Comparison of Perturbed Gait and Cortical Activity Caused by Nociception

JAKOB FRANK
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Master's Thesis

By

Jakob Frank

Department of Electrical and Information Technology
Faculty of Engineering, LTH, Lund University
SE-221 00 Lund, Sweden

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Abstract

The ability to accurately measure pain is very important and can be used when assessing the effect of potential analgesics. The most common method of measuring pain involves reflex tests, where one observes the reflex response of an animal upon painful stimuli. This response is binary, since the observer can only differentiate between if the animal feels pain or not. This rough indication of nociception often leads to poor translation between test results and reality, which could lead to massive complications. In this thesis, two quantitative methods to measure nociception were compared and evaluated. The first method measures the neural activity in the primary somatosensory cortex (SI) and was evaluated through a literature study. The other method analyses the changes in gait exerted by the animal and was evaluated through experiments and video analysis. The first method is rather complex but showed great potential in accurately describing nociception in an animal through the potentials evoked in SI. The gait analysis method uncovered five different parameters which values correlated with increasing nociception. These five parameters together covered different aspects of describing the gait of an animal. The gait method is simpler than the neural recordings method, but it is more limited in the information it provides and in the experiments it can be used in. Comparing these two quantitative methods to the reflex method it is clear that the quantitative methods can provide much more accurate measurement of nociception and therefore help in accurately assessing the capacity of potential analgesics.

Acknowledgements

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Jakob Frank

Contents

List of acronyms

AP	Action Potential
LFP	Local Field Potential
SI	Primary Somatosensory Cortex
UVB	Ultraviolet B
RGB	Red, Green and Blue
DBSCAN	Density-Based Spatial Clustering of Applications with Noise
RH	Right Hind
LH	Left Hind
CNN	Convolutional Neural Network

Popular Science Summary: Measuring pain with gait and the brain

Today, scientists use reflexes to investigate how much pain an animal feels. This thesis has evaluated two different methods which more accurately can measure pain; using the brain's signals and by analyzing an animal's gait.

When pharmaceutical companies develop new painkillers, they need to test their drug repeatedly to make sure the drug's working as intended. One of the most important tests is to make sure that the drug actually has an analgesic (painkilling) effect. This is where pain measurements come in. These pain tests are today performed by looking at the animal's reflex response upon painful stimulation. The weakness of these tests is that they only provide a 'reflex' or 'no reflex' answer, which isn't a great way to evaluate a new potential painkiller. A much better way to measure pain would be to use a method which could accurately describe the level of pain felt by the animal, and not only 'reflex' or 'no reflex'. This would ensure that the analgesic effect of the new drug is confirmed in a more controlled and reliable way.

Two methods to solve this problem have been evaluated, which measure pain in two very different ways. The first method measures the activity in the brain region responsible for sensing pain. Unsurprisingly, this method is quite complicated since it requires sensors to be implanted inside the brain of the animal. Regardless, this method shows great potential in accurately describing the level of pain felt by an animal.

The second method involves analyzing the changes in gait of an animal when it feels pain. If, for example, you burned your foot, you wouldn't want to support your weight as much on that foot, leading to a change in your gait. Similar experiments were performed on rats while their gait was recorded and from analyzing those videos, five different attributes in the animal's gait were proven to change when the pain level increased. This method is simpler than the first one, but it is worse at detecting pain felt in a place other than the paws.

Comparing these two new methods to today's method it's clear that the newer methods can describe more aspects of pain than the older one, providing more pieces of the 'pain-puzzle'. Using these more accurate methods will hopefully enable pharmaceutical companies to faster develop better and safer drugs which will reduce the number of animals needed for testing.

1. Introduction

1.1. Background

Pain-tests are a crucial part of the development of analgesics and the generation of receptive fields. Often, these tests involve tactile stimulation upon which the response of the animal is observed. These qualitative tests only decide if the animal felt enough pain to trigger a reflexive response or not. A quantitative approach, which can decide the amount of pain experienced by the animal, would be preferable, since this would allow for a much more precise evaluation of e.g. analgesics.

1.2. Aim

This project seeks to compare and evaluate two possible quantitative approaches of measuring pain. The first approach consists of analyzing the gait of an animal (rat) by recording its walk before and during increased nociception. The second approach is to measure the cortical activity of the rat before and during increased nociception. The aim is to find parameters that correlate with increased nociception. If such parameters were found, they could replace the qualitative methods of today and vastly improve the speed of mapping receptive fields and the precision of evaluating analgesics. The first approach will be evaluated through experiments to investigate the prerequisites to implement a database of gait parameters which correlate with increasing nociception and to use said database to measure pain. The second approach will be evaluated through literature on previous experiments. The results of these evaluations and the potential of each approach will be compared and discussed.

1.3. Structure of report

The report consists mainly of seven parts; ‘Theory’, ‘Tools’, ‘Method’, ‘Results’, ‘Discussion’, ‘Future work’ and ‘Conclusion’. In ‘Theory’, ‘Tools’ and ‘Method’, the underlying theory, tools required, and methodology used are described. ‘Results’ contain the findings of the performed experiments and analysis. The results are assessed and evaluated in ‘Discussion’, where also the two approaches will be compared. Finally, ‘Future work’ describes the possible improvements that could be implemented while in ‘Conclusion’, the most important findings are summarized.

2. Theory

2.1. Neurophysiology and action potentials

The nervous system is responsible for communicating sensations throughout the entire body. This elaborate system consists of a network of nerve cells (neurons). Neurons resemble other cells in that they have a cell body (soma) containing a nucleus and organelles that are necessary for the survival and function of all cells. To allow the neurons to communicate with each other, the cell branches out and forms dendrites, which are specialized in receiving signals from other neurons (see Fig. 1). The neurons send signals through the axon, which typically is a much longer branch of the cell. The axon is also covered in an insulating layer called myelin, which helps to transfer signals faster. The points of the axon that are not myelinated are called the nodes of Ranvier. A neuron often has many dendrites but only one axon. At the end of the axon is a connection to the next cell, called a synapse, where the signal is transferred by means of direct connections (gap junctions) or, more commonly, by neurotransmitters [1, p. 85].

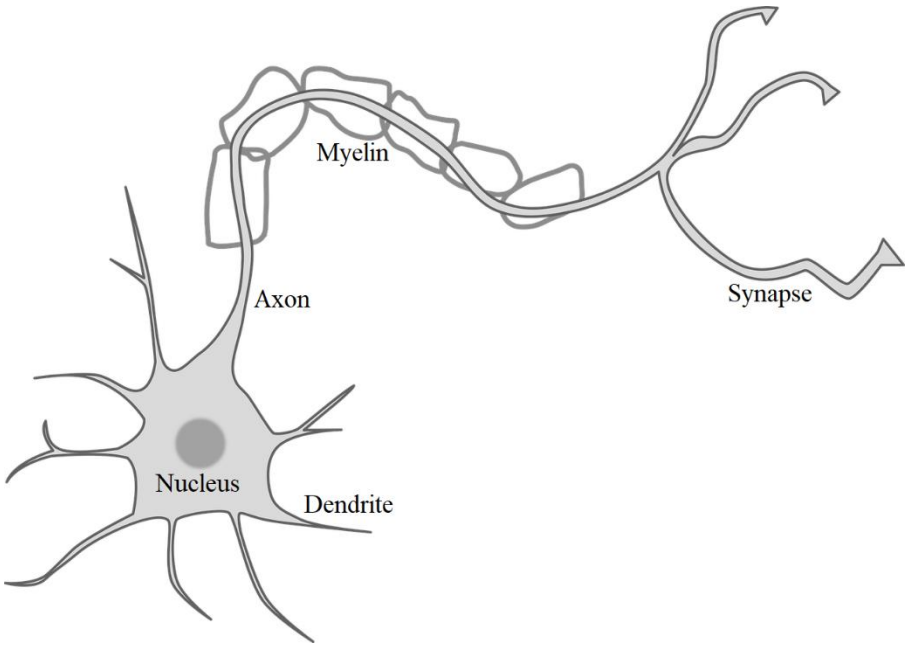


Fig. 1. A neuron with a nucleus (dark gray dot) and dendrites branching out from it. The single longer branch is the axon, which is myelinated (white shapes) and ends in this case in three synapses.

Neurons send electrical signals, called action potentials (APs). APs are generated through various ion currents across the cell's membrane. The ions travel across the membrane through selective voltage-gated ion channels, which are channel proteins gated by the potential across the membrane. In an AP, two ions are the most important: potassium ions (K^+) and sodium ions (Na^+). Naturally, a neuron has a larger concentration of Na^+ outside the cell and a larger concentration of K^+ inside the cell. This natural condition of equilibrium gives rise to a certain potential, called the resting potential which is typically around -70 mV [1, p. 37]. If the cell's potential is increased over a threshold potential, the voltage-gated ion channels are opened and allow for the ion they are specific for to flow across the membrane, dramatically altering the cell's potential. In an AP, the faster Na^+ channels open first, which cause external Na^+ to flow into the cell, increasing the membrane potential (depolarization). Then, the slower K^+ channels open, allowing the internal K^+ to flow out of the cell, decreasing the membrane potential (repolarization). The slower K^+ channels remain open for a longer time, causing the membrane potential to drop even below the resting potential (hyperpolarization). The membrane potential is then restored to the resting potential by active ion pumps. The entire event of an AP takes only a few milliseconds. Thus, the action potential is merely the change in membrane potential due to the opening of voltage-gated ion channels.

The generation of an AP is considered a "all-or-none" event, meaning that either the AP is generated with a standardized waveform (see Fig. 2), or nothing happens, regardless of how large the stimulus is. What regulates if the AP is generated or not is the threshold potential mentioned in the previous paragraph. The AP travels through the cells and their axons via propagation caused by the opening of nearby voltage-gated ion channels due to the depolarization of the cell membrane. The propagation through the axon is called saltatory (jumping), as the nodes of Ranvier are the only places on the axon where ion channels are not covered by myelin. Thus, the APs are regenerated at the nodes of Ranvier and the amplitude of the signal is maintained in a better way.

The Na^+ channels are inactivated for a short time after being opened, meaning that they cannot open again even if the membrane potential is high enough. This period, when the cell cannot fire an AP, is called the refractory period. This enables the AP to only travel in one direction, since the Na^+ channels in the direction from which the AP came cannot open and depolarize the cell. Once an AP reaches a (chemical) synapse, it triggers vesicles filled with neurotransmitters to fuse with the membrane, releasing the neurotransmitters into the space between the pre- and the postsynaptic cell, called the synaptic cleft. The neurotransmitters bind to specific receptors on

the postsynaptic cell, which are coupled to ion channels. Depending on the neurotransmitter, different ion channels are opened, which could depolarize the postsynaptic cell and allow the AP to propagate or hyperpolarize the postsynaptic cell and stop the signal.

Due to the generally standardized waveform of the AP, the information cannot be coded in the amplitude or the duration of the AP. It is rather the frequency of APs that encodes the information [2, p. 30]. A stronger stimulus will increase the frequency of APs, which will trigger a release of more vesicles containing neurotransmitters at the synapse. Even if no stimulus is present, neurons still generate APs randomly with rather low frequency. This 'background' signaling is necessary since the cells can inhibit the signaling of other cells through the synaptic connections. If no background signaling had been present, the cells would not be able to generate less APs, which would mean that only excitatory (increase in generation of APs) regulation would be possible.

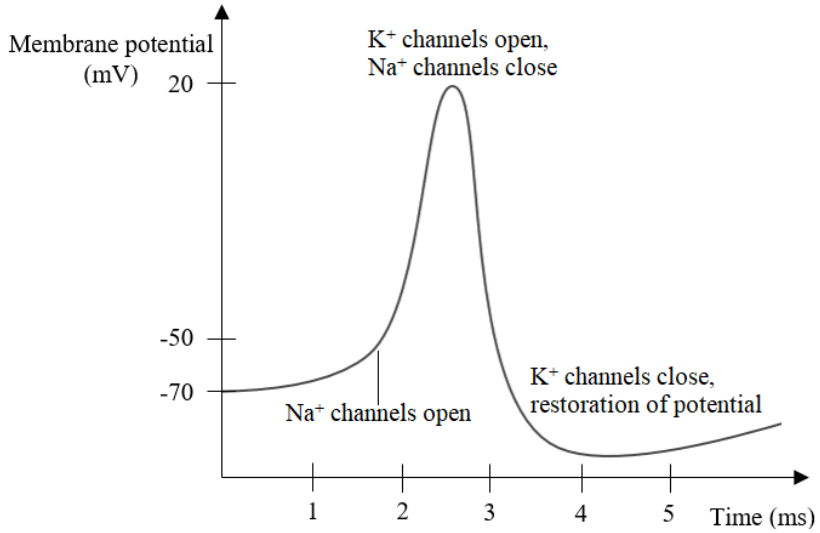


Fig. 2. The standardized waveform of an action potential. The membrane potential initially rests at -70 mV and is then depolarized to the threshold potential at -50 mV. Here the voltage-gated Na^+ channels open and the fast depolarization begins. At the peak (20 mV), the slower K^+ are opened and repolarize the cell below its resting potential. Active ion pumps restore the membrane potential to its initial value. Note that the exact values for membrane potential and time can be different for APs generated by different neurons.

2.2. The sensation of touch and pain

The sensation of touch and pain are quite similarly generated through the stimulation of peripheral receptors which signal through a pathway connected to a certain region in the brain. There are many different types of receptors, responsible for detecting a variety of mechanical, thermal and painful (noxious) stimuli and they are categorized as mechanoreceptors, thermoreceptors, and nociceptors respectively [1, p. 147]. Additionally, there are different types of nociceptors: mechanosensitive nociceptors, thermal nociceptors, and polymodal (responsive to various stimuli) nociceptors. The first two types are so called Aδ nociceptors, meaning that their axons are myelinated and therefore transfer signals more quickly due to increased conduction velocity. The axons of polymodal nociceptors are unmyelinated and they therefore categorize as C nociceptors, which transmit signals slower than their counterpart due to having unmyelinated fibres [2, p. 473-474]. Aδ nociceptors are responsible for the sharp initial sensation of pain, while C nociceptors are responsible for the prolonged, duller sensation of pain. When a nociceptor is triggered by a stimulus, it sends a series of APs along the spinal cord through various relay structures and finally to the primary somatosensory cortex (SI), where the sensation of pain is perceived. This long pathway only consists of three neurons, which are connected by only two synapses.

The SI is topographically arranged, meaning that adjacent areas on the skin are also adjacent in the SI. This arrangement ensures fast processing of a stimulus which triggers adjacent nociceptors since the signal only must travel to one small region in the SI. The area in which a receptor senses a stimulus and therefore generates APs is called its receptive field. Most nociceptors have rather large receptive fields, since it is more important to detect the actuality of pain rather than its precise location [1, p. 165]. Repeated stimulation of a nociceptor can cause it to react stronger even though the strength of the stimulus is not increasing. A receptor which acts this way has been sensitized, and leads to a stronger sensation of, and response to, pain. This increased sensitivity to pain is called hyperalgesia and can be used to increase the response of normally non-noxious stimuli. Additionally, this sensitization can affect areas surrounding the stimulated area, resulting in secondary hyperalgesia. A common way to induce hyperalgesia in animals is through UVB (mid-range UV) irradiation which elicits an inflammatory response in the skin of the affected area. One can then apply a stimulus like a CO_2 laser to the sensitized area to induce a noxious stimulus.

2.3. Today's method of measuring pain

The current method of evaluating nociception involves testing the reflex response of an animal upon experiencing noxious stimuli. Usually a certain area of the rat is sensitized and then stimulated to induce a sensation of pain, upon which the following reflex of the rat is observed. If the reflex is triggered a majority of times during repeated testing, the animal is said to experience pain. This way of testing is rather limited, since the behavioral response relies on observing a reflex which can either be elicited or not. Also, the examined reflexes have been shown to not be specific for nociception, meaning that other non-painful stimuli (e.g. touch) could elicit an equal behavioral response [3]. This non-specific trait of the behavioral response could lead to false positives and therefore a larger uncertainty in the results which could greatly impact the outcome of a validation test of a potential new analgesic. Additionally, many analgesics have a sedative effect which impacts the motor system [4][5]. Since the reflex response heavily depends on the motor system, this indicates that it might be difficult to differentiate between the sedative and the analgesic effect of a drug through behavioral responses. Although the behavioral method is lacking, it is still used due to it being intuitive, cheap and simple to use. Finally, this method is applicable to any part of the body, allowing testers to examine whether an analgesic works locally or all over the body.

2.4. Neural recordings

Neuronal activity is often measured using an electrode array which can take many different forms. The electrode array is implanted into the desired brain region where it can record different signals. It is therefore crucial to be aware of what signals are present in the acquisition system to avoid faulty conclusions. The APs (spikes) are not the only signals that are generated inside the brain. There are in fact many signals present and it is therefore important to know which are interesting and which to omit. This obviously depends on the purpose of the measurement, but in this case 'interesting' signals will be considered signals that describe the activity of cells that respond to stimuli on the paw. This means that the interesting signals in this case will be the spikes coming from the individual stimulated cells. A change in spike frequency for a certain cell corresponds to that cell being stimulated. Thus, the activity of the desired cells corresponds to the measured spikes, but what other signals are present? One is the local field potential (LFP), which is the potential yielded by the summated ion current contribution from many cells in a local volume inside the brain. Mainly the LFP is built from the synaptic currents, but the temporal and spatial distribution of the cells are

very important in the generation of an LFP. Thus, the cells need to be synchronous in their activity and rather closely packed to generate a measurable LFP [6]. By utilizing a filter, one can separate the LFP from the spikes, since the spikes are of higher frequency [6]. Finally, one must also consider the contribution of noise, which is always present. Mainly, noise in neural recordings comes from two sources: thermal noise and spikes from distant cells [7][8]. Thermal noise exists due to the Brownian motion of ions, indicating that ions move randomly and collide with each other. This motion causes brief separations of charges, which results in a small potential. The noise (activity) from distant cells can be hard to filter out since they have the same frequency and waveform as the desired signal. The difference lies in the amplitude, which is much lower for the activity from distant cells. To solve this problem, one usually applies an amplitude threshold, meaning that the signal needs to have higher amplitude than the threshold to be perceived as an actual signal. The signals that do not exceed this threshold are regarded as noise.

2.5. Using neural recordings to measure pain

Two articles were examined and compared, where the aim was to investigate and measure neuronal activity connected to pain in rats [9][10]. Both articles used UVB irradiation to induce hyperalgesia on the heel of a rat's paw and CO_2 laser to cause noxious stimuli. An electrode array was used to record C fibre evoked potentials, which are the potentials created in the SI due to signals from the C fibres. Also, behavioral tests using tactile stimulation were used to confirm and evaluate the method by comparing with the standard approach. Both articles reported a significant increase in the magnitude of C fibre evoked potentials in the SI when comparing the effects of CO_2 laser stimulation before and after inducing hyperalgesia. This increase seemed to be strongest one day after UVB irradiation and then slowly decrease over the following days. In both articles, if the animal was treated with analgesics, the magnitude of the evoked potentials decreased to control levels, meaning that this type of neural recording is sensitive to also measure the effect of analgesia and not only nociception. This allows the method to more accurately determine the effect of analgesics through comparing the magnitude of evoked potentials in SI before and after administering the analgesic. These findings do not rule out other brain regions as key players in detecting and measuring pain, nevertheless they are still important since it has been proven that evoked potentials in SI correlate with perceived pain in humans [11].

3. Tools

3.1. Experiment animals

Laboratory animals are necessary when performing pain-tests, despite the ethical controversy. An ethical permit has been granted which covers the execution of this project. The animals used are Sprague-Dawley rats, which have been accustomed to the lab. The experiments involving the rats are performed by lab workers educated in handling laboratory animals.

3.2. The CatWalk system

To accurately describe the gait of a rat, the CatWalk XT system (Noldus, Wageningen, The Netherlands) is used. It consists of a mounted glass plate acting as a walkway for the rat and a high-speed video camera filming at 100 frames per second from below the glass plate (see Fig. 3). Through the long edge of the glass plate, green light is shining from a light source and the green light is completely internally reflected in the glass plate. When the animal touches the glass plate, the light will scatter and the print will illuminate in green light. Slightly above the glass plate sits a cover, which illuminates red light towards the camera to increase the contrast of the rat and the paw prints. The camera captures the illuminated paw prints and the video can be exported and used in further analysis.

3.3. Sedative system

To sedate the animals during UVB irradiation, the DRE Compact 150 Rodent Anesthesia Machine (DRE Veterinary, Louisville, USA) was used which connects three small tanks containing oxygen gas, nitrous oxide gas and isoflurane gas. Isoflurane gas acts as the anesthetic whereas the other two gases acts as carriers. The system is connected to a tube, which can be lead into a small container where the animal is kept until it is fully sedated.

3.4. UVB lamp

A UVB Narrowband PL-S 9W/01/2P lamp (Philips, Amsterdam, Netherlands) was used to irradiate the lateral side of the right hind paw of each rat. The lamp has a power of 8.6 W and provides light with a wavelength of roughly 300 nm. The lamp was mounted in a frame which was set to the same distance from the rat's paw for each rat.

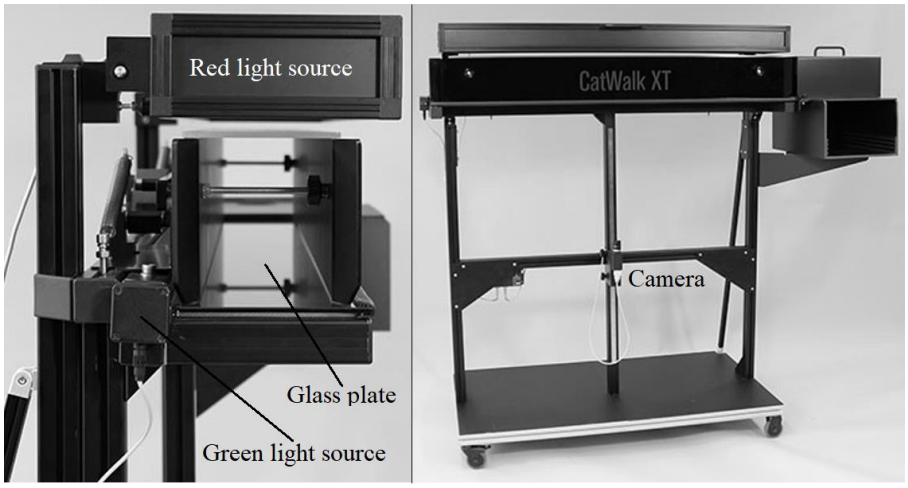


Fig. 3. The CatWalk XT system. Left: The glass plate where the animals are walking is mounted on a frame and green light is shone through the long side of the glass plate. The cover (top) illuminates red light to increase the contrast in the video. Right: The camera is mounted below the glass plate and records the runs. Adapted from [12].

3.5. Laser Doppler monitoring system

The Moor VMS-LDF (Moor Instruments, Axminster, United Kingdom) system was used to validate the inflammatory response during UVB irradiation. This monitoring system works through measuring the Doppler shift on a laser which is shone via an optic fibre through the skin on the desired part of the body. When the laser hits moving objects (e.g. blood cells) the light is reflected with a shift in frequency and is detected by a sensor. The magnitude and frequency of the reflected light is related to the number of moving cells and their velocity [13]. During inflammation, the local blood vessels dilate and the blood flow is increased, which increases the amount of red blood cells present in the blood vessels. The monitoring system can therefore evaluate the effect of inflammation by measuring the frequency and magnitude of the reflected light, allowing the verification of induced hyperalgesia.

4. Method

4.1. Acquiring run data

Six female Sprague-Dawley rats were used for the experiments. The rats were allowed to walk on the CatWalk while being recorded one by one until they were too unresponsive to complete a run in a reasonable amount of time. Light touching directly on the rat or at the immediate surroundings was performed to keep the rats from stopping on the walkway for too long. The glass plate was cleaned using water and dish soap between each animal to remove any dirt left on the surface of the glass plate.

After each rat had completed its runs, Doppler measurements were taken on the right hind paw as control. The flux of blood cells and the mean intensity of the reflected light were measured. The rats were then placed in a small container connected to the sedative system by a tube. Through the sedative system, the input of sedative gas to the container can be controlled. The rats were sedated by a mix of oxygen gas, nitrogen gas and 2% isoflurane. After the rat had been fully sedated, the isoflurane amount was lowered to 1% to maintain the anesthetic effect. During the time of anesthesia, the lateral side of the rat's right hind paw was irradiated with UVB (mid-range UV) light to induce hyperalgesia (see Fig. 4). The UV-lamp's intensity was measured to 17 mW/cm^2 and was mounted to a small frame to keep a constant distance between the skin area and the lamp for each rat. The animals were treated with approximately 1.3 J/cm^2 of irradiation, since this dose has been shown to induce hyperalgesia without causing blisters [9]. The lateral side of the right hind paw of each rat was exposed to the UVB lamp for 15 minutes. The rest of the hind paw was covered by tin foil to protect it from the irradiation. After the UVB treatment was complete, the rats were put back in their respective cages and were freely allowed to wake up.

24 hours later Doppler measurements were taken again on the affected part of the rats' right hind paw to validate the hyperalgesia. The flux of blood cells and the mean intensity of the reflected light were measured. The rats were then allowed to walk on the CatWalk in the same manner as in the control run. 48 hours after the control experiment, another experiment was performed, where the same Doppler measurements were taken yet again and the rats performed CatWalk runs as in the previous experiments. The results from the Doppler measurements are shown in Appendix A.

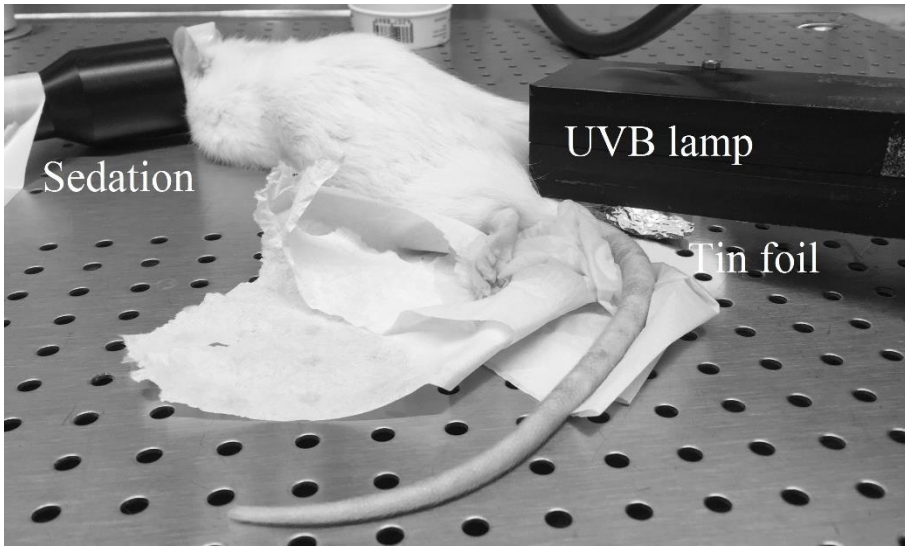


Fig. 4. A rat under anesthesia being UVB irradiated on the lateral side of its right hind paw. The rest of the right hind paw is covered by tin foil to protect it from the radiation.

4.2. MATLAB program for gait analysis

A program was created to analyze all the desired parameters which describe the gait of a rat. The program was to accept one or more video files as input, analyze the gait of the rat and provide a measurement of the pain of the rat by comparing it to a predefined database of parameter values which was to be created through the experiments mentioned above. The intended workflow of the program can be seen in Fig. 5. To first generate the database of parameter values, the last two steps in Fig. 5 were omitted.

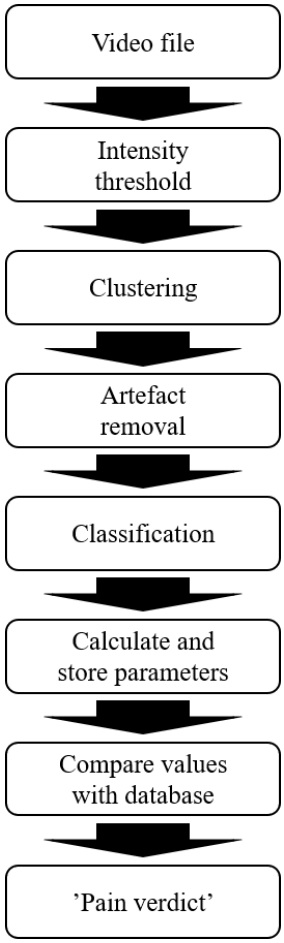


Fig. 5. The workflow of the MATLAB program. To create the database of parameter values, the data gathered from the experiments mentioned in section 4.1 was used and the last two steps of the program was omitted.

4.2.1. Video import and pawprint detection

The experiment resulted in 205 valid runs and their run videos (recordings of the rats running on the CatWalk with green illuminated paw prints, see Fig. 6) were exported and loaded into MATLAB (MathWorks, Massachusetts, USA). The video files were divided into their respective frames, forming a three-dimensional matrix for each frame (in total four dimensions), containing the red, green and blue (RGB) values for the intensities in every single pixel. Since the prints are illuminated in green, only

the matrix corresponding to the green values was used for intensity-based detection of pawprints. The intensity values that exceeded a certain threshold of 40 (determined experimentally using all runs and comparing intensity values) were considered to be part of the prints of the rat's paws and were therefore selected and stored in a new matrix with the same size as the previous matrix, but with only three dimensions. This new matrix now contained only the intensity and location of the detected prints of the rat and possible artefacts. These artefacts could be illuminations caused by junk caught in the fur of the rat or simply other things than the paws of the rat that happened to touch the glass plate during the run. The position, intensity and frame number of every pixel considered to be part of a print was stored in a new matrix.

Fig. 6. A snapshot of a run video of a rat walking on the CatWalk, filmed from below. Due to the red light above the rat, the rat's body is shown in black (zero intensity). The green laser which is shone through the glass plate causes the glass plate to illuminate when the rat walks on it. Two prints are shown in this frame, belonging to the rat's left front paw and right hind paw.

4.2.2. Clustering of pixels

Since every print consists of a multitude of pixels over several frames, a grouping or clustering method is required. A clustering method called Density-Based Spatial Clustering of Applications with Noise (DBSCAN) was used, which clusters the pixel points using a certain algorithm [14]. The algorithm selects a point as the initial cluster point, calculates the distance between the selected point and every other point. If a point is within a certain radius of the selected point, that point gets added to the same cluster as the selected point. Since the algorithm checks the distance between every point to every other point, the amount of calculations quickly adds up (quadratic dependence) if too many points are included. To solve this issue, the clustering algorithm was run in two steps. Initially, the clustering is

performed frame by frame, resulting in a large number of clusters. After this initial step, the clustering is performed yet again, this time with the center-points of the clusters given in the first step as the input. This method will ensure that not too many data points are being used in the clustering algorithm, since it drastically reduces the number of inputs in the first step. Besides determining the radius, one can also select the minimum number of points that are allowed to form a cluster. The points that are not part of any cluster after the algorithm is completed are marked as noise. The radius and minimum number of points were determined experimentally using all runs to 6 and 4 pixels respectively. The radius has to be small enough to not include points which belong to another print, but large enough to be able to cover all the points of a single print. The minimum number of points has to be small enough to allow prints which consist of a small number of points to be clustered, but large enough so that smaller parts of a complete print are not labeled as a separate cluster. What remained after this process were the clustered prints, i.e. groups of pixels in the input video associated with individual pawprints, see Fig. 7, including their position in three dimensions and the number of pixels that form each print.

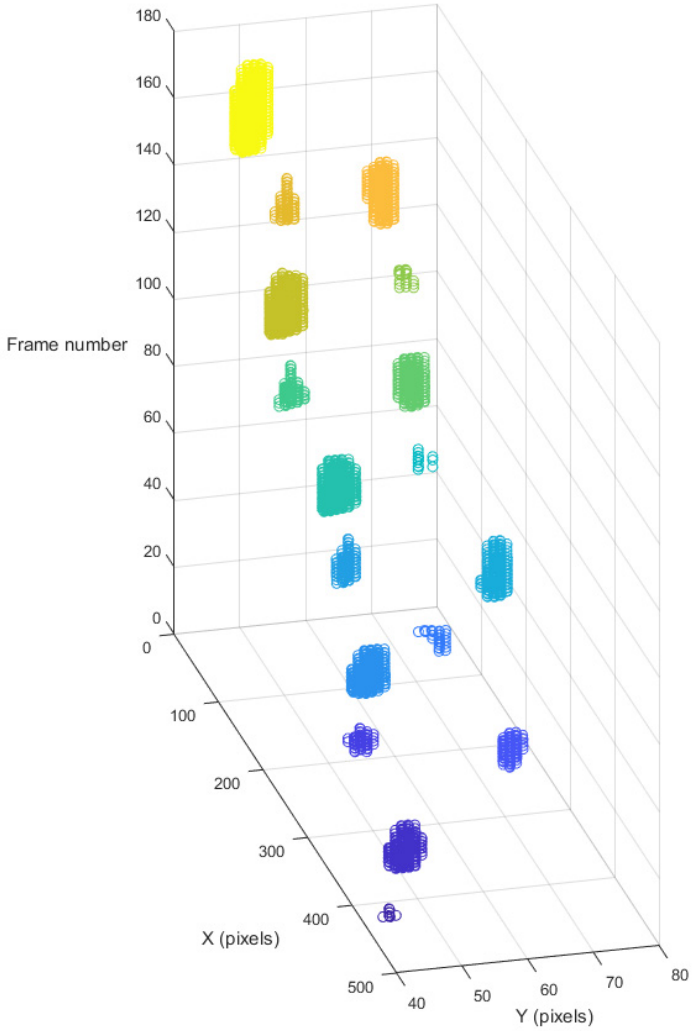


Fig. 7. The clustered prints from the same run as in Fig. 6. Each print corresponds to a cluster with a separate color. In total 17 clusters are shown, which correspond to 17 different prints. The frame number indicates which frame each print was present in. Note the difference in x- and y-scale.

4.2.3. Artefact removal

The possible artefacts were present during the entire video which gave them a very elongated appearance (in the frame dimension) when observing them in a similar fashion as in Fig. 7. These elongated artefacts could also

appear to be "severed" into two or more parts (see Fig. 8), caused by the hand of the lab worker accidentally covering part of the camera while trying to encourage the rat to walk. Since these artefacts also were clustered as normal prints, it was important to be able to separate them from normal prints in order to perform accurate analysis later on.

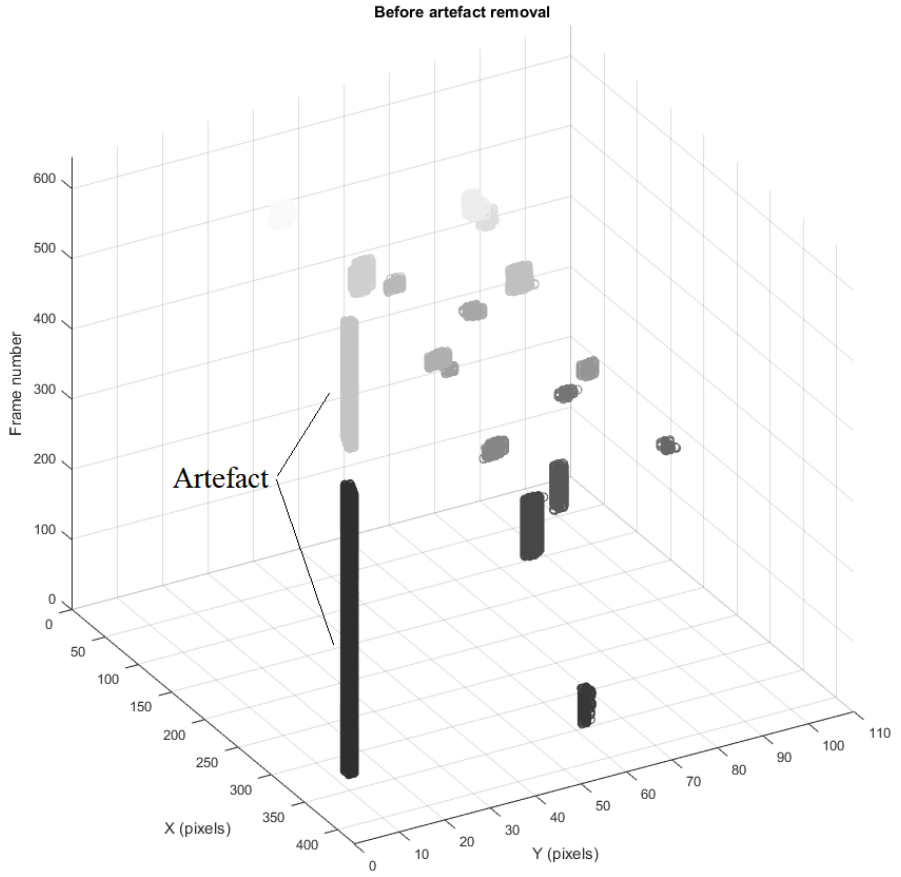


Fig. 8. Clusters from a run containing a severed artefact, which has been separated into two elongated clusters. The elongated and 'constant' shape of the artefacts stand out against the shorter, more irregular normal prints. These clusters will all go through the separation algorithm to detect and remove artefacts. Note the difference in x- and y-scale.

The separation of artefacts and prints was performed in two steps. First, the duration (in frames) and start-time (frame number) were calculated for

each cluster. A plot of duration versus start-time from a run containing an artefact is shown in Fig. 9.

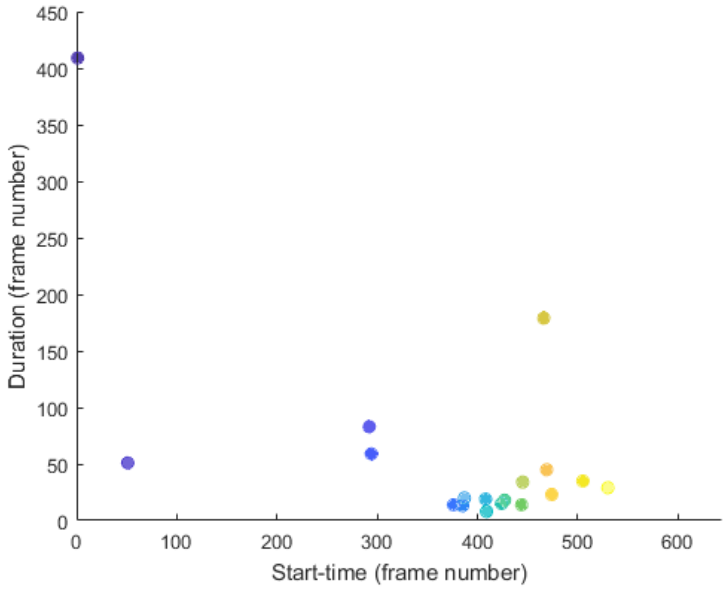


Fig. 9. Duration versus start-time for every cluster for a run containing a severed artefact (same run as in Fig. 8). Each circle represents a cluster and has a different color. The artefact is seen in this figure as the two points with the highest duration. Since the camera filmed with 100 Hz framerate, 100 frames equals 1 second.

From the data shown in Fig. 9 it is possible to determine which clusters are artefacts by looking at how many clusters are present during the duration of one cluster. Consider the first cluster, shown in Fig. 9 with a duration of over 400 frames (4 seconds) and a start-time of 0. If this cluster had been an actual print, belonging to one of the rat's paws, it would not have been possible to witness more than 3 other clusters during the duration of the print, since the rat only has four paws. Since the first cluster has a duration of over 400 frames and more than 3 clusters are present during this time, the first cluster is therefore regarded as an artefact. This process can be exerted on all clusters except the final four, since those clusters cannot have more than 3 clusters present after them. To cover the case where an artefact is present in one of the final four clusters, the second method is utilized.

If an artefact is present in one of the final four clusters, it is because it has been severed from another artefact, as shown in Fig. 8. Here the artefact is divided into two clusters, with an x-value of 400 pixels and a y-value of 10

pixels. The second method calculates the midpoint in the xy-plane for every cluster and compares them to each other. If two or more clusters share the exact same midpoint, they are considered to be severed artefacts. This does pose a risk to eliminate normal prints, but the probability of a rat to put two paws in the exact same place after each other (down to single pixel resolution) is extremely low. All in all, these two methods combined allows artefacts of this nature to be removed, while still keeping the normal prints unaffected. Fig. 10 shows the remaining clusters after the two methods were applied. By comparing it to Fig. 8, it is clear that only the artefacts have been removed by this process.

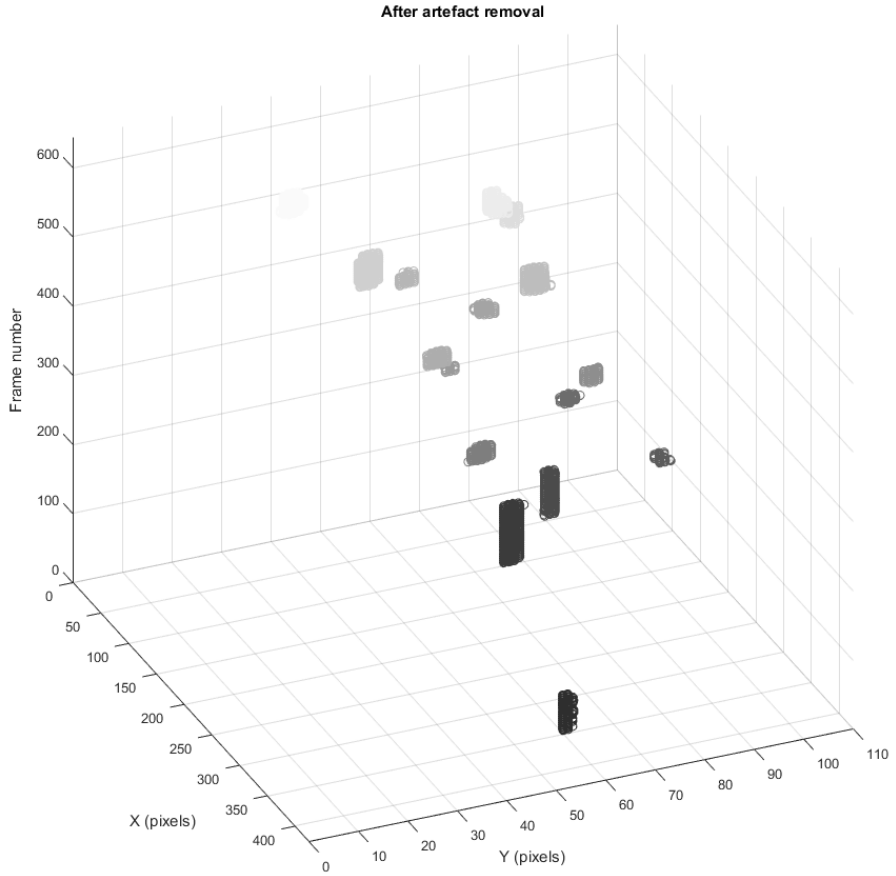


Fig. 10. After the separation algorithm, only the normal prints remain. By comparing the appearance of the prints in this figure and Fig. 8 it is clear that the prints remain unaltered. These clusters will go through the classification process, to determine which prints belong to which paw of the rat. Note the difference in x- and y-scale.

4.2.4. Classification of pawprints

To compare and fully analyze the prints, it is necessary to know which print is connected to which paw. This process, called classification, labels the prints with a number or set of numbers, which correspond to a certain class. In this case there are four classes: right front, left front, right hind and left hind. When all prints are labeled correctly, a complete intensity mapping of every print from every paw during a run has been achieved. The classification process is done in two parts, where the prints are first labeled corresponding to belonging to a front or hind paw and then labeled again corresponding to the prints belonging to the right or left side. This means that a fully classified print might have a label e.g. (1, 2) meaning front plus left, indicating that the print belongs to the left front paw. The first number thus corresponds to the first step (front or hind) and the second number corresponds to the second step (right or left).

The first part of the classification is done by comparing the x-position (the length-dimension of the CatWalk walkway) of two to four simultaneous prints. Depending on the direction in which the rat is walking (right to left or left to right), the comparison in x-position results in different outcomes for the classification. If the rat is walking from right to left in the video, then the print with a smaller x-value will be considered as a print coming from a front paw and vice versa (see top of Fig. 11). After a comparison between two to four prints are completed and the correct labels have been assigned, the classification algorithm selects the next succeeding print and continues the comparisons. This algorithm proceeds until all prints except the first and the last have been classified. The reason why the first and the last print are not chosen to be included by this algorithm is because they will be wrongly classified due to the prints being near the edges of the video. In the beginning when the rat enters the frame of the video, only the front part of the body is visible and therefore also only the front prints. A comparison between the two front prints will classify one of them as a hind print, which is wrong. The same concept is true for the end of the video, where only the hind part of the rat is visible.

When the prints have been labeled as either front or hind, the next part is to determine whether the prints come from the right or left paw. This algorithm works in a similar way, except that it compares two successive hind prints or two successive front prints with each other. Since it is unlikely that a rat will cross its front or hind paws during a walk, this comparison will determine which print belongs to which side of the rat's body. This time, the y-position (the width-dimension of the CatWalk) of the succeeding front or hind prints are compared. As before, the direction of the rat in the video

determines the outcome for the labeling. If the rat walks from right to left and two succeeding front prints are compared, the one with the smaller y-value will be considered a left front print and vice versa. Note that the video is recorded from below the CatWalk, meaning that the rats are seen from below and the direction of the y-axis shown in Fig. 11. The algorithm then selects the next two succeeding front or hind prints and compares their y-position. Since the first and last print were not labeled in the previous algorithm, they cannot be labeled in this one either, since this algorithm relies on the labels from the previous algorithm. Once these two parts have been completed all (but the first and last) prints have been fully labeled and therefore classified into one of the four classes. Now it is possible to compare different types of prints to each other (front prints to hind prints, left prints to right prints, etc.) which is crucial in later gait parameter analysis.

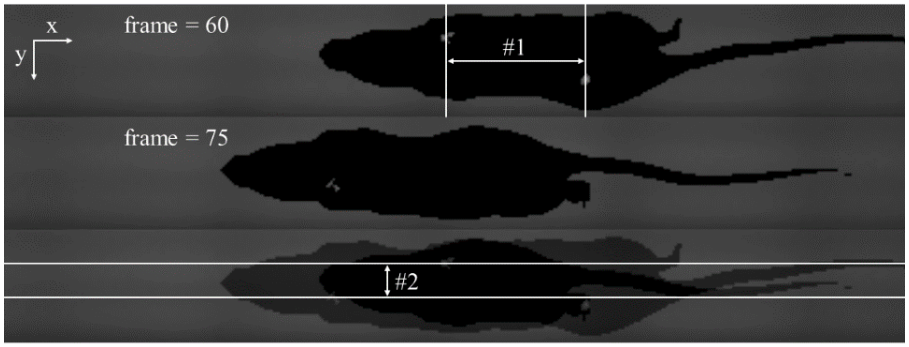


Fig. 11. Top: The same time point in the same run as in Fig. 6 (time point 60). Here, the x-position is compared between the two present prints. Since the rat is walking from right to left, the print with the smaller x-value is deemed to be a front print, and the print with a larger x-value is deemed to be a hind print. Middle: 15 frames later, the next front print is present. Bottom: Overlapping the two timepoints, the second comparison is visible. The y-value of the two front prints are compared. Due to the rat's direction, the print with the larger y-value is deemed to be a right-hand side print and the print with the smaller y-value is deemed to be a left-hand side print. Note that the x- and y-directions are identical in all three images.

4.2.5. Calculate and store parameters

Once the classification step is complete, each print is labeled, and its position and intensity are already known. Now it is possible to extract gait parameters and compare these between different paws which have or have not been UVB irradiated. The following gait parameters and data were

extracted from all the prints through using their position and intensity values. Fig. 12 displays a typical print and how the print length (spread in x-dimension) and print width (spread in y-dimension) were calculated. Fig. 13 shows the max projection of the same print.

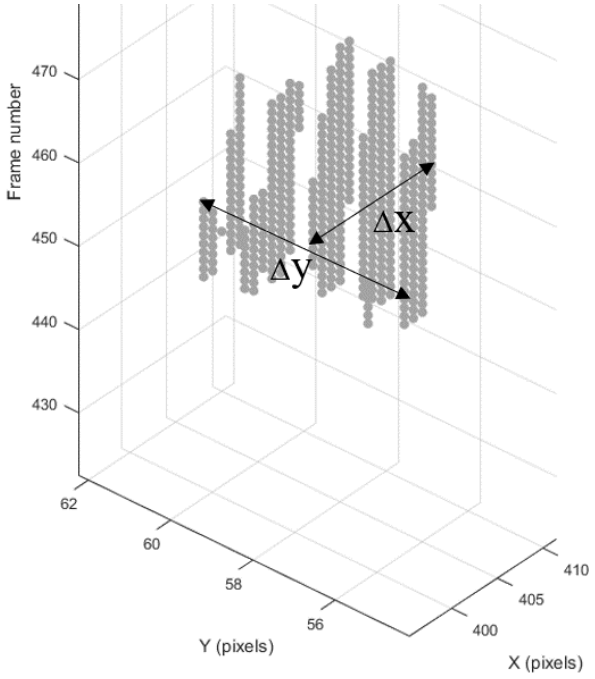


Fig. 12. A typical print shown in the same manner as in Fig. 7. The print length and width were calculated as the spread in the x- and y-dimension respectively. Note the difference in x- and y-scale.

- Mean intensity of all pixels which form a complete print.
- Print length (the print's spread in the x-dimension) in pixels.
- Print width (the print's spread in the y-dimension) in pixels.
- Max projection (a matrix showing the maximum intensity value of each unique pixel forming the complete print).
- Mean area of the part prints (cross-section in the frame dimension) in pixels.
- Max area of the print (the largest area from the part prints) in pixels.
- Total number of pixels which form a complete print.

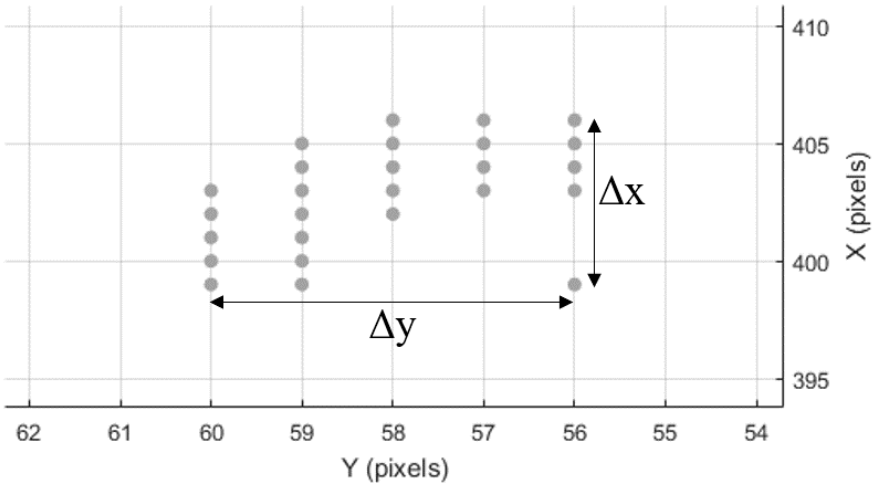


Fig. 13. The max projection of the same print shown in Fig. 12. Each dot now represents the largest intensity value for each pixel that share the same position in the xy-plane. Note the difference in x- and y-scale.

- A normalized value indicating the intensity distribution between the lateral and medial side of a paw, calculated as $\frac{b-a}{b+a}$ where a and b are the summated intensities of the medial and lateral side respectively. This means that the value is bound between -1 and 1, where 1 indicates much more support on the lateral side and -1 indicates much more support on the medial side. If this value is equal to zero, the rat is supporting equally on both sides of the paw.
- A second normalized value indicating the area distribution between the lateral and medial side of a paw, calculated in the exact same way as the previous parameter, except that a and b are now the area of the medial and lateral side respectively.
- The stand time (in seconds), measuring the duration a paw touches the glass plate in one gait cycle (cycle of taking an entire step with one paw).
- The swing time (in seconds), measuring the duration of a paw not touching the glass plate in one gait cycle.

- The mean intensity, image and total number of pixels for every part print which form the complete print.
- The area of the max projection.
- The mean intensity of the max projection.
- The maximum intensity value.

Every print was also labelled with the corresponding date, animal number, run number, print number, class, direction, condition (UVB irradiated or not) and treatment (specifying if this paw was UVB irradiated). These additional labels allowed further comparisons and increased the accuracy in data handling. All data was stored in a table in MATLAB and formed a database to later compare with.

4.2.6. Normality test and boxplots

To compare if there is a significant difference between parameter values for paws which have or have not been UVB irradiated, statistical tests are required. Some comparative statistical tests assume that the data is normally distributed (parametric tests) and others require no assumptions about the sample data (non-parametric tests). Therefore, the data of the various parameters was put through a Lilliefors test, which tests if the data is normally distributed or not. The Lilliefors test works by comparing the distribution of the data to a normal distribution and estimates a goodness of fit. The test compares the outcome of the test to its null hypothesis and can discard this hypothesis if the test outcome is low enough. In a Lilliefors test, the null hypothesis is that the data comes from a normal distribution [15].

Since the data was not shown to be consistently normally distributed (see Appendix B) a parametric test could not be chosen for analysis. Instead, boxplots were made of the data using MATLAB, comparing the right hind paw before and after UVB treatment for every parameter. Also, the right hind paw was compared with the left hind paw, and the difference in parameter value was compared before and after UVB treatment. A boxplot displays the median, the first and third quartiles and the confidence interval for the median (with desired confidence level). Boxplots are used in this analysis to compare different data sets. If the confidence intervals of the medians overlap, the medians are not significantly different (see Fig. 14). To generate boxplots requires no assumption of the distribution of the data sets and is therefore considered non-parametric.

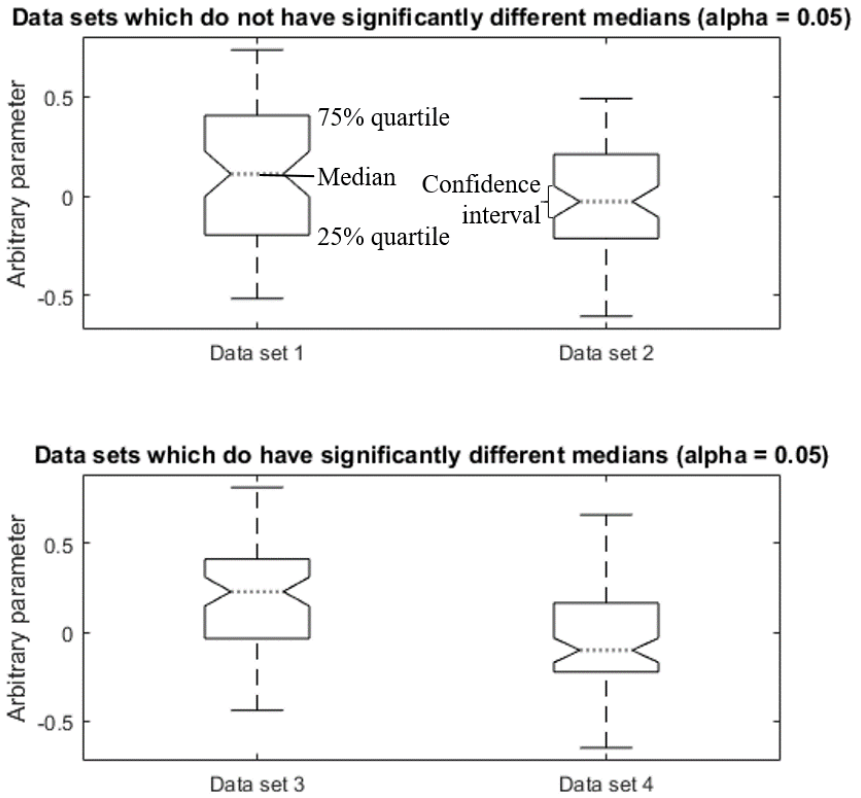


Fig. 14. Example of two boxplots which compares data sets with each other. The first quartile is shown as the bottom of the box, the median is shown as the dotted line and the third quartile is shown as the top of the box. The notches indicate the confidence interval for the median. Top: The confidence intervals overlap, meaning that the medians are not significantly different from each other. Bottom: The confidence intervals do not overlap, which indicate that the medians are significantly different.

Two different comparisons were performed in the analysis. First, the right hind (RH) paw was compared before and after UVB irradiation (24 hours and 48 hours after) for each animal for all available parameters. Second, the right hind paw was compared to the left hind (LH) paw before and after UVB irradiation (24 hours and 48 hours after) for each animal for all available parameters. This second comparison was performed to detect a shift in symmetry, indicating that the rat uses the right and left hind paw differently once it senses pain in one of them. 95% confidence intervals were used for all boxplot analysis.

4.2.7. Exclusion of rats 3,4 and 5

During the analysis of the run videos it was noticed that 48 hours after UVB irradiation, some rats did not touch the glass plate with the right hind paw at all while walking. Even 24 hours after UVB irradiation, it was unreliable to detect the right hind pawprint of these rats during video analysis. This greatly affects the classification process, as the classification is performed through comparisons between simultaneous prints. If the rat seems to only have three paws, the prints will often be wrongly classified and greatly affect the analysis in a negative fashion. Therefore, rat 3, 4 and 5 which did not touch the glass plate with the right hind paw in the third experiment (48 hours after UVB irradiation) were excluded from the analysis. The reason for this behavior could be that the rats are in too much pain to even want to touch the glass plate with the affected skin area, or that the touch is so light that the CatWalk cannot detect the print.

Although the prints belonging to these rats could be classified in the correct way, they still contain important information about the rats' gait. To be able to use some of the information, the maximum intensity was investigated for all paws (not classified) for these rats, to uncover any potential differences before and after UVB irradiation. The maximum intensity was chosen since if the rats did indeed walk on only three paws, the weight distribution should lead to a higher pressure being exerted on the surface, leading to a higher intensity maximum during a run.

5. Results

5.1. Comparing hyperalgesic paw before and after UVB irradiation

Five parameters showed significant results both when comparing the hyperalgesic paw before and after UVB irradiation, and when comparing the hyperalgesic paw with a non-hyperalgesic paw: max area, mean area, swing time, area of the max projection and mean intensity of the max projection. In Fig. 15-19 the results for rat 1, 2 and 6 (the rats that touched the glass plate 48 hours after UVB irradiation) for the right hind (RH) paw comparison are shown with 95% confidence level. Significant differences are implied by stars and deltas, where a star indicates that the right median is significantly larger than the left median. A delta indicates that the right median is significantly smaller than the left median. Outliers are indicated as crosses. Throughout Fig. 15-19 it is seen that 48 hours after UVB irradiation, significant differences in medians can be seen compared to before UVB irradiation.

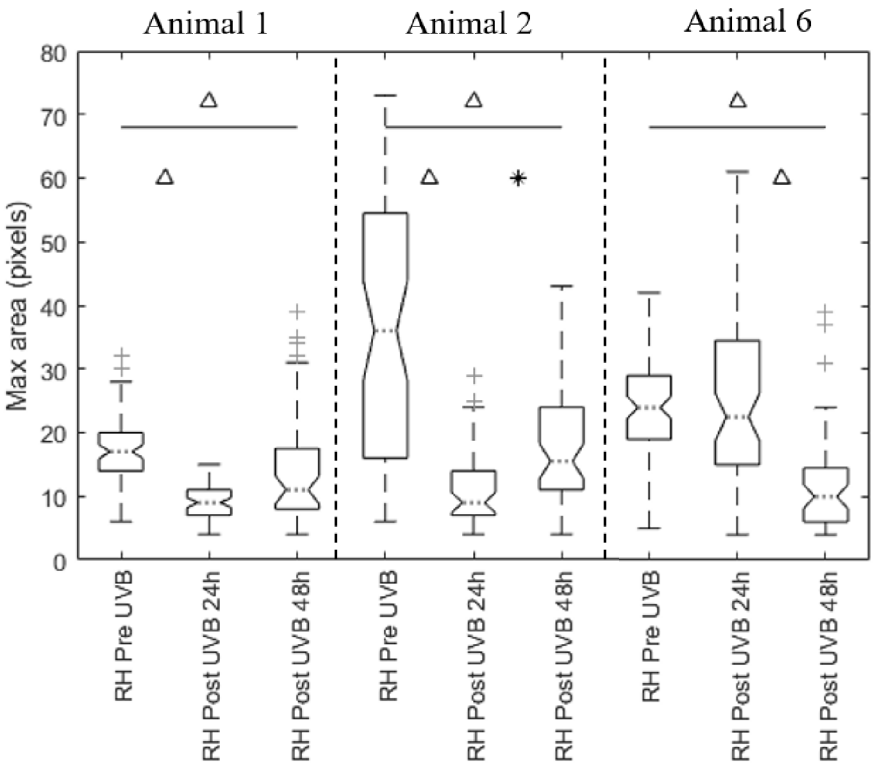


Fig. 15. Max area of rat 1, 2 and 6 measured in pixels. A significant difference before and 48 hours after UVB irradiation is seen in all animals. Delta indicates that right median is smaller than left and star indicates that right median is larger than left (95% confidence level).

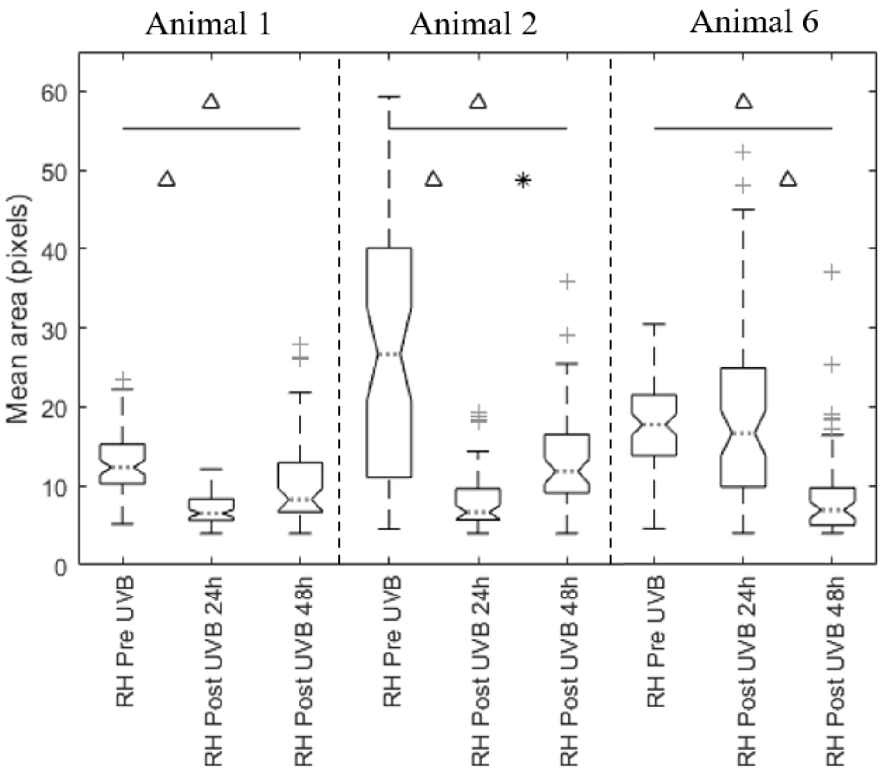


Fig. 16. Mean area of rat 1, 2 and 6 measured in pixels. A significant difference before and 48 hours after UVB irradiation is seen in all animals. Delta indicates that right median is smaller than left and star indicates that right median is larger than left (95% confidence level).

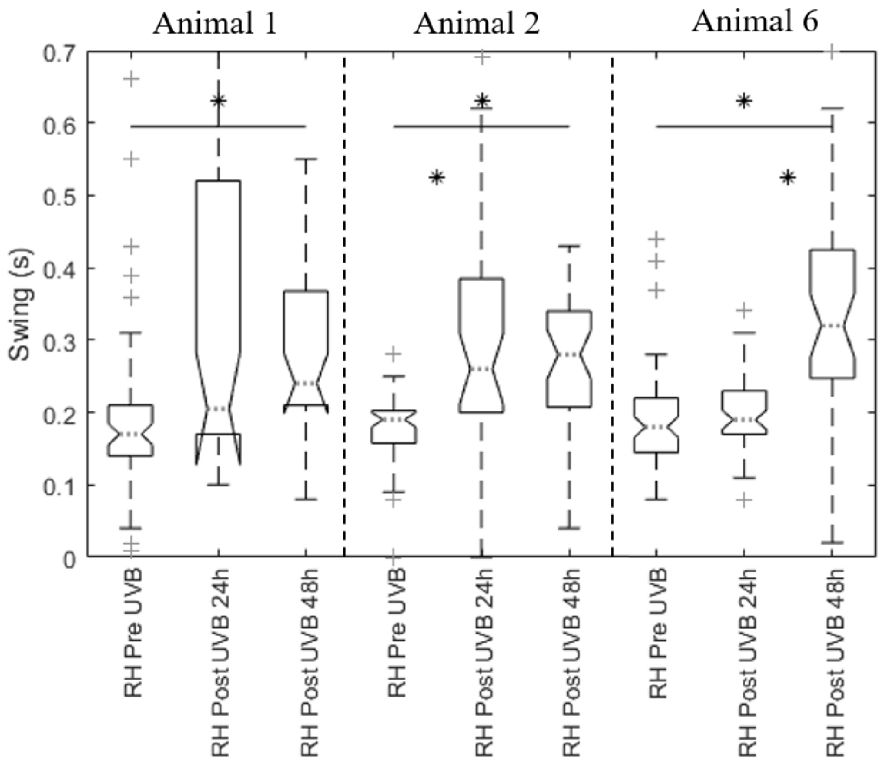


Fig. 17. Swing of rat 1, 2 and 6 measured in seconds. A significant difference before and 48 hours after UVB irradiation is seen in all animals. Delta indicates that right median is smaller than left and star indicates that right median is larger than left (95% confidence level).

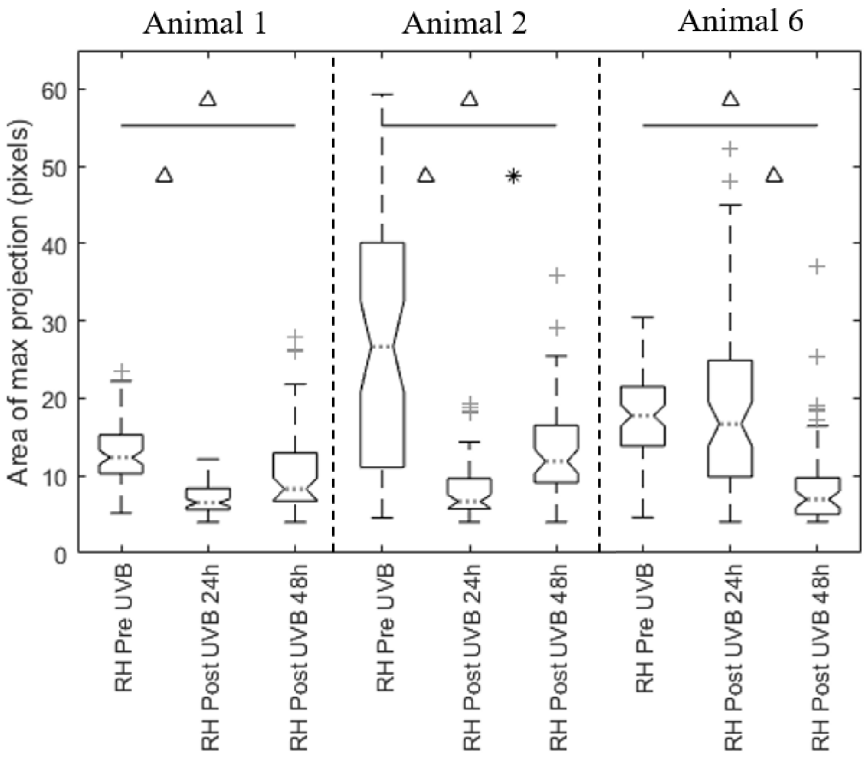


Fig. 18. Area of max projection of rat 1, 2 and 6 measured in pixels. A significant difference before and 48 hours after UVB irradiation is seen in all animals. Delta indicates that right median is smaller than left and star indicates that right median is larger than left (95% confidence level).

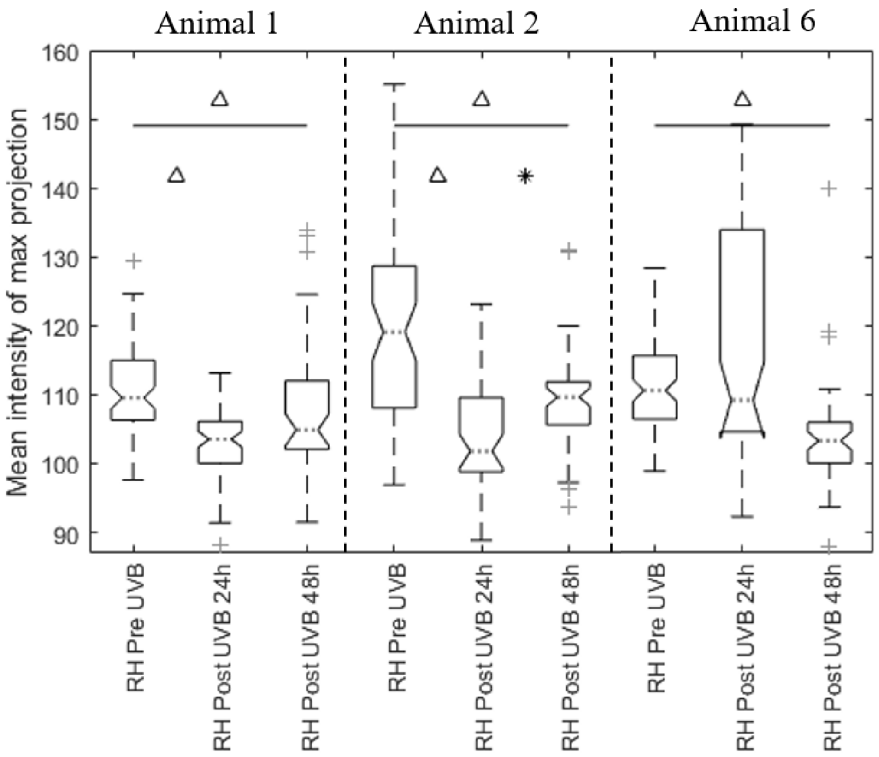


Fig. 19. Mean intensity of max projection of rat 1, 2 and 6. A significant difference before and 48 hours after UVB irradiation is seen in all animals. Delta indicates that right median is smaller than left and star indicates that right median is larger than left (95% confidence level).

5.2. Comparing hyperalgesic and non-hyperalgesic paws

Fig. 20-24 show the results for the right hind paw to left hind paw comparison for the same five parameters as in the previous comparison. Each figure consists of three boxplots, which compare the right hind paw and left hind paw for each animal (A1, A2 and A6) before, 24 hours after and 48 hours after UVB irradiation. From Fig. 20-24 it is seen that there are significant differences between the right hind paw and the left hind paw 48 hours after UVB irradiation for all parameters. Also, these differences are always different (larger versus smaller or vice versa) from the possible differences present before UVB irradiation, which provides a good method of distinguishing the two conditions.

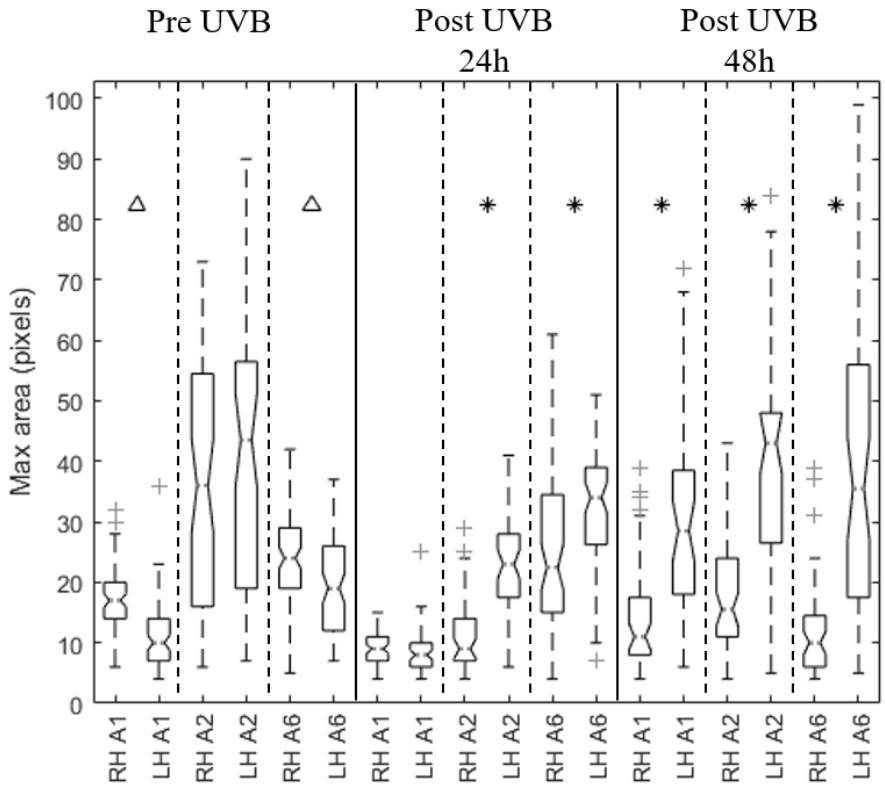


Fig. 20. Max area of rat 1, 2 and 6 measured in pixels. Before UVB irradiation, the area of the left hind paw is smaller or similar to the area of the right hind paw. 48 hours after UVB irradiation, the left hind paw has a significantly larger max area for all animals. Delta indicates that right median is smaller than left and star indicates that right median is larger than left (95% confidence level).

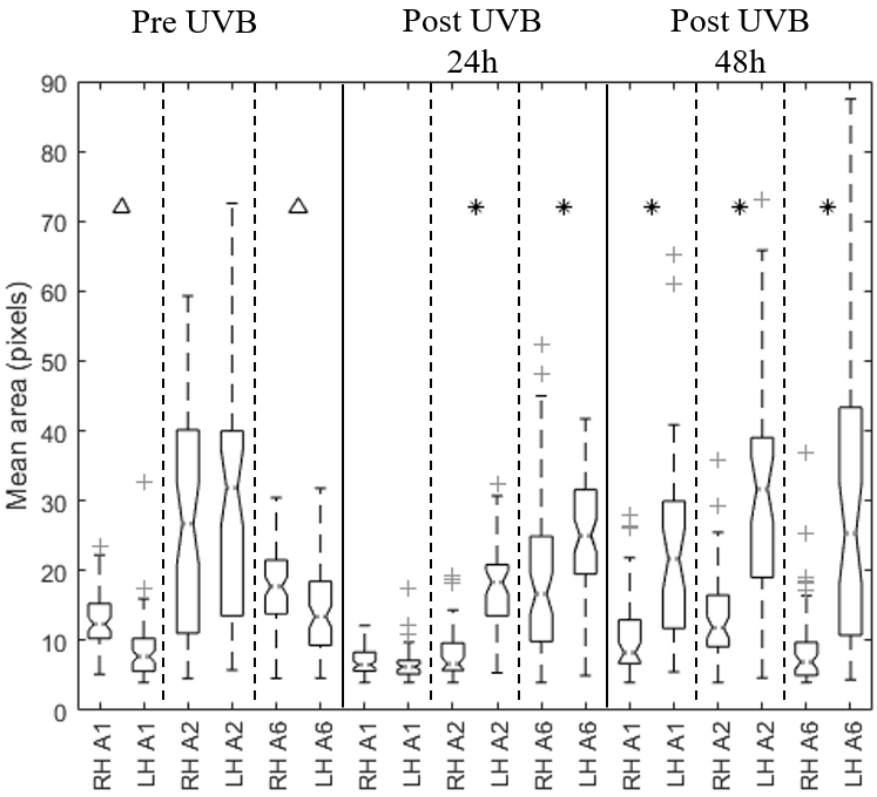


Fig. 21. Mean area of rat 1, 2 and 6 measured in pixels. Before UVB irradiation, the area of the left hind paw is smaller or similar to the area of the right hind paw. 48 hours after UVB irradiation, the left hind paw has a significantly larger mean area for all animals. Delta indicates that right median is smaller than left and star indicates that right median is larger than left (95% confidence level).

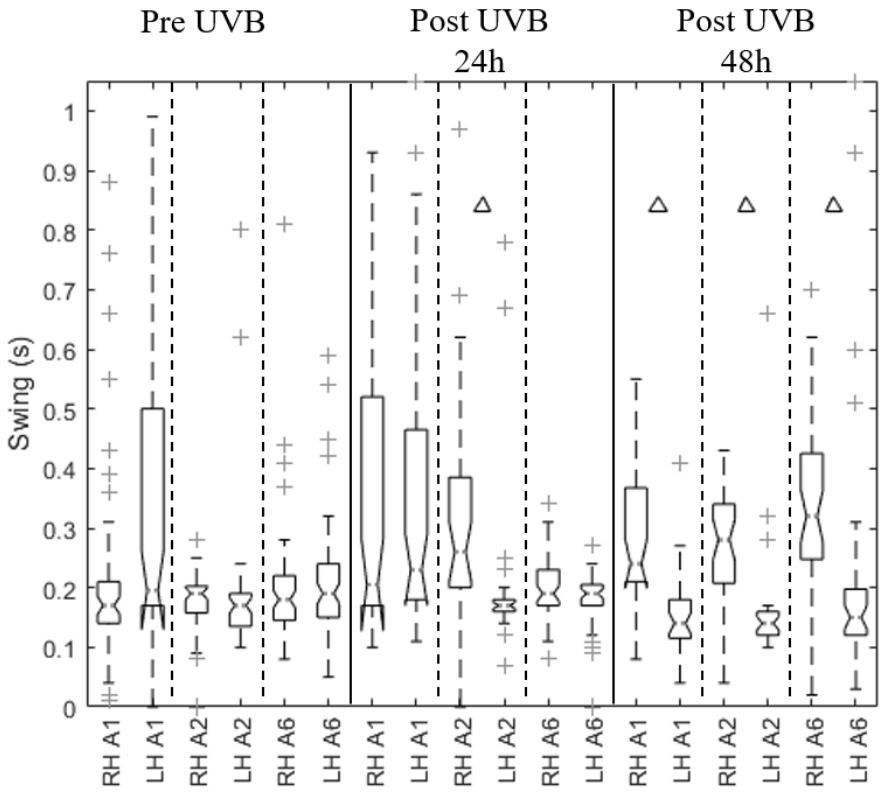


Fig. 22. Swing of rat 1, 2 and 6 measured in seconds. Before UVB irradiation, the swing time of the left hind paw is similar to the swing time of the right hind paw. 48 hours after UVB irradiation, the right hind paw has a significantly longer swing time for all animals. Delta indicates that right median is smaller than left and star indicates that right median is larger than left (95% confidence level).

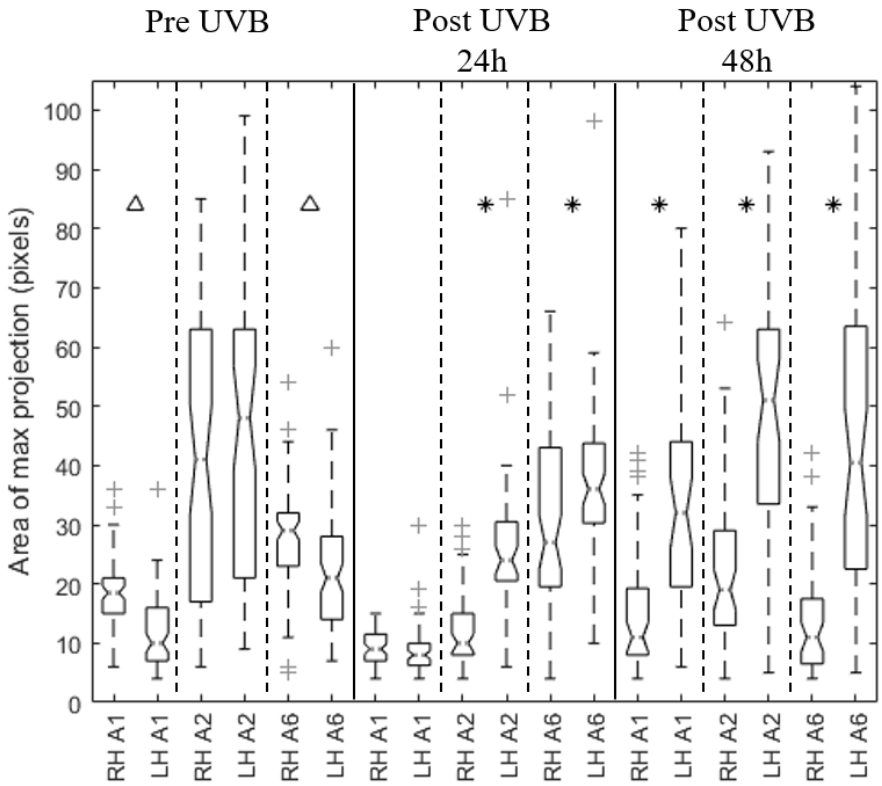


Fig. 23. Area of the max projection for rat 1, 2 and 6 measured in pixels. Before UVB irradiation, the area of the left hind paw is significantly smaller or similar to the area of the right hind paw. 48 hours after UVB irradiation, the left hind paw has a significantly larger area for all animals. Delta indicates that right median is smaller than left and star indicates that right median is larger than left (95% confidence level).

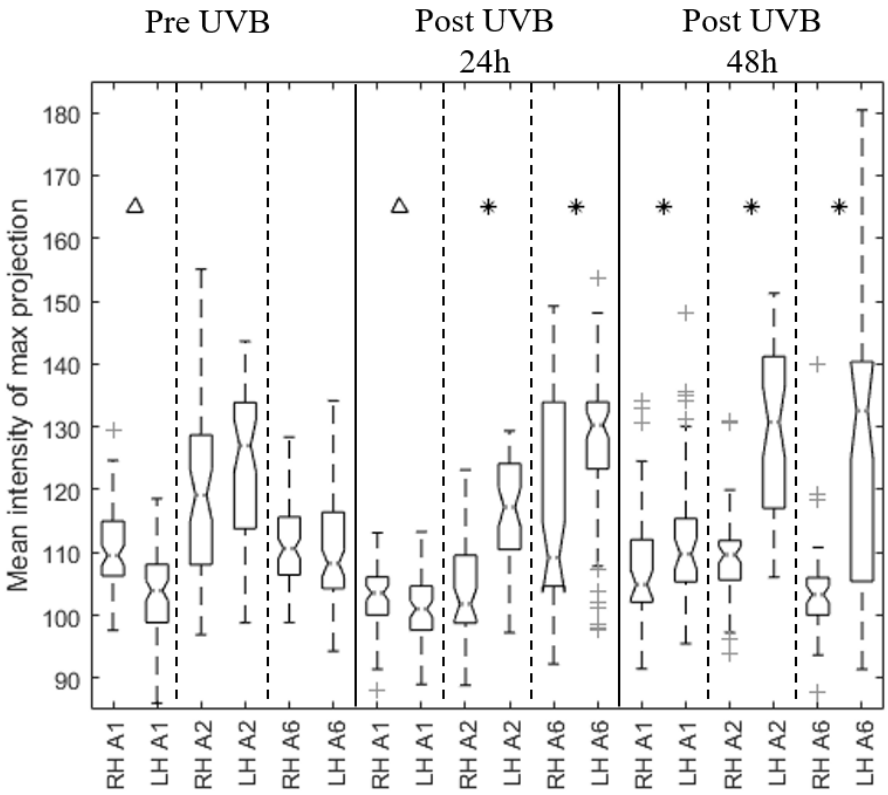


Fig. 24. Mean intensity of the max projection of rat 1, 2 and 6. Before UVB irradiation, the intensity of the left hind paw is significantly smaller or similar to the mean intensity of the right hind paw. 48 hours after UVB irradiation, the left hind paw has a significantly larger mean intensity of the max projection for all animals. Delta indicates that right median is smaller than left and star indicates that right median is larger than left (95% confidence level).

Both comparisons indicate that there are clear, measurable differences in the gait of a rat experiencing pain and a rat which is not experiencing pain. These differences take shape through a longer swing time and a smaller area and a lower intensity of the UVB irradiated paw in both comparisons. All of these findings reflect that the right hind paw has less contact with the glass plate after UVB irradiation than before, which is not surprising considering that the animal experiences pain upon contact.

5.3. Rats 3, 4 and 5

Since rats 3, 4 and 5 did not touch the glass plate with their right hind paw 48 hours after UVB irradiation, they were removed from the main analysis. However, those rats still contain much information which could be valuable for determining levels of pain in an animal. Therefore, the max intensity was examined for all paws (see Fig. 25), where it is seen that for both rat 3 and 5 the max intensity is significantly larger 48 hours after UVB irradiation compared to before UVB irradiation.

Since the results are not consistent for all three animals they cannot be used in any real analysis, but it still shows that there are other parameters that could be significant even for these rats that were excluded from the main analysis. To find these parameters, one must consider that the rats are essentially walking on only three paws 48 hours after UVB irradiation, and how this special gait pattern might affect certain gait parameters. The max intensity should increase in this case since if an animal is only walking on three paws, it distributes all its weight on three paws instead of four, which should then increase the pressure exerted by those three paws.

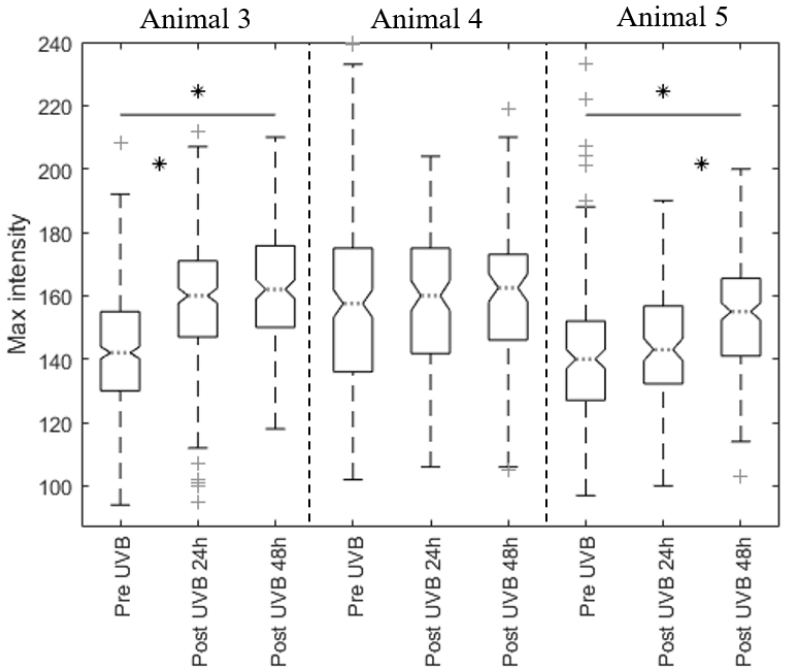


Fig. 25. The max intensity for animal 3, 4 and 5 for all paws. For animal 3 and 5, the max intensity is significantly larger 48 hours after UVB irradiation compared to before. This finding indicates that there are other parameters that might be significant even for these rats that were excluded from the main analysis, and that these rats still contain valuable data. Delta indicates that right median is smaller than left and star indicates that right median is larger than left (95% confidence level).

6. Discussion

6.1. Neural recordings

Since neural recordings require an electrode array to be surgically implanted into the subject's brain, it is crucial to establish that the presence of the electrode array does not affect the outcome of the experiments in a substantial way. One way to determine this is to compare the pain behavioral response using tactile stimulation before and after implantation of the electrode array. In both of the examined articles [9][10] it was concluded that the surgery and implantation of the electrode array did not affect the animal's nociceptive perception.

When inducing hyperalgesia, the sensitization is not restricted to the immediate area, but is also present in the surrounding area (secondary hyperalgesia). This indicates that in order to measure the full effect of nociception, it is important to also be able to measure the contribution from secondary hyperalgesia. In both articles it was noticed that the magnitude of evoked potentials was increased when stimulating both in the immediate (primary) and surrounding (secondary) area of UVB irradiation. It was also noticed that behavioral tests were unable to detect nociception through secondary hyperalgesia [10], discovering a clear flaw in the current standard approach. Thus, neural recordings are sensitive enough to detect the influence from secondary hyperalgesia and can therefore more accurately model the full perception of pain in an animal.

An important aspect regarding evaluation of analgesics is differentiating between the analgesic and sedative effects of a drug since the sedative effects will affect the motor system and therefore alter the reflex response [4][5]. A way of differentiating between the analgesic and sedative effects of a drug was presented in one of the articles [10], where the characteristics of the evoked potentials were examined under different conditions. The presented technique was able to determine whether the examined drugs acted through analgesic or sedative effects, providing a very important tool for analgesic development and analgesic validation. It was shown that morphine has a sedative effect which inhibits the motor response.

In essence, neural recordings are a valuable tool in assessing potential analgesics and measuring nociception in a more quantitative way than standard methods. By expanding the amount of signals recorded more parameters could be found that help describe the perception of pain more accurately. The method has been proven to not affect the animal substantially via the implantation and through the development of better electrode arrays

the potential of more stable and precise recordings is very large. Neural recordings also outclass the behavioral tests by being able to differentiate between sedative and analgesic effects, and also to detect the contribution from secondary hyperalgesia.

6.2. Comparing hyperalgesic paw before and after UVB irradiation

The first comparison (shown in Fig. 15-19) focuses on the absolute changes experienced only in the UVB irradiated paw before, 24 hours after and 48 hours after irradiation. This comparison thus highlights how the irradiated paw is affected and how the rat adapts the gait of this particular paw when experiencing pain in said paw.

6.2.1. Mean and max area

The max and mean area (Fig. 15-16) are lower 48 hours after UVB irradiation for the affected paw for all animals, but a clear positive trend cannot be seen over the entire timeline. For instance, animal 1 has similar max area 24 hours after and 48 hours after UVB irradiation, while animal 6 has lower max area 48 hours after UVB irradiation compared to 24 hours after irradiation. This shows that all three animals act slightly different when adapting their gait during the inflammatory process of 48 hours. The decrease in both max and mean area of the affected paw indicates that the animal does not support its weight as much on said paw after irradiation, due to the pain experienced in that paw.

6.2.2. Swing time

The swing time (Fig. 17) is longer 48 hours after UVB irradiation for all animals and a slight positive trend could be visible over the entire timespan. However, no animal has the same progression over the entire 48-hour period, which matches the findings in the max and mean area results. The increased swing time indicates that the affected paw spends longer time out of contact with the glass surface. This makes sense because the animal will not want to support its weight on the affected paw for a longer amount of time, therefore having it in the air for a longer time instead.

6.2.3. Mean intensity and area of max projection

Fig. 18-19 show that the mean intensity of the max projection and area of the max projection are significantly smaller 48 hours after UVB irradiation. The area of the max projection has the same profile as the other area measurements (Fig. 15-16) whereas the mean intensity measurement differs

slightly. It is not surprising to find such great similarities in the profiles of all the area measurements and the intensity measurement, because when an animal supports its weight less on one paw, it will reduce both the contact area and pressure exerted by said paw.

6.3. Comparing hyperalgesic and non-hyperalgesic paws

The second comparison (Fig. 20-24) highlights the difference between the right hind paw (affected) and the left hind paw. This comparison is interesting because it portraits the shift in symmetry between the two hind paws. The reason for comparing the affected paw with the other hind paw is that they are the most similar to each other and should (in an unaffected animal) behave in a rather similar way. When one of the hind paws are induced with hyperalgesia, the rat should therefore support its weight more on the non-hyperalgesic paw.

6.3.1. Mean and max area

The max and mean area (Fig. 20-21) are significantly lower for the right hind paw than the left hind paw 48 hours after UVB irradiation for all animals. Before UVB irradiation, the left hind paw has a smaller or similar max and mean area compared to the right hind paw. Looking over the entire timespan, there seems to be a positive trend of the max and mean area of the left hind paw increasing whereas the max and mean area of the right hind paw is decreasing. Thus, the animal goes from having similar contact areas on the left and right hind paws (or slightly larger on the right hind paw) to having much larger contact area on the left hind paw. This shift in symmetry indicates an adjustment in the animals' gait where it supports its weight more on the left hind paw rather than the right hind paw due to the increased nociception.

6.3.2. Swing time

The swing time (Fig. 22) is significantly longer for the right hind paw compared to the left hind paw 48 hours after UVB irradiation for all animals. Before UVB irradiation, the swing time is similar for the right and left hind paws for all animals, and a positive trend is seen where the difference increases (where the swing time of the right hind paw increases) over time. Thus, the animal has a similar swing time for both hind paws before UVB exposure and 48 hours later the animals have adjusted their gait to keep the affected paw off the ground for a longer time to avoid pain.

6.3.3. Mean intensity and area of max projection

The area and mean intensity of the max projection (Fig. 23-24) are both larger for the left hind paw compared to the right hind paw 48 hours after UVB irradiation for all animals. The area of the max projection shares the same profile as the other area measurements (Fig. 20-21), as in the previous comparison. The profile of the mean intensity of the max projection is also quite similar to the profile of the area measurements. A clear positive trend is seen in both max projection measurements where the difference increases over time to the favor of the left hind paw. This indicates that the animal has shifted its weight towards the left hind paw rather than the right hind paw, and the animal's left hind paw exerts more pressure than the right hind paw 48 hours after UVB irradiation.

6.3.4. Type of parameters

For both comparisons it has been shown that the selected parameters are suitable for detecting disturbances in an animal's gait when investigating nociception. A quantifiable difference can be measured and therefore used to assess the pain experienced by an animal. Also, the difference in nature of the parameters (area, intensity and swing time) all describe different aspects of the gait of an animal. The area measures the size of the print, the intensity measures the pressure exerted by the paw and the swing time is a dynamic parameter which measures how the animal is moving. This usage of different types of parameters is key to fully realize and describe the complexity of an animal's gait and is a necessity when using gait analysis as a pain indicator.

6.4. Comparing methods

6.4.1. Neural recordings and gait analysis

Neural recordings are measurements of the activity of certain parts of the brain. Since pain is registered and experienced through the brain, it would seem like an optimal method to use when measuring nociception. The method clearly has its advantages, as it theoretically can detect pain originating from any point in the body, assuming that the electrode array is implanted in the correct spot in the brain. Also, neural recordings can detect secondary hyperalgesia, which is important to be able to describe the full concept of nociception. The implantation of the electrode array limits the reproducibility of the method, as it is difficult to guarantee that the electrode array is implanted exactly in the desired location every time. Also, the quality of the electrode array (in terms of e.g. minimizing scar formation, mechanical flexibility in the brain tissue and sensor sensitivity) is crucial for the

measurement, since the electrode array is the measuring element of the method.

Since the neural recordings method measures the actual signals of the brain and not any behavioral aspects, it might be more translational than other methods, provided that the two animals' brains are rather similar in structure and function. In essence, this method is rather complex and highly dependent on the structure and quality of the electrode array, though it has a lot of potential to give a true measurement of nociception from any point in an animal's body.

The gait method is much more simple and straightforward in its concept. It is also more easily reproducible in the sense that there are no priod requirements (such as a working and correctly located electrode array), although it may require some training to make the animals walk consistently in the CatWalk (or any other gait analysis) system. The setup for the method is quite simple and fast, and completing a few runs takes only a couple of minutes. The drawback of the gait method is that it is not specific for detecting pain originating from any point in the body. Although the gait might also be slightly affected by pain stimulation on other parts of the body, this change will most likely be too small to detect using this method. Also, this method measures a behavioral response (changes in gait), which may be affected by other things than increased nociception. If the rat decides to turn around, lie down or stand on its hind legs the outcome of the gait analysis is greatly affected, but not due to increased nociception. This issue highlights the importance of training, so that the animals behave in a predictable and reproducible way in every experiment, so that the variation in the gait parameters in the runs are low.

The neural recordings method and the gait method are quite different in their approach and method, but they both have the potential to measure pain in a graded and quantitative manner. One can consider these two methods as supplementary, where the gait method is faster and easier, but the neural recordings method is complex and more accurate. Depending on the animal, location of pain and desired outcome of the experiment, one method could be more fitting than the other. Additionally, if the nature of the experiments allows it, both methods could be used to confirm and validate the results of each other.

6.4.2. New methods and old (reflex) method

Both the neural recordings method and the gait method are quantitative methods, meaning that they can provide a graded measurement of the magnitude of the pain, rather than simply saying if the animal feels pain or

not. The reflex method (which uses a reflex response) has a binary outcome, which corresponds to if the animal withdrew its paw (felt pain) or not (felt no pain) due to a stimulus. The reflex method has similarities with the gait method, in the sense that it also observes a behavioral response. However, the reflex method is even simpler than the gait method, since observing the reflex response requires no equipment at all, and no post-processing is required. The sheer simplicity of the reflex method is the strongest point of the method, but when accuracy and nuance are more important than fast results, this method is lacking compared to the neural recordings method and gait method. The reflex method could be used as an initial screening to validate if the animal elicits any sort of pain response at all, to later validate with a quantitative method.

6.5. Possible improvements in methodology

6.5.1. Training rats before experiment

A major hurdle in the CatWalk experiments was to make the rats willing to walk across the CatWalk normally. Very often they would stop completely for a while, groom themselves, turn around and walk backwards. When the animal does not walk naturally it will affect the results since some parameters depend on that the animals exercise a ‘normal’, continuous walking pattern. The animals’ behavior could be improved by simply training the rats to walk naturally in the CatWalk before doing the experiments. Doing this would also help the experiments being more stable, since the training process has occurred before the experiments begin and the animal will not adjust its gait during the experiment due to it adapting to the new environment.

6.5.2. Print extraction/detection

The intensity threshold used to detect prints in the video is constant, meaning it will be the same for every video. This may cause issues if one alters the settings of the camera or if the inter-run intensity variance is too high. A dynamic intensity threshold would be preferred to better fit the intensity profile of each specific run but setting this threshold requires further tuning and analysis. Since the camera is mounted to the CatWalk equipment it should not be tampered with and a static intensity threshold was deemed sufficient for this project due to its simplicity. Also, the same lighting should be used in the experiment room during the CatWalk runs to not vary the background intensity levels, which then could effect the print detection process.

6.5.3. Clustering of pixels

The parameters entered into the clustering algorithm are constant, which could cause clustering problems for some videos. This could lead to wrongly clustered prints, meaning that one print could be categorized as two or more clusters, or one cluster could be interpreted as two or more prints. This possible error in turn cascades further into the script and could in turn affect basically every parameter extracted from the run. Although dynamic parameters could theoretically better fit each run, setting these parameters is no trivial task and since each video structure is the same (same distance from camera to glass plate, rats are of generally same sizes etc.) the static parameters should, and seem to be, robust enough to handle this specific clustering problem.

6.5.4. Artefact removal

The second part of the artefact removal revolves around removing clusters that share the exact same midpoint. This theoretically means that prints that share the exact same midpoint will be removed. However, this is extremely unlikely since the midpoint is decided with single pixel resolution. The method was implemented since it was noticed that the artefacts were static during the entire run, making them easy to differentiate from the prints. Considering the effectiveness of the method in combination with the low probability of accidentally removing a print, the method was deemed sufficient.

6.5.5. Classification of pawprints

The first part of the classification method is based on comparisons between simultaneous prints. This means that if one print is not present simultaneously with at least one other print, it cannot be classified. However, that scenario is highly unlikely, since it would correspond to that the rat exerted an extremely alien and novel gait pattern. Additionally, if the rat would walk on only its hind legs for a period of time, the method would wrongly classify one of these prints, since it only compares the position of simultaneous prints. Finally, in the unlikely event that a rat crosses its paws, these prints will be wrongly classified. This method may seem very flawed, but it is actually robust in the sense that if a print is wrongly classified, the method will not necessarily wrongly classify the following prints. Each classification process is independent, meaning that the method can easily correctly classify a print even though the previous one may have been incorrect.

Due to rat number 3, 4 and 5 not putting their right hind paw in contact with the glass surface at all 48 hours after UVB irradiation they were discarded from the main part of the analysis. However, these rats provide a lot of information that could be extremely useful in gait analysis. The reason for excluding them from the main analysis comes down to the classification process, which will wrongly classify prints if not all paws are used during a run. If the classification process could be improved to be accurate regardless of how many paws were used during a run, rat 3, 4 and 5 could also be used in the analysis. The 'absence' of the right hind paw indicates that the animal is in such a high level of pain that it does not even want to touch the glass plate at all with the irradiated paw. This behavior is also an indicator of pain, although it has lost its quantitative nature since it is binary (touching or not touching).

6.5.6. Parameters

The print length, print width and normalized values regarding area and intensity distribution are calculated using pure x- and y-directions. This means that if the rat's print is at an angle from the length of the glass plate, the value will be slightly off. The values could be more accurately calculated if the exact direction of each print was known, but it would still be difficult since the prints are represented by discrete matrices which cannot be separated in every direction.

6.5.7. Camera resolution and sensitivity

Since the video camera which records the run on the CatWalk is the main source of information for the gait analysis, it is of utmost importance. Therefore, having a camera with even higher resolution and sensitivity would enable the gait parameters to be calculated in a more reliable way, since more data (pixels) could be used. Having a higher resolution allows the pawprints to be more accurately described, since the digits of the paw might be visible, and the outline of the pawprint might be more accurately determined. These improvements to be accuracy of the pawprints would help to better quantify the intensity profile of the paw print (max projection) which much of the analysis builds from. Also, increasing the sensitivity of the camera might solve the problem with not consistently detecting the hyperalgesic paws of rats 3, 4 and 5.

7. Future work

7.1. Finalizing the program from the MATLAB script

To create a user-friendly program out of the MATLAB script, the MATLAB App Designer was used. The program was not fully completed, in the sense that no analysis was performed, but the user can still load and process new CatWalk runs to extract the parameters mentioned in section 4.2.5 and visually assess the extracted prints.

In the created program it is possible to load one or more video files from a folder and run the analysis on these video files, extracting the same parameters as in section 4.2.5. The program has three tabs: 'Overview', 'Details' and 'Visuals'. In the 'Overview' tab (see Fig. 26), an overview of the loaded runs is shown with corresponding date, animal number, run number, condition, direction and number of prints.

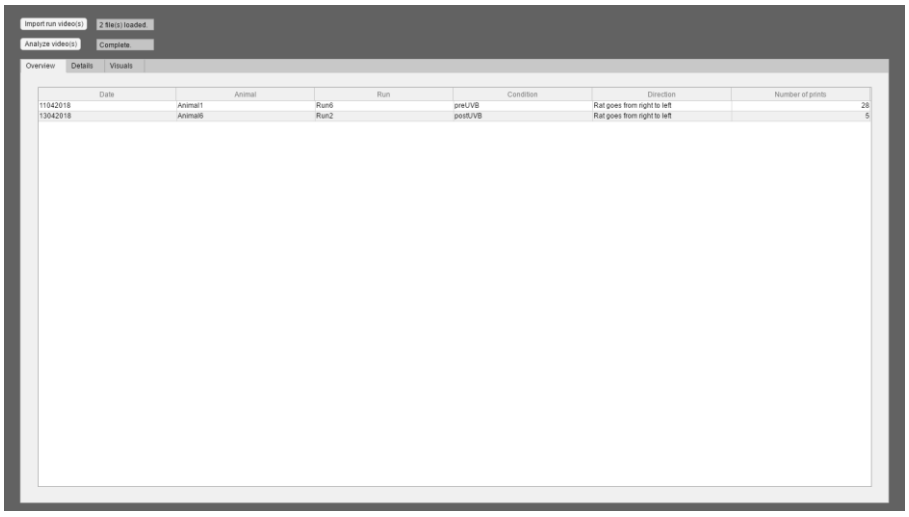
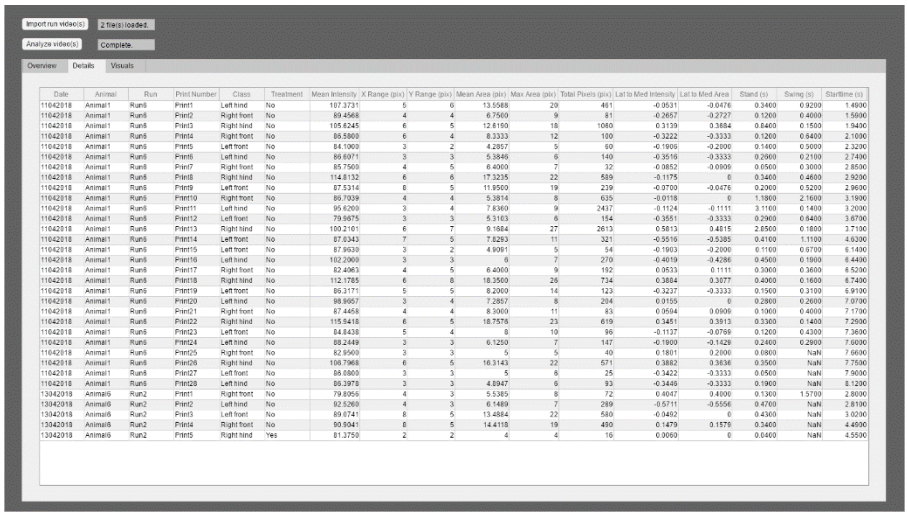


Fig. 26. The 'Overview' tab of the program. Here one finds the general information about the loaded runs. The date, animal number, run number, condition, direction and number of prints are present in this tab. In this case, two runs were loaded, resulting in two rows of information. The resolution in this figure is quite low due to adapting the size of the program window.

The 'Details' tab shows all of the parameter values for each print in an organized table (see Fig. 27). The 'Visuals' tab allows the user to select the desired print by choosing the date, animal number, run number and print number (see Fig. 28). Once the desired print is selected, the max projection

and the part print is displayed in color scale, revealing the intensity in each pixel that formed the print. The user can then use a slider to change the time point to visualize the intensity profile of the print in any time point.



Date	Animal	Run	Print Number	Class	Treatment	Mean Intensity	X Range (pix)	Y Range (pix)	Mean Area (pix)	Max Area (pix)	Total Pixels (pix)	Lat to Med Intensity	Lat to Med Area	Stand (s)	Swing (s)	StartTime (s)
11042018	Animal1	Run6	Print1	Left hind	No	107.3731	5	6	13.5588	29	461	-0.0531	-0.0476	0.3400	0.9200	1.4900
11042018	Animal1	Run6	Print2	Right front	No	89.4568	4	4	6.7500	9	81	-0.2667	-0.2727	0.1200	0.4000	1.5600
11042018	Animal1	Run6	Print3	Right hind	No	105.6245	6	5	12.6190	18	1060	0.3139	0.3684	0.8400	0.1500	1.9400
11042018	Animal1	Run6	Print4	Right front	No	88.5809	6	4	8.3333	12	100	-0.3222	-0.3333	0.1200	0.8400	2.1090
11042018	Animal1	Run6	Print5	Left front	No	84.1069	3	2	4.2857	5	60	-0.1909	-0.2000	0.1400	0.5000	2.3290
11042018	Animal1	Run6	Print6	Left hind	No	86.6971	3	3	5.3846	6	140	-0.3516	-0.3333	0.2660	0.2100	2.7490
11042018	Animal1	Run6	Print7	Right front	No	85.7569	4	5	6.4000	7	32	-0.0852	-0.0909	0.0500	0.3000	2.8590
11042018	Animal1	Run6	Print8	Right hind	No	114.8132	6	6	17.3235	22	589	-0.1175	0	0.3400	0.4600	2.9290
11042018	Animal1	Run6	Print9	Left front	No	87.5314	8	5	11.9500	19	239	-0.0700	-0.0476	0.2900	0.5200	2.9690
11042018	Animal1	Run6	Print10	Right front	No	86.7039	4	4	5.3814	8	635	-0.0118	0	1.1800	2.1600	3.1890
11042018	Animal1	Run6	Print11	Left hind	No	95.6269	3	4	7.8360	9	2437	-0.1124	-0.1111	3.1100	0.1400	3.2090
11042018	Animal1	Run6	Print12	Left front	No	79.9675	3	3	5.3103	6	154	-0.3551	-0.3333	0.2900	0.8400	3.6790
11042018	Animal1	Run6	Print13	Right hind	No	100.2101	6	7	9.1884	27	2613	0.5813	0.4815	2.8500	0.1800	3.7190
11042018	Animal1	Run6	Print14	Left front	No	87.0343	7	5	7.6293	11	321	-0.3516	-0.5385	0.4100	1.1100	4.6390
11042018	Animal1	Run6	Print15	Left front	No	87.9939	3	2	4.9091	5	54	-0.1903	-0.2000	0.1100	0.6700	5.1490
11042018	Animal1	Run6	Print16	Left hind	No	102.2069	3	3	6	7	270	-0.4019	-0.4286	0.4500	0.1900	5.4490
11042018	Animal1	Run6	Print17	Right front	No	82.4063	4	5	6.4000	9	192	0.0533	0.1111	0.3000	0.3600	5.5290
11042018	Animal1	Run6	Print18	Right hind	No	112.1785	6	8	18.3509	26	734	0.3884	0.3077	0.4900	0.1600	5.7490
11042018	Animal1	Run6	Print19	Left front	No	89.3171	5	5	8.2000	14	123	-0.3237	-0.3333	0.1500	0.3100	6.9190
11042018	Animal1	Run6	Print20	Left hind	No	88.9957	3	4	7.2857	8	204	0.0155	0	0.2800	0.2600	7.0790
11042018	Animal1	Run6	Print21	Right front	No	87.4458	4	4	8.3000	11	83	0.0594	0.0909	0.1000	0.4000	7.1790
11042018	Animal1	Run6	Print22	Right hind	No	115.5418	6	5	18.7576	23	619	0.3451	0.3913	0.3300	0.1400	7.2590
11042018	Animal1	Run6	Print23	Left front	No	84.8438	5	4	8	10	96	-0.1137	-0.0769	0.1200	0.4300	7.3690
11042018	Animal1	Run6	Print24	Left hind	No	89.2449	3	3	6.1250	7	147	-0.1909	-0.1429	0.2400	0.2800	7.6090
11042018	Animal1	Run6	Print25	Right front	No	82.9569	3	3	5	5	40	0.1801	0.2000	0.0800	NaN	7.6690
11042018	Animal1	Run6	Print26	Right hind	No	108.7968	6	5	16.3143	22	571	0.3882	0.3636	0.3500	NaN	7.7590
11042018	Animal1	Run6	Print27	Left front	No	86.8869	3	3	5	5	25	-0.3422	-0.3333	0.0500	NaN	7.9090
11042018	Animal1	Run6	Print28	Left hind	No	89.3978	3	3	4.6947	6	93	-0.3446	-0.3333	0.1900	NaN	8.1290
13042018	Animal6	Run2	Print1	Right front	No	79.8056	4	3	5.5385	8	72	0.4047	0.4000	0.1300	1.5700	2.8090
13042018	Animal6	Run2	Print2	Left hind	No	82.5269	4	3	6.1489	7	289	-0.5711	-0.5556	0.4700	NaN	2.8190
13042018	Animal6	Run2	Print3	Left front	No	89.6741	8	5	13.4884	22	580	-0.0492	0	0.4300	NaN	3.0290
13042018	Animal6	Run2	Print4	Right front	No	90.9041	8	5	14.4118	19	490	0.1479	0.1579	0.3400	NaN	4.4690
13042018	Animal6	Run2	Print5	Right hind	Yes	81.3759	2	2	4	4	16	0.0060	0	0.0400	NaN	4.5590

Fig. 27. The ‘Details’ tab of the program. Here one finds all the parameter values for each print of the loaded runs. The resolution in this figure is quite low due to adapting the size of the program window.

Further work would involve completing the program by using the parameters explained in section 5 and comparing them with new parameter values generated by new runs loaded into the program. By doing this, the program could assess whether the new rat would more likely correspond to being untreated (no UVB stimulation, feeling no pain) or UVB irradiated (experiencing pain). By using all five significant parameters presented in 5.1 and 5.2, a combination of some, or only one of them, one could get an indication of the level of pain experienced by an animal and present this information to the user in a simple way. However, further data processing (such as normalization) might be required to be able to fully compare different animals with each other. Implementing these changes (last two steps in Fig. 5) would complete the program and allow the user to quickly and easily determine the pain level of one or multiple animals at once.

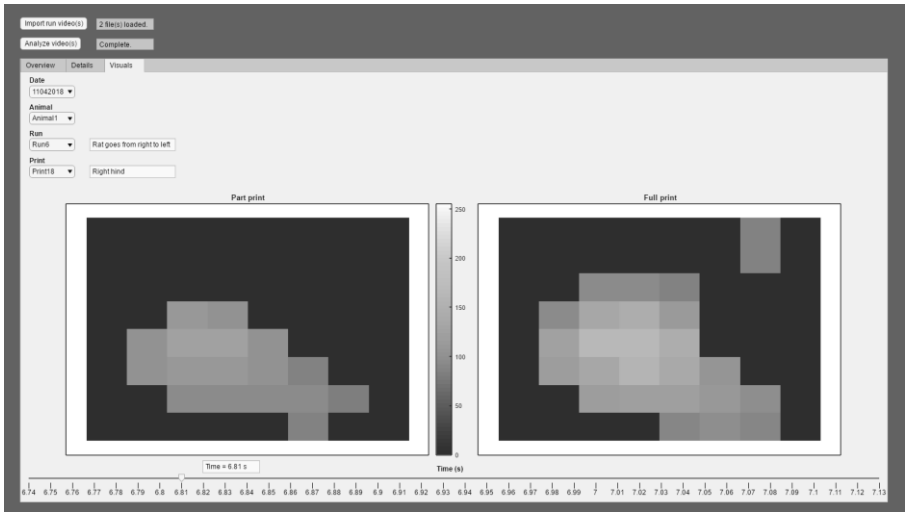


Fig. 28. The ‘Visuals’ tab of the program. Here one can select the desired print from the date, animal, run and print drop-down menus (shown in top left). Then it is possible to see the max projection (right side) and the temporal part print (left side) and slide through the entire duration of the print and see how the part print changes (slider shown at bottom). The resolution in this figure is quite low due to adapting the size of the program window.

7.2. Relative measurements

The comparisons presented in section 5.1 and 5.2 related the right hind paw (hyperalgesic) to itself before and after UVB irradiation and to the left hind paw, respectively. To fully utilize all the data, more relative comparisons could be made. For instance, to compare the ratio of parameter values between hind paws and front paws would use all the available data and give an interesting measurement of the parameter value distribution relative the front and hind side of the animal. More similar relative measurements could be performed to try and find comparisons that utilize as much data as possible and being able to detect differences before and after UVB irradiation. Although, one should be careful to not overcomplicate the ways of comparing, since it will be harder to interpret what the results imply, and one could therefore draw faulty conclusions from them.

7.3. Replace classification method with neural network

Since the classification step is one of the most important steps in the process of analyzing the run videos, it is crucial that it works efficiently and

with a low error rate. The current classification method (described in section 4.2.4) utilizes relative comparisons, which requires simultaneous prints. One way to improve the classification method would be to implement a convolutional neural network (CNN), which specializes in classifying images with excellent results [16]. A CNN is trained to find special features on a larger amount of images to be able to separate the input images into different classes. The CNN is able to learn what features to look for to be able to do this separation, which makes it easy to use. A CNN would therefore not require simultaneous prints to be able to classify the prints and would therefore be a fitting method to replace the current classification method with. Additionally, increased camera resolution and sensitivity would further enhance the classification abilities of the CNN since it would provide a more complete visualization of the pawprints, which would aid the CNN in its classification process.

8. Conclusion

The aim of this thesis was to evaluate two quantitative approaches of measuring pain. The neural recordings method was evaluated through analyzing two articles that performed similar experiments of using neural recordings to measure pain. It was found that in both articles that the magnitude of C fibre evoked potentials correlated with increased nociception. Additionally, this method was able to detect and measure secondary hyperalgesia and a method to separate between the sedative and analgesic properties of a drug was examined. The neural recordings method has a great potential to be extremely accurate and thorough in its pain analysis, but it suffers from being very reliant on the quality and implantation of the measuring equipment and the complexity of the technique.

For the gait analysis method, five parameters were found that were significantly different 48 hours after UVB irradiation compared to before UVB irradiation. These parameters were consistently different when observing the effect on the right hind paw before and after irradiation, but also when comparing the left hind paw to the right hind paw before and after irradiation. These five parameters cover measurements of the area, intensity and the swing time of the print, which in all gives a rather complete description of the characteristics of the print. For the animals that were excluded in the main analysis (due to them not touching the glass plate with the right hind paw), other parameters might still be useful when trying to measure the pain experienced by an animal through its gait. The gait method is rather simple and straightforward, but it is not specific for pain originating from anywhere in the body. To create a functioning program which is able to assess an animal's pain by comparing a new run with an existing database, further data processing might be required to be able to compare different animals with each other.

Comparing these two quantitative methods to today's method of observing reflex responses, it is clear that the quantitative methods are superior in accuracy, sensitivity and its ability to describe nociception to a fuller extent. Although, the reflex method is the simplest of them all, which has allowed it to be used for a long time. Therefore, one must consider the type of experiment that is to be performed and what the purpose of the research is when choosing the appropriate nociception evaluation method. Additionally, one could combine these different techniques to get a more complete assessment of the pain experienced by the animal.

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Appendix A: Doppler measurements

Table 1. The Doppler measurements recorded from the lateral right hind paw of the rats before, 24 hours after and 48 hours after UVB irradiation. The flux measurements indicate that the UVB irradiation caused sustained hyperalgesia through an inflammatory response.

	Flux (perfusion unit)			Mean intensity (arbitrary unit)		
Rat #	Pre	24h	48h	Pre	24h	48h
Rat 1	310	620	670	119	123	118
Rat 2	410	595	525	117	142	108
Rat 3	245	706	820	127	116	107
Rat 4	320	670	780	123	123	139
Rat 5	260	715	860	121	139	135
Rat 6	210	475	850	128	116	119

Appendix B: Normalization tests

Table 2. The results from the Lilliefors tests conducted on the acquired data. The first column indicates the animal number, the left or right hind paw and if the experiment was performed before, 24 hours after or 48 hours after UVB irradiation. 1 indicates that the null-hypothesis was discarded and 0 indicates that the null-hypothesis was not discarded. The first row indicates which parameters are being examined and is further explained below.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
1R	0	1	1	0	0	1	0	0	1	1	1	0	0
1R+	0	1	1	1	1	1	1	0	1	1	1	0	0
1R++	1	1	1	1	1	1	1	1	1	1	1	1	1
1L	0	1	1	1	1	1	0	0	1	1	1	0	0
1L+	0	1	1	1	1	1	0	1	1	1	1	0	0
1L++	1	1	1	0	0	1	0	0	1	1	0	1	1
2R	1	1	1	1	1	1	0	1	1	1	1	0	1
2R+	1	1	1	1	1	1	0	0	1	1	1	1	0
2R++	1	0	1	1	1	1	0	0	1	0	0	1	0
2L	1	1	1	1	1	1	1	1	1	1	1	1	1
2L+	1	1	1	0	0	1	0	0	1	1	1	0	0
2L++	0	1	0	0	1	1	0	0	1	1	0	0	1
3R	1	1	1	1	0	1	0	1	1	1	0	0	1
3R+	0	1	1	1	1	1	0	1	1	1	1	0	0
3R++	0	1	1	0	0	1	0	0	1	1	0	0	0
3L	0	1	1	0	1	1	0	0	1	1	1	0	0
3L+	1	1	1	1	1	1	1	0	1	1	1	1	0
3L++	0	1	1	0	0	1	0	0	1	1	1	0	0
4R	1	1	1	1	1	1	0	0	1	1	1	1	0
4R+	0	1	1	0	0	1	0	0	1	1	0	0	0
4R++	1	1	1	0	0	1	0	0	1	1	0	0	0
4L	1	1	1	1	1	1	0	0	1	1	1	1	1
4L+	1	1	1	0	0	1	0	0	1	1	0	0	0
4L++	0	1	1	0	0	1	0	1	1	1	0	0	0
5R	0	1	1	0	0	1	1	1	1	1	0	0	1
5R+	1	1	1	1	1	1	0	1	1	1	1	1	1
5R++	0	1	1	0	0	1	0	0	1	1	0	0	1
5L	0	1	1	0	0	1	0	0	1	1	1	0	0
5L+	1	1	1	0	1	1	0	0	1	0	1	0	1
5L++	0	1	1	0	0	1	0	0	1	1	1	0	1
6R	0	1	1	0	0	1	0	0	1	1	1	0	0
6R+	1	0	1	1	1	1	0	0	1	1	1	1	1
6R++	1	1	1	1	1	1	0	1	1	1	1	1	0
6L	0	1	1	1	1	1	0	0	1	1	0	1	0
6L+	1	1	1	0	0	1	0	0	1	1	0	1	1
6L++	1	1	1	0	0	1	0	0	1	1	0	1	1

The parameters listed in the first row in Table. 2 as P1-P13 represent the following:

- P1: Mean intensity.
- P2: Print length.
- P3: Print width.
- P4: Mean area of the part prints.
- P5: Max area of the print.
- P6: Total number of pixels.
- P7: Lateral-medial intensity distribution.
- P8: Lateral-medial area distribution.
- P9: Stand time.
- P10: Swing time.
- P11: Area of max projection.
- P12: Mean intensity of max projection.
- P13: Max intensity of max projection.

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