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Microneurography: Recordings from single neurons in human peripheral nerves

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Over the last 50 years, the technique of microneurography has provided us with valuable insights into activity in human nerves. Microneurography allows us to record from the axons of single neurons in various peripheral nerves across the body. This approach has provided a wealth of information on peripheral nerve signals, especially the encoding of touch in mechanoreceptive afferents in humans. As these single neuron recordings are performed in conscious, healthy human participants, their activity can be directly linked with the resultant perceptual processes. This chapter will focus on how to setup, use, and apply microneurography, to make single unit recordings from mechanoreceptive afferents to study the sense of touch. Only a handful of laboratories in the world carry out single unit microneurography, due to the many challenges faced when recording from human nerves (e.g., ethical, practical, mental), and it takes extensive training to become competent in the technique. The co-founder of the technique (Åke Vallbo) rightly states that the method is safe, as long as the experiments are performed with care and consideration, and he emphasizes that a highly considerate attitude is required during microneurography [1]. Overall, we will explore how microneurography developed to the practice it is today, how to use it safely and effectively, and provide avenues for future development and investigations with the technique.

Key words: Microneurography, single unit, mechanoreceptor, mechanoreceptive afferent, tactile, touch, human, peripheral nerve

1. INTRODUCTION

“Microneurography is, in principle, a very simple technique, but in practice is quite demanding.” Åke Vallbo (2018) [1]

Microneurography began in 1965 at the University Hospital in Uppsala, Sweden, where Karl-Erik Hagbarth and Åke Vallbo established a technique where axonal activity in human peripheral nerves could be registered using percutaneously-inserted, metal, needle electrodes [2,3]. It was not the first time that human nerves had been recorded from, yet previous attempts involved other approaches, such as surgical nerve exposure [4] or using glass electrodes [5]. Although technology for data acquisition and processing has advanced since these early recordings (e.g., computers), the practical aspects of the approach (electrodes, amplifiers) remain virtually unchanged. This is in part due to the attention to detail and dedication of these two scientists. They tested and refined the technique extensively on themselves, assessing various nerves and electrode types to be used for recording. The initial excitement of hearing their own neuronal activity – albeit it an extremely weak signal – must have been extraordinary. Still to this day, microneurographers take great pleasure and privilege in listening to such responses. Hagbarth and Vallbo went through a long process of overcoming conceptual, medical, ethical, and technical obstacles including electrode types and insertion techniques, as covered in the comprehensive history documented by Vallbo [1]. Multi-unit microneurography (e.g., recording efferent sympathetic activity [6]) is conducted in a number of laboratories across the world, where the activity of many axons is recorded simultaneously, while only a small number of laboratories conduct the much more challenging approach of recording from a single axon.

Microneurography is a highly manual skill; there are many technically-demanding factors to consider, and it takes years to learn to practice it safely and effectively (Note 4.1). Additionally, there is a human being at the end of your electrode, making interpersonal skills and effective communication with the participants critical to its success (Note 4.1). Thorough training is essential in learning microneurography, where it is best to work for a number of years in an established group [1] to learn how to conduct the technique efficiently and safely, and there are always new situations and difficulties that are encountered. It is a ‘clean’ procedure, but it is not conducted under sterile conditions, as there is no surgery or cutting involved. Rather, a fine needle electrode is gently manually-inserted through the skin of a participant by light pushing. This is usually pain-free, although occasionally, the electrode may generate a sensation of pressure on the skin, which occasionally causes some discomfort, but this may be avoided by shifting the insertion site a few millimeters. Once the electrode has been successfully placed within a nerve, single unit responses are searched for from individual myelinated A β or unmyelinated C-fiber axons (Note 4.2).

There are many steps to consider in a microneurography experiment, from a well-organized experimental set-up to neural signal analysis (Notes 4.1, 4.3-9). There are also many stages where the technique can fail to generate usable data, which is one of the reasons so few single unit microneurography research groups exist. One issue before starting any experiments is to have ethical approval. This is an extremely important step, for safety of both the participant and experimenter. As the skin barrier is

crossed, microneurography is treated as an invasive approach that poses some level of risk (e.g., transmission of infection, tissue damage), which often entails high levels of ethical consideration, including medical surveillance, auditing (e.g., of consent forms, data collected), and insurance. Here we aim to outline the potential ethical and technical difficulties encountered during microneurography, as although it can produce insightful data, it is notoriously challenging.

Many peripheral nerves can be accessed, yet some are easier to locate and achieve a stable intraneural position for recording (e.g., the median nerve at the wrist is superficial, relatively large, and at a clearly defined anatomical location Notes 4.10-11). Deeper and smaller nerves, such as the antebrachial nerves innervating the forearm, can be located, but this is extremely challenging (Note 4.11). Figure 1 shows a diagrammatic representation of many of the nerves that have been accessed in microneurography. Some nerves, such as the median and peroneal, are commonly used, but for others, such as the nerves in the face, only a few of papers have documented recordings (e.g., [7]).

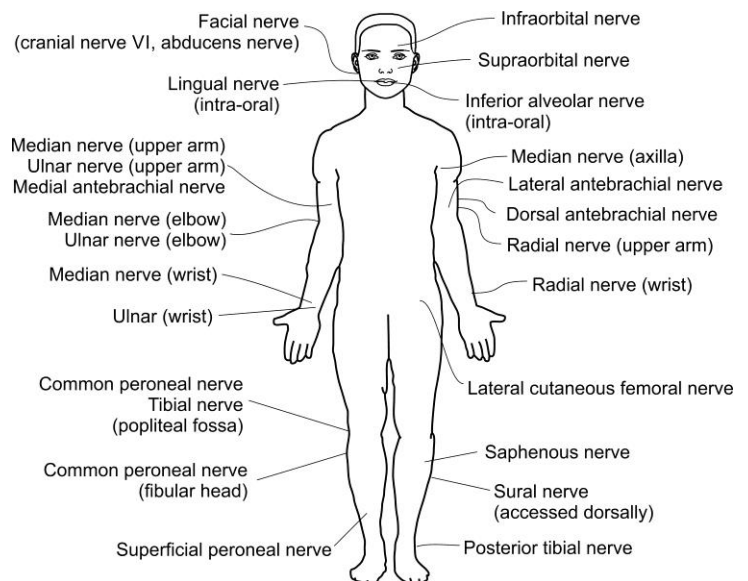


Figure 1: Nerves typically accessed in single unit microneurography.

The median nerve of the arm and the peroneal nerve of the leg commonly accessed in microneurography, as these are large nerves, relatively easy to find, and have many sensory afferents. Note that a lot of microneurography is carried out on the left side of the body and these locations are for demonstration. Example references for each nerve: facial (cranial nerve VI, abducens) [8], supraorbital [9], infraorbital [7,10,11], lingual [12–15], inferior alveolar [14–17], median - axilla [18], upper arm [19–26], elbow [27,28], wrist [18,25,26,29–32], ulnar - upper arm [20,33,34], elbow [35], wrist [18,32,33], radial - upper arm [36–42], superficial branch at the wrist [25,32,35,43,44], lateral antebrachial [45–52], dorsal antebrachial [45–48], medial antebrachial [20], lateral cutaneous femoral [53], common peroneal (fibular) [35,36,54–61], superficial peroneal [62], tibial [35,60,61,63,64], posterior tibial [65], saphenous [66,67], sural [68,69].

Single unit microneurography includes the recording of individual afferent mechanoreceptive, thermoreceptive, and nociceptive signals, as well as muscle and joint afferents (see also other chapters in this book). Even some single unit efferent sympathetic [70,71] and motor [72] recordings have been published. However, the majority of single unit studies have focused on cutaneous mechanoreceptive afferents and the sense of touch in the hand, which is predominantly conveyed by thickly myelinated, A β axons (Note 4.2) [for a classic review, see 10], although mechanoreceptors in hairy skin have also been investigated [e.g. 11–14]. There is some single unit work on cutaneous thermoreceptive and nociceptive afferents, predominantly from unmyelinated C-fibers [for a review, see 15] or occasionally thinly myelinated A δ afferents [75,76] (Note 4.2). Further, research has also focused on subcutaneous muscle and joint afferent signals, using recordings from both the upper and lower limbs (e.g. [34,41,77–82], for further information, see also [83]). One of the reasons that many studies have recorded from cutaneous mechanoreceptive afferents is that they are numerous, relatively easy to identify and maintain recordings from, and play an important role in interacting with our external environment in our sense of touch.

Fundamentally, the practical approach to microneurography has not changed much over the years from its initial development in the 1960s [35], but there is potential for development. Nerves are typically found through experience in human anatomy (Note 4.4, 4.9) and/or in using electrical skin stimulation to find the course of the nerve and the best access point in a given participant. One recent development is the use of ultrasound-guided microneurography, where an ultrasound system can be used to visualize the nerves before implantation and even during intraneural manipulation [62,84–86]. Accessing nerves in microneurography experiments can take many hours (Note 4.7), but ultrasound can dramatically cut down this time, directly visualizing nerves and electrode implantation [62]. Further technical developments have been made in the recording apparatus, in low-amplitude nerve stimulation (even during neuroimaging) [29], and in the robotic stimulators compatible with nerve recording [19,24,45,46,77,87–92].

The application of single unit intraneural microstimulation (INMS), which is an extension of single unit microneurography, has also driven the development of microneurography systems that may be used concurrently with neuroimaging [29]. Experiments in the 1980s showed that it was possible to selectively stimulate one, single myelinated A β mechanoreceptive afferent axon, using a few microamps of current, and produce a ‘quantal’ tactile percept. It is possible to demonstrate that a quantal evoked sensation can be linked to the highly-controlled stimulation of a single mechanoreceptive afferent, when appropriate methodology is used [93–96]. Very few papers exist using single unit INMS, and this technique has been questioned over the years [97–99], but it continues to be used and has been extended into investigating not only the perceptual quality of the quantal sensation evoked (e.g., vibration in a fast-adapting type I afferent, or pressure in a slowly-adapting type I afferent), but also to image brain responses. Initial studies combined electroencephalography (EEG, chapter in this book) with single unit INMS [100] and 3Tesla (T) functional magnetic resonance imaging (fMRI, chapter in this book) with single unit INMS [101]. These studies paved the way for further investigations using magnetoencephalography (MEG) [29,30] (chapter in this book) and ultra-high field 7T fMRI [29,31]. These neuroimaging environments are challenging to conduct INMS in. Strong magnetic fields require minimizing the use of metal in the fMRI scanner and dealing with problems associated with long cables. The MEG environment has a surprisingly high level of electrical interference, mitigated only by careful positioning of participants. These challenges led to the development of a system by Glover and colleagues [29] that allows high quality and high-resolution single unit recordings, low-current intraneural stimulation, and many added safety features. Although detailed information about single unit INMS is beyond the remit of the current chapter, this is an extension of the recording technique detailed here, and the above references provide information on the equipment and approach required for such experiments.

Many microneurography laboratories have relied on home-built hardware, to amplify, filter, and acquire signals; however, these systems are now many years old and safety requirements are more stringent, meaning that new systems are required. There are few options to purchase a commercially-available microneurography system, but the NeuroAmp EX (AD Instruments; New South Wales, Australia) is available, and many multi-unit microneurographers use the Nerve Traffic Analyzer from Absolute Design (Solon, IA; formerly Iowa Biosystems). The SC/Zoom system, used for numerous

experiments in Sweden, was developed at the University of Umeå, but is not available commercially. Other openly-available options include using a modified Open Ephys system (Open Ephys initiative, <https://open-ephys.org> [62]) or a system with custom designed amplifiers and stimulators initially constructed to use during microneurography with neuroimaging [29]. These systems are currently being combined and adapted for various types of open microneurography by the authors, as previous systems have had somewhat limited functionality for these kinds of specialist experiments. Making these systems standardized, simple to use in combination with ultrasound, and more widely available should help increase adoption of the technique, by flattening the learning curve and decreasing the technical demands.

2. MATERIALS

2.1 Experimental set-up

- An adjustable, comfortable chair (e.g., a dental chair), preferably reclinable (Note 4.6).
- A sturdy, solid frame to stably support the investigated body region (e.g., arm/leg rest), supplemented with padding and vacuum cushion supports as appropriate.
- Preparation equipment: disinfectant (e.g., 70% alcohol, which may be combined with other substances, such as 2% chlorhexidine or iodine), tissue, small compresses, skin tape (~1 cm diameter), very fine marker pens.
- Means of electrode sterilization, for example by dry heat, gas, or UV light.
- Environmental requirements (Note 4.12). Microneurography is best conducted in a temperature-controlled environment, under standard conditions (e.g. ~22°C). This aids in keeping the participant and their skin at a comfortable temperature.
- Electrical shielding. It is ideal to conduct microneurography in a shielded room (e.g., metal in the walls to provide a Faraday cage) with good mains grounding. This helps minimize external electromagnetic interference, as small signal amplitudes are recorded in microneurography. This is not a strict requirement, and microneurography is possible in more ‘hostile’ electrical environments, including in MEG or MRI scanners where there are more sources of interference [29–31,101]. However, interference from these and from other nearby equipment may be unpredictable and make identification of single unit recordings more challenging due to masking of the nerve signals.

2.2 Experimental equipment

2.2.1 Electrodes

An insulated, high impedance, active recording electrode (length dependent on the nerve investigated (Notes 4.9-11) is required, in conjunction with an uninsulated electrode as a reference (~3 cm long) that is inserted subcutaneously nearby (~5 cm away). An uninsulated stimulating electrode, which is the same length as the recording electrode, may be useful during an electrical search for the nerve, where the identified nerve depth can be a precise reference for the recording electrode insertion. This approach can help find deep nerves, where the nerve is initially located via this stimulating electrode (using up to 2 mA current), then the recording electrode is inserted nearby, parallel and slightly distal to this, which avoids searching using high current stimulation with the recording electrode (typically limited at 250 μ A), which decreases its impedance if used repetitively. FHC (Bowdoin, ME) is the only commercial maker of microneurography electrodes, although earlier experiments used homemade electrodes [1]. Electrodes can be bought with specific requirements (e.g., length, pin termination, impedance), but it is also possible to modify them by cutting to size and attaching pin terminations. Even if they are sterilized, it is not recommended to re-use electrodes between participants.

2.2.2 Microneurography system

The nerve activity should be amplified and filtered, with an audio output of the signal sent to speakers and/or on-ear headphones (which are useful to hear the activity clearly; Note 4.12) and the signal displayed via recording hardware/software that allows real-time processing and visualization of signals, along with data saving. The microneurography system should optionally incorporate an electrical stimulator for nerve localization or intraneural stimulation [31, 53, see below].

- *Participant safety*. As with other physiological recording techniques (e.g. EEG, EMG), participant isolation is important in microneurography. Participants should always be adequately electrically-isolated from the

external sampling or stimulation equipment. This can be done, for example, by optically isolating signals at the preamplifier head stage (e.g., in the SC/Zoom system), by isolating and battery-powering the entire recording system [29], or by having a specific optically isolated output of the nerve signal in the main amplifier [102].

- **Grounding.** As in standard electrophysiological methods, such as electromyography (EMG), a participant ground is used to reduce electromagnetic interference, such as via a silver plate next to the skin with a saline-soaked tissue or a gel-based pad adhesive electrode. Additionally, it is important that the experimenter, who will be touching the electrodes and participant, is connected to the participant ground, to avoid the possibility of static shocks.
- **Amplification.** Microneurography signals are very low amplitude. It is uncommon to see nerve signals in excess of 50 μV peak amplitude. Signals must therefore be amplified significantly. A signal gain of more than 100,000 times, usually at 10 $\mu\text{V}/\text{V}$, is necessary to visualize potentials on most recording systems.
- **Filtering.** Filtering allows the effective removal of external interference with minimal effects on nerve signals. Bandpass filtering typically between 0.2-10 kHz is generally adequate, and a typical bandpass range is 0.3-3 kHz. Additional notch filters at the mains supply frequency (50/60 Hz) may be useful when the system is used in combination with mains powered equipment. Filtering should ideally be implemented in hardware for real time output [102]. Alternatively, this can be performed in software [29], but precise, real-time output is difficult to achieve, which can make listening to and visualizing the signals challenging, as a small, yet noticeable, delay is introduced.
- **Audio.** An audio system of sufficient quality to hear the activity in real-time greatly helps with identification of nerve signals and with identifying/troubleshooting interference. Using high-fidelity audio equipment (speakers or headphones; Note 4.12), nerve activity (high frequency 'whooshing'), electrical interference (commonly 50/60 Hz mains hum), and concomitant motor unit activity (2-5 Hz 'thumping' noise) are easily and readily distinguishable by an experienced experimenter, more simply than by visualization alone. Additionally, when performing a detailed characterization and mapping of receptive fields, it is not possible to simultaneously watch a display.
- **Signal visualization.** It is advantageous to display and process signals in real-time, in addition to having the audio signal. On-line processing can be used to detect individual spikes based on their shape and amplitude. A simple method for doing this is using a histogram of local peak amplitudes [103]. This allows a simple effective display of the goodness-of-separation from the noise floor or between afferents. After appropriate thresholding of spike activity, a triggered window showing the detected spikes can be useful to distinguish between different afferents. If multiple afferents are present, these may differ slightly in shape or amplitude, but this difference may not be audible. Unlike cortical neuronal recordings, where spike shapes may differ significantly between neurons, in peripheral nerves this may not always be the case. Additional signs can be used to identify signs of multiple afferents in a recording, including the amplitude summation that can occur when multiple afferents fire synchronously or detected spikes firing at non-physiological instantaneous firing rates with intermittent extremely high frequency instantaneous spikes (>500 Hz). These signs, along with the identification of different receptive properties (adaptation, threshold etc.) or spatial separation of receptive fields can help identify different afferents that may not be easily separable during analysis of the signals. Special care should be taken to note indicators of the presence of any additional afferents, even if the amplitude or shape separation seems sufficient, since this can be invaluable during the analysis.
- **Data acquisition.** After amplification and filtering, nerve signals are normally saved digitally. A sampling frequency of >10 kHz is adequate for visualizing and differentiating impulses, since the recordings are only used as a method to time-stamp the spikes and produce a binary output of the response. An important point to consider is the simultaneous acquisition of other signals alongside nerve activity. Nerve signals can have sub-millisecond timing accuracy, and this accuracy may be necessary for some stimulus-linked sampling. Many acquisition systems are capable of performing such simultaneous acquisition of external signals alongside the nerve activity at sufficient frequencies, including Spike2 (CED, Cambridge,

UK), SC/Zoom, LabChart (AD Instruments; New South Wales, Australia), Nerve Traffic Analyzer, National Instruments DAQ with LabView (NI, Austin, TX) MATLAB (Mathworks, Natick, MA) [30] or Python, and Open Ephys [62].

2.2.3 Equipment for finding the nerve

- **Extraneural electrical stimulation.** A constant current stimulator (up to 2 mA) that is suitable for nerve localization (i.e. finding the approximate trajectory of the nerve) may be used, either via transcutaneous electrodes or a percutaneously uninsulated needle electrode. This can either be integrated with the microneurography amplifier (e.g. SC/Zoom system) or use an external stimulator (e.g. AD Instruments). Such a large current is too great to use to achieve nerve penetration, as this could be painful if applied near the nerve. Therefore, it is advisable to have another means of electrical stimulation with a maximum output of 250 μA to avoid this possibility. This lower range of electrical stimulation can be used with a percutaneously-implanted electrode (an uninsulated stimulation electrode or the insulated recording electrode); however, great care should be taken when using electrical stimulation close to the nerve. Search currents that are required for extraneural stimulation with high impedance recording electrodes require high voltages (up to 50 V) or may not be deliverable, so caution should be taken in system design and use. An important principle to always adhere to is to decrease the stimulation current to 0 μA before starting stimulation, then increase this gradually to the point of sensation. The window between no activation of any nerve fibers and the recruitment of the majority of the nerve can be small. If the electrodes (needle or transcutaneous) are moved while stimulating, this could lead to a sudden, surprising, and sometimes painful recruitment of a large number of fibers. When using the needle electrodes special care should be taken, since current thresholds can vary more substantially than transcutaneous stimuli and participant movement from a shock will be more problematic. What is a barely perceivable stimulus extra-neurally (80-100 μA) would be intolerable intra-neurally and this risks involuntary contractions and potential nerve damage. Additionally, for safety the system should be easily controllable/reducible or switched off by the experimenter.
- **Intraneural electrical stimulation.** An intraneural stimulation system may be used to perform INMS, within the nerve. This system should be designed to deliver a small current (up to $\sim 10 \mu\text{A}$) and special care needs to be taken in its design. The system must have the capability to switch between recording and stimulating modes, easily, quickly, and safely, with full control to reduce/turn of the stimulation by the experimenter. It is possible to have a system that is suitable for both nerve search (up to $\sim 250 \mu\text{A}$) and intraneural stimulation [95]. However, because of this wide range in current thresholds, such systems need to be well-designed to safely switch between these ranges depending on the intended stimulus. Electrical artefacts can be generated from switching between recording and stimulation [29], which can be painful for the participant when in the nerve. Precautions can be taken to avoid this, such as 'soft' analog switches that gradually switch the electrodes from the amplifier to stimulator. In general, using high impedance electrodes for recording single unit activity requires voltages of <0.25 V for INMS, using constant current stimulation in the low μA range to evoke sensations [29]. Constant voltage stimulators may be used for INMS [96]; however, constant current is recommended where possible, as it gives greater control and relative invariance with potential electrode impedance changes, which is more predictable, safer, and stimulation intensities can be compared across experiments.
- **Ultrasound.** Consider the use of an ultrasound system to help map the path of the nerve before electrode insertion and for guiding the electrode to certain nerves [36,62,84–86]. The characteristics of the ultrasound system should be selected carefully, since there will be a tradeoff when choosing the ultrasound frequency used in the probe. Greater frequencies will provide greater levels of detail, so they can image smaller nerves, but will not be able to image deep tissue. Clinical systems for local anesthesia are suitable, but are bulky and expensive, and there are now several providers of compact portable systems that are sufficient to visualize nerves and convenient for experimenters to use. However, care should be taken when using ultrasound in real time, since pressure needs to be applied to

visualize the nerve and this may risk bending the needle. Selecting a site with good solid subcutaneous support (e.g. peroneal nerve at mid-calf level [62], radial nerve just above the elbow [36]) mitigates this possibility.

2.2.4 In-experiment tools

Various tools are useful during microneurography, such as assorted tweezers (Note 4.13), small (~5 cm) measuring stick for measuring the depth of the electrode (subtract the exposed length from the total electrode length), tape measure for measuring the distance between the afferent receptive field and recording electrode, glasses or screens to shield the participant's view if needed during stimulation, camera with a suitable macro lens (for general experimental photographic and video documentation, as well as precise records of mapped receptor locations), and documents or templates for taking notes and information in a standardized manner.

2.2.5 Mechanical skin stimulation equipment

This can range from basic equipment, required to activate and identify units (e.g., wooden stick, brush), to more specialized tools to precisely define a receptive field, such as a range of calibrated monofilaments, typically from 0.04 to 750 mN, with sufficient gradations to perform suprathreshold mapping of mechanically-sensitive afferents. Once an afferent has been located and characterized manually, more complex robotic devices can be used to stimulate a single unit in a more specific, automated, and controlled manner [19,24,45,46,77,87–92].

2.2.6 Issues with external interference

Electromagnetic interference may be produced by specific pieces of equipment, but this is often unpredictable and it is difficult to know the extent without testing it. It is found commonly with power transformers, which can introduce significant mains noise (50/60 Hz oscillations), but additional factors including room lighting and even environmental conditions may introduce issues (Note 4.14). Lights can be turned off or replaced with battery powered sources, but the weather is more difficult to account for. It is important to evaluate new equipment, since interference can be unpredictable and specific to each device, and there may be certain ways to mitigate this. In principle, it is best to battery-power all equipment, where possible, since this eliminates the most common source of interference (mains electricity) experienced during microneurography. In cases where this cannot be done, a notch filter (see filtering section) can be added to mitigate this. For testing equipment, a simple initial test is to use the system with the electrodes in a conductive medium (e.g., a piece of fruit), in a phantom (e.g., saline filled bottle) grounded to a participant, or inserted superficially in the skin at the intended recording site. This is sometimes necessary, as participants may act as an antenna, or couple noise capacitively into the recording.

METHODS

3.1. Experimental setup: accessing the nerve

3.1.1. Welcome the participant

Provide information about the nature of the study (Note 4.15) and ask the participant to read and sign the informed consent form to participate. Make sure that they have eaten beforehand and have been to the toilet. Participants should be advised to avoid caffeine consumption on the day of the experiment. Caffeine can make the participant more prone to movement and nervousness, which is not ideal during microneurography, and it is a diuretic. To reduce the potential for discomfort subjects should also always be clearly informed that they are free to terminate the experiment at any time.

3.1.2. Installation of the participant

Seat the participant in a comfortable position (Note 4.3, 4.5). Cushions and/or foam padding can be used where required and a vacuum cushion (covered by a towel or tissue) can support and immobilize the body part under investigation. Plan the position of the participant in advance, according to the nerve to be accessed. Particular attention may be paid to the angle and pronation of joints, since the nerve can be more or less accessible based on the position of muscles and tendons, when joints are at a particular angle. Having detailed knowledge of the anatomy of the area under investigation is important, as this can inform decisions about participant positioning (Figure 2). Minor adjustments in the position of the participant and cushioning to maximize comfort are recommended, even if this takes some time, as once they are in position, it is often not possible to make further adjustments without

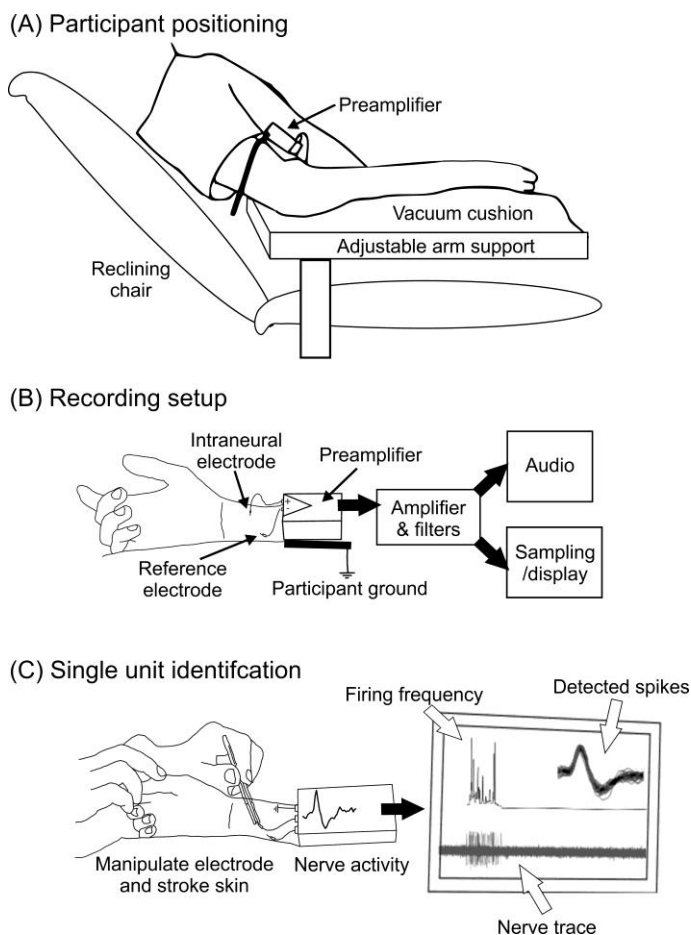


Figure 2: Typical setup and procedure in a microneurography experiment.

(A) The participant is positioned comfortably in a way that is specific to the nerve being investigated (here for the median nerve at the wrist). (B) The recording setup, where the recording electrode is placed intraneurally, the reference electrode is inserted superficially into the skin nearby, and the participant is grounded. The signal from the recording electrode can then be amplified and filtered, as well as listened to and visualized. (C) Single unit responses are searched for and identified.

affecting, and sometimes even abandoning, the experiment (Note 4.3). Some participants may find particular positions uncomfortable, for example, supination of the wrist makes median nerve recordings more straightforward, but may not be tolerable for long periods (Note 4.5). Simple factors that may also cause discomfort include the wires connecting the grounding plate or bumps in the cushioning. If these occur at a problematic location (e.g., median epicondyle of the humerus at the elbow) over a number of hours, pressure may lead to numbness or loss of sensation that is unrelated to the microneurography itself. In general, if possible, it is good practice to form the cushion with the subject in place, get them to remove their arm/leg and check for such problematic bumps/wires, and then replace it.

3.1.3. Instructions for the participant

At this stage, describe the basic outline of the experiment and how it will progress: palpation of the skin to locate the best place to insert the electrode, electrical localization of the nerve if used, insertion of the electrode, registering activity, and unit stimulation. It is critical to instruct the participant to notify the experimenters of any sensations or discomfort experienced, since these can act as a significant guide toward the success or failure of the experiment (Note 4.5). Projected sensations, such as tingling in the skin, can indicate proximity to the nerve, whereas discomfort local to the electrode insertion site is typically a result of pressure on subcutaneous structures (e.g., pressure on blood vessels, ligaments or tendons), but may also be due to pressure on the perineurium. These sensations may not be problematic per se, but repeated pressure on these structures may lead to sensitization and increased discomfort, so should always be avoided. Additionally, the participant should be instructed that large or sudden movements are not permitted or advisable during the experiment.

Slow controlled movements of non-immobilized body parts may be possible, but it is critical that the participant informs the experimenter before these are made, so that preparations such as retracting the electrode away from the nerve, can be made, if necessary (Note 4.3). The participant should be prepared that there will be portions of the experiment where they are required to be absolutely still and as relaxed as possible, that is, while recording activity from a single afferent (Note 4.15); however, staying completely rigid for the entire experiment can be tiresome and straining, so it is important to allow some small movements where possible, for comfort. A relaxed subject is a priority; anything that produces tension may also induce muscular motor unit activity, which can be of a much larger amplitude than the nerve signal and mask recordings. Such activity is often seen in recordings from nerves in the arm and is due to the position of the shoulders and neck. The activity is characterized by a large-amplitude, relatively low frequency (2-5 Hz) 'thumping' response that is extinguished when the participant relaxes or the shoulder is slightly repositioned.

3.1.4. Electrode location

First, the participant ground should be attached and positioned. Then, the ideal site for electrode implantation can be located (Note 4.4). Palpate for anatomical landmarks indicating the position of the nerve, such as muscles and tendons. Nerves typically run in neurovascular bundles through soft tissue, and it is sometimes possible to feel these, for example, the large median nerve in the upper arm or the peroneal nerve at the back of the knee. Electrical stimulation of the skin surface with blunt ball-type electrodes results in a sensation that is projected to the nerve innervation territory (typical threshold is usually a few milliamps), so this can be used to identify the location of particular nerves/branches in an individual subject. Alternatively, ultrasound can be used to help directly locate nerves and identify optimal insertion sites on the skin for the best chance of nerve access. Once identified, it is advisable to mark a planned site for electrode implantation on the skin using a fine ink pen. At this point the skin at the site of insertion should be properly disinfected.

3.1.5. Electrode implantation

Electrodes must be sterilized and handled with care to avoid contamination and the risk of introducing infections as well as damage to the insulation at the tip. They are easily held using non-locking forceps or blunt tweezers (Note 4.13). It is good practice to insert the electrode at an appropriate angle for nerve access, as this initial insertion will guide the path of the electrode. The electrode should be gripped relatively close to the tip (~10 mm), to prevent bending on insertion. At this stage, warn participants of the insertion and have other experimenters in the room to attend to them and watch for signs of adverse reactions (Note 4.1, 4.5, 4.6).

3.1.6. Experiments

During experiments, it is advisable to work as a team (Note 4.15). Microneurography is demanding and with several experimenters it is possible to recognize early signs of vagal reactions more quickly (Note 4.6) and deal with these before syncope/fainting occurs. The primary experimenter may be extremely focused on electrode positioning and characterization of the recording.

3.1.7. Participant management

Managing the participant is important during all stages of the experiment (Notes 4.1, 4.5-7, 4.15), it is advisable to keep the participant engaged and attentive for what can be a period of several hours, both whilst locating the nerve and finding recordings; they must not be allowed to sleep. This allows you to pay attention to any sensations experienced by the participant, which is vital for participant safety and may successfully guide the course of the experiment.

3.1.8. Finding the nerve

Once the electrode has been inserted, it is good practice to note and keep track of the depth in the tissue (Note 4.16). This can be done by knowing the length of the electrode, and simply measuring the exposed length with a small ruler. Multiple strategies may be used in order to successfully guide the recording electrode towards the nerve, depending on the experiment, but these include: anatomical knowledge and experience (Notes 4.4, 4.8, 4.9, 4.11), the use of a search electrode, stimulation through the recording electrode, and ultrasound guidance. Small movements of the electrode should be made while stroking or tapping the afferent innervation territory. When in very close proximity to the

nerve, background mass activity discharge may be heard during touch of the innervation territory (a near-instantaneous 'whooshing' sound, while stroking the skin) and/or the participant may experience projected paresthesia, such as a tingling at the innervated patch of skin. Since these reported sensations may act as a significant guide for the experimenter, participants should be properly instructed to expect these and to inform the experimenter when they are experienced, as this can guide positioning to achieve a juxta-neural position.

Once the electrode appears to be located in close proximity to the nerve, it is best to make small, distinct, manual adjustments to penetrate the nerve. Firm, but controlled, movements are helpful to make the penetration swiftly and smoothly, as the participant can experience both increased projected and local discomfort or strange pressure sensations (Note 4.5), and at this point there is a particular increased risk of a vagal reaction (Note 4.6). These sensations usually reduce in intensity once a successful nerve penetration has been made, and at this point the precise electrode depth should be noted, by measuring the length of exposed shaft. This measurement can be very useful as a reference point for moving within the nerve, and a depth for relocating the nerve if the intraneural position is lost (Note 4.16). Achieving nerve penetration is sometimes challenging, as the electrode needs to be centered on the nerve to perform this successfully. It may often happen that the electrode appears to pass the nerve on either side, and this step of the technique can be improved by re-angling and carefully using extra neural stimulation through the recording electrode (up to 250 μ A), to search for a position with a minimal threshold for projected sensations. This allows iterative steps to be made to refine the positioning and improve the chances of penetration, particularly for small nerves.

3.2. Single unit recordings

3.2.1. Checking electrode quality

When attempting single unit recordings, a critical factor in success is the electrical impedance of the electrode. If the impedance is too high, background discharges will not be audible as a guide, and finding afferents is more challenging. If impedance is too low there is a higher chance of multiple afferents in the recording. Electrode impedance can be measured at 1 kHz before the experiment, to know the pre-insertion impedance, and during the experiment, which can act as a good indicator of recording quality. Monitoring the impedance can help throughout the experiments and this can be estimated using low current stimulation, even while recording intra-neurally [29]. In general, successful recordings from myelinated mechanoreceptive afferents are obtained using electrodes with impedances of 100-500 k Ω . In addition to the intermittent monitoring of impedance during the experiment, it is useful to monitor the noise floor in recordings. Drops in the noise levels in a stable electrical environment can indicate a reduced impedance, so this should be checked. When moving the electrode, particularly when encountering touch tissue layers, it is possible the electrode tip may get damaged. This often results in a substantial reduction in impedance, which will be seen in the noise floor (sudden drops when encountering a tough layer) and will be reflected in the measurements. If the electrode impedance falls below this range, it is wise to change electrodes to improve the chances of obtaining a successful recording.

3.2.1. Knowing your way around a nerve

Once an intraneural position has been achieved, it is important for the experimenter to be able to judge the relative magnitudes of electrode movements within the nerve. There are several ways to judge this. Mass activity heard when stroking the skin may only appear at focal sites, such as on a single finger or phalange, which will normally change as the electrode is advanced. Additionally, while adjusting the electrode, the participant may experience projected paresthesia to a particular location, often seen in combination with an audible discharge. It is also advisable to get subjects to report these, since there are sometimes coordinated shifts in the region of paresthesia and the region where activity can be evoked. One advantage of lower median nerve recordings at the wrist is that the nerve is rather flat and is organized more topographically, i.e., to the radial side, you can find recordings nearer the thumb, and to the ulnar side, you find recordings going to the fourth finger, where in between lies a topographic relation of the hand. This topography is not found in other nerves to the same extent, which is even true for more proximal access sites for the median nerve (e.g., above the elbow).

3.2.2. Searching for individual afferents

The region of mass activity, and the areas in which participants experience projected sensations, will be the most likely site to find a single afferent

recording, so searching should mainly be focused here. A thorough skin search should be conducted throughout recordings, but particularly when the electrode appears to have changed position. To excite receptors and identify recordings, appropriate stimulation should be delivered to the innervation territory of the nerve or fascicle under study, according to the experiment and afferent type sought for the study. The way the search is conducted will determine which types of afferents are identified since only activated afferents will be observed. For example, tactile afferents may be readily searched for by stroking the skin, muscle or joint afferents may be searched for by gently moving the body part or palpating the muscle, whereas locating nociceptors may require pinching of the skin or high intensity electrical stimulation. Thermoreceptors, which have been less studied in microneurography, require a different strategy, where static or dynamic mapping using thermal stimulation is required.

3.2.3. Identifying afferents during recording

Experimenters should be tuned to what the activity from single afferents both looks and sounds like (Note 4.17). Single afferents will commonly have large signal amplitudes, with a 'popping' quality, with high frequency clicks generated by each spike. The pitch of this activity (rate of pops or clicks) can approximately indicate the firing frequency. This should be recognized by the experimenter as a prompt to conduct a thorough search and characterization to confirm whether this is a single afferent.

3.2.4. Verifying recording quality

Once a putative single afferent has been identified, the region should be thoroughly mapped to identify its response properties, and search for signs of multiple afferents in the recording. This is advisable to do as swiftly as possible, since recordings may only be transient, yet reliable characterization is also important. Improperly identified receptors or multiple afferents present in the recording may lead to uninterpretable data. At this point, all the features of the afferent response that have been used in its characterization (e.g. force sensitivity, receptive field size, sensitivity to remote stimuli, regularity of firing etc. [73]) should always be noted. It can also be good to record data during the classification (e.g., sustained responses to check for variability in the firing of slowly adapting afferents), since this can be re-visited for subsequent checking/verification. Afferents are all individual and unique, and there may be cases in which classification is slightly ambiguous/problematic. It is important that any ambiguity/uncertainty is clearly documented during recordings, along with the reasoning, since this has the potential to impact upon the group level results (Note 4.18), often separated by afferent class.

3.2.5. Begin the protocol and monitor

Once the identity of the afferent has been confirmed and this has been adequately characterized, the experimental phase and protocol may commence, depending on the chosen parameters (Notes 4.18-19). Once the experimental protocol has begun, it is good practice to continually monitor the nerve signals and watch for changes such as a decreasing single unit amplitude, although it can occasionally increase (Note 4.19). Whilst recording, it is possible for the electrode position to slip slightly. This may happen spontaneously but can be minimized by the participant sitting very still, yet relaxed, before commencing recording. If this is suspected, it is advisable to re-verify the receptor properties, where possible during the experimental protocol. It is possible that the recording electrode may spontaneously slip between different afferents, and this may not always be apparent from the spike shape/amplitude.

3.3. After the experiment

It is important to debrief the participant when the experiment has finished. One particular point is to remind participants of the possibility of temporary after-sensations in the projected region of the nerve that was accessed, which may be felt for up to a couple of weeks [102,104]. They should also be advised to refrain from putting pressure on the implantation site or surrounding tissue (e.g., by palpating it or doing weight-training) for a few days. Blood vessels are occasionally damaged by the electrodes, but the point of electrode insertion may bleed a little on removal of the electrode due to damage of a superficial vessel, but this is rare and there are not usually any issues at this site. Occasional bruising can occur around the site of implantation, and any continued redness, hotness, or unpleasant sensations must be followed up and the participant should be informed to contact the experimenters in the case of any issues.

4. NOTES

1. It is extremely important how you conduct yourself in front of the participant. This can influence the whole experiment and the participant must always be put first. This is also true for many approaches, such as in TMS (see TMS chapter in this book). Microneurography involves *in vivo* single unit recording, and sometimes things do not go well (e.g., loss of single unit recording during skin stimulation), but the experimenter must always be aware that the participant will hear everything they say and may worry about it. You should not, for example, say that it is a 'bad' recording.
2. Large-diameter (~10 µm) myelinated axons are the least difficult to record from, due to the large amplitude signal, yet it is possible to record from small-diameter (~1µm) unmyelinated C-fibers. Only a handful of recordings have been made from thinly-myelinated axons (Aδ, ~3 µm) (cold Aδ [75,76] and vellus hairs [43]), possibly due to a combination of a thin axon and insulating myelination along the majority of the fiber making recordings less likely.
3. It is important that the participant is seated very comfortably. They may be there for a number of hours and they must not move significantly once the electrode has been implanted. Very small, slow and controlled movements may be possible before the electrode is in the nerve, but the participant should be instructed to always alert the experimenter of this before doing anything. Small adjustments that are not disruptive to the electrode positioning can be very helpful for the participants (e.g., taking the foot rest up or down slightly) at points where the stability is not critical (i.e., between single unit recordings), but these should always be performed carefully.
4. Individual humans have many variations in their skin anatomy and physiology. Sometimes participants may have extremely hard or elastic skin, and this can make implanting the electrode challenging, as well as hindering the application of tactile stimuli. Some people may sweat more than others on their hands too – and this is normal.
5. Discomfort. At all times, pain and discomfort should be minimized. If the participant is uncomfortable for any reason, this is unethical and the experiment is less likely to be successful – discomfort will increase EMG activity, and sweating. At the slightest hint of discomfort, the experimenter should actively decrease it, for example by changing electrode position. Additionally, it is best to instruct participants to report any feelings of numbness, tingling or paresthesia not associated with electrode movements as soon as possible. These may be indicators of excessive pressure on the nerve that could lead to lengthened or stronger after-sensations and should be avoided.
6. Fainting may occur during microneurography. If this happens, it is typically through a psychological reaction during the implantation in the skin or when the electrode touches a nerve where particularly strong paresthesia or deep pressure sensations may be experienced. The experimenter must be trained to recognize signs that this may happen, remain calm, and look after the participant. This is why it is recommended to have at least two microneurographers in the room, to watch for signs of this and to help. 'At risk' participants for psychologically induced fainting may appear particularly nervous or uncomfortable, and particular attention should be paid. If the participant shows signs of a vagal reaction (sweatiness, blanched skin, feeling 'strange', feeling warm, becoming less responsive), the electrode(s) must be quickly and carefully removed as soon as possible, then the participant can be reclined. If a vagal reaction occurs, once recovered, it is advisable that the participant leaves the chair, takes a walk, drinks some water, and maybe eats something small and sugary, such as a piece of fruit. At this stage, a longer break can be useful if the reaction seems to have been psychologically triggered. Once this has happened, the participant may restart the experiment, if they want, as the experiment often proceeds without issue afterwards, but caution should be taken. If it happens a second time, the experiment should be stopped. The same issues are possible in TMS (See TMS chapter in this book).
7. It is possible that you may not successfully record from a peripheral nerve during an experiment. This can be disheartening for both the experimenter and the participant. However, the experimenter should reassure the participant that this is normal and there is nothing 'wrong' with them.
8. Depending on the mechanical properties of the skin, which can differ substantially between subjects, introducing the electrode may be smooth and straightforward, but can be more challenging. The electrodes are not sharp, so will not insert as easily as a hypodermic needle, but instead part the tissue using blunt force which can be more unpredictable. Participants with particularly elastic skin can make electrode insertion difficult, since the skin may deform extensively without the electrode penetrating. Electrode insertion should be firm, applying sufficient constant pressure until the skin is penetrated. In some cases, this takes some time, but the electrode may apply pressure for extended periods time. Applying excessive pressure or any lateral force during insertion, can bend the electrode, which will make positioning challenging and unpredictable. Experimenters should try to be particularly attentive to the possibility of electrode bending at this stage, and throughout the experiment, and become accustomed to the sensation that this has happened.
9. Individuals show variations in their nerves - you may come across a nerve that may not follow the textbook description. This is not abnormal and is a part of the diversity you find in human physiology. Some examples are that the lateral antebrachial nerve that innervates the dorsal forearm can innervate skin well above the cubital fold (i.e., at locations proximal to the electrode) and also reach well into the glabrous skin of

the thumb. Another example of finding afferents proximal to the level of the electrode is when recording from the peroneal nerve at the level of the knee, responses can be found into the thigh. Responses from cutaneous afferents above the level of the electrode do not typically extend more than ~5 cm proximal, yet it is clear that nerves do not just go 'down' (i.e., distally) in their innervation.

10. The median nerve, as accessed ~3 cm proximal to the wrist, is one of the easiest nerves to access and record from. It is between 5-10 mm deep and is relatively large (~5 mm diameter) and flat (~3 mm thickness) [105].
11. Typical nerve depths range between 8 mm (median nerve at the wrist, therefore a 15 mm electrode is typically used) to much deeper nerves, up to ~40 mm deep (e.g., the median at the mid-upper arm, where a 50 mm electrode may be used).
12. Noise-cancelling sound systems like those typically used in headphones can actually filter out some of the nerve signal. Further, wireless systems, although highly practical, may suffer from intermittent interference and have a small, yet noticeable, delay.
13. The addition of heat-shrink thermoplastic to the end of tweezers or forceps can be useful in non-rigidly gripping the electrodes, allowing simple control of pressure applied to the electrode.
14. External interference. Stimulators may produce electromagnetic interference that is dependent on the distance from the recording electrode. This may be minimized by choosing a more proximal recording electrode position, increasing the distance to the stimulator, or by better electrical shielding. Further, environmental issues may occur occasionally during low humidity weather, where static electricity can be an issue. It can mean that the participant gets a static shock when the experimenter touches them, which is not pleasant and may make the participant move. This is one reason why it is best practice to ground the experimenter as well, by attaching an adhesive gel electrode to the experimenter's skin and connecting it to the participant ground. Also, it is not ideal to conduct microneurography in storms (electrical hazard) and even in extreme weather (it can be unsettling, distracting or worrying for all).
15. Microneurography experiments can be long and boring – for the participant and the experimenter! This is also why it is recommended to have at least two microneurographers present, to alternate positioning the electrodes and keeping the participant engaged (awake). The participant may want to sleep or rest, but this is not recommended, as they may jerk awake suddenly, risking nerve damage from electrode movement. Further, the non-experimenting microneurographer can subtly watch the participant for potential cues of fainting or sleeping. This can also be indicated by monitoring concurrent electrodermal activity.
16. The electrode locations where sensations are experienced can be kept as a reference to know the depth at which the nerve is encountered, or at which subjects may be prone to experiencing sensations (e.g., pressure on blood vessels). This can also be used to keep track of the location of any significant tissue fascia layers, indicated by difficulty in advancing the electrodes and sudden drops in electrode impedance. These layers of tissue may be difficult to penetrate, and passing through repeatedly may damage the electrode, significantly reducing the impedance and reducing the possibility of isolating high-quality recordings. By identifying and recording the depth of these, it is often possible to adjust the electrode without passing back through these layers.
17. It can be very difficult to stimulate the receptive field of a single unit. Often, the receptive fields are very small and may fall anywhere within the innervation territory of the nerve. These can be hidden from a big tactile stimulator when located near a skin fold or on curved skin. This is very true of the skin on the fingers, where the fingers themselves can also get in the way of each other. It is best to design stimulators which can be flexibly positioned to access as much of the innervation territory as possible, since searching for afferent recordings on a particular skin site such as the tip of the finger may be challenging and time consuming, and many other single unit recordings may be wasted in this case.
18. The number of *participants* you need for a full dataset is not a good guide in microneurography; it is the number of *single units* that counts (i.e. the variance across units, rather than across participants). You may use 10 participants and get no data; whereas you may have a very productive experiment on one participant and gain data from 15 single units.
19. Spike shapes. Most recordings from myelinated afferents are biphasic, with a prominent positive going peak, while most unmyelinated afferent recordings are triphasic, with a prominent negative going peak [1,102]. However, more complex spike shapes may be observed. When pressure is exerted on the myelinated afferents, these may begin to show a double peaked spike [106], thought to be due to slowing induced by a partial conduction block local to the electrode. At this point, the second peak may permanently or intermittently disappear due to a full conduction block. These recordings can then appear as two different spike shapes, but actually originate from the same fiber. Care should be taken to adequately verify and note this by stimulating the receptive field, and checking if this is consistent and not due to the presence of additional afferents. These kinds of details should be noted during the experiments, as these can be very useful during the signal analysis.

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