, whereas C-fibers occupied the central part of laminae II and a small part of laminae I [12]. The figure below depicts the neuronal circulatory in the dorsal horn.

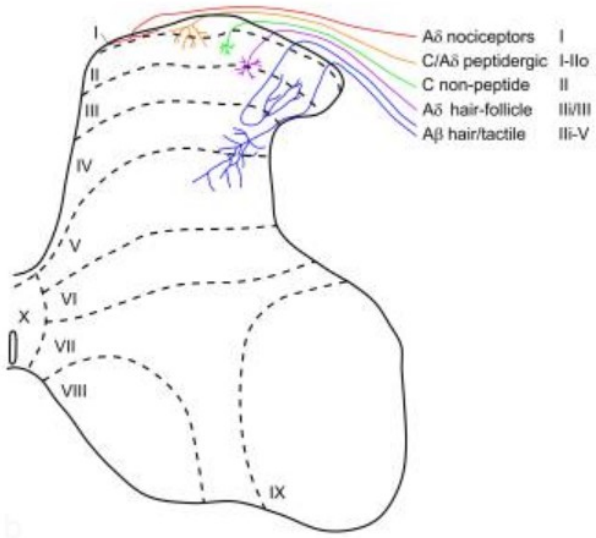


Figure 2.2: Neuronal circuitry in dorsal horn, retrieved from [12]

Within the dorsal Horn, there are two types of neurons associated with the processing of the nociceptive signals: nociceptive specific (NS) neurons which respond to the high intensity stimuli and, wide dynamic range (WDR) neurons which respond to a more broader range of stimuli. These neurons will only synapse with specific types of fibers. For instance, WDR neurons in laminae V synapse with $A-\delta$ and $A-\beta$ fibers whereas NS neurons in laminae I synapse with $A-\delta$ and C-fibers [13]. Both NS and WDR are second order neurons which allow to transmit the nociceptive signal from the spinal cord to the Thalamus through the anterior and lateral spinothalamic tract. The nocicpetive signal then will be carried to the somatosensory cortex via the third order neurons.

2.2 Nociceptive Flexion Reflex

The Nociceptive Flexion Reflex (NFR) has been demonstrated as a reliable tool in the study of pain. The NFR is assessed by measuring the bicep femoris muscle activity using a technique to vary the stimulation intensities applied on the sural nerve at the location of the lateral malleolus. Following stimulation of the sural nerve will generate two different reflex responses. RII is the short-latency, lower amplitude, and corresponds to a tactile reflex whereas RIII is the long-latency

and higher amplitude, and corresponds to a nociceptive reflex. The intensity of stimulation to elicit the RIII reflex (NFR) is assumed as a good indication for the pain sensation [4], [9], [6]. The NFR is a polysynaptic spinal withdrawal reflex that is stimulated following activation of nociceptive $A-\delta$ afferents. The magnitude of the nociceptive flexion reflex is correlated with the intensity of perceived pain [5]. The mechanism to perceive pain is so that the $A-\delta$ afferent neurons transmit the nociception to the dorsal horn and then the second order neurons (smaller afferent) may transmit the signal to the brain through the ascending pathway and eventually the reflex response will be transmitted back with the help of the efferent neurons through the descending pathway. According to the studies of Rhudy and France [6],[5], NFR is elicited if at least one substantial peak occurs between (90-150ms) post-stimulation window relative to the (-65, -5ms) pre-stimulation baseline interval. The interval of NFR occurrence was shortened to prevent any contamination by the RII reflex.

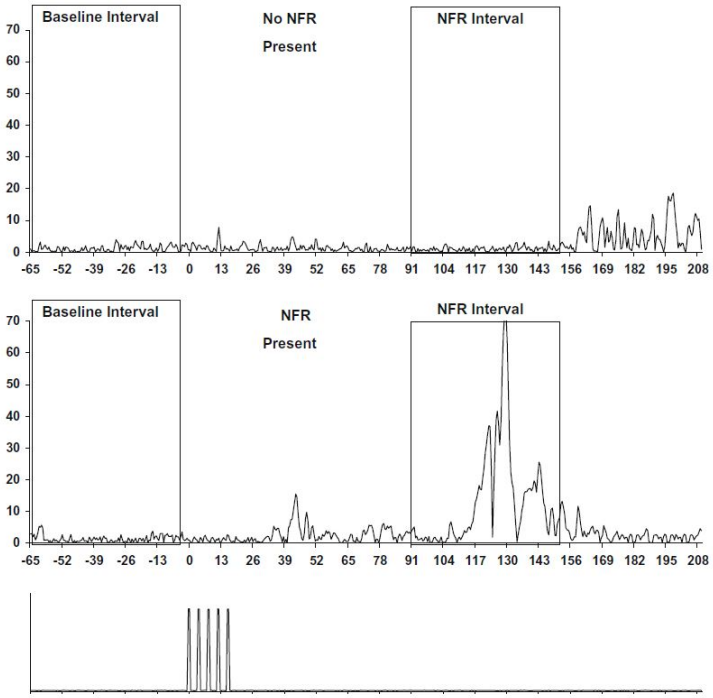


Figure 2.3: NFR Interval, retrieved from [6]

According to the definition of NFR, the stimulation does not elicit an NFR in the bicep femoris muscle in the top panel. However, the bicep muscle shows higher activity during the NFR interval in the middle panel, hence the NFR is present. The bottom panel illustrates the onset and offset of the five pulses stimulation.

2.3 Multi-Threshold-Tracking Algorithm,(MTT)

Following the understanding of the pain transmission mechanism and the NFR occurrence, an implementation to elicit the NFR should be discussed. The multiple threshold tracking (MTT) is an adaptive technique to generate a new set of stimulation based on the last perceived stimulation, developed by the BSS group at University of Twente [14]. The LabView software is the interface for the MTT algorithm. This algorithm allows to provide different sets of stimulation that are varied in some parametric properties. For instance, the Number of Pulses (NOP), Inter-Pulse Interval (IPI) and the Pulse-Width (PW).

Originally, for each pair of stimulation, the MTT algorithm starts with the early subject's pain perception during the familiarization step. Then, the whole set of stimulation is changed based on reflex detection. However, within the set, stimulation can be randomly chosen. During the experiment, if the reflex is detected from HDsEMG, the whole set of next stimulation will decrease (even if the largest stimulation within the updated set is still higher than the lowest stimulation in the previous set) and if the reflex was not recognized, the intensity of the next set of stimulation would be higher to activate the NFR (even if the smallest stimulation within the updated set is still smaller than the highest stimulation in the previous set). Ultimately, the MTT keeps tracking the threshold value when it changes during the measurement.

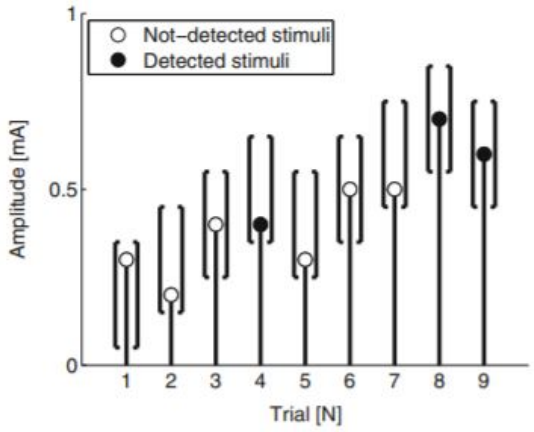


Figure 2.4: Stimulus selection using the MTT algorithm
[14]

An example of the stimulus selection procedure is depicted above. The brackets represent the set within which a stimulus can be randomly chosen. A not-detected stimulus results in an increase of the set while a detected stimulus results in a decrease of the set.

To activate the $A-\delta$ fibers, the **NociTRACK AmbuStim** stimulator generates electrical stimulation, which is capable of working together with the MTT algorithm. A set of bipolar electrodes are usually required to only elicit the relevant fibers. The other kinds of electrodes such as the intra-epidermal electrical stimulus electrodes will elicit not only the small nociceptive fibers ($A-\delta$ and C fibers) but also some large, non-nociceptive fibers (for example $A-\beta$ fibers)

which are irrelevant for the pain assessment [15], [16].

2.4 HDsEMG signal

Investigating the biomedical signals will benefit the analysis of the human body process. Relevant to this study, EMG signals will be acquired while the electric potential generated by the motor units **(Motor-Unit-Action-Potential,MUAP)** during the neuromuscular activity (contraction/relaxation) is measured. The EMG signal is controlled by the nervous system and it is dependent on the anatomical and physiological properties of muscles. For instance, EMG signals are often used to estimate muscle activation, muscle force and also to analyze some neurological properties such as the reflex response of a single motor unit. With the aim of measuring the reflex response, the interference surface electromyogram signal (sEMG) is the most common technique. However, it has been demonstrated that the single motor unit recordings are needed for an accurate analysis of inter-neuronal circuits at the spinal level [17].

The behavior of individual single motor unit is often studied with the use of intramuscular EMG (iEMG) signals. However, This technique is invasive and has high selectivity which then limits the number of accessible motor units to analyze. Therefore, due to the difficulties using iEMG signal and the necessity to analyze single motor unit in the study of reflex, the technique to record high-density EMG (HDsEMG) signals and then decompose the retrieved signal to single motor units has been recommended [17].

2.5 Decomposition Algorithm, Convolutive Blind Source Separation

With the necessity of analyzing the single MU in the study of reflex, exploring an appropriate decomposition method is still debatable. Analyzing the activity of Motor Units (MUs) provides a sight into the spinal circuitry. The activity of motor neurons reflects into the innervated muscle fibers, and therefore, the discharge timing of muscle fibers reveals the discharge pattern of the motor neurons. Consequently, recording the HDsEMG signal from muscle fibers will provide information about Motor Units' properties like their firing rate, MU action potential (MUAP), etc.

According to our research question, one of the aims of this assignment was to explore the feasibility of using the HDsEMG signals to identify nociceptive reflex in a single MU. However, this would be carried out by decomposition of the HDsEMG signal. The decomposition algorithm is called Convolutive Blind Source Separation, which is a combination of fastICA Separation Vectors (with the general idea of sparse sources and not independent sources), and a source estimation refinement step based on Convolution Kernel Compensation (CKC) algorithm [18].

The primary assumption to develop the decomposition algorithm is that, the HDsEMG signals can be described as a convolutive mixture of a series of delta functions (spike trains, s_j),

representing the discharge timing of MUs and the impulse response (h_{ij}), representing the MU action potential. Thus, the mathematical representation of this assumption is:

$$x_i(k) = \sum_{l=0}^{L-1} \sum_{j=1}^{n} h_{ij}(l) s_j(k-l) + n_i(k) \qquad i = 1,...,m, k = 0,...,D_R$$

Where $x_i(k)$ is the i'th EMG channel, k is the discrete time, D_R is the duration of recording, h_{ij} is the action potential of j'th MU recorded in channel i, s_j the spike train of j'th MU, L is the duration of action potentials, l is the convolution indices, n_i is the additive noise and n and m are the number of active MU and the number of channels, respectively. s_j can be extended to include the original sources and the delayed version of each source. x_i also is usually extended to contain each observation and the delayed version of each observation. The extended model in the matrix form defined as below:

$$\bar{x}(k) = \bar{H}\bar{s}(k) + \bar{n}(k)$$

Where $\bar{x}$ is the extended observation, $\bar{H}$ is the extended impulse response and $\bar{s}$ is the extended matrix of sources. After extension, the algorithm is developed via three steps:

- **Whitening**: The whitening matrix (W) is calculated using Singular-Value-Decomposition method. The purpose of whitening is to scale the standard deviation to be equal to one. The whitened source is then retrieved as:

$$\bar{s} = W\bar{x}$$

- **fastICA or fixed point algorithm**: This segment is implemented with defining the separation vectors (w_i) and estimate the sources which elicits this pattern. Afterward, the spareness of each source were calculated and the loop to adjust the separation vector (w_i) was continued until a certain error threshold has been reached. This segment is called as fixed point algorithm which is based on the sparsity of each source. The pseudo code for this part is as below:

  - Initialize the separation matrix, B
  - Initialize the separation vectors $w_i(1)$ and $w_i(0)$
  - Estimate the source elicits from each separation vector: $s_i = w_i^T * Z$
  - While $|w_i(n)^T w_i(n-1) - 1| < TOL$
    - Find the sparseness of each source ($g(x) = \frac{d(G(x))}{dx}$ is a measure of sparseness): $g[w_i(n-1)^T * Z]$
    - Update the separation vector:

$$w_i(n) = E(Zg[w_i(n-1)^T * Z]) - E(g[w_i(n-1)^T * Z])w_i(n-1)$$

- **CKC algorithm**: This segment is implemented with finding peaks algorithm in the estimated sources and classify those peaks using the k-means classification to distinguish between the different Motor Units Pulse Train. This part improves the estimation of each source because there is a probability that the fixed point algorithm converges to an unreliable estimation. This step has to be continued until the Coefficient of Variation (CV) of each source does not have variability greater than the defined threshold. The pseudo code is as below:
  - Estimate source generates pattern w_i, $\quad s_i = w_i^T * Z$
  - Divide sources in two groups of high peak and low peak and estimate the pulse train of each,
  - Find the CV of pulse train for higher peaks,
  - Update w_i until a good threshold for CV reaches.

Finally, a **silhouette measure (SIL)** was computed on the estimated source after the third step. This measure was defined as a difference between the within-cluster sum of point-to-centroid distances and between-cluster sum of points-to-centroid distance. The SIL is a metric to calculate the goodness of clustering. The source will be accepted as an active MU, if the SIL measure is greater than a specific threshold considered as 0.80 or 0.85 in our experiments.

The decomposition algorithm requires some input parameters and slight modifications, which will be discussed in the Methodology section.

CHAPTER 3

METHODOLOGY

Based on acquired knowledge and the collected literature, a Methodology section should be defined to answer the proposed research questions. The methodology pipeline has consisted of six parts that will be discussed in detail in this chapter.

3.1 Part One: Experiment Setup

3.1.1 Study Population

To investigate the feasibility of NFR detection in online and offline manners, we invited 10 subjects to this study. This study is ethically approved (see the A.1), and the subjects were informed about the procedure in advance. For each subject, a consent form, a subject's information form and an experiment's information letter were provided and they signed the consent form to confirm that they understood the protocol and corresponded to the inclusion criteria. Each session will take place approximately 2 hours, including the time spent on preparation and round-up. During each experiment, the HDsEMG data is recorded using the grid electrode and the occurrence of NFR is evaluated right after the experiment. According to the pilot experiments, the NFR happens quite later than 90-150 ms that was mentioned in literature [6],[5]. If the occurrence is later than what is expected (roughly around 100ms late), the measurement will be repeated again with the delayed NFR online detection algorithm.

3.1.1.1 Experiment Inclusion Criteria

To be able to participate in the experiment, subject must be eligible for these criteria:

- Providing a signed, written informed consent (it is necessary that subjects also read the subject information form),
- Age between 18 and 60,
- Being healthy without a medical history of acute pain or related muscles,

3.1.1.2 Experiment Exclusion Criteria

Exclusion from experiment are due to:

- Refusal during the study,
- Skin problems at the site of the pain sensitivity measurement,
- Pain complaints at the time of the experiment or unable to undergo the painful measurement,
- A medical history of chronic pain,
- Acute pain at the time of the measurement (NRS >3),
- Excessive consumption of alcohol (>4 units of alcohol) or within 24 hours before the experiment,
- Consumption of pain-blocking or muscle relaxing medicines (methocarbamol, gabapentin, etc.) within 24 hours before the experiment,

3.1.2 Experiment Instruments

The required equipment is provided in the table 3.1:

Component	Quantities	Information
Computer	1	With Labview, TMSi-Refa Polybench and Matlab 2020
Stimulator	1	NociTrack-Ambustim,(0-40mA) intensity
Trigger generator	1	Capable to connect with stimulator and Refa-amplifier
Stimulation electrodes	1	One pair of anode and cathode Ag/AgCl electrodes
Ground electrode	1	Ag/AgCl electrode
Amplifier	1	TMSi-Refa 128 channels amplifier
Measuring(HDsEMG)electrode	1	A set of 8*8 semi-disposal electrode
Surgical Bed	1	For participants to lay prone while doing the experiment
Electrode Gel	1	Apply between the active layer and HDsEMG electrode
Required cables	–	To connect all of the instruments

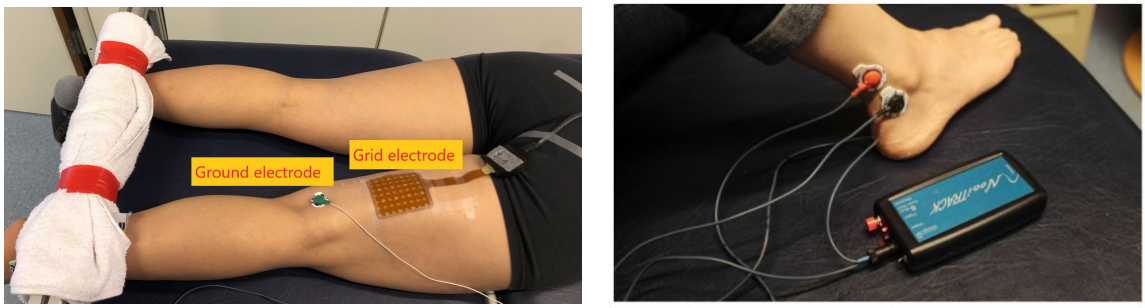
Table 3.1: Required Instruments

3.1.3 Experiment Execution

3.1.3.1 Subject's Position

As it was discussed in the previous chapter, 2 and according to studies of Rhudy and France [6], stimulating the sural nerve on the location of lateral malleolus may elicit a Nociceptive Flexion Reflex (NFR) that can be measured on the bicep femoris muscle. To elicit the sural nerve, a pair of Ag/AgCl electrodes are located on the lateral malleolus while the subject is laid prone on a surgical bed and is positioned so that his leg is passed under the bar. Moreover, the muscle activity from the bicep femoris will be recorded using a 8x8 grid-array HDsEMG electrode to determine the NFR. To avoid interference of HDsEMG signal with noise, the ground electrode

will be placed on the bicep femoris tendon, the junction between the knee and the bicep muscle itself. Later on, to measure the MVC, the subject will be asked to push his heel against the bar to the upside to generate a flexion on his muscle. To clarify the experimental process more, the pictures of recording and stimulation sites are provided below:



((a)) Location of HDsEMG electrode and the ground electrode

((b)) Location of the stimulation electrode and the NociTRACK AmbuStim stimulator

Figure 3.1: Recording location and Stimulation location

The subject's position creates less interference to the HDsEMG electrodes as much as possible and has been confirmed to be used in generating NFR [6]. The stimulation is applied so that the anode electrode (red) is placed approximately 2cm superior to the cathode (black).

3.1.3.2 Electrical Stimulation Settings Determination

Stimulation is two sets of 35 stimuli each and a total number of 70 stimulation. Based on the pilot experiment that was done for this study, to finish the experiment, on average, subjects can tolerate 70 stimuli. The first set of stimulation consisted of 5 rectangular pulses of 1 ms duration and 3 ms inter-stimulus interval. The same setting was designed for the second set of stimulation but with 7 rectangular pulses. Previous studies demonstrated that this stimulation setup is adequate to generate an NFR in the desirable location [19]. The stimulus types are selected according to the MTT algorithm randomly but with the same number of times. The values of the next stimuli are also decided by the MTT algorithm. The electrical stimulation is applied to the subjects using the simple pair (one anode and one cathode) of Ag/AgCl electrodes.

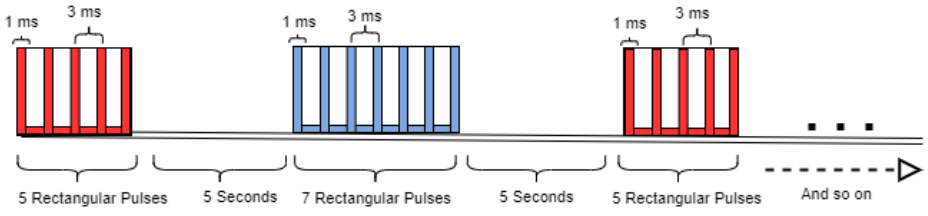


Figure 3.2: Electrical stimulation settings, Two sets of rectangular pulses

3.1.3.3 Familiarization and Initialization steps

This experiment is designed to provide first a familiarization step for the subject to get acquainted with the stimuli. The magnitude of stimuli will gradually increase when the subject presses the button, but will not exceed over 30mA. When the subject releases the bottom, the process will stop automatically. After the familiarization, the procedure will continue to the initialization of the experiment. Similar to the familiarization, the subject has to hold the stimulation button until he can feel tickling or ache at the stimulation site. However, this value of stimulation will be defined as the initial threshold for NFR for the next step of the experiment. It should be mentioned that as we have two sets of stimuli, the subject needs to repeat this step twice to define the initial values for both sets. After the subject completed this step, LabView's interface will be opened to start the experiment. More information about the experiment setup can be found in the "Experiment Protocol", section "V.Experiment Procedure" attached to the A.1.

3.1.3.4 HDsEMG signal recording

During this study, High-density EMG signals are recorded continuously with a sampling frequency of 2048Hz using an 8x8 grid-array electrodes (CE-certified) located in the bicep femoris muscle. This has been done using both sizes of small (4mm inter-electrode distance) and large (8.5mm inter-electrode distance) grid electrodes. The large grid electrode records greater muscle fibers (which might be irrelevant to the bicep femoris muscle) whereas the small grid electrode locates precisely on the identified muscle and it records the exact muscle activity. The HDsEMG signals are then amplified with a TMSi 138-channels Refa EMG amplifier (CE-certified). The HDsEMG signals and the trigger codes are recorded on a dedicated desktop computer running TMSi Polybench software.

Figure 3.3: TMSi small and large grid electrodes

[20]

3.1.3.5 Maximum Voluntary Contraction Force (MVC) Determination

The motor unit mechanism underlying the Maximum Voluntary Contraction Force (MVC) will be varied compared to when the muscle is at rest. 55% of the EMG activity in the bicep femoris muscle during daily life activation occurs at an intensity of 10% of MVC [21]. Hence, there is an assumption that the nociceptive reflex response in a single MU would be more detectable by employing a percentage of MVC during the entire experiment [6]. To calculate the MVC, first, the EMG signal envelop is captured. This aim will be accomplished by low-pass filtering the signal and then apply the full-wave rectification to the signal. An alternative approach is also using the definition of the root mean square (RMS) value within a window that slides across the signal.

Later, the MVC is defined as the maximum of obtained RMS values in each window [22]. (assume that $x(t)$ is the EMG signal and $x_m(t)$ is the mean of the signal)

- The RMS of signal: (T_w= width of each window in second and Nw=number of points)
  - y(t)=($x(t)$-$x_m(t)$)^2
  - z(t)=sqrt((Sum of y(t) from $t - T_w/2$ to $t + T_w/2$) / N_w)
- MVC is the highest RMS in each window,

During the experiment, to measure the maximum voluntary contraction force, the subject will be asked to contract his/her bicep femoris muscle three times by pushing up his/her heel against the bar with the maximum force. The MVC is then measured by taking the average of HDsEMG's amplitude in all three contractions and a line with a certain percent of MVC has appeared on the screen that subject has to keep this amount over the entire experiment using his visual feedback. In the beginning, the study decided to stay at 10% of MVC during the entire experiment. However, the decomposition results revealed that 10% is not adequate to extract the activity of each MUs individually. Therefore, the level of contraction was increased up to 20% of MVC (see figure 3.4)

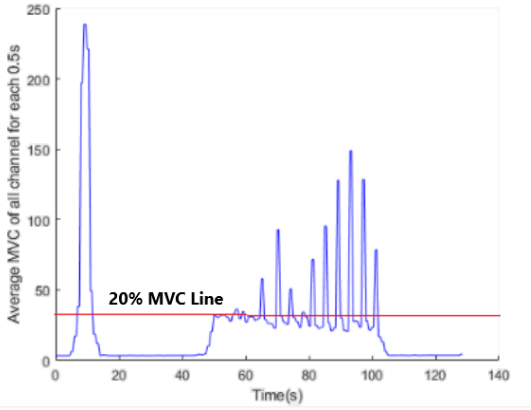


Figure 3.4: The Maximal Voluntary Contraction Force (MVC) and the 20% MVC line. The first high peak is the average of three contractions and from 47seconds the real experiment starts.

3.1.3.6 MTT Algorithm

As was mentioned before in chapter 2, the MTT algorithm decides the value of the next stimulus. The stimulation is given after every 5 seconds and randomly between the two sets. The stimulus will continuously increase for a step size of 0.4 mA if the reflex response is not detected, and it would be decreased by the 0.4 mA step if a reflex is detected. Consequently, there is a need for an online NFR detection algorithm which will be discussed in section 3.1.3.7. The HDsEMG signals are recorded while the MTT protocol modifies the stimulation intensities and the NFR online detection algorithm is executed concurrently.

3.1.3.7 NFR Online Detection

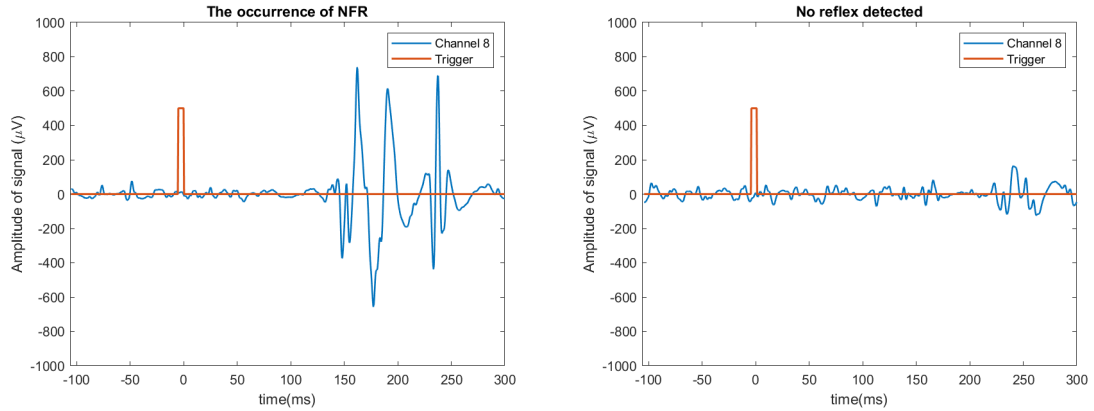
Scoring Criteria and Cut Point Determination: According to studies of Rhudy and France [5], among all scoring criteria to identify the occurrence of NFR, three of them (the first three criteria in the table 3.2) performed the best. The evaluation is based on the area under the Receiver Operator Characteristic (**ROC**) curve, which is $\geq 0.97\%$ for all of these three criteria. The table below represents the mathematical definition used for these criteria:

Scoring Criteria	Mathematical Definition
Baseline Adjusted NFR Interval Peak	(NFR interval peak - baseline mean)
NFR Interval Peak	peak voltage in NFR interval
NFR Interval Peak z-score	(NFR interval peak - baseline mean) / baseline SD
NFR interval z-score	(NFR interval mean - baseline mean) / baseline SD

Table 3.2: The best criteria to identify the NFR occurrence

These criteria were used to consider an NFR present where the EMG activity between the 90ms-150ms post-stimulation shows at least one substantial peak relative to the activity of baseline from -65ms to -5ms pre-stimulation. However, in this study, the aim was to focus on the two standardized criteria (NFR Interval Peak z-score and NFR Interval z-score) because of their generalized property. Although the area under the ROC curve for NFR Interval z-score is less than the other three criteria, the cut point for this criteria is more stable. Therefore, these two criteria, NFR Interval Peak z-score and NFR Interval z-score respectively with the stable cut-points of 10.32 and 1.38, represented by [5] are used for the NFR online algorithm.

Adjustment in NFR Detection In the real experiment, the occurrence of NFR might be approximately 100-150 ms late. This can be explained by the difference in the setting of the experiment, the equipment, or the height of the subject which directly relates to the length of their afferent nerve and thus to their nerve conduction velocity. After, the first set of experiments, if the appearance of NFR was delayed, the measurement will be again repeated with a minor adjustment to detect the NFR between 150-250 ms post-stimulation. The figure below represents the difference between the activity of signal when the reflex is present and when it is absent.



((a)) Visualization of single channel when reflex is detected

((b)) Visualization of single channel when reflex is not detected

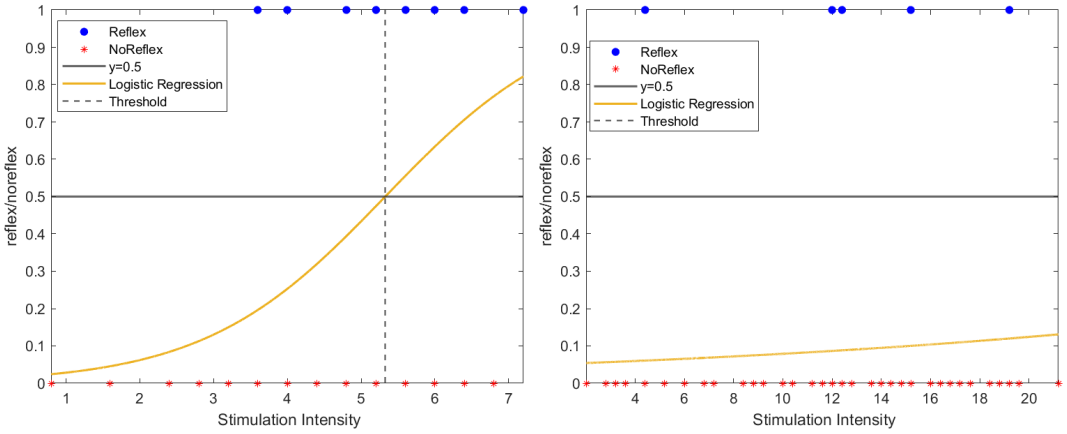
Figure 3.5: Reflex V.s No-Reflex. To have clearer visualization, the amplitude of the trigger was depicted 500 times larger. This reflex was detected by the NFR Interval z-score criteria, a choice that was justified in section 3.1.3.7.

3.1.3.8 Analysing Inclusion Criteria

The subjects who we include in our analysis, have to meet two criteria:

- Subject has to belong to the responder group, who showed reflex during the experiment.
- Subject has to pass his NFR threshold, ensuring that he received at least one stimuli stronger than his pain tolerance.

However, one of the possible ways to determine the NFR threshold is using a psychometric function that establishes the relationship between the stimulation intensity and the induced response (reflex or no reflex). The probability of reflex occurring will be estimated via a logistic regression model, implemented with a generalized linear model that applies a logit link function. The threshold regarding the NFR is then determined for the probability of 50% in this curve.



((a)) An example of passed NFR threshold **((b))** An example of not passed NFR Threshold

Figure 3.6: NFR threshold passed V.s NFR threshold not passed. The reflex responses occurrence in the right panel have the probability less than 0.5 which would result in not passing the NFR threshold.

3.2 Part Two: Data Processing

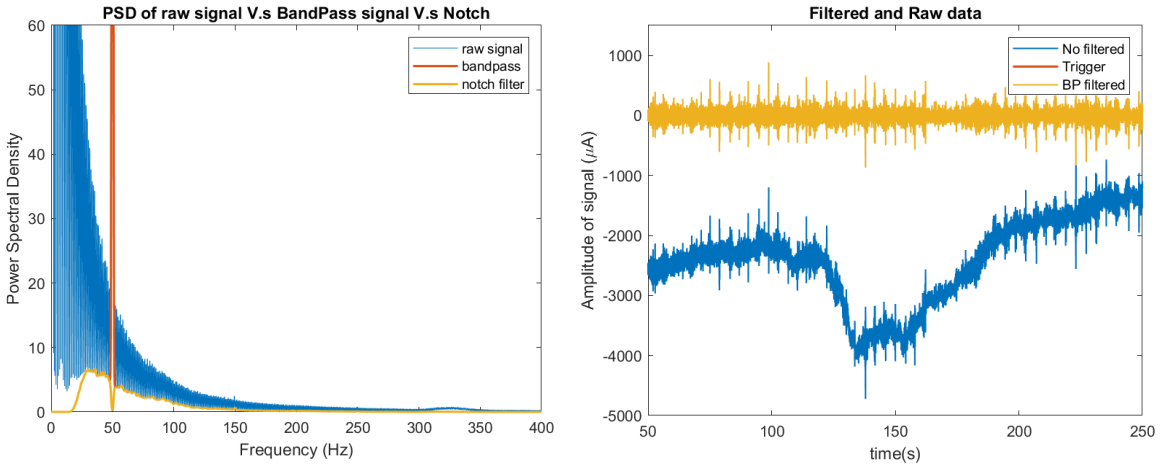
3.2.1 Preprocessing

With the aforementioned information, during the experiment, raw HDsEMG signal is measured. However, in pre-processing step, raw data has to be modified to remove the background noise, movement artifacts, urban electricity frequency and the stimulation frequency intervened with EMG signal.

3.2.1.1 Filtering

To avoid aliasing, a sampling rate of at least two times higher than the cutoff frequency must be chosen $f_{nyquist} = \frac{1}{2} f_s \geq f_{max}$. The sampling frequency, f_s with the TMSi-Refa amplifier is 2048Hz, therefore the frequency range of approximately 0-1000Hz can be measured with the

device. However, according to **Seniam** [23], a fourth order digital bandpass filter (Butterworth filter) with high cutoff frequency of 300Hz to remove high frequency noises, and a low cutoff frequency of 20Hz to remove baseline drift associated with movement and any DC offset, is preferred. Moreover, a notch filter to remove the urban electricity interference at 50Hz is applied to the data. The mechanism of filtering is depicted in time domain (right figure) and the PSD in frequency domain (left figure).



((a)) Power Spectral Density of raw, BP and notch filtered

((b)) Raw data V.s BandPass Filtered

Figure 3.7: Visualization of Raw and BP signal in time and frequency domain

3.2.1.2 Stimulation Artifacts Removing

The stimulation artifact rises quite next to the stimulation trigger in some of the measurements. There is an assumption that this artifact will impact the outcome of the decomposition since it will destroy the signal quality. To remove this kind of artifact, an epoch of 30ms after the offset of each stimulation was smoothed with the average of the rest of the signal between the following stimulation. This method of smoothing was then applied to the entire signal, to increase the signal quality. Below, the filtered signal, the stimulation instant, and the signal after removing the stimulation artifact are plotted. The yellow and blue signals are coincident where the stimulation artifact is removed.

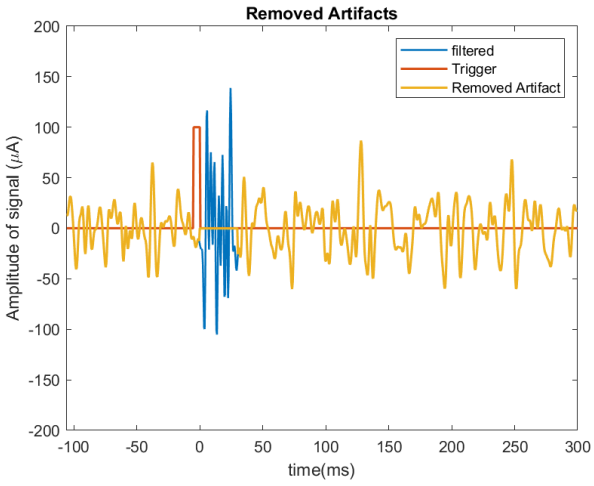


Figure 3.8: Stimulation Artifact Smoothing

3.2.1.3 Signal Normalization

To bring the activity of all of the HDsEMG channels in the same range and remove the DC bias, the signal is normalized to zero mean and unit variance. Throughout this method, the mean activity over all of the channels is subtracted from the activity of each channel and then the result is divided by the standard deviation of the signal. The mathematical representation is defined as:

$$X_j(k) = \frac{(X_j(k) - Xm(k))}{std(X_j(k))}$$

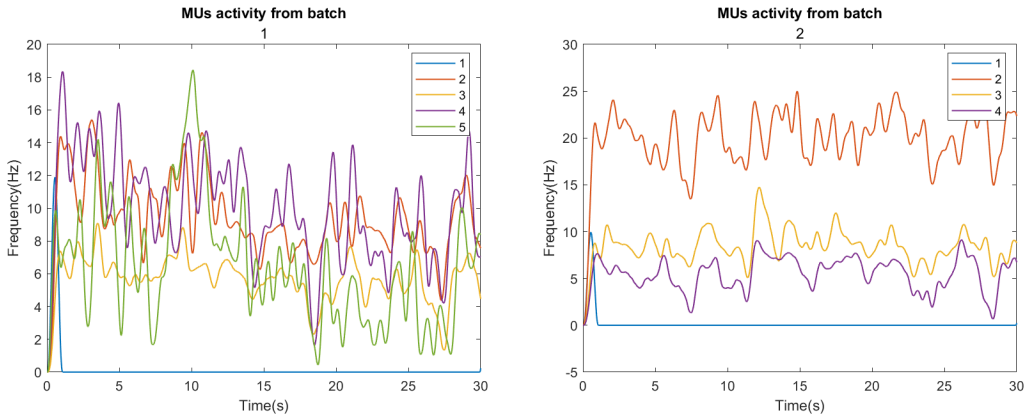
Where $X_j(k)$ represents the activity of j'th channel and k'th sample of time, $Xm(k) = \frac{\sum_{j=1}^{NChannel} X(k)}{Nchannel}$ represents the mean activity over all of the channels throughout the time and $std(X_j(k))$ represents the standard deviation of all channels in the specific sample of time.

3.2.2 Segmentation of the Composite Signal (detecting MUAPs)

The first step to decomposing a HDsEMG signal and detecting the activity of MUs is to choose a fixed length section that is subsequently assumed to contain a significant MUAP contribution [24]. It is assumed that after the stimulation starts, some MUs are activated to respond to this elicitation. Therefore, the appropriate candidate section was supposed to be in the range of 5 seconds before the beginning of the first stimulation until 5 seconds after the offset of the last stimulation. However, due to the long measuring (70 stimuli in total), the behavior of MUs are not constant along the time. This is because the signal section may, in fact, be comprised of a superposition of two or more MUAPs together with a period of isolated MUAP or even a significant noise spike. Thus, is it preferred that the candidate segment divides into shorter batches, for instance, 30 seconds with 10 seconds overlap in between. The 10 seconds overlap was implemented to avoid missing the firing of MUs in the transient section from one batch to the other batch.

3.3 Part Three: Decomposition Algorithm Parameters Determination

To run the decomposition algorithm efficiently, the number of iterations in which the sources are updated, the threshold to compare with the SIL measure, and the sampling frequency must be defined. The higher number of iterations allows for more precise exploring to extract spike trains. In this assignment, 100 and, in some cases, more iterations were used. Furthermore, a lower threshold leaves the algorithm less restricted about the validity of the extracted spike trains. This was chosen as 0.85 or 0.8 most of the time. However, due to the inconstant and unstable behavior of MUs along the time, it is preferred that the decomposition algorithm frequently executes for batches of 30 seconds with 10 seconds overlap.



((a)) Firing of extracted spike trains from batch 1 **((b))** Firing of extracted spike trains from batch 2

Figure 3.9: The out put of decomposition algorithm, executes for two 30 seconds batches with 10 seconds overlap

The output of decomposition algorithm 3.9 is the smoothed firing of each extracted spike train within a specified batch. The x axis represents the time in seconds and y axis represents the smoothed discharge rate of each extracted spike train.

3.4 Part Four: Merging Algorithm

Several spike trains will be extracted from each batch following the decomposition algorithm. Some of these spike trains are activated not only during a particular batch but also during the entire experiment. However, they will be frequently considered as a new extracted spike train in the subsequent batch. To avoid the repetition of spike trains, a merging algorithm is developed to check the similarity between each pairs following two consequent batches. Employing the merging algorithm would achieve only the unique spike trains, which are later considered as unique MUs.

This algorithm calculates the cross correlation (**using the command XCORR in matlab**) between each pair of spike trains in consequent batches along with the duration of their overlap. Two spike trains assumed to be a unique MU, if the threshold defined as $\boldsymbol{TH} = \frac{\boldsymbol{max(c)}}{\boldsymbol{k}}$ (where **C=CorrCoeff, the area of overlapping** and **k=the total area of firing for each spike train**) is higher than 50% and not equal to 100% which only happens if two signals are white noise. The output of the merging algorithm is the matrix of paired MUs in each batch (**MPairs**) and also the merged MU firing activity. The result of an example merging algorithm with 2 batches and the correlated pairs in each batch with the value of their correlation is provided in table 3.3.

Batch	Correlated pairs	Percent of correlation
Batch 1 and Batch 2	pair 2 and pair 3	89.6552 %
	pair 3 and pair 4	83.0189 %

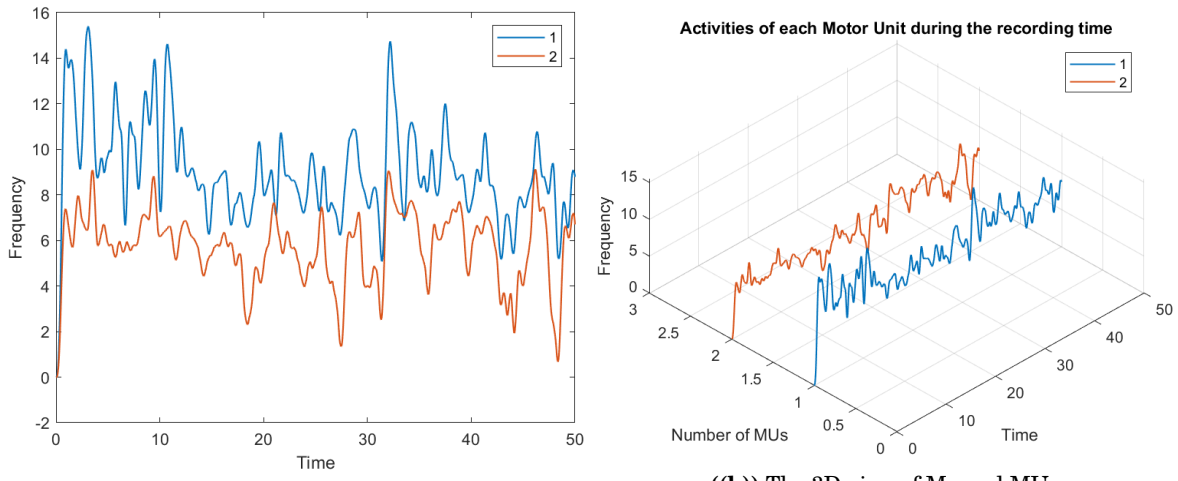
Table 3.3: Correlated Pairs in each Batch

The table 3.4 confirms that each row is an unique merged MU which is generated from the similarity between the indicated pair represented below each batch. For instance, the merged MU1 is the result of the correlation between pair2 in batch1 and pair3 in batch2, and the merged MU2 is the result of the correlation between pair3 in batch1 and pair4 in batch2.

MU	Batch 1	Batch 2
MU 1	2	3
MU 2	3	4

Table 3.4: Merged MU (MPairs) generated from correlated pairs in each batch

The firing activity of these two merged MUs will be stored to evaluate the quality of each of them separately in the next step.



((a)) The 2D view of Merged MUs

((b)) The 3D view of Merged MUs

Figure 3.10: Two Merged MUs

3.5 Part five: MUs Selection Algorithm

In the previous section 2.5, the decomposition algorithm was discussed in detail. However, a routine estimate of the decomposition accuracy is still lacking. Several deflections in decomposition algorithm may happen to identify the decomposed MU spike train. Some of the decomposed MUs may reflect the discharge pattern of stimulation, some of them may fire quite less which means they are only partially activate or they may fires in an irregular discharge patterns with a lot of silence period in between. All of these conditions, results in an unreliable decomposed MU spike train.

However, according to [25], the Coefficient of Variation for the Inter-Spike Intervals ($\boldsymbol{CV_{ISI}}$) of the identified MUs, is correlated with the decomposition accuracy. Therefore,$\boldsymbol{CV_{ISI}}$ can be

used as a potential tool to ensure that the decomposed MU spike train is reliable. To apply this metric to identified MU, the actions below were taken:

- The merged MUs spike train was divided in batches of 10 seconds with 5 seconds overlap,
- For each batch the $\boldsymbol{CV_{ISI}}$ was calculated. (where $\boldsymbol{TI}$ represents the instant time of firing).

$$\boldsymbol{ISI = diff(\frac{TI}{sFreq})} \quad \boldsymbol{CV_{ISI} = \frac{std(ISI)}{mean(ISI)}}$$

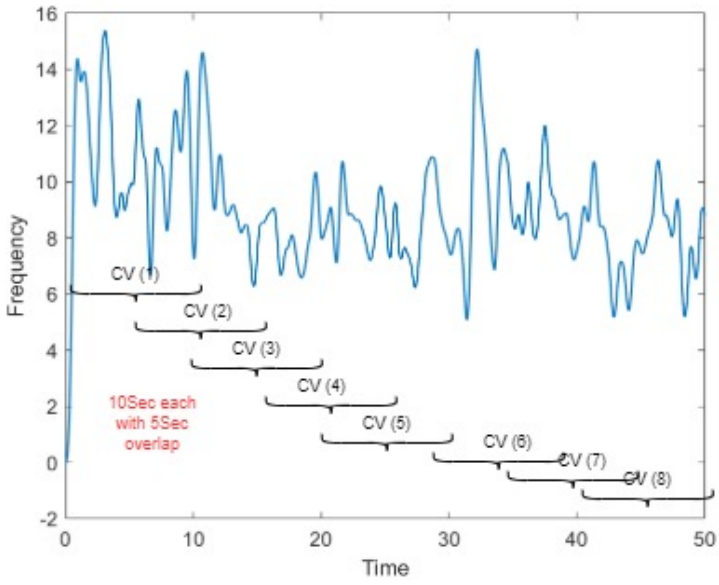
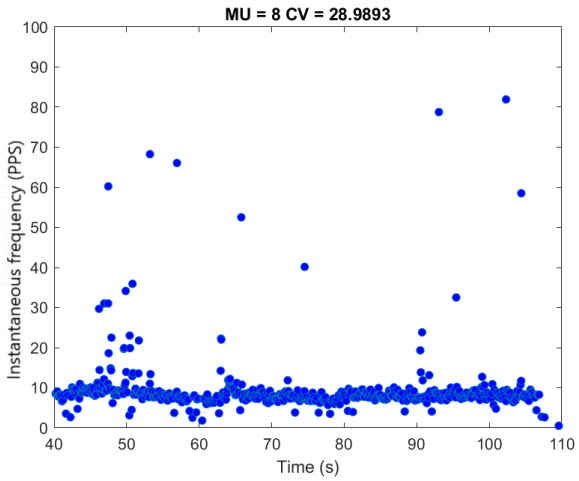


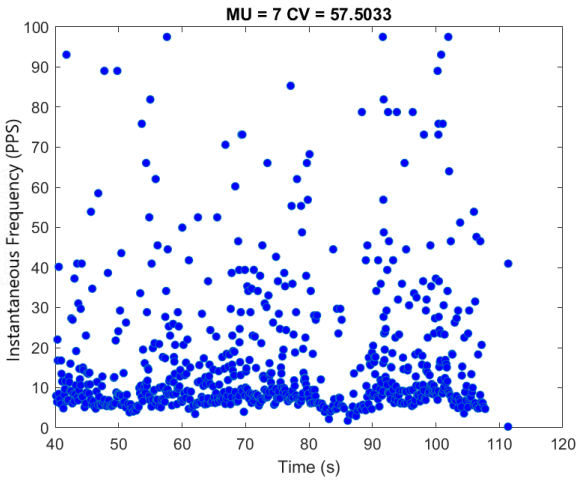
Figure 3.11: Coefficient of Variation for each batch of 10 seconds with overlap of 5 seconds

- According to literature [18], discharge patterns with $\boldsymbol{CV_{ISI}}$ greater than 50% must be excluded from the analysis. To allow more flexibility for MU qualification, 80% of all of the calculated CV within the entire batches (in the case above only 6 CV) are required to be smaller than 55%. This criteria permits for less restriction to assess the good MUs.

The instantaneous discharge rate for each MU is then plotted to confirm the accuracy of a good MU selection algorithm based on visual inspection. The more the instantaneous discharge rate graph is aligned with a straight line, the more reliable MU spikes train and the less $\boldsymbol{CV_{ISI}}$ are.



((a)) The instantaneous DR for a selected MU with average CV=28.98



((b)) The instantaneous DR for a non selected MU with average CV=57.50

Figure 3.12: Selected MU discharge rate V.s Non selected MU discharge rate

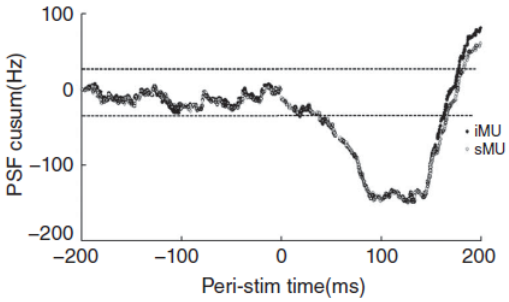
3.6 Part Six: Offline NFR Detection

As indicated in chapter 1, One of the aims of assignment is to detect the offline nociceptive reflex response from a single MU. To accomplish this goal, two metrics of peri-stimulus-time histogram (PSTH) and the peri-stimulus frequencygram (PSF) are defined. The PSTH represents the number of MU spike occurrence in a particular bin width around each stimuli whereas the PSF method represents the instantaneous discharge rates of single MU against the time instant of stimulus [17]. However, in most of the cases, the PSTH and PSF are presented together with their **Cumulative SUM**. The cumulative sum of PSTH and PSF is a sensitive technique for detecting subtle changes in the mean level of spike occurrence/discharge rate that was obscured by the variability in each individual bin [26],[27],[28],[17]. The mathematical representation of the **CUMSUM-PSTH** and **CUMSUM-PSF** is as below:

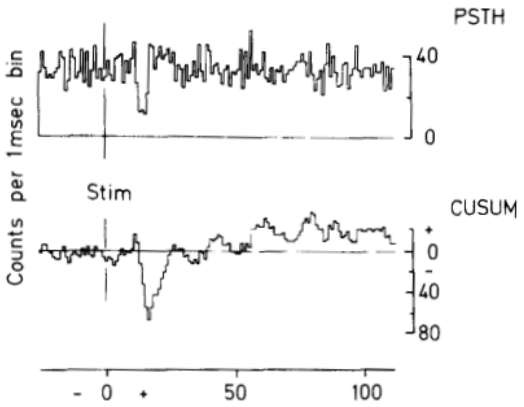
$$CUMSUM_{PSF} = \sum_{t=pre}^{t=post} (x(t) - M), \qquad CUMSUM_{PSTH} = \sum_{n=1}^{n=Nbins} (y(n) - K)$$

Where as the M is the mean pre-stimulus discharge rate, x(t) is the instantaneous discharge rate at time t, K is the mean pre-stimulus spike occurrence and y(n) is the number of spike occurrence within each individual bin.

A significant **inhibition/excitation** reflex was occurred only if there is a substantial **Negative/Positive** change in the slope of CUMSUM-PSF or CUMSUM-PSTH after the onset of stimulus. The horizontal dashed line are the minimum and maximum changes in pre-stimulus course used to determine the significance of reflex response.



((a)) The CUMSUM-PSF representing an inhibitory reflex



((b)) The PSTH and CUMSUM-PSTH representing an inhibitory reflex

Figure 3.13: CUMSUM-PSF and CUMSUM-PSTH [27]

3.6.1 Reflex Properties Evaluation

Defining some properties for a single extracted MU, would guide us to have more evidence about the NFR occurrence. Reflex latency, reflex prevalence, and the average stimulus intensity are the metrics used for this purpose. The reflex latency is the time interval between the onset of stimuli and the first turning point that can be obtained from the plot of CUMSUM-PSF [28]. However, to define the two other properties, each stimulus contribution to a reflex for a single MU must be evaluated. The average stimuli intensity is the mean of all of the stimuli intensities that contribute to a reflex, and the reflex prevalence is the percentage of the stimuli that contribute to an excitatory reflex. The stimulus-wise contribution method is implemented by considering a window surrounding each stimulus (1 second post-stimuli and 2 seconds pre-stimuli) and fitting a polynomial curve on the ***IDR*** to follow the change in the instantaneous frequency after the onset of the stimulus.

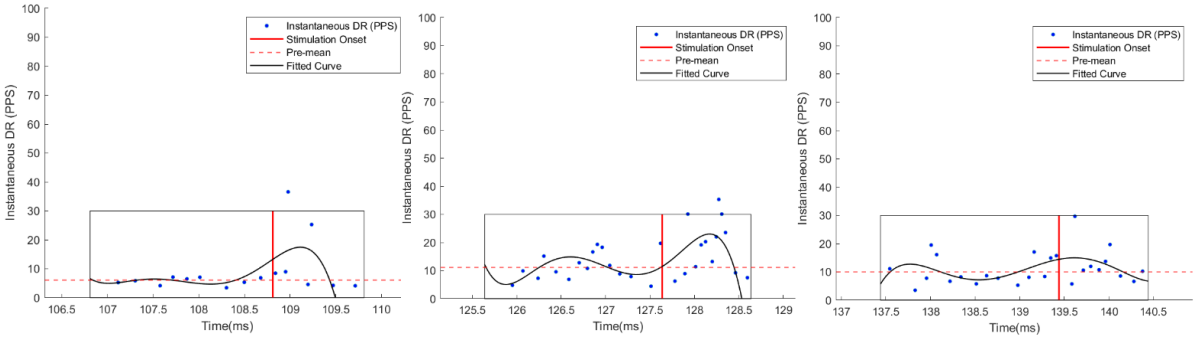


Figure 3.14: Stimulus-Wise contribution for three consequent stimulation in a single MU. 2 of 3 stimuli showed excitatory reflex. So, the reflex prevalence is 2/3=66.66% and the stimuli intensity is the average of two first stimulation intensities that generated a reflex. The outliers of 50Hz from post-stimulation and 28Hz from pre-stimulation are removed. The fitting curve is a polynomial order 5.

3.7 Methodology Overview

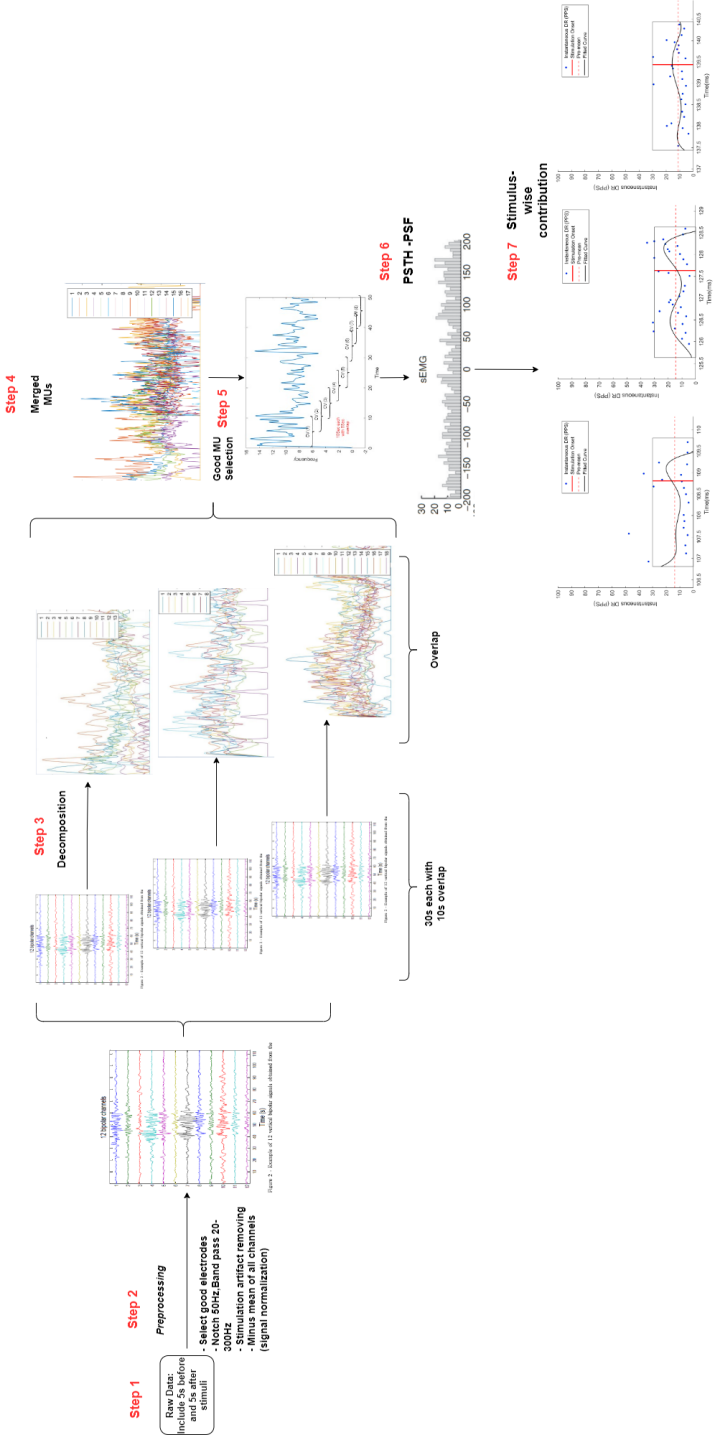


Figure 3.15: Methodology Overview

Chapter 4

Results

4.1 General Results

4.1.1 Experiment Results

As it was decided on 3.1.1, 10 subjects were invited to participate in this study. Table 4.1 reflects the results of online NFR detection in the total 10 subjects concerning how many stimuli they received and, among them, how many reflexes they showed. Some demographic characteristics were also added to the table.

Subject	Gender	Age	Height (cm)	Num of stimulation	Num of detected reflex
Sub 1	F	26	165	32	0
Sub 2	M	27	179	70	7
Sub 3	F	29	180	13	3
Sub 4	M	26	180	70	28
Sub 5	F	25	157	33	0
Sub 6	F	28	161	4	3
Sub 7	M	24	170	49	13
Sub 8	M	23	182	32	0
Sub 9	F	25	160	33	0
Sub 10	M	24	181	70	19

Table 4.1: Subjects' information, Number of received stimuli and Number of detected reflexes

Among the participants, subjects number **1, 5, 8 and 9** did not respond to the sural nerve stimulation. The MTT algorithm kept increasing the stimulation intensity until it reached its

maximum of 40mA. Then, the protocol was stopped with the researcher's interference despite not receiving the total number of stimuli. Besides, subjects **3, 7** reported that the stimulus was no longer durable, and they stopped the experiment due to intolerable pain. Furthermore, data from subject number **6** was excluded from the analysis due to her low pain threshold. 75% of the stimulus she received elicited reflexes which makes the study unable to determine a precise pain threshold.

4.1.2 Statistical Analysis

In the statistical section, we investigate a statistical test to evaluate whether our subjects' height or gender will influence the probability of inducing the NFR reflex. The factor age was excluded from our analysis since our subjects are relatively from the same age group (mean-age = 25.7, std-Age = 1.8886). To achieve our goal, a logistic regression model considering three different height groups (Height-group1 (156cm-165cm), Height-group2 (166cm-175cm), Height-group3(176cm-185cm)) and the gender (female=0, male=1) as factors and the number of reflexes as the dependent variable, was build in SPSS. The model is supposed as below:

$$\ln\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 * SEX + \beta_2 * Height1 + \beta_3 * Height2 + \beta_4 * Height3$$

Tests of Model Effects

Source	Type III Wald Chi-Square	df	Sig.
(Intercept)	57.570	1	.000
Sex	.008	1	.929
Height	6.889	2	.032

Events: DetectedStimuli
Trials: Stimuli
Model: (Intercept), Sex, Height

((a)) Wald-Chi Square test

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval Lower	Upper	Hypothesis Test Wald Chi-Square	df	Sig.
(Intercept)	-3.497	.5860	-4.645	-2.348	35.598	1	.000
[Sex=1.00]	.060	.6753	-1.263	1.384	.008	1	.929
[Sex=.00]	0[a]	.	.	.	.	.	.
[Height=3.00]	2.293	.8813	.565	4.020	6.766	1	.009
[Height=2.00]	2.418	.9509	.554	4.281	6.464	1	.011
[Height=1.00]	0[a]	.	.	.	.	.	.
(Scale)	1[b]						

Events: DetectedStimuli
Trials: Stimuli
Model: (Intercept), Sex, Height
a. Set to zero because this parameter is redundant.
b. Fixed at the displayed value.

((b)) Wald-Chi Square parameters

Figure 4.1: Wald-Chi Square test, with significance level of $\alpha = 5\%$

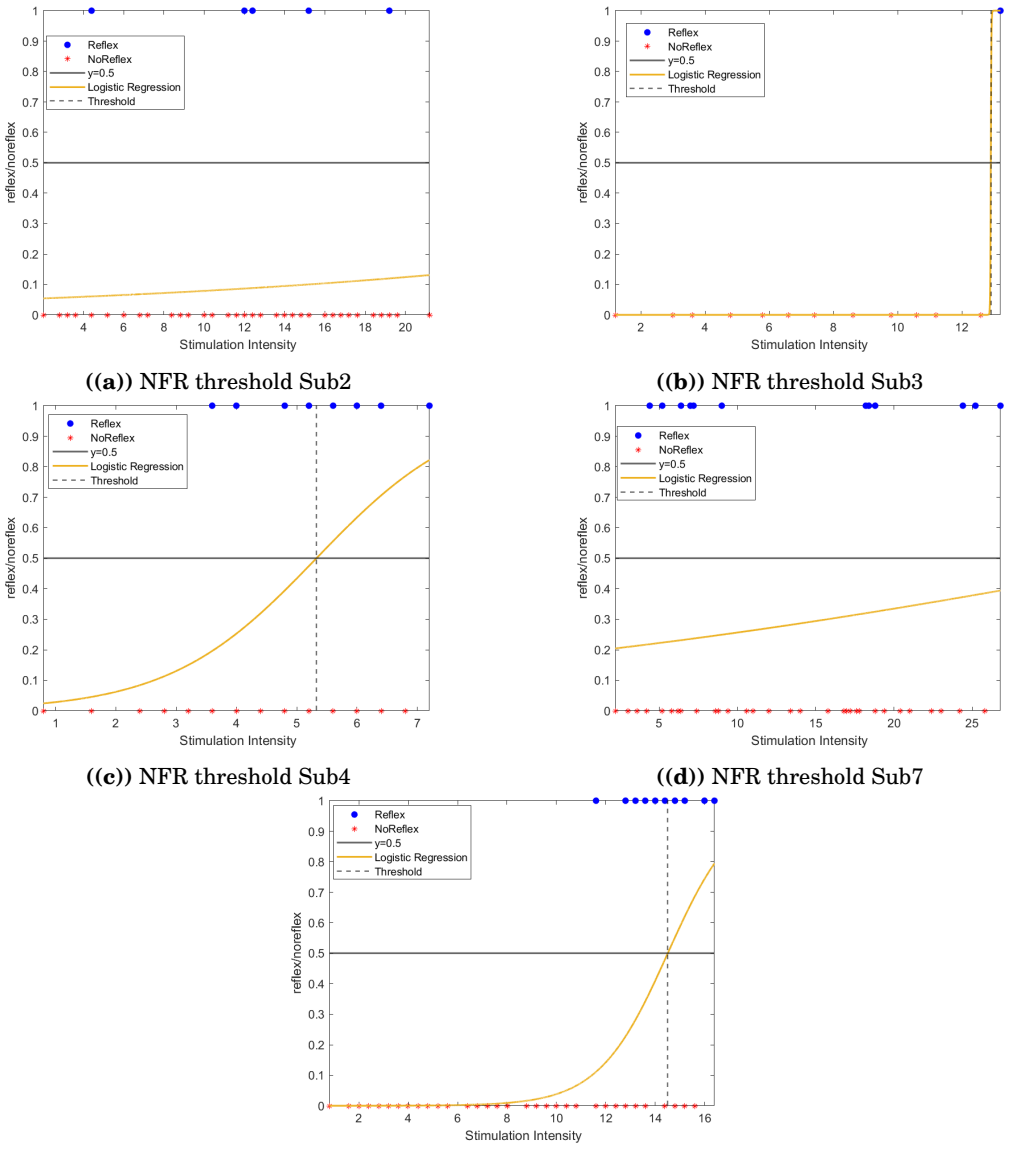
The null hypothesis $H_0 : \beta_1 = 0$ against the alternative hypothesis $H_1 : \beta_1 \neq 0$ is tested to investigate the effect of "Gender" on the probability of inducing NFR. From table, obtaining $Wald = 0.008$, we fail to reject the null hypothesis considering ($\alpha = 5\%$ and $Wald > 3.841$ (df=1)). Again, to investigate the impact of "Height" on the number of reflexes, the null hypothesis $H_0 : \beta_2, \beta_3, \beta_4 = 0$ against the alternative hypothesis $H_1 : \beta_2, \beta_3, \beta_4 \neq 0$ is tested. Since the P-value < significance-level (0.032 < 0.05), we will reject the null hypothesis, and we conclude that the factor "Height" has an impact on the probability of inducing a reflex.

4.1.3 Nociceptive Detection Threshold

According to 3.1.3.8, to evaluate the eligibility of our subjects for the rest of the analyses, the nociceptive threshold (table 4.2) and the logistic regression curve (figure 4.2) only for subjects who showed NFR during the experiment session, are presented below:

	Subject 2	**Subject 3**	**Subject 4**	**Subject 7**	**Subject 10**
NFR Threshold(mA)	–	–	5.32	–	14.50

Table 4.2: Nociceptive Threshold (mA)



((a)) NFR threshold Sub2

((b)) NFR threshold Sub3

((c)) NFR threshold Sub4

((d)) NFR threshold Sub7

((e)) NFR threshold Sub10

Figure 4.2: Nociceptive detection curve and the NFR threshold for subjects who showed reflexes

The results confirm that achieving the nociceptive threshold in some subjects is impossible. The reflex responses have the probability less than 0.5 and the logistic regression curve could not be always fitted appropriately (For instance the case 2,3 and 7). Therefore, the rest of the analysis will be limited only to subjects 4 and 10, who showed reflexes, and meantime, they have a rational nociceptive threshold.

4.2 Decomposition Results

As we mentioned in 4.1.3, only subjects number 4 and 10 are eligible for the rest of the analysis. The table 4.3 and 4.4 reflect the total number of batches for each subject, the total number of extracted spike trains from each batch, and also the total number of unique MUs after employing the merging algorithm. The merged MUs are the results of similar pairs between two subsequent batches.

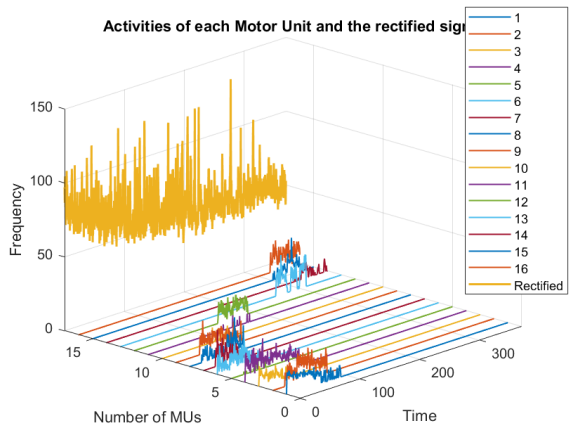
Subject	**Signal Length(Sec)**	**Batch Number**	**Total Extracted Spike Train**	**Total Merged MU**
Subject 4	378.78	18	85	16
Subject 10	290.48	14	86	14

Table 4.3: Decomposition Results

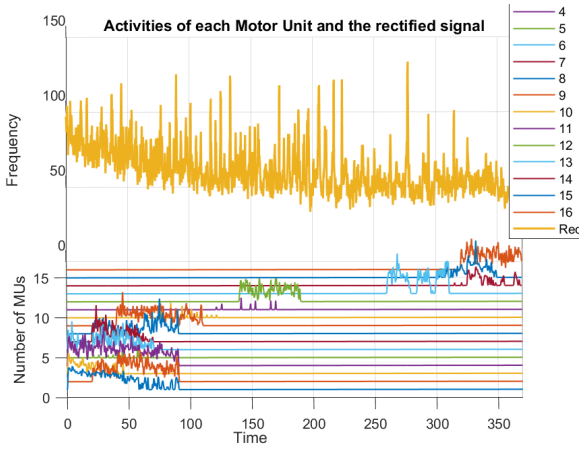
Batch	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Number of Spike Train per Batch,Subject 4	10	10	9	8	4	3	1	5	4	1	2	4	1	3	4	2	9	5
Number of Spike Train per Batch,Subject10	11	11	10	13	4	7	6	5	7	7	1	1	2	1	–	–	–	–

Table 4.4: More details about the extracted spike trains per each batch

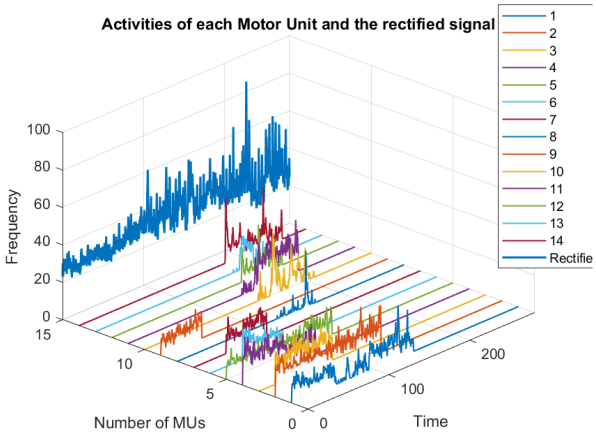
The results obtained from the tables 4.4 and 4.3 indicate that longer experiment and more number of batches, does not necessarily result in more extracted spike trains. The figure 4.3 illustrates the results of merged MUs after employing the decomposition and merging steps for our two selected subjects. Each color demonstrates a particular MU's smoothed firing rate (in Hz), and in the background, the rectified raw HDsEMG signal (in mV) is plotted.



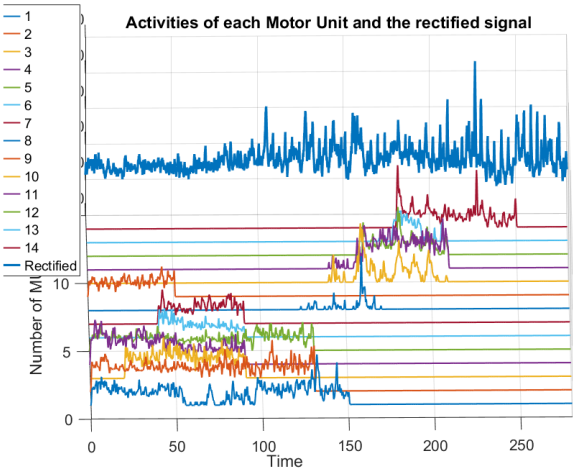
((a)) Merged MUs subject 4, upward view



((b)) Merged MUs subject 4, forward view



((c)) Merged MUs subject 10, upward view



((d)) Merged MUs subject 10, forward view

Figure 4.3: Two views of merged MUs for subjects 4 and 10

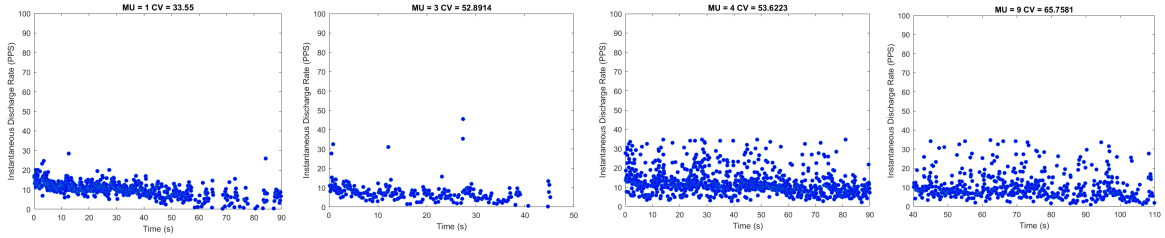
The MTT algorithm keeps increasing the stimuli intensity until the reflex occurs. Therefore, proceeding with the experiment, overall, the stimulation intensity would be higher, and more significant nociceptive reflexes would occur. We expected to see more activated MU when the reflexes were bigger. However, despite the substantial NFR reflex that appeared, the number of extracted MUs was mainly reduced. The plots of 4.3 also confirm the fact that at the beginning of the experiment, more MUs are activated, whereas this changes when the experiment proceeds. Two possible arguments for this observation will be provided in chapter discussion, section 5.2.

4.3 Good MUs Selection Results

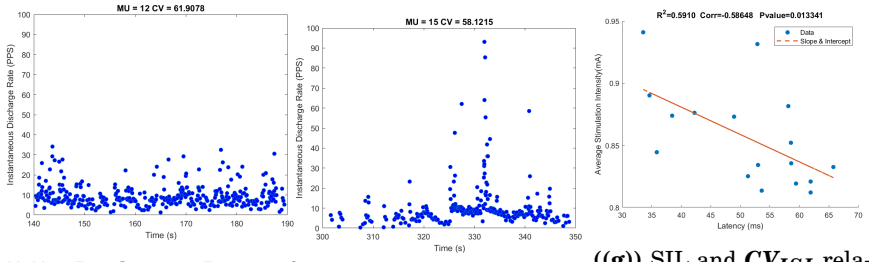
The table 4.5 reflects the result of qualified MUs with their $\boldsymbol{CV_{ISI}}$ values and also their $\boldsymbol{SIL}$ values to indicate the accuracy of the decomposition algorithm for each merged MU. The graph of Instantaneous Discharge Rate ($\boldsymbol{IDR}$) against the time for qualified MUs for subject 4 is also depicted in figure 4.4.

Subject	Qualified MUs	CV_{ISI}	SIL
Subject 4	MU1	33.55	0.9411
	MU3	52.89	0.9316
	MU4	53.62	0.8137
	MU9	65.75	0.8327
	MU12	61.90	0.8122
	MU15	58.12	0.8817
Subject 10	MU1	53.00	0.8343
	MU2	35.85	0.8446
	MU4	51.29	0.8253
	MU5	34.61	0.8903
	MU6	38.45	0.8740
	MU7	42.24	0.8763
	MU8	58.61	0.8357
	MU9	48.94	0.8732
	MU11	59.43	0.8194
	MU12	61.90	0.8210
	MU14	58.56	0.8522

Table 4.5: Qualified MUs with their CV_{ISI} and their SIL value



((a)) Discharge Rate of MU1 **((b))** Discharge Rate of MU3 **((c))** Discharge Rate of MU4**((d))** Discharge Rate of MU9



((e)) Discharge Rate of MU12 **((f))** Discharge Rate of MU15 **((g))** SIL and CV_{ISI} relation

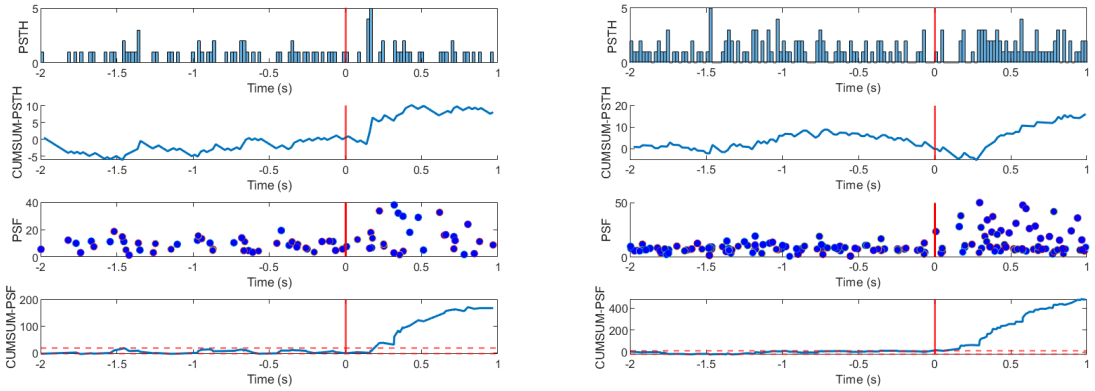
Figure 4.4: Good MUs extracted from subject 4

According to the table 4.5 and figure 4.4(g), the CV_{ISI} and SIL measures are negatively correlated (corr=-0.5864,Pvalue=0.01334,R^2=0.5910), which means that the better the MU is (less CV_{ISI}), the more reliable spike train it has (higher SIL value, obtained from the decomposition algorithm). Regarding the figure 4.4, the qualified MUs are those that their IDR plot against the time is aligned or partially aligned with a straight line. However, the selection method can

not distinguish between the extracted MUs resulting from the decomposition error and those with abrupt frequency changes because of their brief reflex instant. To avoid removing the MUs improperly, we will also visually inspect the activity of MUs, which results in qualifying some MUs despite having the $\boldsymbol{CV_{ISI}}$ greater than 55. (see table 4.5)

4.4 Offline NFR Detection in a Single MU

The process of NFR detection is assessed with the definition of CUMSUM-PSTH and CUMSUM-PSF [28],[27],[26],[17]. However, in our study, this method must be implemented only for the stimuli that induce reflexes. The NFR is a polysynaptic with-drawal flexion reflex that will elicit an excitatory reflex in the bicep femoris muscle. The slope change in CUMSUM-PSTH and CUMSUM-PSF plots for each MU must indicate the occurrence of an excitatory reflex which is desired. The figure 4.5 indicates the behavior of two qualified MUs from two different subjects.



((a)) PSTH and PSF of an excitatory MU(Sub4-MU6) **((b))** PSTH and PSF of an excitatory MU(Sub10-MU14)

Figure 4.5: PSTH, CUMSUM-SPSTH, PSF and CUMSUM-PSF. Bin width is 10ms, the pre-stimulus time is 2 seconds and the post-stimulus time is 1 second which contains the standard NFR time occurrence (150-250ms post-stimuli). The constant pre-stimulus mean (k-level) was subtracted from the data. The vertical red line shows the onset of stimulation and the two dashed lines in CUMSUM-PSF limits the activity of pre-stimulus within the $+\boldsymbol{\delta}$ and $-\boldsymbol{\delta}$.

As the figure 4.5 illustrates, both plots show an excitation after the onset of stimuli as the slopes of CUMSUM-PSTH and CUMSUM-PSF increase. It should be noted that the CUMSUM-PSF and CUMSUM-PSTH are the average behavior of MU, consisting of all of the stimuli which induce reflexes. From the CUMSUM-PSF plot, the reflex type (inhibitory or excitatory) and the reflex latency can be obtained. However, the NFR prevalence in a single MU is calculated by evaluating the $\boldsymbol{IDR}$ of MU regarding the window surrounding each stimulus.

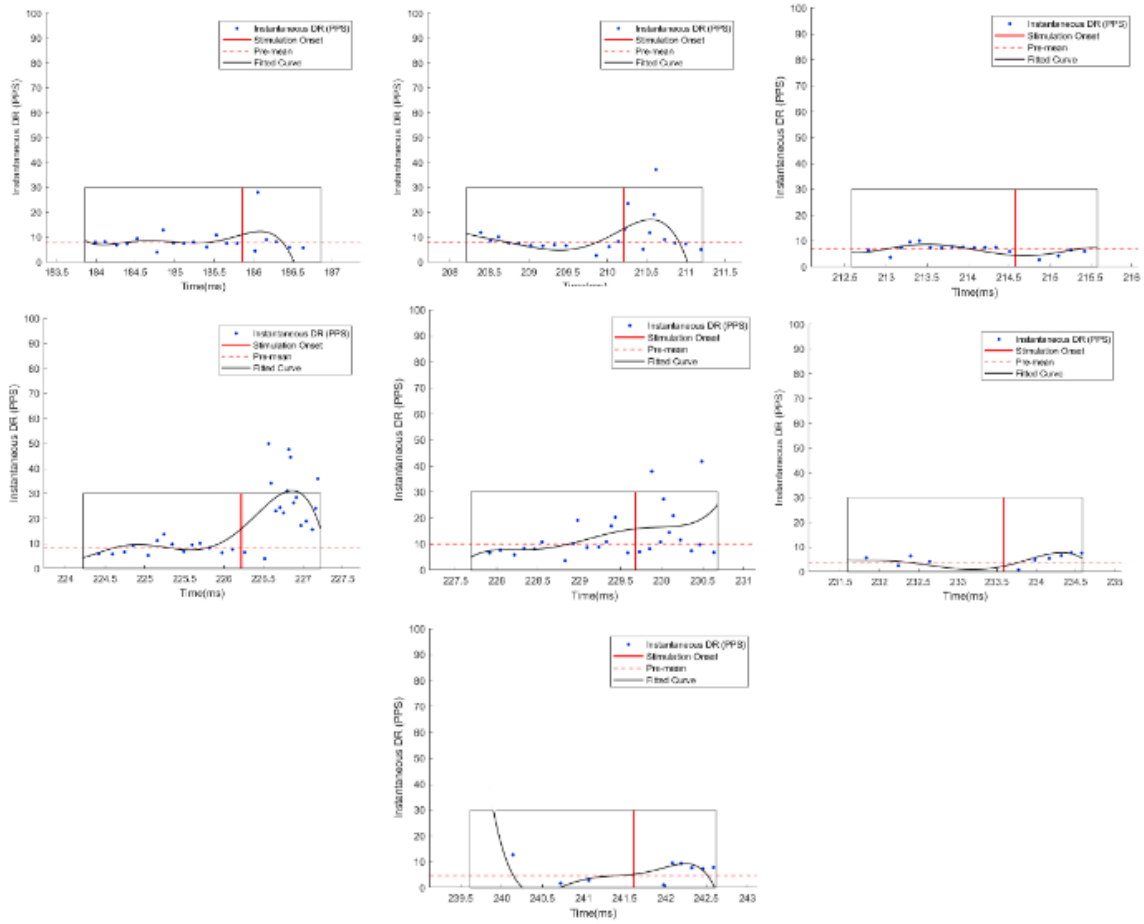


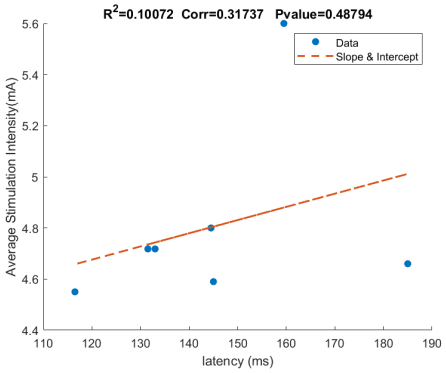
Figure 4.6: Each reflex stimulus contribution-Subject10-MU14. The plot of spike occurrence, the surrounding window around each stimulus (1 second post-stimulus and 2 seconds pre-stimulus), and the polynomial order 5 to fit the data and show the trend of the instantaneous frequency in both pre-stimulus and post-stimulus time are showed in the graph.

The number of stimuli that contribute to a reflex in each MU, the reflex prevalence, the reflex latency, the average stimuli intensity activating each MU, and the MU type are reported in the table 4.6. Two eligible subjects have 30 MUs in total after merging, regardless they are qualified or non-qualified MU. Among them, 19 MUs contain at least one stimulus that induces a detectable reflex ($\boldsymbol{Prevalence \neq 0}$ and defined) despite having no significant behavior. The rest may belong to MUs which do not have any spike occurrence in their post-stimuli course (**No post-stimuli IDR**), or they do not contain any stimuli inducing reflexes at all (**No reflex**). In conclusion, $\frac{\mathbf{19}}{\mathbf{30}} * \mathbf{100} = \mathbf{63.33\%}$ of MUs respond to our stimulation. More details are presented in the table 4.6.

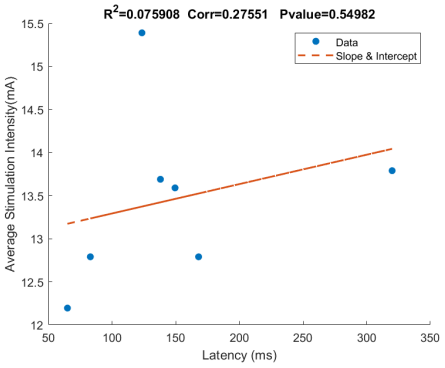
Results from figure 4.7 indicate that there is no linear correlation neither between the stimulation intensity and the reflex prevalence nor between the stimulation intensity and the reflex latency. Therefore, the reflex latency and how prevalent it is, are not influenced by the average stimulation intensity inducing that reflex.

Subject	MU	Num of reflexes	Prevalence	Latency (ms)	Stimulation Intensity(mA)	MU type
Subject 4	MU1	6	33.33%	–	5	No significant behavior
	MU2	6	83.33%	133	4.718	Excitation
	MU3	1	–	–	–	No post-stimuli IDR
	MU4	6	83.33%	116.5	4.55	Excitation
	MU5	4	–	–	–	No post-stimuli IDR
	MU6	4	75%	185	4.66	Excitation
	MU7	4	50%	145	4.59	Excitation
	MU8	6	50%	144.5	4.8	Excitation
	MU9	8	62.5%	131.5	4.718	Excitation
	MU10	6	–	–	–	No post-stimuli IDR
	MU11	4	–	–	–	No post-stimuli IDR
	MU12	4	100%	15	4.79	Excitation
	MU13	2	50%	–	7.2	No significant behavior
	MU14	1	0%	–	–	No significant behavior
	MU15	2	50%	–	5.6	No significant behavior
	MU16	1	100%	159.5	5.6	Excitation
Subject 10	MU1	3	66.66%	65	12.195	Excitation
	MU2	2	50%	168	12.79	Excitation
	MU3	0	–	–	–	No reflex
	MU4	0	–	–	–	No reflex
	MU5	2	50%	83	12.79	Excitation
	MU6	0	–	–	–	No reflex
	MU7	0	–	–	–	No reflex
	MU8	4	0%	675	–	Late Inhibition
	MU9	0	–	–	–	No reflex
	MU10	4	75%	660	13.59	Excitation
	MU11	4	100%	138	13.69	Excitation
	MU12	2	100%	320	13.79	Excitation
	MU13	2	50%	149.5	13.59	Excitation
	MU14	7	85.71%	123.5	15.39	Excitation

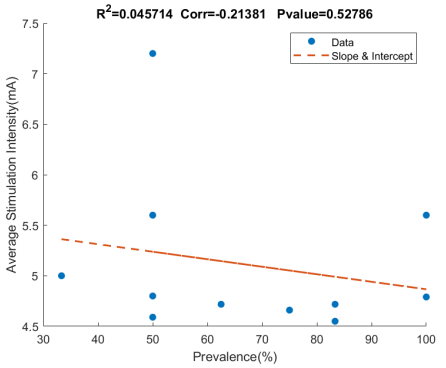
Table 4.6: Number of reflexes, Reflex Prevalence, Reflex Latency, Stimulation Intensity and the Mu type for all the MUs of both selected subjects.



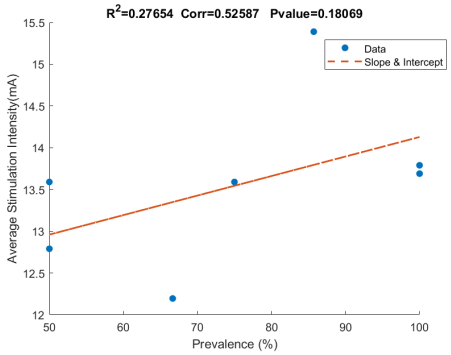
((a)) Stimulation-Latency relation,subject4



((b)) Stimulation-Latency relation,subject10



((c)) Stimulation-Prevalence relation,subject4



((d)) Stimulation-Prevalence relation,subject10

Figure 4.7: There are no significant linear correlation between Stimulation intensity with prevalence and latency. P>0.05 and R^2<0.5 in all of the cases.

One of the possible reasons why not all of the MUs elicited an excitatory reflex may lie in the error of the decomposition algorithm for that particular MU. To investigate the influence of decomposition error on successfully detecting an excitatory reflex, table 4.7 reports the SIL values for both MUs, which showed excitation (group=1) and MUs which failed to show the excitation (group=0). A Shapiro-Wilk test (figure 4.8) was applied to both groups to check whether the data is normally distributed.

	No Excitation	**Excitation**
SIL value (%)	82.82	94.11
	81.37	94.11
	82.06	89.11
	81.14	88.31
	81.94	86.23
	83.27	82.06
	81.22	95.20
	82.52	88.17

Table 4.7: SIL values for MUs which are classified as an excitatory MU and the rest that do not belong to the excitatory group,(these data are only for subject4)

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	reflex	Statistic	df	Sig.	Statistic	df	Sig.
SIL	.00	.180	8	.200*	.934	8	.558
	1.00	.211	8	.200*	.922	8	.445

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Figure 4.8: Shapiro-Wilk test

As for the both groups the $\boldsymbol{P-value > 0.05}$, we fail to reject the null hypothesis ($\boldsymbol{H_0 : F(t) = \phi(\frac{t-\mu}{\delta})}$) and we conclude that both groups are normally distributed. Therefore, the Two-sample t-test can be applied because of normally distributed data. The Two sample t-test (figure 4.9) indicates that there is a significant difference between the mean of the two groups ($\boldsymbol{P-value < 0.05}$). Thus, the null hypothesis is rejected.

Group Statistics

	reflex	N	Mean	Std. Deviation	Std. Error Mean
SIL	.00	8	82.0425	.78316	.27689
	1.00	8	89.6625	4.53820	1.60449

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
SIL	Equal variances assumed	12.100	.004	-4.680	14	.000	-7.62000	1.62821	-11.11216	-4.12784
	Equal variances not assumed			-4.680	7.417	.002	-7.62000	1.62821	-11.42670	-3.81330

Figure 4.9: The results of Two sample t-test

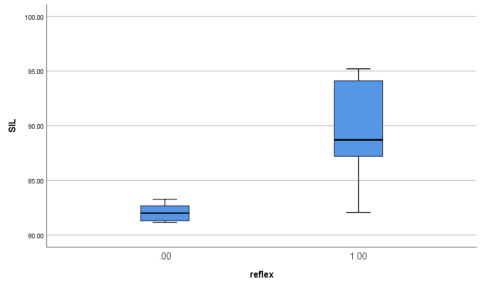


Figure 4.10: The Box-Plot to indicate the difference between the mean of two groups

These results conclude that the failure for detecting an NFR at single MU might be due to the errors which come up from the decomposition algorithm.

CHAPTER 5

DISCUSSION AND FUTURE WORKS

5.1 General Discussion

During this study, NFR at a single MU level was obtained non-invasively by recording the HDsEMG signals from the bicep femoris muscle. Online NFR detection was evaluated successfully while the MTT-NFR application executes. However, the different minor result in this part is that using the proposed setup, the NFR occurs later, around 150-250 ms post-stimuli rather than 90-150 ms, which was mentioned in the literature. This can be related to the height of our intended subjects which may decrease the Nerve Conduction Velocity (NVC) and thus influences the pace of responding to the stimulation [29]. It can either result from the different settings and equipment used in this particular study.

Furthermore, according to [30], between 8-15% of the subjects are non-responders to the sural nerve elicitation. However, in our study, the non-response rate is 40%, which is quite high. It should be considered that our study involved only a population of healthy young adults (mean-age = 25.7, std-Age = 1.8886). In people with chronic pain conditions, the NFR threshold is routinely reduced compared to healthy participants. Thus, our results concerning the presence of non-responders may not be generalized to populations with a history of chronic pain. We recommend for **future works** to explore a method for excluding the non-responder subjects before doing the long experiment's protocol. This can be done by doing the sural nerve stimulation initial testing to evaluate the chance of showing the NFR during the actual experiment. Besides, in some cases, the sural nerve stimulation is uncomfortable, which disables our subjects to continue the experiment until they pass the NFR threshold. This is indeed the opposite of the theory that the NFR is considered as an objective tool for pain assessment because in those cases, the subject felt an endurable pain although he did not pass his NFR threshold. For instance, the

majority of our subjects, regardless they showed reflex or not, mentioned that **"The electrical stimulation does not feel comfortable to stand until the end of experiment"**. **Future studies** can implement an alternative setting with stimulation over the foot's medial arch while the subject is standing. According to [30], this setting would lower the rating of participant discomfort and will elicit a larger NFR while the subject is standing. Thus, the NFR threshold obtained from this setting would be more indicative for pain assessment. Comparing the results of two different settings was not applicable for this study due to the limited time. With all the aforementioned obstacles, only two subjects were eligible to analyze in this research which did not provide significant validity to rely on the obtained results. **Future studies** can improve the accuracy of the results by expanding the dataset to include more eligible subjects.

5.2 Detailed Discussion In Decomposition Results

As we discussed in 4.2, the decomposition algorithm can not extract the MU spike train appropriately, especially at the end of the experiment. This can be explained in two ways:

1-The nature of MUs: One of the possible reasons for this phenomenon would lie in the existence of diverse MU types in a single muscle spindle. α motor neurons and the MUs themselves vary in size. Small α motor neurons innervate relatively few muscle fibers and form small motor units, which are called **slow motor units** and are important for activities that require sustained muscle contraction. Despite the small MUs, the large motor neurons innervate larger, more powerful motor units that are easily fatigued. These units are called **fast fatigable (FF) motor units** and are especially important for brief exertions [31]. According to the concept called **size principle**, MUs are activated in order of size from smallest to largest depending upon the required force. As the experiment proceeds, because of keeping the flexion position continuously, the subject becomes more fatigue. The fatigue would decrease the recruitment threshold for high threshold MUs which would result in activation of more fast-fatigable MUs [32]. These MUs stay active only for a short time, and their activity will suddenly drop. This would prevent the merging algorithm from merging the MUs at the end of the experiment successfully, and consequently, less number of merged MUs are extracted when the experiment is closer to the end.

2-Decomposition algorithm error The other possible reason for this phenomenon comes from the error of the decomposition algorithm. This algorithm performs a specific step in estimating a possible MU spike train, which takes the highest peak of the signal and compares the similarity with the rest of the signal to cluster the high peaks and low peaks in two groups. Therefore, when significant reflexes occur in the HDsEMG signal, the algorithm picks the high reflex and tries to explore a similar source to this peak. However, due to the brief reflex period, there is no similarity between the rest of the signal and the reflex. Thus, the algorithm iterates infinitely between the

high reflexes without finding any possible MU spike train. Consequently, this would reduce the number of extracted MUs when the reflexes are significant.

Relying on these explanations, we can not confirm that the decomposition algorithm extracts the MUs correctly. We strongly recommend for **future works** to explore the most relevant and appropriate decomposition algorithm for reflex studies.

5.3 Detailed Discussion In Offline NFR Results

NFR is a polysynaptic withdrawal flexion reflex in the ipsilateral limb that excites the flexor muscle groups to remove the elicited part from the nociceptive stimulus [33]. In this study, the bicep femoris muscle is contracted when the knee joint is flexed during the 20% MVC. Hence, the bicep femoris would behave as a flexor muscle, and it is expected that the stimulation over the sural nerve elicits an excitatory reflex in this muscle. According to results obtained from section 4.4, the excitation must also be observed from the graph of CUMSUM-PSF. However, this is not the case in all of the results. Sometimes an inhibitory reflex would appear, which can be explained by the lower discharge rate in that particular stimulus due to the subject's fatigue or wrongly stimulating the tibial nerve, which will induce an inhibitory reflex. However, the other possible reason for this observation could be the error of deficient extracted MU spike train from the decomposition algorithm, which might reduce the activity of MU in its post-stimuli course.

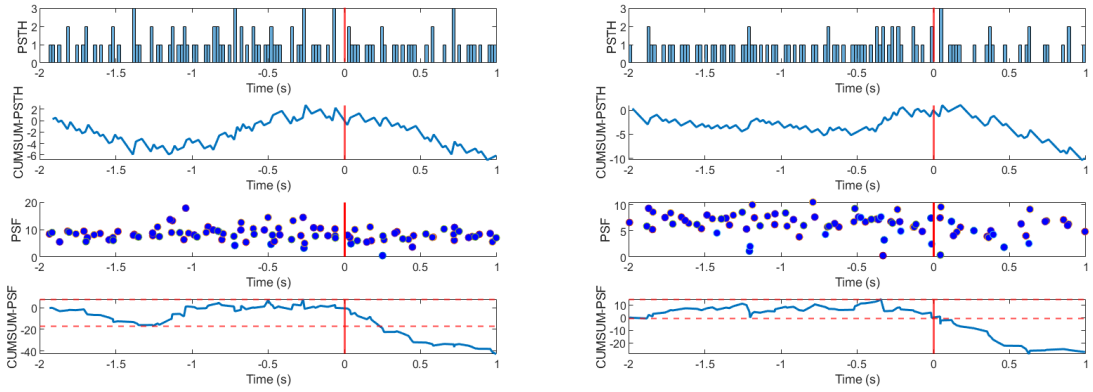


Figure 5.1: Two examples of inhibitory MU

CHAPTER 6

CONCLUSION

Chronic pain is a worldwide issue that affects a million patients. The Nociceptive Flexion Reflex is a reliable tool for studying pain which is correlated with the pain threshold. The lack of knowledge about the mechanism of nociception at the MU level leads to investigating the feasibility of NFR detection in a single MU.

Implementing a suitable and rational experiment setup contributes to both online and offline NFR detection. The combination of NFR detection algorithm using the NFR Interval Z-score with MTT method was executed successfully. Additionally, our proposed decomposition algorithm was capable of extracting single MUs for further offline NFR analyzing. The offline method was qualified to determine the NFR occurrence, the MU type, the reflex prevalence, and also its latency. However, there are still failures to explain the relationship between the stimulation intensities with the other reflex properties and also to explain the exact reason for an inhibitory reflex happening.

In conclusion, the study detected the NFR online in 60% of the subjects (excluding those who are not responders). 63.33% of the single MUs, extracted from two eligible subjects confirmed the NFR occurrence by employing the offline method. Furthermore, there was a significant difference in SIL-values between MUs that showed excitation and those that did not show. Overall, the decomposition algorithm accuracy might influence the chance of detecting an excitation in a single MU.

APPENDIX

A.1 Experimental protocol document

EXPERIMENTAL PROTOCOL

Evaluation of Nociceptive Flexion Reflex (NFR) via an Adapted multi-threshold tracking (MTT) Protocol

(November 2021)

Authors:

Sahel Akbari (S2569280), Luan Duong (S2236117)

Page 1 of the exprimental protocol

PROTOCOL TITLE: Evaluation of Nociceptive Flexion Reflex (NFR) via an Adapted multi-threshold tracking (MTT) protocol.

Short title	NFR detection using the MTT protocol
Date	10-5-2021
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Content of experimental protocol

I. Background

Chronic pain, defined as the unusual sensation of the pain continuing beyond the normal healing phase [1], has received much attention with its adversity to human health. However, the clinical assessment for chronic pain is still complicated and difficult due to the large variation of the patients' sensations to pain. Quantifying this sensation is thus crucial in the diagnosis and long-term treatment for chronic pain. One of the possible methods to measure pain is using Nociceptive Flexion Reflex (NFR), or R3 reflex, which is believed to be relatively stable, stimulus-induced and physiological spinal response. However, NFR does not always appear with pain unless small diameter, myelinated A-delta fibers are activated. This threshold for maximal reflex response is considered similar to the threshold for chronic intolerable pain. A number of studies have also confirmed that there is a linear correlation between this NFR threshold and the subjective perception of pain [2]. Hence, our study focuses on establishing an analysis framework to determine the NFR threshold at the spinal cord level, specifically for a single motor unit.

Previous studies confirmed that inducing the lateral malleolus in the location of the sural nerve pathway while the subject maintaining a standardized angle of knee flexion will generate a nociceptive flexion reflex (NFR) in the bicep femoris muscle [3]. However, our research wants to further confirm and analyze this reflex appearing at a single motor unit level. To achieve this purpose, we utilize the recording of high-density surface EMG (HDsEMG), which has been used to investigate and decompose the muscle activity into each motor unit [4]. Thus, our research will be done in an experimental context to confirm the feasibility of using HDsEMGS in detecting a single motor unit activity during pain threshold navigation. The participants in the experiment are stimulated at the lateral malleolus, and the HDsEMG signals will be recorded from the bicep femoris muscle to detect NFR. Stimulating the sural nerve will generate two types of responses. The first one (RII) is of short-latency, low threshold, and corresponds to a tactile reflex. The second one (RIII) is of longer latency and higher threshold and corresponds to a nociceptive reflex [2]. The Multi-Threshold-Tracking (MTT) algorithm is used on HDsEMG data to determine the NFR threshold and the value of the next stimuli. If the threshold has been reached, the amplitude of stimuli decreases, and when the reflex is not detected, the amplitude of the next stimuli would be higher. The MTT keeps tracking the threshold value when it changes during measurement

II. Objectives

To confirm the feasibility of using HDsEMG in quantifying the pain sensation among patients, we need to achieve the following purposes from the experiment:

- The ultimate purpose is to investigate the response threshold in the single motor unit (MU). To achieve this primary objective, several subjects will be asked to participate in the electrical stimulation experiment and the HDsEMG signals of their responses will be recorded. The retrieved HDsEMG data will be decomposed into single MUs for each individual participant, and then will be further analyzed to understand the pain response activities. The protocol will be designed to support this main objective.
- The study then can be divided into two stages in the MU analyzing process: to estimate the distribution of the MU's reflex gain, and to identify the NFR threshold in the individual unit scale. For the first aim, we will classify MUs based on their behaviors, in order to investigate the synaptic organization at the spinal level responding to the pain irritation. The second aim in estimating the individual MU's pain response threshold will be tackled by the offline Multi-Threshold-Tracking (MTT) algorithm.

III. Overall study design and plan

This experiment will take place at the University of Twente within one session per participant. Each session will take approximately 2 hours, including the time spent on preparation and round-up. Each session's procedure will be presented as follows. Please note that the duration of each phase is provided as an estimation. More detailed information can be found in the section "Experiment Procedure".

1. **Introduction (5 minutes):** The subject will be provided with information about the experiment and asked for consent to participate in the study.
2. **Preparation (15 minutes):** The subject will be connected to the EMG equipment and the nociceptive stimulation device (NOCITRACK).
3. **Familiarization (10 minutes):** The subject will be familiarized with the stimuli. In this phase, the subject can get used to determine whether a stimulus exceeds the stimulation threshold, and learn how to behave during the experiment.
4. **Experiment (60 minutes):** The experiment, in which HDsEMG signals will be measured for a variety of nociceptive stimuli to investigate the occurrence of Nociceptive Flexion reflex (NFR).
5. **Round-up (10 minutes):** The subject will be disconnected from the equipment and debriefed.

IV. Study population

A. Intended research population and recruitment strategy

Only healthy subjects older than 18 years old and below 60 years old who volunteered for the testing are eligible for recruitment in the present study. In addition, the participants do not have shown a history of acute pain in any muscles in their leg. However, due to the nature of the study which is not biased only for a specific community or population, there are no vulnerable participants for this study. There are 10 participants expected for this program. Participants are recruited from our research groups or students from our university. However, to balance the demographic properties (gender/age), random selection could be used. To include the participants who are volunteers to participate in this study, we will invite people from our research group or within our close contacts. Furthermore, an email with an introduction to the study together with a consent form will be sent to the volunteers.

B. Inclusion criteria

1. With a signed, written informed consent (it is necessary that subjects also read the subject information form),
2. Age between 18 and 60,
3. Being healthy without a medical history of acute pain or related muscles,
4. Negative Covid-19 test result or QR-code of having two doses of vaccination.

C. Exclusion criteria

1. With refusal during the study,
2. Skin problems at the site of the pain sensitivity measurement,
3. Language problems,
4. Diabetes,
5. Implanted stimulation device,

6. Pregnancy,
7. Pain complaints at the time of the experiment or unable to undergo the painful measurement,
8. A medical history of chronic pain,
9. Acute pain at the time of the measurement (NRS >3),
10. Excessive consumption of alcohol (>4 units of alcohol) or within 24 hours before the experiment,
11. Consumption of pain-blocking or muscle relaxing medicines (methocarbamol, gabapentin, etc.) within 24 hours before the experiment,

V. Experiment procedure

A. Material and Methods

1. HDsEMG signal

High-density EMG signals are recorded continuously with a sampling frequency of 2048Hz using an 8x8 grid-array electrodes (CE-certified) located in the bicep femoris muscle. HDsEMG signals are amplified with a TMSi 136-channel Refa EMG amplifier (CE-certified). The HDsEMG signals and the trigger codes are recorded on a dedicated desktop computer running TMSi Polybench software. During the experiment, the subject lay prone on a padded table while he is positioned so that his knee is bent at a 45-degree angle from the center of the bar. The subject's position creates less interference to the HDsEMG electrodes as much as possible and has been confirmed to be used in generating NFR. The subjects are asked to press the response button and hence receive stimuli.

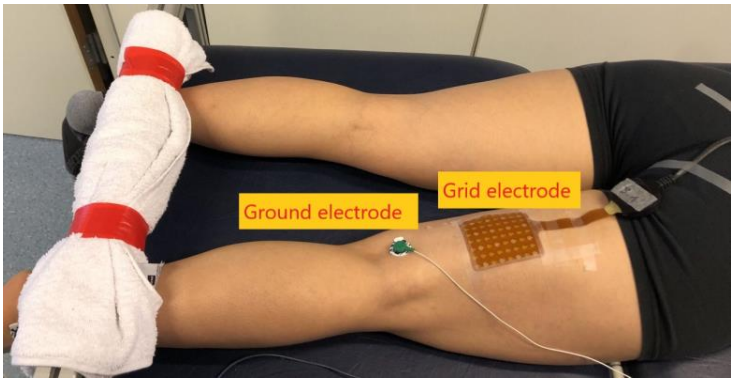

Figure 1: Location of the HDsEMG recording electrode and the position of the subject

2. NociTRACK AmbuStim stimulator

NociTRACK AmbuStim stimulator will apply interchangeably and randomly two sets of 150 stimuli (in total 300 stimuli) with different pulse trains. The subject only receives the stimuli when he/she presses the button on the stimulator, and thus the subject is always able to stop the experiments whenever he/she feels uncomfortable. Each stimulation from the first set consists of five pulses with a duration of 1ms and an interpulse duration of 3ms, while each stimulation in the second set consists of seven pulses with the same duration as the first set. Previous studies showed that this stimulation setup is adequate to generate an NFR in the desirable location. By doing so, NFR is defined as a mean EMG response in the 90–150-ms post-stimulation interval that exceeded mean EMG activity during the 60-ms prestimulation baseline [5]. The stimulus types are selected according to the MTT algorithm randomly but with the same number of times. The values of the next stimuli are also decided by the MTT algorithm. The stimuli will increase for a

step size of 0.4 mA (or decrease by a step size of 0.4 mA) depending on if the reflex responses are not (are) detected.

All of the stimuli are applied to the lateral malleolus in the location of the sural nerve, using bipolar Ag/AgCl electrodes, where the anode (red) is placed approximately 2cm superior to the cathode (black).

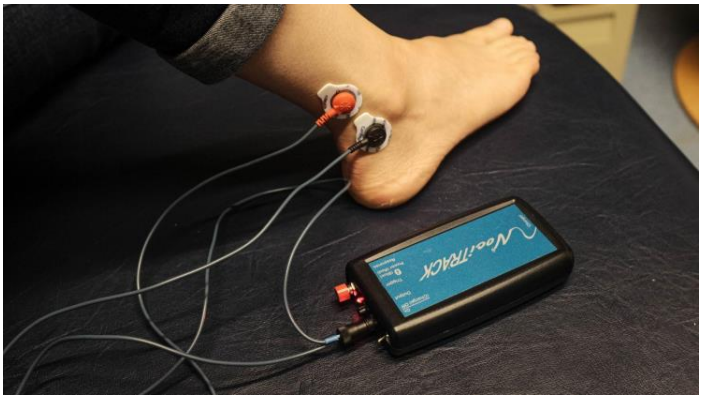

Figure 2: Location of the stimulation electrode and the NociTRACK AmbuStim stimulator

3. Multi Threshold Tracking (MTT) algorithm

The Multi-Threshold-Tracking (MTT) algorithm is used on the high-density EMG (HDsEMG) data to determine the value of the next stimuli. The subject first decides his/her initial amplitudes of stimulation where he/she can feel the excitation (for both sets of stimulation). These values are set as the initial thresholds for NFR. During the experiment, if the reflex is detected from HDsEMG, the amplitude of the next stimuli will decrease and the NFR threshold is updated as this stimulation value; and if the reflex was not recognized, the amplitude of the next stimuli would be higher to activate the NFR. The amplitude of the stimuli would change with the step size of 0.4 mA. The MTT keeps tracking the threshold value when it changes during the measurement.

B. Experiment execution

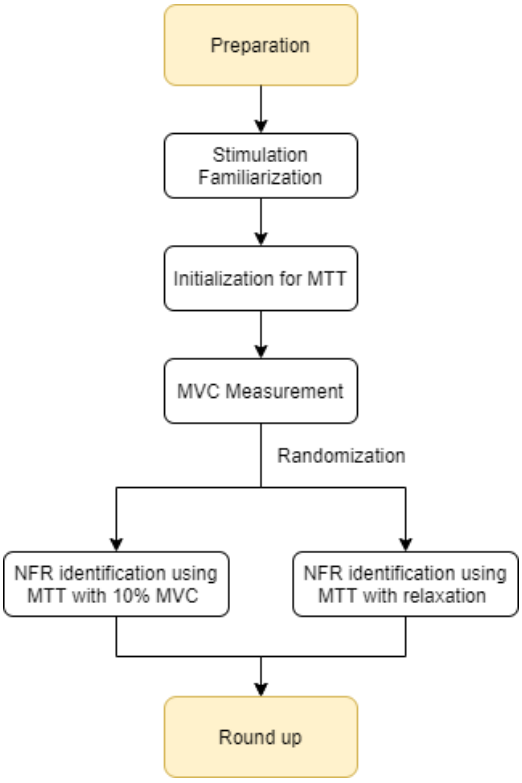

Figure 3: Overall flow chart of the experiment

1. Preparation of the subject for the experiment

Before the arrival of the subject, all experimental equipment is ready and easy to reach: the padded table will be placed in the measurement room, HDsEMG 8x8 grid electrodes are cleaned and sanitized with alcohol, the electrode gel is applied smoothly between the electrode surface and the active layer, NociTRACK AmbuStim stimulator has to be charged before the session starts and all of the cables (trigger cable and amplifier cables) has to connect to the PC and the other devices. More information on the setup can be found in the ***Appendix.***

Before starting the procedure, the subject will be asked to read and sign the informed consent form. The researcher will evaluate the subject's eligibility for participation according to the inclusion criteria. The subject is strongly recommended to wear shorts during the experiment to prevent the artifact from touching the electrode surface with his clothes. The location of the simulation (lateral malleolus) and the recording site (bicep femoris) are cleaned with alcohol and the scrub gel is applied to decrease the skin resistance. In addition, the subject will be asked to shave the location of the stimulation and recording site to further eliminate the artifacts in the signals

According to the new Covid-19 rules and regulations (November 2021), the subject and the researcher have to wear a face mask during the experiment and keep a 1.5-meter distance. The subject is also required to show Corona pass before the experiment begins.

2. *Familiarization and initialization of the stimuli*

At the beginning of the experiment, the subject's ID number will be saved. LabView interface of the system will be connected to the stimulator. The first step of the experiment will serve for the subject to get acquainted with the stimulation process. The magnitude of stimuli will gradually increase when the subject presses the button, but will not exceed over 20mA (only for familiarization, in the MTT processes is 30mA). The subject can release the button when he/she gets familiarized with the stimuli and the process will stop automatically.

After the familiarization, the procedure will continue to the initialization of the experiment. Similarly to the familiarization, the subject has to hold the stimulation button until he can feel tickling at the stimulation site. However, this value of stimulation will be defined as the initial threshold for NFR for the next step of the experiment. It should be noted that as we have two sets of stimuli, the subject needs to repeat this step twice to set the initial values for both sets. After the subject finishes this step, another LabView's interface is opened to start the experiment.

3. *NFR threshold determination*

During the experiment, we will assess the detection threshold for pain in the subject's hamstring. This will be done by stimulating the lateral malleolus, in the location of the sural nerve pathway with weak electrical stimuli using bipolar Ag/AgCl electrodes. The muscle activity from the bicep femoris will be recorded using a 8x8 grid-array HDsEMG electrode to determine the NFR. The measuring electrodes are implemented at the beginning of this step and last until the session is completed. The ground electrode will be placed on the subject's knee. This experiment consists of two main stages and the second stage has two different setups, simply to compare the differences in protocols and to decide which method is more optimizing in analyzing signals. The subject will be arbitrarily assigned to each set-up randomly depending on our analysis. The experiment stages are as follows:

<u>Determining the maximum voluntary contraction (MVC) of the subject</u>

To measure the maximum voluntary contraction force, first, the subject will be asked to contract his/her bicep femoris muscle three times by pushing up his/her heel against the bar with the maximum force. The subject is positioned by lying prone on the table with his/her knee touching the table surface with the knee flexion angle of 45 degrees against the center of the bar. The MVC is calculated by taking the average of HDsEMG's amplitude in all three contractions.

<u>Determining the NFR threshold</u>

This step of the experiment has two different setups:

a. *The subject has to keep the 10% of MVC during the whole experiment*:

   After the subject finished the previous step, a new window will appear with a line indicating the 10 percent of his MVC. However, if the subject is assigned to this setup, he/she will need to keep his/her contraction to reach this percentage until the end of our experiment.

b. *The subject has to be in a relaxed and natural position during the whole experiment:*

   Different from the previous case, the subject will be asked to relax during the whole experiment. The pre_NFR data (contracting muscle to generate the MVC, without applying the stimuli) will be used as a validation for the rest of the experiment.

Subjects are randomized into different cases, then the first stimuli will be given based on the previous initialization step. The LabView shows the two sets of the stimuli (each has 150 stimuli) separately in two different screens. The stimuli will be given after every 5 seconds, and randomly between the two sets. The intensities of stimuli continuously increase/decrease if NFR cannot/can be detected using the MTT algorithm. The maximum of stimuli, however, cannot be over 30mA.

4. Experiment round-up

After completing the experiment, two steps will be taken to finish the session:

Data storage confirmation

It is important to confirm the data collection after the experiment so all the files are saved in an accessible location. The researchers can confirm the subject understood that he/she can always alter or not save his/her data through the research, and announce that the dataset will be stored and manually deleted after the study under the General Data Policy Regulation.

Rounding-up and clean

After saving the data files, all of the devices (Stimulator, PC, Amplifier, ...) have to be turned off and disconnected from the subject. The electrodes can be removed from the subject's body. The stimulation electrodes (Ag/AgCl) are disposed of and discarded after the experiment. The recording grid electrode will be cleaned with alcohol and reused in the next experiments. The subject can change his/her clothes and take a shower downstairs (ZH-109) if he/she wishes. He/she also has the opportunity to receive the result of the data analysis after the experiment if he/she explicitly indicated in the consent forms.

VI. Handling of adverse events

A. The general handling

With the selection of the subjects, no adverse effects are expected on the participants. The procedure is the standard procedure that has been carried out in the BSS group. The stimulation is only activated when and only when the participant pressed the button. Besides that, "stop" button is always placed on the interface to immediately cease the experiments.

B. In case of serious unexpected adverse events

In the case of serious adverse effects, the phone number of the technician of the laboratory and the emergency section of the University of Twente are provided on the lab's wall in front of the subject's sight. In addition, the laboratory is equipped with a fire extinguisher capsule. A first aid kit is also available in the laboratory.

Reference used in the Experimental protocol

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Appendix of the experimental protocol

A. Required Materials

Components	Quantities	Information
Computer	1	One computer with LabView 2014 SP or higher, MTT-EMG stimulation software (build in LabView) and with Matlab 2020 or higher, TMSi-REFA Polybench Designer, and a custom-made TMSi-REFA Polybench recording application. The computer should have at least the following specifications: - Windows 7 64-bit, or higher - Intel Core i5 1.6 GHz, or higher - 8 GB RAM, or more - Bluetooth adapter
Stimulator	1	NociTRACK AmbuStim single-channel stimulator, capable of generating a minimum current of 0 mA and a maximum current of 30 mA.

Trigger generator	1	Arduino-based trigger generation system, which can be connected to the computer (input) via USB A to B cable, connected to the NociTRACK AmbuStim stimulator (output 1) via a BNC cable and connected to the EMG amplifier via a DB25 parallel cable
USB A to B cable	2	1 Cable for connection of the trigger generator to the computer, 1 Cable for connection of the TMSi fiber-to-USB converter to the computer
BNC cable	1	Cable for connection of the trigger generator to the NociTRACK AmbuStim stimulator. Should have a length of at least 2 meters
Parallel cable	1	Cable for connection of the trigger generator to the EMG amplifier. Should have a length of 1-2 meters
Stimulation electrode	1	(one pair of bipolar electrodes) Ag/Cl cathode (black) is placed between the outer ankle and the heel
Ground electrode	1	Ag/Cl anode (red)
Amplifier	1	TMSi_Refa, 128 channels amplifier
Medical power supply	1	TMSi_Refa power supply with on-off switch, to supply electricity to the amplifier
Fiber-to-USB converter	1	TMSi_Refa optical fiber-to-USB converter
Optical fiber	1	TMSi_Refa optical fiber used to communicate between the amplifier and the computer via the TMSi_Refa fiber-to-USB converter
Measuring electrodes(HDsEMG electrodes)	2	Set of 8x8 semi disposal grid electrode
Electrode Gel	1	Sufficient layer of Gel between the measuring electrode surface and the active layer
Comfortable chair	1	Comfortable chair for participants to relax during the experiment. The chair should be able to move the footrest

Tissues and Towers	10	For the subject to use in cleaning after the experiment

B. Step-by-step Protocols

Preparation of experimental setup and materials

a. *General Preparation*

Inform the subjects to bring shorts or wear pants which are easy to take off.

b. Material Preparation

Before session, make sure that the following materials are ready and easy to reach:

- Arm chair (charged)
- HDsEMG 8x8 grid electrodes and amplifier cables
- NociTRACK AmbuStim stimulator (charged) of TMSi
- Bipolar stimulation electrodes ((Ag/AgCL) of TMSi
- Desktop
- Scrub gel
- Trigger device

c. Connect

Before session, make sure that the equipment are connected:

- Computer to TMSi_Refa Amplifier
- Stimulator cables to Nocitrack
- Measurement electrode cables to TMSi Amplifier
- Trigger cable to amplifier
- Trigger cable to Nocitrack

Experiment execution

d. Reception of the subject

Meet with the subject at the entrance of the lab. Be present at the entrance 10 minutes before the start of the session. Give the subject a hard copy of the information letter and make sure that they are eligible for the inclusion criteria.

e. Preparation of the subject for the experiment

- Explain to the subject what is going to happen during the experiment:
- Use Scrub gel and a tissue to clean the measurement and stimulation sites
- Scrub the electrode gel between the measuring electrode and the active layer
- Explain to the subject where the electrodes for simulation and recording are placed (In lateral malleolus and bicep femoris)

Familiarization and initialization of the stimuli

a. In the beginning, explain to the subject that stimulation will be made for familiarization of stimuli procedure:
    - "The first measurement will just serve to get acquainted with the stimulation process. The magnitude of stimuli will increase when you press the button. However, it is not possible to have stimulation larger than 20mA so you can be relaxed".
    - "You can release the button when you get familiarized with the stimuli. If you release the button, the stimuli will stop automatically"
b. Save the subject's ID number and press 'Connect' in the LabView interface to connect to the stimulator. When the subject is ready, start the familiarization via the LabView interface by pressing 'Stimulate' and tell the subject that he/she can start by pressing the response button.
c. After the familiarization, we can continue to the initialization of the experiment. Explain that:
    - "The second stimulation will be served to determine the initial reflex threshold. Thus, you have to hold the stimulation button until you can feel tickling at the stimulation site. This threshold will serve as the initial level of stimulation for the next step of the experiment. We have two sets of stimulation for this experiment so you will need to do this twice. "
d. Press 'Start' and record the initial reflex threshold (twice). After the subject releases the button, another LabView's interface is opened and the "Start Experiment" button on the LabView can be pressed.

The experiment

Determining the maximum voluntary contraction (MVC) of the subject

a. Explain the experimental procedure to the subject:
    - "We want first to determine the maximum contraction of your muscle. You first need to contract your bicep femoris muscle several times. EMG signal will be recorded from these contractions"
    - "Then, a new window will appear with a line indicating a certain percentage of MVC. You will need to hold your contraction to reach this percentage, and you need to keep until the end of our experiment (through the next phase of recording NFR)."
b. Open and run the Matlab interface.
c. Indicate the subject can contract the muscle, and press continue after the subject finishes contracting. Repeat for several times.

Recording the NFR threshold

Patients can be asked into do **one** of the following guideline:

a. A new interface came up with a certain percentage of MVC line shown on the graph. Ask the subject to hold the contraction until the intensity of the EMG reaches this line. Then tell the subject to keep this contraction till the end of the next step.
b. Ask the subject to keep the leg relaxed on the padded table.

Then :

a. Explain the experimental procedure to the subject:
    - "Stimuli will be given to see if a NFR can be detected. The stimuli will be given after every 5 seconds and the intensities continuously vary, either decreasing or increasing depending on if NFR can be detected (but the maximum of stimuli cannot be over 30mA). There will be two sets of total 300 stimuli."

- “The MTT algorithm will follow the amplitude at which a reflex is given and the HDsEMG signal will be recorded into separated data.”
- “You should hold the response button as long as possible. When you feel uncomfortable, you can always release the button and the stimuli will be stopped.”
- “Each experiment will take approximately in 30 minutes”

b. Press “Start Experiment”. Indicate the subject may now press the button and start the Procedure.
c. Press the “Stop” button in LabView to stop the program. Press the “Stop Recording” button in the recording application.

Experiment round-up

Data storage confirmation

a. Confirm the HDsEMG data is recorded and saved in separate text files.
b. Inform the subject: “The experiment was completed successfully.”

Rounding-up and clean

a. Turn-off the stimulator and disconnect the subject from all cables.
b. Instruct the subject:
   - “You can take off the electrodes.”
   - “The smaller electrodes can be thrown in the garbage, the bigger electrodes you can give to me.
   - “In case it is necessary, you can change back downstairs in the shower, (ZH-109)
c. Ask the subject if he/she would like to be informed about the result of the experiment and provide the contact information in case that he was eager to receive the results.
d. Put all the equipment back and recharge the armchair and the stimulator.

APPENDIX B

ABBREVIATIONS

Symbol	Description	First use
NFR	Nociceptive Flexion Reflex	Sec 2.2
HDsEMG	High-Density-Surface-EMG	Sec. 2.4
MU	Motor Unit	Sec. 2.4
MUAP	Motor Unit Action Potential	Sec. 2.4
MTT	Multi-Threshold-Tracking-Algorithm	Sec. 2.3
MVC	Maximum-Voluntary-Contraction-Force	Sec. 3.1.3.5
BSS	Blind-Source-Separation Decomposition Algorithm	Sec. 2.5
PSTH	Peri-Stimulus-Time-Histogram	Sec. 3.6
PSF	Peri-Stimulus-Frequencygram	Sec. 3.6
CUMSUM – PSTH	Cumulative Sum PSTH	Sec. 3.6
CUMSUM – PSF	Cumulative Sum PSF	Sec. 3.6
$\boldsymbol{CV_{ISI}}$	Coefficient of Variation-Inter spike Interval	Sec. 3.5
NS	Nociceptive Specific Neurons	Sec. 2.1.1.2
WDR	Wide-Dynamic-Range Neurons	Sec. 2.1.1.2

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