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Quantification of motor behaviour in freely moving rodents

by

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Abstract

Systems evaluating potential treatment methods for Parkinson's disease and chronic pain conditions, using rodent experimental models, are highly needed. Currently used systems are aiming to evaluate these kinds of treatment methods by analyzing the animal's motor behavior. These systems either generate binary data or multifaceted data for when the rodent is spatially limited or data for when the rodent is less spatially limited, i.e. moving in an open field, that are less faceted. In this thesis a setup was built by combining setups from the currently used systems, to enable high faceted data for when the rodent is moving in an open field. Further, the data generated from this setup was interpreted by evaluating and comparing image analysis methods, using two different software. The interpretation of the data aimed to generate parameters describing the rodent's motor behavior by detection and classification of the rodent and its various body parts and paws. The first image analysis method is based on blob detection and was evaluated using MATLAB. This method resulted in insufficient parameter values, but with potential for development regarding the evaluation of the pressure load of the paws. Further, this method showed great potential in classifying the rodent's body parts and paws. This method therefore has the potential to be used with the built setup as a tool in motor behavior analysis, such as gait pattern and body posture analysis. The second image analysis method is deep learning based segmentation, object tracking, and classification and was evaluated using DeepLabCut. This method resulted in insufficiency in the detection and classification of the rodent's paws, but showed potential in the detection and classification of the body parts. This method therefore has the potential to be used as a tool in motor behavior analysis when analyzing the rodent's body posture and movement pattern, for instance, in combination with the first method.

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Contents

1	Introduction	10
1.1	Background	10
1.2	Aim	11
1.3	Structure of the report	11
2	Theory	12
2.1	Rat gait	12
2.2	Pain	13
2.3	Parkinson's disease	14
2.4	Methods for measuring motor behavior	14
2.5	Frustrated total internal reflection	15
2.6	Image analysis	16
2.6.1	Blob detection & classification	16
2.6.2	Kalman filter	17
2.6.3	Deep learning based segmentation & object tracking	17
2.7	Ethics	18
3	Methods	19
3.1	Setup & data collection	19
3.2	Image analysis in MATLAB	19
3.2.1	Background subtraction & noise reduction	19
3.2.2	Blob detection	20
3.2.3	Temporal correction	23
3.2.4	Feature extraction	23
3.2.5	Paw print validation	23
3.2.6	Determination of directions	23
3.2.7	Classification of paws	25
3.2.8	Extraction of video sequences	26
3.2.9	Correction of classes within frames	26
3.2.10	Stance linearity & footprint clustering analysis	27
3.3	Deep learning based segmentation, object tracking & classification of body parts	28
3.3.1	Image processing	28
3.3.2	Labeling & training	28
3.3.3	Determination of directions	29
3.3.4	Classification	30
3.4	Deep learning based segmentation, object tracking & classification of paws	30

3.4.1	Image processing	30
3.4.2	Labeling & training	30
3.4.3	Classification	30
4	Results	32
4.1	Setup & data collection	32
4.2	Image analysis in MATLAB	32
4.2.1	Classification	32
4.2.2	Stance linearity & footprint clustering	35
4.2.3	Area features	36
4.3	Deep learning based segmentation & object tracking	37
4.4	Deep learning based segmentation, object tracking & classification	38
5	Discussion	39
5.1	Setup & data collection	40
5.2	Image analysis in MATLAB	40
5.2.1	Classification and gait analysis	40
5.2.2	Body posture	41
5.2.3	Movement patterns	42
5.2.4	Stance linearity & footprint clustering	43
5.2.5	Area features	43
5.2.6	Method improvements	44
5.2.7	Kalman filter	45
5.3	Deep learning based segmentation & object tracking	46
5.4	Deep learning based segmentation, object tracking & classification	46
5.5	Ethics	47
6	Conclusion	48

List of Figures

2.1	(A) Snell’s law. (B) Frustrated internal reflection.	15
2.2	Illustration of the principle frustrated internal reflection in a touch screen [13].	16
3.1	The setup and the electrical stimulator. (A) The electrical stimulator (white arrow) for future experiments with the setup below. (B) The setup seen from above. The white arrow is here showing the placing of the camera, below the glass plate. (C) The setup seen from the side, where 1. is the plexiglass box, 2. is the glass plate with attached LED strip and 3. is the camera placed on a stand. . .	20
3.2	The workflow of the MATLAB program, showing the most essential steps of the segmentation.	21
3.3	Noise reduction, artifact removal and blob detection. (A) Original image. (B) Image after cropping, noise reduction and artifact removal. (C) Blob detection of the rat’s body. (D) Blob detection of the rat’s paws, zoomed in image. (E) Blob detection of the rat’s paw parts, zoomed in image.	22
3.4	Illustration determination of the rat’s direction. The cross is the body’s centroid and the dashed arrow along with the long solid arrow is the body’s major axis. The angle a is approximate -45° , indicating the body is being directed northeast. However, the directions (small arrows) of the paws is suggesting the rat is being directed southwest, which will be determined as the rat’s direction.	25
3.5	Illustration showing relative angles. The cross is the body’s centroid, the center arrow is its directional vector. The two smaller arrows are directional vectors from the rat’s body’s centroid to the paws’ centroids. The angles a and b are the relative angles, where a is approximately 45° (the rat’s right side) and b being approximately -135° (the rat’s left side).	26
3.6	(A) A graphical example of stance linearity showing the anterior extreme position (AEP), intermediate positions (IP) and posterior extreme position (PEP) with an appurtenant curve fitted using the least square method, as well as a linear line from AEP to PEP during one stance phase. (B) Graphical view of total mean squared error over the time period frame number 1 to 300, i.e. during 10 seconds of a video recording, showing plateaus when the rat is sitting or standing still.	27
3.7	Screenshot of DeepLabCut’s graphical user interface, during labeling process.	29

3.8	(A) The workflow of the DeepLabCut software in combination with the MATLAB program. (B) The workflow of the DeepLabCut software.	31
4.1	Scatter plot showing paw prints appurtenant the four paw classes; right hind paw, left hind paw, right front paw and left front paw divided according to the paw prints pad area and its angle relative the rat's body.	33
4.2	Histograms showing duration and number of touchdowns of each paw class; 1 = right hind, 2 = left hind, 3 = right front, 4 = left front. (A) Duration of the paw classes, counted in number of frames, for all sequences. (B) As (A), but for only the sequences where the rat is walking. (C) Number of touchdowns of the paw classes, for all sequences. (D) As (C), but for the walking sequences.	34
4.3	Graphs showing: (A) Total mean squared error plotted against frame number 1-300. (B) Total IP-distances of all paws plotted against frame number 1-300. (C) IP-distances of right hind paw versus left hind paw plotted against frame number 1-300. (D) Mean squared error of each paw plotted against time.	36
4.4	Graphs showing IP-distances plotted against time in deciseconds: (A) IP-distances of right hind paw plotted against time. (B) IP-distances of left hind paw plotted against time. (C) IP-distances of right front paw plotted against time. (D) IP-distances of left front paw plotted against time.	37

List of Tables

3.1	Overview of features extracted or calculated from the blob detection.	24
4.1	Table showing duration of the paws appurtenant the respective paw class, in relation to all paw classes, during all sequences respective walking sequences. Also, the number of times the paws appurtenant the respective paw class is used, in relation to all paw classes during all sequences respective the walking sequences are shown. .	33
4.2	The average stance and swing time in milliseconds for respective hind paws, calculated from video sequences where the rat is walking.	34
4.3	Foot print clustering of each respective paws, of their AEP respective PEP, i. e. the standard deviation of the positions relative the body for the AEP respective the PEP, in degrees.	36
4.4	Area features of the paws and pad areas of each paw class.	37

Populärvetenskaplig sammanfattning: Kvantifiering av motoriskt beteende hos råttor i fri rörelse

Cirka 10 miljoner människor världen över lever med Parkinsons sjukdom och cirka 40 % av världens befolkning lever med långvarig smärta. Den här avhandlingen handlar om att implementera ett system för att hjälpa till att utvärdera behandlingar för dessa tillstånd, såsom djup hjärnstimulering (DBS).

System som används för att förbättra DBS, det vill säga elektrisk stimulering av djupa hjärnstrukturer, för att behandla patienter som lider av Parkinsons sjukdom och för att utvärdera DBS som en potentiell behandling för långvarig smärta är mycket nödvändiga. Aktuella tester involverar analys av gnagares motoriska beteende vad gäller till exempel gångmönster och kroppshållning när djuret påverkas av smärta eller experimentella modeller av Parkinsons sjukdom, samt när de djupa hjärnstrukturerna hos djuret stimuleras. Nackdelen med dessa tester är att de antingen ger begränsad information om djurets motoriska beteende, eller ger mångfacetterad information om det motoriska beteendet, men där djurets motoriska beteende istället begränsas på grund av uppställningens design.

I detta examensarbete har ett system implementerats som inte begränsar djurets motoriska beteende, där en råtta rör sig i ett öppet fält. Vidare har tre metoder för att hämta mångfacetterad information från denna uppställning implementerats, utvärderats och jämförts. Uppställningen genererar en videoinspelning av råttan som rör sig i det öppna fältet och de tre metoderna involverar bildanalys av videoinspelningen. Den första metoden innefattar bildanalys baserad på blob-detektering och de två andra metoderna innefattar bildanalys baserad på djupinlärningsbaserad segmentering.

Jämförelser av dessa tre metoder visar att metoden baserad på blob-detektering är den mest kompatibla med den implementerade öppet fält-uppställningen. Kombinationen av öppet fält-uppställningen och blob-detekteringsmetoden ger mångfacetterad information om råttans motoriska beteende när den rör sig relativt obegränsad. Detta system kan därför ge mer exakta undersökningar av råttans motoriska beteende än system som används idag.

Metoderna baserade på djupinlärningsbaserad segmentering visade otillräcklig noggrannhet. Utvärderingarna av dessa metoder visade dock på potential för utveckling och de kan därför fungera som ett potentiellt komplement till systemet.

Att använda systemet bestående av den implementerade uppställningen med bildanalys baserad på blob-detektering kommer förhoppningsvis att förbättra utvärderingar av DBS som en behandling, använt för sig självt eller möjligen i kombination med djupinlärningsbaserad segmentering.

1

Introduction

1.1 Background

About 10 million people worldwide live with Parkinson's disease (PD) and about 40 % of the world's population experience long term pain at some point in life. Commonly used treatment methods for PD are medication as well as traditional deep brain stimulation (DBS). These treatment methods often improve the quality of life for the patient, but with the cost of undesirable side effects. The traditional pain treatment is by medication, often opioids. This treatment can have undesirable side effects that can be expensive for both the patient as well as society. Evaluations to improve DBS as a treatment method for PD, as well as evaluations of DBS as a potential treatment method for long term pain, are therefore highly needed. Evaluations to improve DBS as a treatment method for PD can be performed by inducing an experimental model of PD using a unilateral 6-hydroxydopamine (6-OHDA) lesion. This is a lesion, where the placing of toxin in the brain area of dopamine cell bodies or their ascending fibers is done, to cause a lateralized destruction of dopamine neurons and lateralized loss of striatal dopamine innervation [4]. Further, evaluations of DBS as a potential treatment method for long term pain can be performed by inducing pain in a rodent using nociceptive stimulus.

Evaluation of a pain condition or 6-OHDA lesion in rodents with subsequent treatments can then be performed by studying the rodent's motor behavior. A behavioral assessment evaluating motor deficits in rodents, e. g. rats, in experimental models of Parkinson's disease, is the cylinder test. This test method evaluates the forelimb use asymmetry in the rat by placing it in a glass cylinder and measuring the number of times it rears up and touches the cylinder wall [1]. However, the rat, and therefore its natural behavior, is limited by the cylinder glass and the test requires manual, human evaluation, which is time consuming. Also, this kind of test evaluates only quantity and not quality regarding the rat's use of its forelimbs.

To examine how motor behavior in rats is affected by pain or experimental models of Parkinson's disease and how it can be normalized with different types of treatments, a system called Catwalk XT can be used. With this system, the rats' gait pattern can be characterized by filming the rat from below when it passes over a glass plate, through a small tunnel [2]. This system can give information about, for instance, the pressure distribution of the rat's paws in addition to quantitative measures. However, this system requires the rat to be trained of going in the tunnel and it also requires the rat to be able to move relatively unimpeded.

These requirements constitute a major limitation in cases where the spontaneous behavior of the rats in open field is to be examined.

An open field setting, used to evaluate possible side effects of deep brain stimulation with microelectrode clusters, has been performed by placing a rat provided with an accelerometer and two LED's in a box and filming it from above [4]. This setting gives movement data with information about the rat's position in a less limited environment. However, this setting lacks information about the rat's gait pattern and the pressure distribution of its paws.

A system that enables time stamped information of the rat's position, posture and loading of paws in open field would therefore be desirable. This could enable a more truthful evaluation of treatment of Parkinson's disease and pain treatment, such as stimulation in deep brain structures.

1.2 Aim

In this project, the aim is to implement a system where the behavior of rats, in open field, can be analyzed by means of video recording and image analysis. Further, the aim is to evaluate and compare different image analysis methods, using two different software; image analysis using MATLAB based on blob detection, as well as image analysis using DeepLabCut based on deep learning based segmentation, object tracking and classification.

1.3 Structure of the report

The report consists mainly of the parts 'Theory', 'Methods', 'Results', 'Discussion' and 'Conclusion'. In 'Theory' and 'Method' the underlying theory and methods used are described. 'Results' contains findings made when analyzing the methods. In 'Discussion', these findings are evaluated and the analyzed methods and possible improvements of them are discussed. A summation of the findings is done in 'Conclusion'.

2

Theory

2.1 Rat gait

To analyze a rat's motor behavior, its gait pattern can be analyzed and characterized. A characteristic trait in the gait pattern of rats is that its paw's initial contact with the surface, when initializing stance phase, is typically evenly distributed over the entire paw. This can be compared with, for example, humans whose foot's initial contact with the surface is typically distributed only over the heel. During the stance phase, the pressure over the paw is then gradually moved forward to the toes before the paw is lifted. This parameter is typical of rats, regardless of walking or running speed. Parameters that are affected by walking or running speed are step length (distance between the position of initial contact of one paw and the position of initial contact of the next paw), which increases with increased speed, and the stance time (time period when one or more paws are in stance phase), which decreases with increased speed.

A parameter that can be used to distinguish walking from running is the duty factor, defined as the total stance time for all the paws of the entire step cycle divided by the time period of the entire step cycle. If the value of this parameter is equal to or higher than 0.5 the rat is considered to be walking and at values lower than 0.5 it is considered to be running. In order to distinguish different movements in detail, methods for detecting and determining the number of paws being in stance phase at the same time can be used. A rat can have four different gaits and in addition three different combinations of paws in stance phase. Walking is characterized by three paws being in stance phase at the same time. Running can be divided into three different categories; trot, amble and gallop. Trot is characterized by two diagonal paws in the stance phase simultaneously and amble is characterized by two lateral paws in stance phase simultaneously. Whether a rat is most likely to show amble or trot has been shown to be individual. Some individuals prefer trotting and others ambling. Galloping is characterized by one paw at a time in stance phase, followed by no paw being in stance phase (aerial phase). If the rat is standing still, this is characterized by four paws being in stance phase. Furthermore, both hind paws or both front paws can be in stance phase at the same time, either because the rat jumps or because it cleans or eats, amongst other things. Also, the parameters swing time and stance time can be used by themselves or in relation to each other when analyzing gait. Swing time is the time elapsed between one of the paw's time for take-off until the same paw's next

touch down. Stance time is the time elapsed throughout a paw in stance time, i.e. the time elapsed between the paw being in anterior extreme position (AEP) until it is in posterior extreme position (PEP).

Additional parameters that can be measured to analyze gait patterns are stance linearity index and footprint clustering. Stance linearity index is a measure of the difference between the actual stance trail of the paws and a smear of that stance trail. The stance linearity index can reflect the amount of body wobbling during stance phase, since paws in stance phase can be seen as the paws being anchored at the glass plate, relative to the body. Footprint clustering corresponds to the standard deviation of the mean values of the coordinates of AEP, i.e. the position of the paw (relative to the body) in touchdown, and PEP, i.e. the position of the paw (relative to the body) in take-off. This is a measure of how much spatial variation there is in the position of the paws, measured to all the times they are in AEP respectively PEP, which gives a cluster. A large cluster gives the result that the variation is large and vice versa. For rats, this parameter has been shown to be greater for PEP than AEP [5].

2.2 Pain

Around 40 % of the world's population experience long term pain at some point in life. The most commonly used treatment method for this condition is medication, such as opioids which relieves the pain. However, the use of opioids often comes with side effects with a negative impact on the patient and a great cost to society. Nociceptive pain occurs through the stimulation of specialized nerve cells, nociceptors, in an organ or tissue. The area from which each individual nociceptor can be activated is called the receptive field. Stimulation of a receptive field activates the nociceptor, which via the spinal cord leads the nerve signal to the somatosensory cortex (SI) in the cerebral cortex. Nociceptors can be of myelinated type, $A\delta$ -fibers, which gives a high conduction velocity. Nociceptors that are not myelinated are of C-fiber type, which has low conduction velocity. $A\delta$ nociceptors are therefore responsible for initial, sharp pain that gives the body signals of possible danger, while C-fiber nociceptors are responsible for long term, blunted pain.

Furthermore, SI is topographically organized, meaning the nociceptors being activated by adjacent areas in a tissue or organ also activates adjacent areas in SI. This allows rapid processing of stimuli of adjacent nociceptors. Most nociceptors have relatively large receptive fields, as it is of greater importance and benefit that the brain registers pain in itself than it registers the exact location from where the pain occurred. Repeated stimulation of a nociceptor may cause a greater outcome, although stimuli do not increase. This is called hyperalgesia and provides a stronger sensation of pain and can also affect areas around the stimulated area, resulting in secondary hyperalgesia [6]. A way to induce hyperalgesia in animals is to burn the skin with ultraviolet B (UVB) irradiation followed by applying stimuli in the form of a CO_2 laser, causing secondary hyperalgesia, to the area of interest [7]. This can also be analyzed and measured by applying UVB irradiation to the pad of a rat's paw followed by an evaluation test of how its gait pattern possibly is affected in terms of use of the paw and/or pressure load of the paw.

2.3 Parkinson's disease

Over ten million people worldwide suffer from Parkinson's disease (PD), a progressive neurodegenerative movement disorder caused by the deprivation of dopaminergic neurons in a part of the brain called substantia nigra. This can cause tremors, bradykinesia, dystonia, postural instability, dyskinesia and festination amongst other symptoms. All these symptoms can have an impact on the movement, e.g. gait pattern, in the person having the disease. An available treatment method for those living with PD is deep brain stimulation (DBS), where deep brain structures involved in motor functions are stimulated by electrical impulses through electrodes [3].

2.4 Methods for measuring motor behavior

Current methods for evaluation of motor behavior under the influence of nociceptive pain and pain treatment include either testing the response of an animal in form of reflexes or analyzing the movement pattern of the animal. The first mentioned method is limited, as the animal's response is binary, i.e. it either does or does not occur. In addition, it has been shown that this type of response is not specific to pain, as other non-painful stimuli such as touch also can give rise to the same response in the animal [8]. A current method used to examine experimental models of PD and subsequent therapeutic effects of antiparkinsonian treatments in rodents is the cylinder test. Here, the rodent is placed inside a glass cylinder where it is video recorded from above and from the side. The video recording is then evaluated and scored by an experimenter, counting the number of times the rodent rears up and touches the cylinder wall with its right respectively left forelimb or both forelimbs at the same time. The procedure is then repeated after the rodent is injected with 6-OHDA unilateral in the striatum, substantia nigra pars compacta to simulate PD and again after the rodent has antiparkinsonian treatment. Since it has been shown that unilaterally lesioned rodents exhibit the preferential use of the forelimb ipsilateral (i.e. opposite side relative to) the lesion, the cylinder test can be used to examine possible forelimb use asymmetry [1]. Although the cylinder test gives easily interpreted results with high accuracy, it, like the previously mentioned method, only gives binary data, describing whether the rodent uses a forelimb or not. Also, it lacks information regarding the rodent's motor behavior when moving freely.

Other methods to examine motor behavior in rodents, under the influence of pain or 6-OHDA lesion, are the CatWalk XT and MouseWalker systems, by utilizing frustrated total internal reflection and image analysis. In the CatWalk XT and MouseWalker systems a rat is filmed from below as it moves on a glass or plexiglass plate through a tunnel [2] [5]. This method enables examination and analysis of the motor behavior of the rat in an unaffected state versus when it is affected by pain or an experimental model of PD, possibly in combination with subsequent pain relief or PD treatment. It also gives information about, for instance, the pressure load of each paw. However, this system partially restricts the rat from moving freely since, in order to obtain meaningful and valid data, the rodent needs to comply to specific requirements when carrying out the task of moving across the plate. To examine DBS as a treatment and how it can be im-

proved studies have been made including examination and evaluation of rodents, e. g. rats, in an open field. A proven method is to place a unilateral 6-OHDA injected rat in a relatively large box. The rat is provided with a head-mounted 3-axes accelerometer, provided with two LED's, allowing detection of the head's position and direction. The rat is video recorded from above and the video recording is evaluated and scored, using measurements of the displacement of the rat's head during electrical stimulation of deep brain structures. A large displacement score is associated with the rat moving freely, i.e. reversing the symptoms induced by the unilateral 6-OHDA injection [4]. This method gives information about the rat's movement pattern when moving freely. However, this method does not provide any information on the rat's gait pattern, the pressure load on each paw or the placement of the body relative to the paws.

2.5 Frustrated total internal reflection

When light beams reach an interface between two media with different optical densities, the light beam can be transmitted, reflected or both. If the light travels from a medium, m_1 , with higher optical density, n_1 , to a medium, m_2 , with lower optical density, n_2 , the light will be reflected if the angle of incidence of the light is greater than the critical angle for total reflection, where the critical angle, θ , is given by Snell's law, see equation 2.1.

$$\theta = \sin^{-1}\left(\frac{n_2}{n_1}\right) \quad (2.1)$$

In equation 2.1 n_1 is the optical density of the medium having the highest optical density and n_2 is the optical density of the medium having the lowest optical density (figure 2.1a).

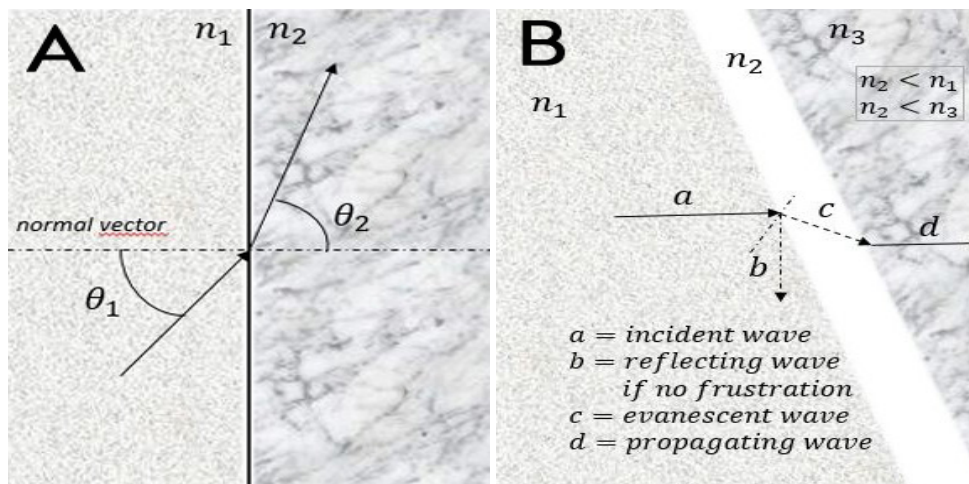


Figure 2.1: (A) Snell's law. (B) Frustrated internal reflection.

If the incident light strikes the interface in such a way that no light in the beam is transmitted, but all light is reflected back into medium m_1 , total internal reflection occurs. If a third medium, m_3 , with higher optical density than medium m_1 is close enough to the surface of medium m_1 , the light will no longer be fully reflected but will be partially transmitted. Medium m_3 is then being hit by some

of the light due to evanescent wave coupling (figure 2.1b). This phenomenon is called frustrated total internal reflection and is used, for instance, in certain touch screens, where incident light from the medium having the highest optical density is calculated to determine the location of the object touching the screen (figure 2.2). The closer medium m_3 is to medium m_1 , the more the light will be transmitted across the interface and vice versa. This enables an estimate of the distance between medium m_1 and m_3 by measuring the light intensity of the light transmitted and thus illuminating medium m_3 [11]. Furthermore, this can be used to estimate the paw use and pressure load of a rat's various paws in systems like CatWalk XT (Noldus Information Technology 2022).

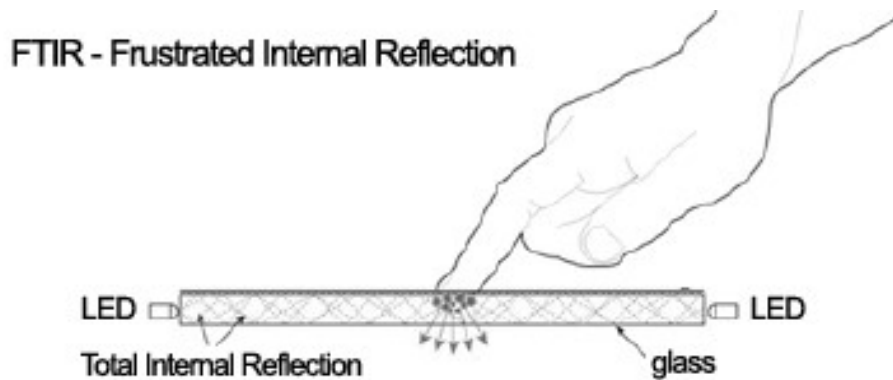


Figure 2.2: Illustration of the principle frustrated internal reflection in a touch screen [13].

2.6 Image analysis

2.6.1 Blob detection & classification

A video recording can be seen as a chronologically ordered sequence of time stamped images (frames). Frame-by-frame analysis refers to the analysis of the video recording frame by frame. For the detection of objects (e.g. paw prints and body) in an image, blob detection can be used, by analyzing the image in order to detect regions that show similarities. These similarities can be values related to the pixel values of the image, which can be translated as the color of the pixel. Further, the color of the pixel often is described as the intensity of the pixel in the RGB dimension, where R means red, G means green and B means blue. This method of detection can be seen as segmentation of the image, which divides the pixels of the image into two sets; background and foreground pixels.

Effective segmentation requires a clear difference between the pixel values being background and pixel values being foreground and therefore parameters, such as contrast and saturation in the image, may need to be adjusted. When possible, the settings of the video camera and also external factors, such as lighting, can be adjusted. Thereafter, one or more threshold values, indicating values for which the algorithm is to sort as background respectively foreground, are evaluated and

optimized. When the image is segmented into background and foreground, the foreground may be filtered to reduce noise and other disturbances in its pixel values. When the pixel values belonging to the foreground are optimized, the object or objects are detected and ready to be analyzed and classified [9].

Analysis of the detected objects can consist of defining features and values of these in order to divide similar objects into different subgroups, called classes, by classification. Features of an object can be shape, size, pixel values, position in the image and position relative to other objects in the image. Classification can therefore be used in examining the motor behavior of rats by analyzing a video recording showing a rat in motion using the frame-by-frame method, where the rat's various body parts, such as paws, tail and body, each belong to different classes. Furthermore, the rat's different paws can be distinguished and defined separately by analyzing their respective position to the rat's body in combination with other features. This can give the paws four different class affiliations; right hind, left hind, right front or left front paw.

2.6.2 Kalman filter

To enhance the ability to track and follow a detected object, which belongs to a certain class, over time, the Kalman filtering method can be used. The center of the object in a frame is determined and the Kalman filter is then used to predict the position of the object's center in the next frame, which is done by recursive prediction and correction. First, the movement of the object is detected, second, the object is tracked in the next frame and third, the movement of the object is analyzed. This allows the same object to be tracked and followed throughout the video recording and reduces the risk of the object being detected and classified as two or more objects during the video. Kalman filtering also enables the detection, classification and tracking of more than one object at the same timestamp of the video. Factors increasing the risk of incorrect detection, classification and tracking of an object are light changes and noise in the image, complex movement patterns of the object and complete or partial loss of the object [9]. An example of this could be the paws of a rat, which are completely lost from the frame when the paw is in swing phase during walk, if FTIR-enabled detection is used. Also, the paws will be partly lost during parts of the stance phase, since not the entire area of the paw is touching the glass plate throughout the entire stance phase. This will lead to the paw print changing form, visually.

2.6.3 Deep learning based segmentation & object tracking

Another potential method for detecting and tracking an object in a video recording is by deep learning based segmentation and object tracking. This can be done using, for instance, DeepLabCut (DLC) [10]. DLC is a software package using deep learning, developed to be used for tracking movement patterns in animals. Deep learning is a subcategory of machine learning, which is a method of teaching a computer to solve a task, by training the computer to detect and store information from input data, without the computer being programmed with rules for that particular task. Furthermore, deep learning is a method that is based on

algorithms using many process layers with complex structures to solve the task. Deep learning is a common method in image classification.

Using DLC, image analysis such as segmentation and blob detection of the frames is not required. The workflow is instead structured in such a way that the user selects a number of video files containing video recordings of the same or similar type of object which are then to be detected and tracked in one or more video recordings. A subset of the frames is extracted from the video files and with the help of DLC's graphical user interface, the object and possibly the various parts of the object can be classified by providing objects and/or sub-objects with labels. The deep learning network is then trained and its performance is evaluated. The procedure is repeated until a satisfactory result is achieved. The inference can then be used to detect and track objects and/or sub-objects in novel video recordings [10].

As when using blob detection to classify objects in an image the deep learning network requires a clear difference between the pixel values being background and pixel values being foreground. Therefore, parameters such as contrast and saturation in the image, may need to be adjusted. When possible, the settings of the video camera and also external factors, such as lighting, can be adjusted.

2.7 Ethics

The principle '3R' is an important application when working with experimental animals. 3R is an abbreviation for the words "replace", "reduce" and "refine". Replacement aims to replace animal experiments with a method or a combination of several methods where animals are not used, in case it gives a result with an equal or greater amount of information. Reducing aims to reduce the number of animals used in an experiment or to get more information from one and the same animal without increasing the animal's suffering, in case it gives a result with an equal or greater amount of information. Refining aims to reduce pain or other sufferings in an animal in an experiment, in case it gives a result with an equal or greater amount of information [12].

3

Methods

This project aims to set up a system that enables time stamped information of the rat's position, posture and loading of paws in an open field by combining setups used in CatWalk XT and MouseWalker with the open field setting described in section 2.4. Furthermore, three different methods will be compared in the subsequent image analysis.

3.1 Setup & data collection

The setup, see figure 3.1a-c consists of a glass plate of dimension 80·80 cm and thickness 1 cm resting on top of a table in steel, where the glass plate forms the tabletop 73 cm above the floor. A LED strip is attached around the edges of the glass plate using duct tape, making the light rays from the LED strip only propagate into the glass plate and not the surroundings. The color of light of the LED is selected green. Underneath the table, in the middle of the glass plate and 17 cm above the floor, a GoPro Hero8 Black is placed. A box with the outer dimensions of 28·52·52 cm and 1 cm thick walls is placed on top of the glass plate. The box consists of four walls of plexiglass, covered in matte black paperboard, with no bottom or top. The rat is placed inside the plexiglass box and a dark cloth is placed above the box to create a dark background. The reason why a black cloth is used and not a lid is so that the electrical stimulator, see figure 3.1a, can be used in future experiments. The room in which the setup is placed is dimmed out and data collection is made by video recording at 30 fps of the dimension 2160 · 3840. After about five minutes, the data collection is stopped by turning the camera off.

3.2 Image analysis in MATLAB

A program is created in MATLAB R2021b (The Mathworks, MA, USA) to analyze the collected data by loading the video file, read it frame by frame and follow the workflow seen in figure 3.2.

3.2.1 Background subtraction & noise reduction

To reduce static disturbance in the images caused by, for instance, dirt on the glass plate, a background subtraction is made, where an average value of all frames in a

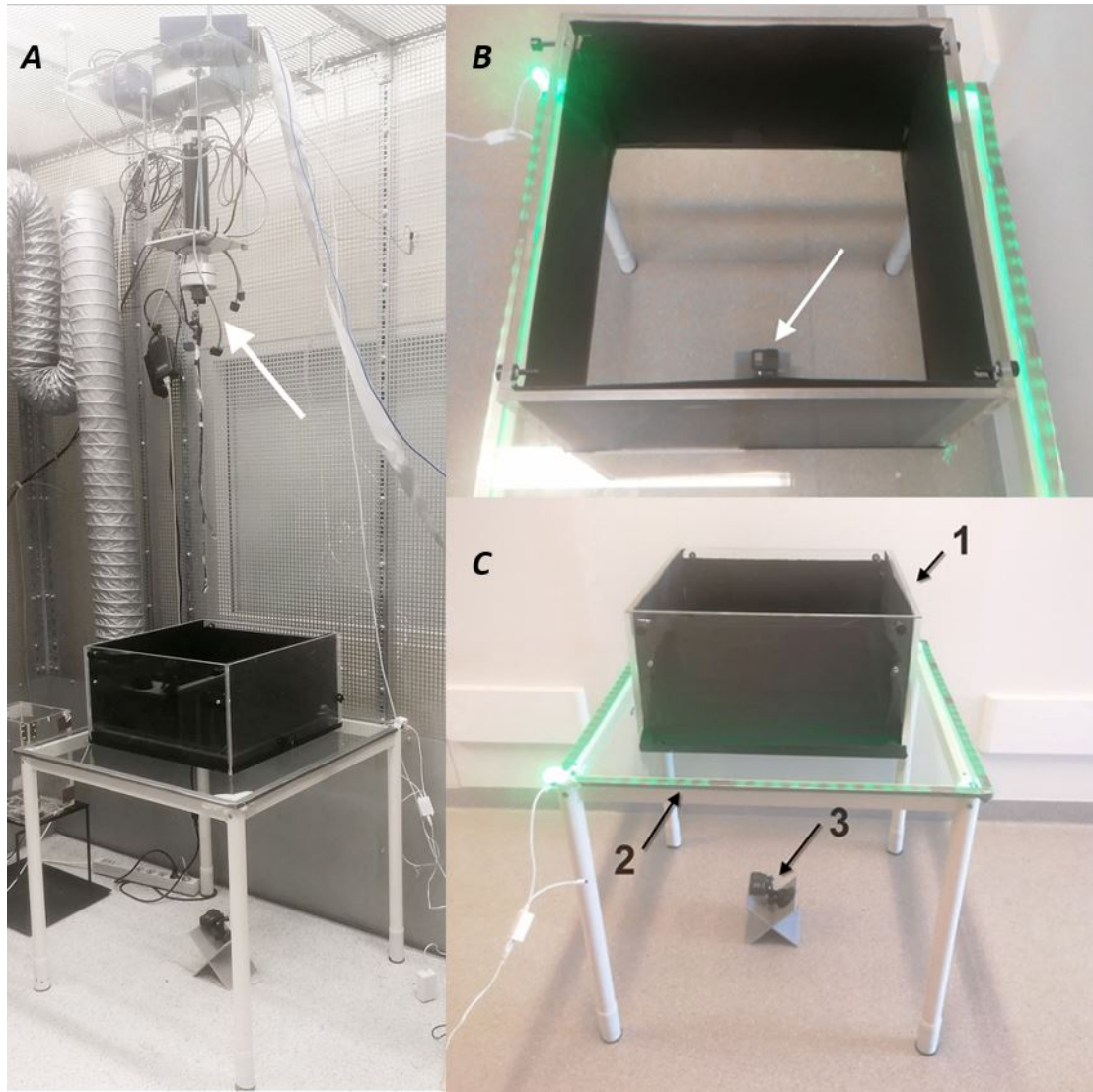


Figure 3.1: The setup and the electrical stimulator. (A) The electrical stimulator (white arrow) for future experiments with the setup below. (B) The setup seen from above. The white arrow is here showing the placing of the camera, below the glass plate. (C) The setup seen from the side, where 1. is the plexiglass box, 2. is the glass plate with attached LED strip and 3. is the camera placed on a stand.

video recording is subtracted from each frame. To reduce the risk of interference caused by objects outside the plexiglass box, each frame is cropped to dimensions determined by visual analysis of the video recording, see figure (figure 3.3b). Furthermore, noise reduction is done with an area opening operation, removing objects less than a certain size.

3.2.2 Blob detection

The blob detection is done in three levels; detection of the rat's body, its paws, and its paw parts, using different threshold values. The rat's body does not touch the glass plate, but is at such a distance from the glass plate that some of the light beams from the LED are subject to frustrated internal reflection. This means the pixels within the region of the rat's body have the highest intensity in the green

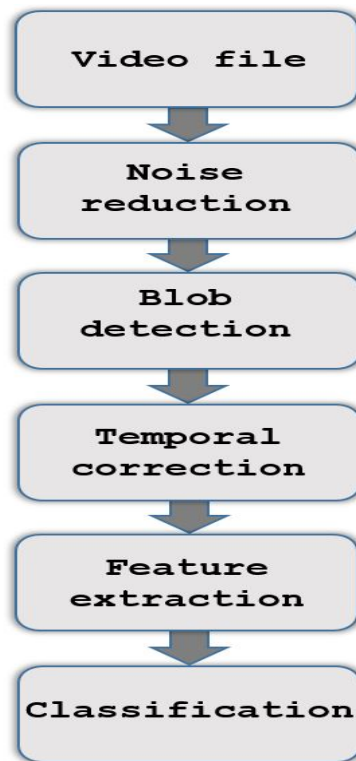


Figure 3.2: The workflow of the MATLAB program, showing the most essential steps of the segmentation.

dimension, albeit relatively low intensity. In order to detect the area forming the rat's body, a subtraction is therefore made, where the grayscale of each frame is subtracted from the green color band of each frame. Since the rat's body is visually light green, the frames are then binarized with a relatively high threshold value and filling operations may be performed. Having each frame containing only one blob, see figure (figure 3.3c) the region being the rat's body, features of the region are determined and saved, such as area, coordinates of the region's center of mass and orientation of the region's major axis.

The next level of detection is the detection of the rat's paws. The area of the paws is here defined as the area of the pad, toes and adjacent areas as they touch the glass plate or are at a significantly shorter distance from the glass plate than the rat's body is. This means the pixels within the region of the paws have a relatively high intensity in the green dimension, but also in the grayscale and therefore no extraction of the green color band is required. Instead, the frames are binarized directly from grayscale with around 20 times higher threshold than the threshold used when detecting the rat's body, see figure (figure 3.3d). In the same way as for the region of the rat's body, the features of the regions forming the paws are determined and saved. Each paw print is also assigned an identity number (paw ID) chronologically ordered.

The third and last level of detection is the detection of the different parts of the paws, which are defined as pads and toes touching the glass plate. The pixels within these regions therefore have higher intensity than some of the pixels within the regions of the body or the entire paw. To determine and save properties of the paw parts, a mask is made on each frame, one paw ID at a time. The mask consists of pixels with pixel values equal to zero and the pixel values of the pixels inside

the region of the paw are equal to or above zero. The frame is then binarized with a threshold value approximately 1.4 times higher than the threshold value of the paw detection, see figure (figure 3.3e). For each paw part, features of the region are determined and saved. Also, the paw parts are connected with its belonging paw ID.

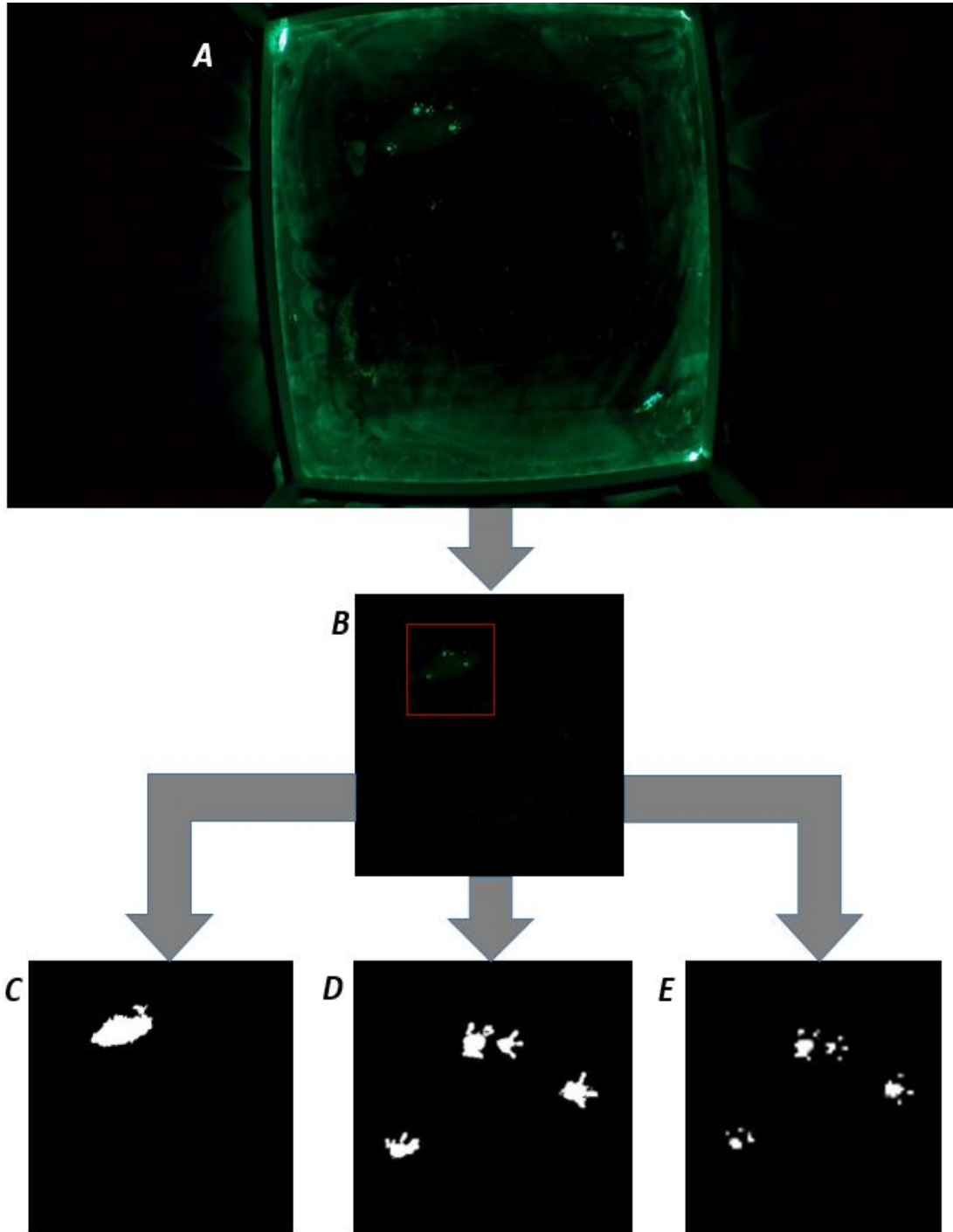


Figure 3.3: Noise reduction, artifact removal and blob detection. (A) Original image. (B) Image after cropping, noise reduction and artifact removal. (C) Blob detection of the rat's body. (D) Blob detection of the rat's paws, zoomed in image. (E) Blob detection of the rat's paw parts, zoomed in image.

3.2.3 Temporal correction

The paw prints' identity numbers are corrected and grouped so that paw prints present in several consecutive frames are having the same identity number. A paw print is defined as one and the same paw print if the coordinates of the mass center of the paw print in the current frame are within the range of the boundary box coordinates for one of the paw prints in the previous frame. If this is the case, the paw prints in question are classified with one and the same paw ID.

3.2.4 Feature extraction

During the blob detection features of the different regions were determined. These features alone and features calculated and determined using these primary features were saved as data in MATLAB tables. All features and their definition are listed in table 3.1.

3.2.5 Paw print validation

Paw IDs belonging to paw parts having a total area of zero are removed, since those prints hence are actually not paw part prints, but (falsely) detected paw prints. For the same reason, paw IDs located within a large distance from the region of the body are also removed. Paw IDs that are present in only one frame throughout the whole video are also removed. These prints are most likely not paw prints, since a duration of one frame in a video recorded in a frame rate of 30 fps equals a duration of only 33 ms.

3.2.6 Determination of directions

To determine the rat's orientation in each frame, a combination of orientations is used, which constitutes the orientation of the rat's body together with coordinates for the different parts of the paws, see figure 3.4. Orientation of the rat's body is determined on the basis of the body region's major axis relative to the horizontal axis, i. e. the x-axis, which gives a value between -90° and 90° . A value of -90° or 90° corresponds to the body's major axis being directed perpendicular to the x-axis in a northern respectively southern direction. A value of 0° corresponds to the major axis being directed perpendicular to the y-axis, i.e. in the eastern or westerly direction and so on. This value is converted to directional vectors, but these directions do not tell where the front end of the body is relative to the rear end of the body. To determine whether the body, for instance, is directed in a northern or southern respectively eastern or western direction, the direction of the paws is determined.

The direction of the paws is determined by setting the mean value of the centroids of the toes in relation to the value of the centroid of the pad, where the former value is assumed to be closer to the front end of the rat than the latter value. Once the direction of each paw print is determined, the direction within a paw ID is determined by adding and normalizing each paw print belonging to the paw ID, so that one and the same paw ID is having the same average directional vector of each frame it is present in. Thereafter, the directional vectors of each paw

Table 3.1: Overview of features extracted or calculated from the blob detection.

Features, body	
Area	Area of the body
Centroid	Center of geometry of the body
Orientation	Angle between x-axis and major axis of the body ranging from -90° to 90°
Direction	Directional vector of the body using its orientation
Features, paws	
Bounding box	Position and size of the smallest box containing the paw
Contour area	Area inside the contour of the paw
Centroid	Center of geometry of the paw
Features, paw parts	
Direction	Directional vector of the paw from pad to toes
Pad area	Area of the paw's pad
Total Area	Total area of all paw parts present in a paw print
Major axis length	Maximum major axis length of the pad within a paw ID
Minor axis length	Minor axis length corresponding to major axis length
Collected direction	Resultant of all paw directional vectors within a frame
Features, rat	
Direction	Direction of the rat; direction of the body and collected direction of paws to flip the directional vector if needed within a frame
Relative direction	Directional vector from the centroid of the body to the centroid of each paw within a frame
Relative angle	The angle between the directional vector of the rat and the relative directional vector of each paw within a frame
AEP	Position of paw relative to the position of the body in paw touchdown
PEP	Position of paw relative to the position of the body in paw take off
IP	Intermediate positions, i.e. the paw's positions between the stance phase's AEP and PEP
MSE	Mean squared error of the AEP, IP and PEP to a line fitted by the least square method in a stance phase
TMSEpF	Total mean squared error of the MSE of the paws present in a frame
IP-distance	Distances from the intermediate positions to the linear line extending between the stances phase's AEP and PEP
Footprint clustering	Variability in AEP's and PEP's respectively, for each paw class

ID being present in one and the same frame are added and normalized, so that the paws are having a collected direction in each frame. The collected direction of the paws in a frame is then combined with the directional vector of the body in that same frame to get the rat's direction.

To determine where a paw is located relative to the body the directional vector from the body's centroid to the paw's centroid is determined, named the relative directional vector. Thereafter, the relative angle between the body's directional vector and the relative directional vector is determined. This parameter can have values between -180° and 180° , where values closer to 0° indicate the paw print is close to the rat's front end and a value closer to -180° or 180° indicates the paw print is close to the rat's rear end.

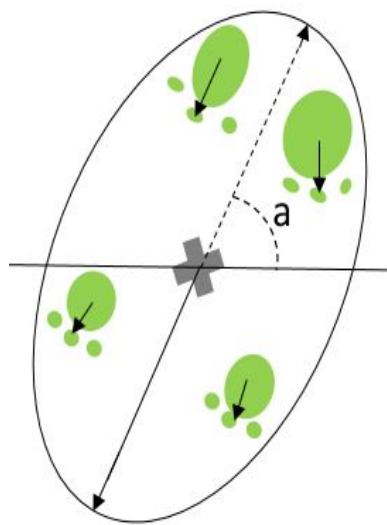


Figure 3.4: Illustration determination of the rat's direction. The cross is the body's centroid and the dashed arrow along with the long solid arrow is the body's major axis. The angle a is approximate -45° , indicating the body is being directed northeast. However, the directions (small arrows) of the paws is suggesting the rat is being directed southwest, which will be determined as the rat's direction.

3.2.7 Classification of paws

The paw prints can be divided into four classes according to whether they belong to the right hind, left hind, right front or the left front paw. To determine whether a paw print is belonging to a right or left paw its relative angle is used. If its relative angle is $< 0^\circ$, the paw print is classified as belonging to a left paw and if its relative angle is $> 0^\circ$, the paw print is classified as belonging to a right paw, see figure 3.5. Whether the paw print belongs to a front or hind paw is determined by the size of its pad area. A pad area above a certain value leads to the paw print belonging to a hind paw and a value below that certain value leads to the paw print belonging to a front paw. Once all paw prints are classified, each paw print belonging to the same paw ID is corrected if necessary. This is done by assigning each paw print in that paw ID the most commonly occurring class affiliation within the paw ID.

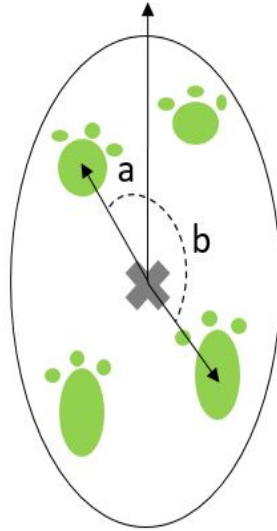


Figure 3.5: Illustration showing relative angles. The cross is the body's centroid, the center arrow is its directional vector. The two smaller arrows are directional vectors from the rat's body's centroid to the paws' centroids. The angles a and b are the relative angles, where a is approximately 45° (the rat's right side) and b being approximately -135° (the rat's left side).

3.2.8 Extraction of video sequences

Since gait patterns can mainly be analyzed when the rat moves in a gait, the frames showing the rat sitting down or standing still are discarded. Sitting is defined as the region of the rat's body being below a certain value in area. To determine if the rat is standing still or moving in a gait, frames are extracted and analyzed in sequences of 10 consecutive frames. Whether the rat moves in a gait or stands still within the frame sequence is defined by the average displacement of the coordinates of the body mass center between two consecutive frames. If the average displacement, in either the x - or y -direction, exceeds a certain value, all frames within the sequence are saved to a separate table only containing features of frames where the rat is walking. Otherwise, all frames within the sequence will be left out from that separate table.

3.2.9 Correction of classes within frames

Once the data exclusively contains frames visualizing the rat moving in a gait and the paw prints are classified, the data is searched and corrected if needed. The classified paw prints are analyzed one frame at a time to see if any frame contains an irrational set of paw classes. For instance, a frame cannot contain more than four paw prints, more than two left paw prints, more than two hind paw prints and so on. In case a frame has five or more paw prints, the paw print with the smallest pad area is removed. In case a frame has more than two left, right, hind respectively front paws the duplicates' features are compared to each other and the most likely to be false are compared to the other paw classes in the frame. If the frame has a paw print with features close to its opposite class, this paw print and every paw print in the paw ID it belongs to is corrected.

3.2.10 Stance linearity & footprint clustering analysis

The parameter relative directional vector is used to determine, i.a. the parameters anterior extreme position (AEP) and posterior extreme position (PEP) and also different measures of stance linearity, based on the parameter stance linearity index. To get a graphical overview of the amount of body wobbling over time the total mean squared error per frame (TMSEpF) is computed for each frame. To get the TMSEpF the MSE of each stance phase is first calculated. A stance phase is defined as the AEP to the PEP including all the intermediate positions (IP). The coordinates of the AEP, PEP and IP are all fitted to a line, using the method of least squares, see dashed line in figure 3.6a, and the MSE of the stance phase is then calculated. This gives an average value of the linearity in this particular stance phase, i.e. the amount of body wobbling relative to the paw being in stance phase. The TMSEpF is then calculated by adding the MSE of all paws being in stance phase in this particular frame. In addition to TMSEpF giving a value to the total amount of wobbling in a frame, it also gives the possibility to graphically estimate time periods of when the rat is sitting or standing still. This arises due to the fact that a rat sitting or standing still during a certain number of frames is equal to the rat being in the one and same stance phase during that number of frames. Therefore, this time of periods can be identified in a plot as plateaus when TMSEpF is plotted against time or frame number. Figure 3.6b is an example of this, showing the TMSEpF plotted against time, e.g. frame number 1-300. To estimate possible body

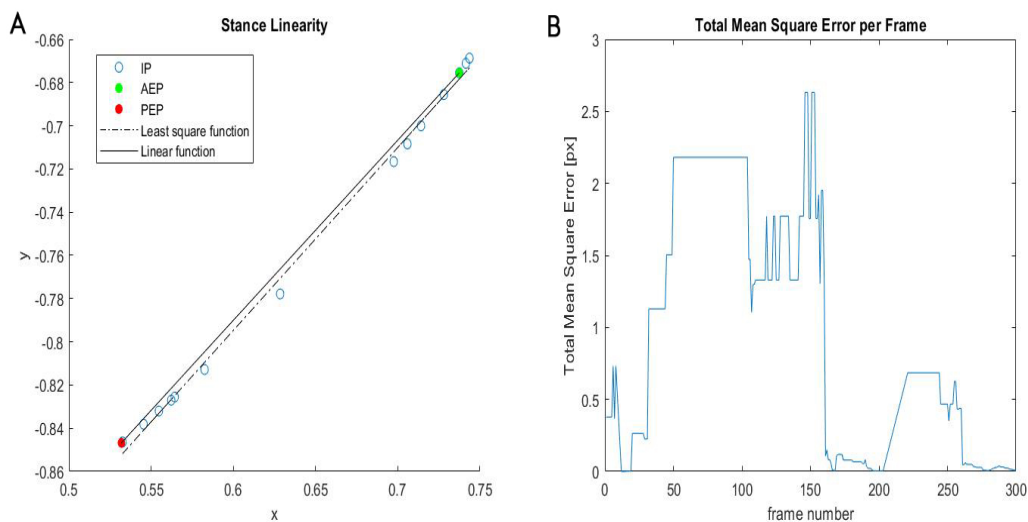


Figure 3.6: (A) A graphical example of stance linearity showing the anterior extreme position (AEP), intermediate positions (IP) and posterior extreme position (PEP) with an appurtenant curve fitted using the least square method, as well as a linear line from AEP to PEP during one stance phase. (B) Graphical view of total mean squared error over the time period frame number 1 to 300, i.e. during 10 seconds of a video recording, showing plateaus when the rat is sitting or standing still.

wobbling during a period of time where the rat is sitting or standing still in one stance phase, the spatial variation is calculated by computing the distances of its different IP paw prints to a linear line drawn between the stance phase's AEP and PEP, see solid line in figure 3.6a. The IP-distances are calculated using equation

3.1:

$$d = \frac{|ax_1 + by_1 + c|}{\sqrt{a^2 + b^2}}, \quad (3.1)$$

where d is the IP-distance, i.e. the distance between an intermediate position, positioned at coordinates (x_1, y_1) , to the linear line, $ax + by + c = 0$, going from the AEP to the PEP. These distances can be used to estimate total body wobbling per frame during frames where the rat is sitting or standing still. They can also give an estimation of which paw is causing which amount of wobbling, i.e. give an estimation of tightness of motor control in that particular paw limb.

The parameter footprint clustering, which can give an estimation of tightness of motor control in touchdown respective take-off of the respective paw limbs, is calculated using a paw's every AEP's respective every PEP's during a period of time. This gives a cluster of the paw's AEP's respective PEP's, hence the name. The footprint clustering of a paw is then calculated by computing the standard deviation of the respective clusters, using their relative angles in these positions.

3.3 Deep learning based segmentation, object tracking & classification of body parts

Deep learning based segmentation is done with DeepLabCut (The A. and M.W. Mathis Labs) (DLC) via Anaconda (Anaconda Inc), followed by analysis and classification in MATLAB, see the workflow in figure 3.8a.

3.3.1 Image processing

The image processing is done using MATLAB. To increase the run time efficiency during the training process in DLC, the video recording is downsized to 1920·1080-pixel format and reformatted from a MP4 file to an AVI file. To reduce the risk of interference caused by objects outside the plexiglass box, each frame is cropped to dimensions determined by visual analysis of the video recording. Also, the brightness in each frame is increased, to enhance the possibility of the software identifying, detecting and following the rat's body.

3.3.2 Labeling & training

The labeling process is done using DLC's graphical user interface. A new project is created in DLC and the processed video recording is imported. Approximately every 40 frames are extracted, which are then provided with labels for the rat's head, belly, rear end and four paws respectively, see figure 3.7. The labeled frames are saved and checked visually before a training data set is created. The training data set is trained with 150.000 iterations. Once the data set is trained, it is evaluated visually and by using values for parameters of the loss function, extracted from DLC. In case the values for training and test errors are not satisfactory, the training data set is trained with additional iterations or the training data set is expanded with additional frames. Otherwise, another video recording is analyzed in DLC, which has undergone downsampling, an increase of brightness and reformatting to an AVI file. Then, using DLC, a novel video is created with labels of

the analyzed video with an appurtenant H5 file containing information about the different labels' position in each frame and their likelihood of being present in a certain frame at that certain position.

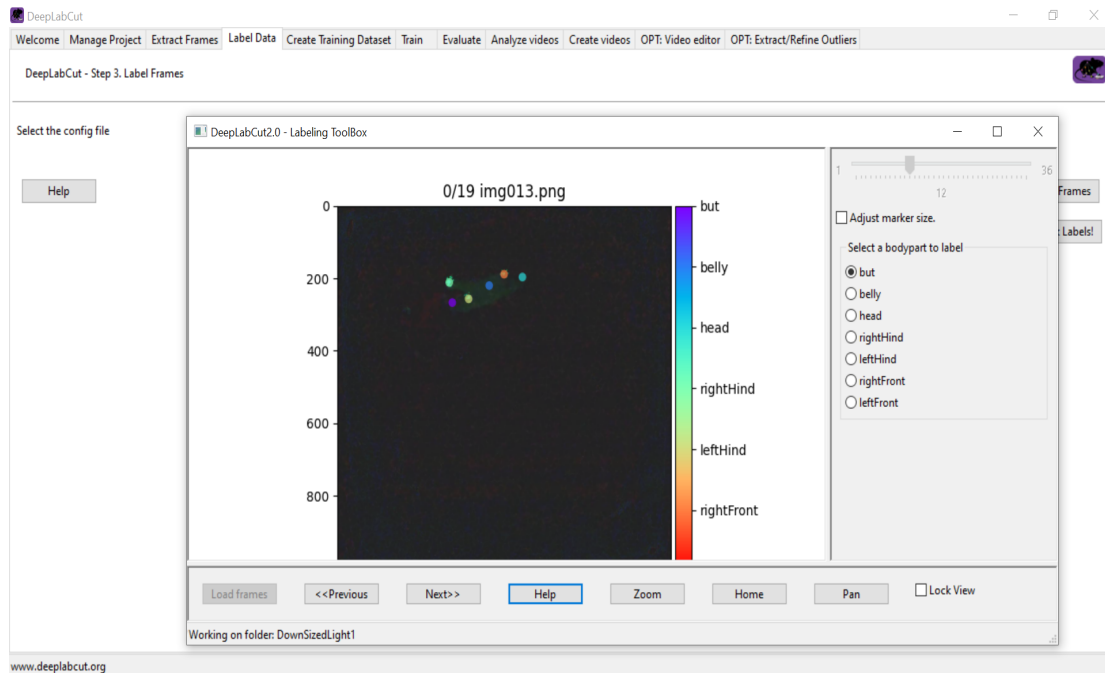


Figure 3.7: Screenshot of DeepLabCut's graphical user interface, during labeling process.

3.3.3 Determination of directions

The H5 file is reformatted to a CSV file using python via Anaconda and the CSV file is loaded in MATLAB. The CSV file now contains coordinates of the positions for all body parts respectively, frame by frame, as well as a likelihood value. All values appurtenant the rat's belly, head or rear end are added to a table. The likelihood value is a measure of the likelihood of a certain body part being in a certain frame at a certain position, valued in a range from 0 to 1. All body parts' labels having a likelihood below 0.9 are assigned the average coordinate- and likelihood values of the body part in question found in the neighborhood frames. Then, body parts' labels still having a likelihood below 0.9 are removed from the table. The directional vector of the rat is determined by the number of labeled body parts present in the frame in the order of priority:

1. Three labels present: resultant of the directional vectors of head relative to the belly, belly relative to the rear end and head relative to the rear end in the frame in question.
2. Two labels present: belly relative to the rear end or else the head relative to the rear end or else the head relative to the belly, depending on which of the body parts has a label in the frame of question.

In those cases a frame contains less than two labeled body parts, the directional vector is given by the direction of the neighborhood frames.

3.3.4 Classification

The respective paws are classified as in the previous section, using relative angles and size of pad area. The relative angle is the angle between the directional vector of the body and the directional vector of the paw print relative to the body, where the directional vector of the paw print relative to the body is imported from the table made in the previous section. The angle of the paw print relative to the body is then used to determine whether each paw print belongs to a right or left paw. Also, as in the previous section, each paw print is determined to belong to a front or hind paw using the paw prints size of the pad area, where the pad area is imported from the table made in the previous section. When all paw prints are classified each paw print belonging to a certain paw ID is corrected if needed, making all paw prints within the same paw ID belonging to the same paw class. At last, correction of classes within frames is done if needed, as in the previous section.

3.4 Deep learning based segmentation, object tracking & classification of paws

Deep learning based segmentation and classification is done with DeepLabCut (DLC) via Anaconda, see the workflow in figure 3.8b.

3.4.1 Image processing

Image processing of the video recording is done according to the procedure described in the previous section, see section 3.3.1.

3.4.2 Labeling & training

Labeling and training of the data are done according to the procedure described in the previous section, see section 3.2.2. A data set is created, trained and evaluated and a novel video is created with labels of the analyzed video with an appurtenant H5 file containing information about the different labels' position in each frame and their likelihood of being present in a certain frame at that certain position, as in section 3.2.2.

3.4.3 Classification

The H5 file is reformatted to a CSV file using python via Anaconda and the CSV file is loaded in MATLAB. All values appurtenant the rat's paws, being the coordinates and likelihood of each detected paw print frame by frame, in the CSV file are added to a table. All paw prints' labels having a likelihood below 0.9 are removed from the table.

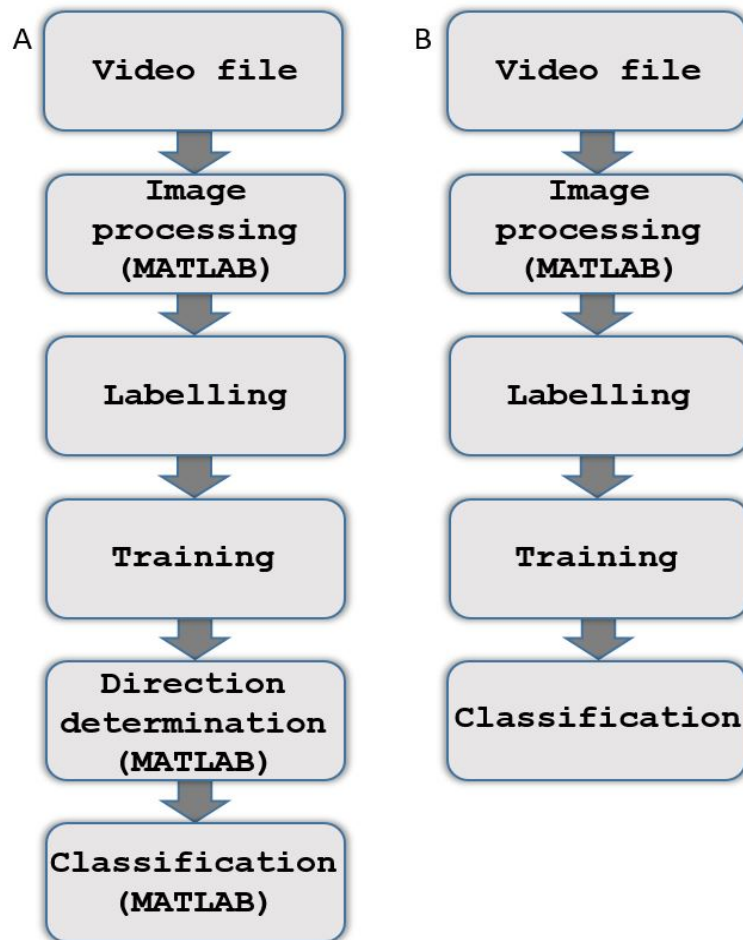


Figure 3.8: (A) The workflow of the DeepLabCut software in combination with the MATLAB program. (B) The workflow of the DeepLabCut software.

4

Results

4.1 Setup & data collection

The setup worked and generated data as intended. The rat was able to move unhindered and video recordings of high image quality were generated.

4.2 Image analysis in MATLAB

The main milestone of this project is to be able to use any of the proposed methods to classify the paws with satisfactory accuracy.

4.2.1 Classification

The image classification method done by analysis in MATLAB, described in section 3.2 resulted in approximately 93 % accuracy in classifying a detected paw print ID belonging to the correct paw class, with the results of manual classification as ground truth. The error rate of 7 % included 0 % of incorrect front or hind classification, approximately 6 % of incorrect right or left classification and 1 % of incorrect detected body parts. As described previously, the classification of paws as left or right paws and front or hind paws was based on the pad area and the relative angle. A class division according to these terms can be seen in figure 4.1, where the paw prints' relative angles are plotted against their pad areas. This plot shows the paw prints form 4 more or less distinguished clusters. In particular, the front and hind paw prints are showing a clear division; below respective above approximately 800 pixels in area, which is the pad area threshold value chosen for this particular rat and video recording. A division can also be seen between right and left paw prints; above respective below 0° , although it is not as distinct as between the front and hind paw prints. Whether a paw print's pad area is equal to or greater than 800 px is decided based on the requirement that the paw print's paw ID contains at least one paw print with a pad area equal to or greater than 800 px. This requirement causes some of the hind paw prints to be visually outside their proper cluster. Also, the paw ID correction, described last in section 3.2.7 and the correction of classes within frames, described in section 3.2.9 causes paw prints to be visually outside their proper cluster, hence the overlap of clusters representing left and right paw prints in figure 4.1. The duration of the respective

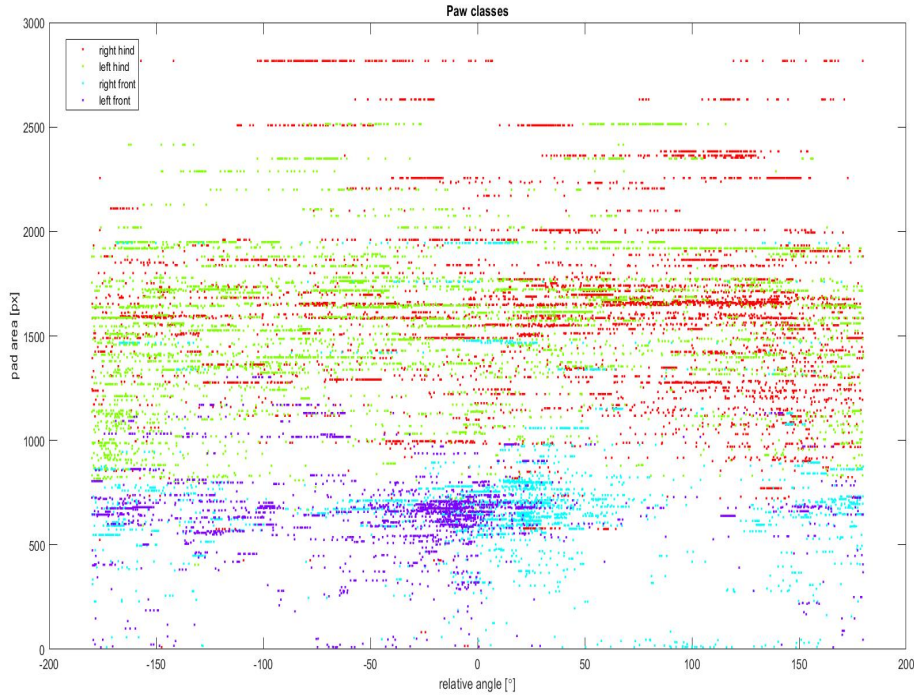


Figure 4.1: Scatter plot showing paw prints appurtenant the four paw classes; right hind paw, left hind paw, right front paw and left front paw divided according to the paw prints pad area and its angle relative the rat’s body.

paw classes relative to each other, i.e. the total number of frames a paw appurtenant a certain paw class is being present in relation to the number of frames all paws are being present in total is shown in table 4.1. The duration and number of

Table 4.1: Table showing duration of the paws appurtenant the respective paw class, in relation to all paw classes, during all sequences respective walking sequences. Also, the number of times the paws appurtenant the respective paw class is used, in relation to all paw classes during all sequences respective the walking sequences are shown.

Duration [%]	All sequences	Walking sequences
Right hind	33.90	29.35
Left hind	34.07	34.80
Right front	16.78	18.27
Left front	15.25	17.59
Number of touchdowns [%]	All sequences	Walking sequences
Right hind	19.25	25.71
Left hind	18.53	26.60
Right front	32.14	22.70
Left front	30.08	25.00

touchdowns of each paw class are also shown in histograms (figure 4.2a-d). Here, ‘number of touchdowns’ is the number of paw IDs belonging to the paw class in question and ‘duration’ is the total number of frames a paw belonging to the paw class in question is present, not in relation to all paw classes. Also, here 1 equals right hind, 2 equals left hind, 3 equals right front, 4 equals left front. The aver-

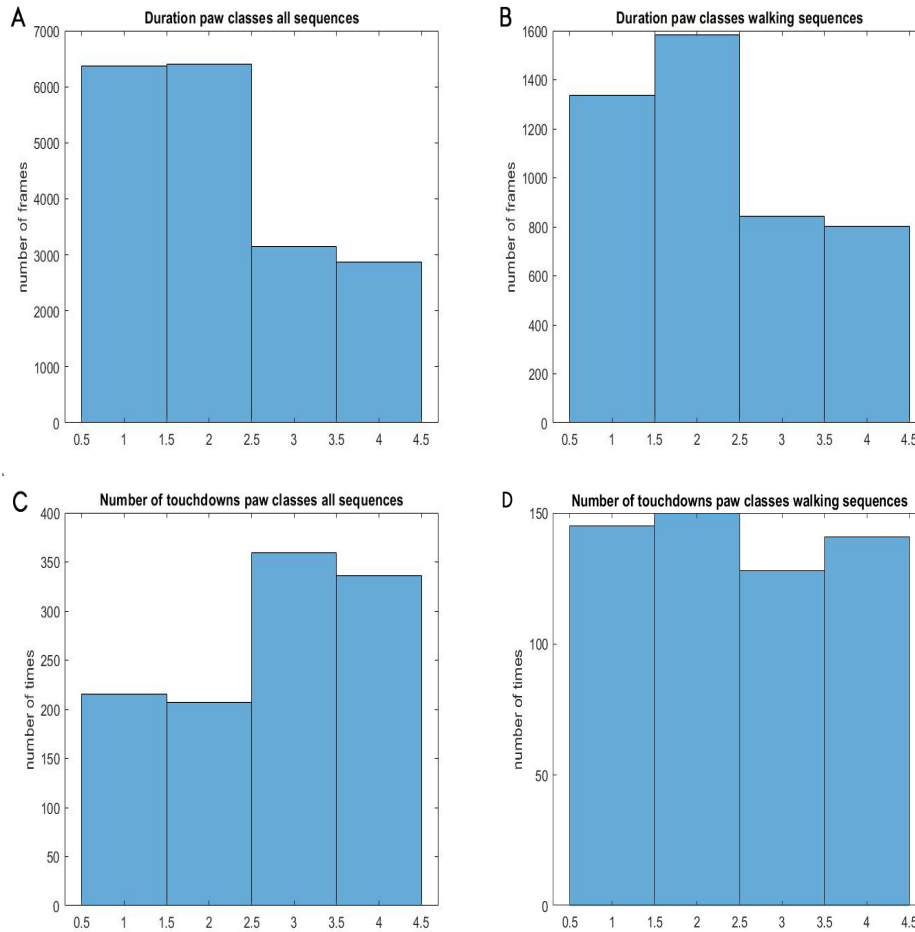


Figure 4.2: Histograms showing duration and number of touchdowns of each paw class; 1 = right hind, 2 = left hind, 3 = right front, 4 = left front. (A) Duration of the paw classes, counted in number of frames, for all sequences. (B) As (A), but for only the sequences where the rat is walking. (C) Number of touchdowns of the paw classes, for all sequences. (D) As (C), but for the walking sequences.

age stance and swing time, mentioned in section 2.1, was calculated for the hind paws during walking sequences. Corresponding values for the front paws were not calculated, due to insufficiency in detection. The stance time is computed by dividing a paw class' duration by the number of touchdowns for that paw class, divided by the frame rate of the video recording in milliseconds. The swing time is the difference in frame number from a paw class' paw ID to the paw class' next paw ID, divided by the frame rate of the video recording in milliseconds. These calculations resulted in the values shown in table 4.2.

Table 4.2: The average stance and swing time in milliseconds for respective hind paws, calculated from video sequences where the rat is walking.

	Right hind	Left hind
Stance time [ms]	307	352
Swing time [ms]	133	140

4.2.2 Stance linearity & footprint clustering

The stance linearity analysis resulted in the parameter total mean squared error per frame (TMSEpF), which is graphically shown in figure 4.3a (and also in figure 3.6b), where TMSEpF is plotted against time over the time period of frame number 1 to 300. This graph shows the total measure of stance linearity per frame for all paws being present in that frame and the time periods where the rat is sitting can be seen as plateaus. Here, stance linearity is based on curve fitting by the least square method and the measurement of linearity for each paw print present in a stance phase is the mean squared error of this paw print relative to the fitted line. To get measures of which of the paws contribute more or less to the total mean squared error over time, the mean squared error of each paw over time is plotted, see figure 4.3d. In these calculations, it is not taken into account whether the respective paw prints are an AEP-, IP- or PEP-print, which leads to every stance phase having the same total mean square error throughout the entire stance phase. This in turn will give rise to a graphical plateau when the one and same stance phase occurs over several frames in a row, as when the rat is sitting or standing still.

The average total mean square error (TMSE) and each respective paw's average mean squared error (MSE) throughout the video recording was calculated which resulted in the numbers:

- Average TMSE = 0.2205
- Average MSE, right hind = 0.2976
- Average MSE, left hind = 0.4621
- Average MSE, right front = 0.0863
- Average MSE, left front = 0.0359

Note, this approach gives a linearity measure partly including linearity when the paws are actually not in stance phase, but in swing phase, since the MSE of the AEP-print and PEP-print is included in the calculations.

If instead the stance linearity is based on a line fitted from the stances phase's AEP-print to its PEP-print and the measure of that stance phase's linearity is calculated by computing the distances from each IP-prints to the fitted line (IP-distance), this will result in data possibly varying throughout an entire stance phase. An example of IP-distances is shown in figure 4.3b, where the total IP-distances is plotted against time over the time period of frame number 1 to 300. In figure 4.3c the IP-distances over the same time period as in figure 4.3b is plotted, showing the IP-distance per paw. In this graph, only the hind paw's IP-distances are shown, since the front paws are not present in video sequences where the rat is sitting down.

A graphical view of stance linearity, in terms of IP-distances, for all respective paws is shown in figure 4.4a-d, where IP-distances of each paw are plotted against time.

The parameter footprint clustering of each respective paw was calculated by computing the standard deviation of the paw's AEP- respective PEP-positions relative the body throughout the video recording, using the relative angles for the AEP- and PEP-prints. This resulted in the values seen in table 4.3.

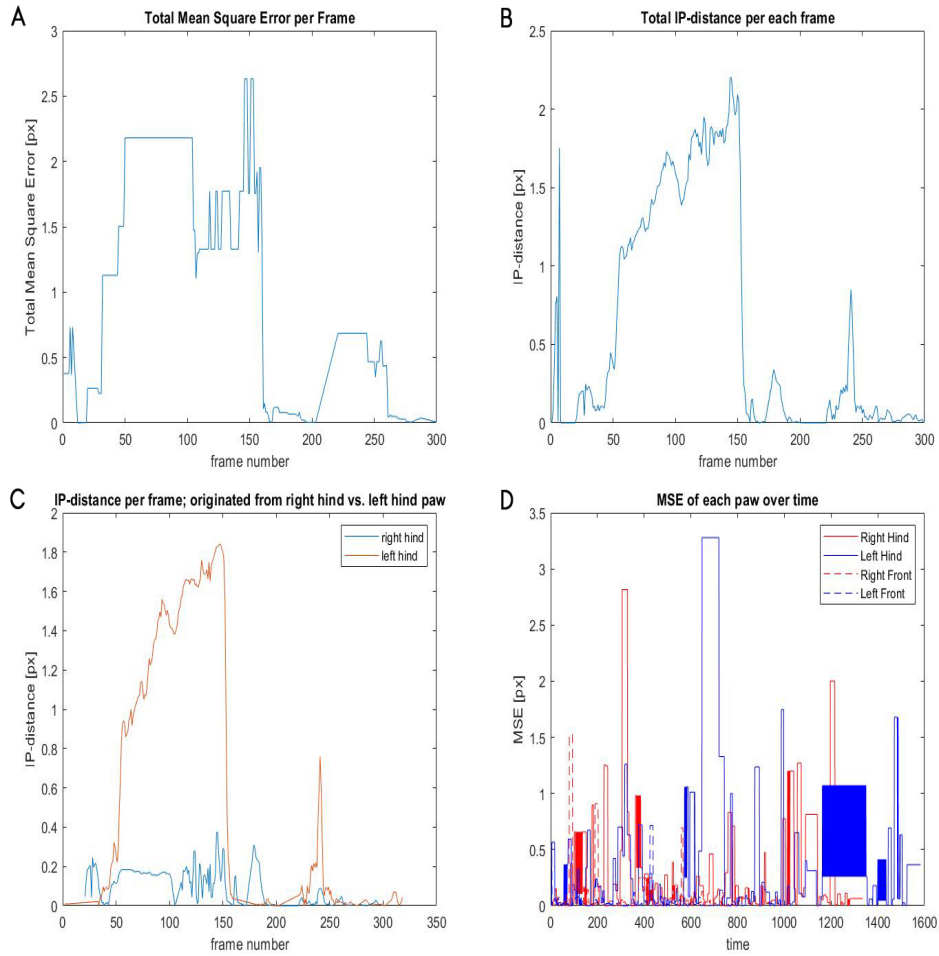


Figure 4.3: Graphs showing: (A) Total mean squared error plotted against frame number 1-300. (B) Total IP-distances of all paws plotted against frame number 1-300. (C) IP-distances of right hind paw versus left hind paw plotted against frame number 1-300. (D) Mean squared error of each paw plotted against time.

Table 4.3: Foot print clustering of each respective paws, of their AEP respective PEP, i. e. the standard deviation of the positions relative the body for the AEP respective the PEP, in degrees.

	AEP [°]	PEP [°]
Right hind	98.92	112.50
Left hind	102.22	114.16
Right front	98.91	106.14
Left front	98.49	116.60

4.2.3 Area features

Values of features regarding the area of the paws and the paw parts, which can be of use when analyzing the pressure load of the rat's paws, are shown in table 4.4. In this table, the different features are divided into the four paw classes for comparison. The parameter named pad area is determined by a paw ID's largest occurring paw part area. The parameter named total area is the total area of a paw ID's paw parts, being present in one frame.

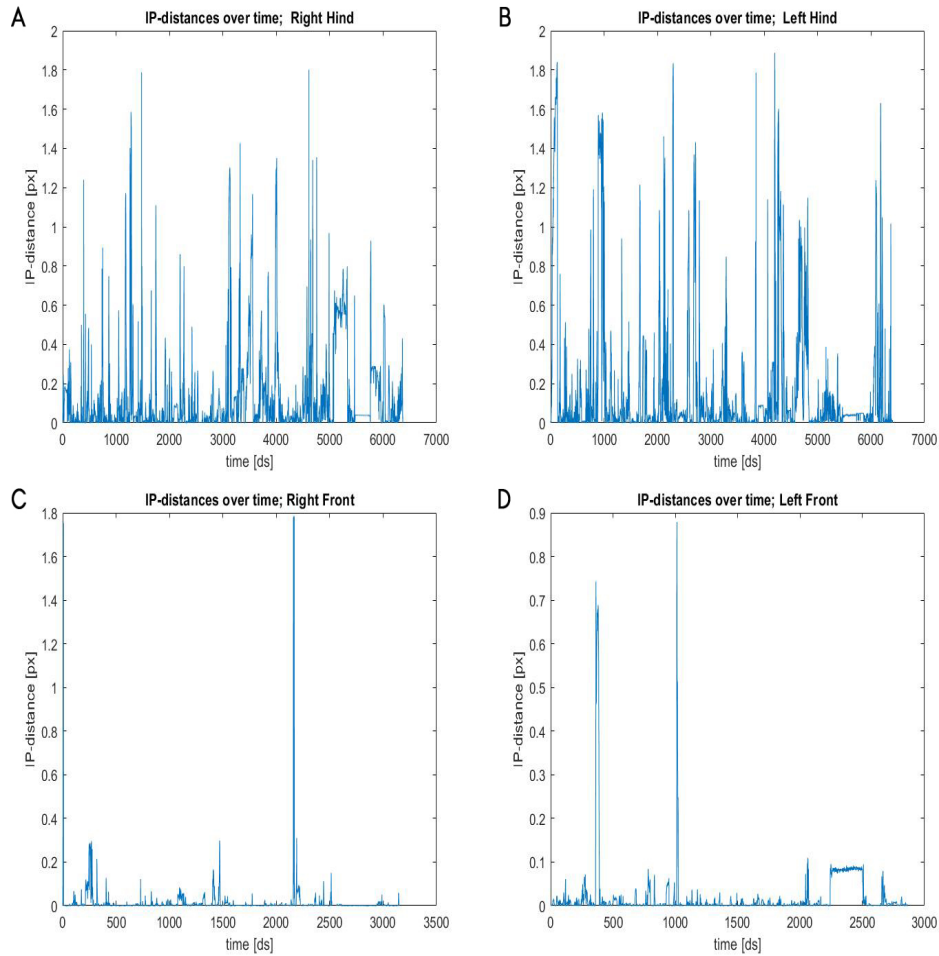


Figure 4.4: Graphs showing IP-distances plotted against time in deciseconds: (A) IP-distances of right hind paw plotted against time. (B) IP-distances of left hind paw plotted against time. (C) IP-distances of right front paw plotted against time. (D) IP-distances of left front paw plotted against time.

Table 4.4: Area features of the paws and pad areas of each paw class.

	Right hind	Left hind	Right front	Left front
Maximum total area [px]	2994	3193	2041	1303
Average total area [px]	1459	1431	681	637
Maximum pad area [px]	2816	2514	1945	1303
Average pad area [px]	1649	1598	739	667

4.3 Deep learning based segmentation & object tracking

The deep learning method done by deep learning based segmentation and object tracking in DeepLabCut (DLC), described in section 3.3 resulted in an accuracy of classifying a detected paw print ID belonging to the correct paw class of approximately 67 %, with the results of manual classification as ground-truth. The error rate of 33 % included 0 % of incorrect front or hind classification, approximately 32 % of incorrect right or left classification and 1 % of incorrect detected body parts. Due to the accuracy being significantly lower using this classification method, no

further analysis was done.

4.4 Deep learning based segmentation, object tracking & classification

The deep learning method done by deep learning based segmentation, object tracking and classification in DeepLabCut (DLC), described in section 3.4 resulted in an accuracy of classifying a detected paw print ID belonging to the correct paw class of approximately 30 %. The method also generally resulted in a low detection rate of the paw prints, in particular the paw prints belonging to the front paws. Due to the accuracy and detection rate being significantly lower using this classification method, no further analysis was done.

5

Discussion

The available methods to evaluate pain affected or 6-OHDA lesioned rats with subsequent treatments described in section 2.4 each has its pros and cons. The cylinder test is executed with a simple setup, requiring small resources and provides data easy to interpret. However, the interpretation of the data is time consuming and requires an examiner. Further, the data only gives information about the number of usage for the respective paw when the rat is rearing up and touches the cylinder wall. Thus, the data does not contain any information about, for instance, pressure load on the paws, the rat's body posture, gait pattern or other motor behaviors.

The method used in the systems CatWalk XT and MouseWalker provides data that is less time consuming than the cylinder test, at least in relation to the information given, and does only partly require an examiner in the interpretation process. These systems can result in data containing information regarding the rat's gait pattern, the pressure load on its respective paws and the number of usage of its respective paws. However, a disadvantage of these systems is that the rat has to be trained to go through the tunnel, for example done by luring the rat with treats a certain number of times until the rat voluntarily goes through the entire tunnel without stopping or turning around. This training process may give a disadvantage to some extent for being somewhat time consuming and mainly it gives the disadvantage of the rat showing a forced instead of its normal motor behavior when passing through the tunnel and the fact that the rat and therefore its motor behavior is limited by the walls of the tunnel is also creating a limited motor behavior. Also, the CatWalk XT system is provided with an appurtenant software, which the user needs to use when analyzing the data. This software cannot be switched out, which means that the user cannot combine the system with other possibly wanted systems and cannot calculate possibly wanted parameters not being preprogrammed in the software.

The method used in the open field setup has the advantage of providing data generated by the rat in a practically unrestricted environment. It also allows the user to combine the setup with other possibly wanted systems. However, this method only generates data regarding the rat's motor behavior in general, as its displacement, and lacks information about the pressure load of the paws and gait pattern.

This project therefore aimed to investigate the possibility of a system that enhances evaluations of rats when affected by pain or 6-OHDA lesion and possible

treatments of these conditions. This was done by combining some of the currently used methods and setups and evaluating the possibility to analyze the data by blob detection and classification, using MATLAB, and deep learning based segmentation, object tracking and classification, using DeepLabCut.

5.1 Setup & data collection

The setup worked and generated data as intended. The data was at first to be collected using a web camera, having the same image resolution and frame rate recording as the GoPro later on used, but this generated video recordings having too low reproduction of the LED inside the glass plate and too high reproduction of the LED propagating into the surroundings. This may have to do with differences in the cameras' exposure, whereon this parameter can be of importance for the setup. Also, the alternative of having background light, above the rat, was tested, but was discarded later on.

5.2 Image analysis in MATLAB

5.2.1 Classification and gait analysis

The main step stone of this project is to be able to use one or several of the methods described in chapter 3 to classify the paws with satisfactory accuracy as this is a direct necessity when evaluating pain or 6-OHDA lesion in rats and treatment methods for these conditions. When using gait analysis as a tool in these evaluations it is important that the paw prints are correctly classified to enable identification of how the rat uses its respective paws and identification of any difference in paw use after pain exposure or 6-OHDA lesion with subsequent treatment. An accuracy of 93 % may be considered high enough in this reference, if one assumes the future amount of data being analyzed is sufficiently large and thoroughgoing.

The plot shown in figure 4.1 has paw prints located outside their visually proper clusters. The paw prints being correctly corrected from being appurtenant to a front paw to being appurtenant to a hind paw or vice versa can exemplify the deficiency of the blob detection. However, it needs to be taken into account that, at least a part of the paw prints being classified as hind paws even though their pad area is below 800 pixels is because of the paw's whole surface not touching the glass plate throughout the whole stance phase. The paw prints being accurately corrected from being appurtenant to a right paw to being appurtenant to a left paw or vice versa can exemplify the fact that a rat's paw not necessarily is located on the same lateral side as it is attached to the body, but is sometimes crossed to the contralateral side of the rat's body.

In figure 4.1 the clusters of front respective hind paws also show a somewhat distinctiveness in their relative angles. The approach of classifying the paw prints belonging to a front or hind paw dependent on their relative angle was therefore also tested, but this generated a lower accuracy whereupon this approach was discarded. There is a possibility that this approach could generate a higher accuracy by enhancing the method of detecting and determining the position of the rat's body location. The histogram in figure 4.2d is showing an insignificant difference

between the four paw classes, which is indicating a classification accuracy high enough. This statement is strengthened by comparing the histograms in figure 4.2a and figure 4.2b, where the relation between hind paw duration and front paw duration is significantly higher in figure 4.2a than in figure 4.2b. This is a result of figure 4.2a, in addition to walking sequences, also containing sequences where the rat is sitting down and only touching the glass plate with its hind paws giving hind paw prints a significantly longer duration than the front paw prints. This is further substantiated by comparing figure 4.2a and figure 4.2c, since it shows the increased proportion of hind paw duration in figure 4.2a (compared to figure 4.2b) not being caused by a higher detection ability in favor of hind paws in sitting sequences.

The histograms in figure 4.2b and figure 4.2d shows just a slight over representation of hind paw print touchdown, but a significant over representation of hind paw print duration. This would indicate the rat taking as many steps using its front paws as its hind paws, but the stance phase being shorter of the front paws than the hind paws. This is not likely, instead, this is indicating possible improvement of the setup and/or the blob detection, since the hind paw prints are clearly more visible and/or better detected. Note that the data in these histograms are extracted from the sequences where the rat is moving, the significant over representation of hind paw print duration can therefore not be explained by the fact that the rat is only using its hind paws when sitting down.

Parameters related to duration and number of touchdowns are stance and swing time, see table 4.2. These parameters were only computed for the hind paws, given the indication of the front paw print's lack of detection. However, the stance and swing time can be parameters of use when analyzing the rat's gait. A rat affected by pain in one of its paws can give rise to a decrease in stance phase time of that paw, due to the rat being less willing to put pressure load on the paw. For the same reason, this could give rise to an increased stance time duration of some or all the other paws not being affected by pain. Also, the swing phase could be affected by pain experiences, where the swing phases of the paws not being affected by pain most likely would decrease as a direct cause of the pain affected paw's stance time being decreased. The swing phase of the affected paw would most likely be unaltered or possibly increased. A general and significant difference in these two parameters between the respective paws could also indicate a general limping or abnormal gait pattern, which in turn can indicate the rat being in pain in general or being affected by, for instance, a 6-OHDA lesion or an electric pulse stimulation.

5.2.2 Body posture

When the rat is sitting down, the use of its paws is of less importance, since there is no gait pattern to analyze. However, video sequences of the rat sitting down can be of use in terms of analyzing a 6-OHDA lesioned rat's body posture since it, in sitting positions, may have a tendency of showing axial dyskinesia, typical of this condition [14]. This project does not include rats that are affected by anything, but only rats in their normal state. Therefore, an evaluation of this typical body posture has not been performed, but the project has generated the parameters mean squared error in a stance phase (MSE), total mean squared error per

frame (TMSEpF) and intermediate position distance (IP-distance) as suggested parameters to use in such an evaluation. The TMSEpF can be seen as a graphically, timestamped representation of whether the rat is sitting, standing still or moving, where a sitting sequence will be seen as a plateau in the graph. The value of the TMSEpF in this plateau can further indicate a high or low total axial displacement of the rat, which may indicate the rat showing axial dyskinesia or not. The MSEpF may further be used in combination with an analysis of the IP-distances of that plateau, to identify possible frames where the rat shows signs of axial dyskinesia, or other movement characteristics, such as tremors. Further, as mentioned in the previous section, a comparison of the histograms in figure 4.2a and figure 4.2b shows an increased relation between hind paw duration versus front paw duration as a result of the histogram in figure 4.2a has sitting sequences included. This increase can give a measure of how inclined the rat is to sit down during a video recording. This measure in itself can give an estimation of if and how the rat is affected by, for instance, pain or a 6-OHDA lesion.

5.2.3 Movement patterns

The video sequences showing the rat not sitting nor fully walking, but showing its intermediate steps when switching from sitting to walking or vice versa have the lowest accuracy in classification of the paws with the method used in section 3.2. This is most likely due to rats having a tendency in placing their front paws in another direction than the hind paws when going from sitting to standing, before starting to walk. This tendency causes the direction of the rat to be wrongly classified since the used method partly uses the direction of the paws to determine the direction of the rat. As these kinds of video sequences do not contain a (full) gait pattern to analyze they can be removed from the gait analysis, for instance using the method described in section 3.2.8. However, the sequences showing this kind of movement can be of use when analyzing the motor behavior during the evaluation of pain or Parkinson's treatments since a rat affected by pain or a 6-OHDA lesion often prefers to use the paws contralateral to the lesioned or pain affected side. This behavior can give rise to the rat turning more often to the unaffected side than to the affected side [4]. Therefore, the data generated in these video sequences can be of use since it contains information on the hind paws' direction and position and the front paws' direction and positions, which in turn can generate information about which side the rat may prefer to turn.

Also, the histogram in figure 4.2c may serve as a basis for analyzing the rat having a possibly preferred usage of one of its front paws in these video sequences, by comparison of the number of touchdowns of the respective front paws (bar 3 respective 4). From this point of view, these video sequences may give measurements similar to those generated from a cylinder test. However, the histogram presented in figure 4.2 is most likely deceptive regarding the comparison between bars 3 and 4, since these are generated from the classification method based on the rat's body's direction, which resulted in a low accuracy in the sequences where the rat is between sitting and walking. To get a more truthful comparison between the number of touchdowns made by the right respectively left front paw another classification method, for instance, one where the direction of the paws is used would be necessary. In addition to the video sequences where the rat is between sitting

and walking, data appurtenant video sequences showing the rat walking also can contain information about a possibly preferred turning side, through the use of how the rat's direction is changing over time.

5.2.4 Stance linearity & footprint clustering

As mentioned in section 5.1.2 the parameters (TMSEpF, MSE and/or IP-distance) may be used as a tool when analyzing possible body axial rotation/dyskinesia in a 6-OHDA lesioned rat. These parameters, alone or in combination with each other, also may be a possible tool when analyzing the rat walking as these parameters can give an indication of the amount of body wobbling. The amount of body wobbling may increase versus decrease when the rat is affected by pain or a 6-OHDA lesion versus when the rat is affected by treatment.

Also, the parameter footprint clustering may be of use in gait analysis since it can give measurements regarding the rat's tightness of motor control in its respective paw limbs. The values presented in section 4.1.2 show a tighter motor control of the rat's front paw limbs (AEP) than in its hind paw limbs (PEP), which is a general characteristic of rats [5]. The values are also indicating this particular rat has tighter motor control in its right body half than in its left body half. This sort of comparison may be a tool in future gait analysis, since these values may change when the rat is affected by pain or 6-OHDA lesion and subsequent treatment.

Which one of these parameters or which combination of these parameters is being of use in a gait and/or body posture analysis is to be further evaluated by comparing data of an unaffected rat with data generated when the rat is affected by pain or 6-OHDA lesion.

5.2.5 Area features

A readout from the table 4.4 gives that the maximum total area is equal to the maximum pad area for the left front paw, which is not a reasonable result. This is indicating a threshold value set too high in the paw part detection, leading to a lack of detection of the left front paw's toes alternatively a threshold value set too low, leading to the toes and pad area being merged together to one blob. Comparing these two values of the left front paw with the corresponding values of the right front paw though is indicating that the threshold value is set too high if assuming the rat's front paws and pads are of approximately the same size.

The paws were classified as belonging to a hind paw class or front paw class according to their pad area. This threshold value was set to 800 px and the values regarding pad area in table 4.4 are strengthening this to be a correct set threshold value. However, the maximum pad areas of the front paws are significantly higher than 800 px, which is indicating the classification corrections made in sections 3.2.7 and 3.2.9 are a necessity. From the table 4.4 one can also read that the parameter called average total area may be of use to classify the paw prints belonging to a front or hind paw, by itself or possibly in combination with the parameter average pad area.

5.2.6 Method improvements

Possibly useful parameters that can be extracted from every sequence in the video is the amount of the paw area and also which of the paw parts are being loaded during each stance phase. If a paw has a paw print area that significantly decreases after the paw is UVB irradiated, this can be an indication of the rat experiencing pain in that particular paw and vice versa when the rat is experiencing pain relief. Furthermore, an analysis of which of the paw part's area has decreased can give an indication of where the rat experiences the pain originates from. These parameters were determined during the method described in section 3.2.2 as pad area, total area and contour area, see table 3.1.

The resulting values of the first two parameters, mentioned in chapter 4, may be a representation of these parameters to some extent. However, these values are probably insufficient in terms of being of use in an experienced pain- and pain relief-evaluation, since the paw part detection is insufficient in some of the frames, where paw parts visual to the human eye are not detected. This statement is also strengthened by a readout of table 4.4, which gives that the maximum total area is equal to the maximum pad area for the left front paw. This is mainly due to the threshold value of paw part detection being optimized in favor of the correct classification of the paw classes. Choosing a lower threshold value led, in some cases, to one or more paw parts being detected as one blob. This in turn led to these merged paw parts being detected as a false pad with a false pad area resulting in the paw print being classified as belonging to a false hind paw.

The last-mentioned parameter, contour area, is probably also insufficient in terms of being of use in an experienced pain- and pain relief-evaluation. Again the threshold value was optimized in favor of correct paw classification and to be able to have too many true undetected paw prints the threshold value was set relatively low. This led to parts of the rat, for instance, its head and tail tip, being falsely detected as paws. The detected blobs appurtenant the tail tip was easily identified as false paw prints due to their large distance from the body. However, a method to identify a blob being appurtenant the body or the head was not made. Choosing this threshold value relatively low also made the paw area include more parts of the paw than the paw parts actually touching the glass plate. This is the reason why this parameter is called contour area instead of paw area and is seen as a tool to detect areas of possible paws and assign them the correct paw ID, rather than a tool to be used when the actual value of the paw is of interest, as in a pain- and pain relief-evaluation.

Possible ways to improve these four parameters include changes and optimizations of the setup and of the program written in MATLAB. Examples of properties deriving from the setup can be the camera resolution, the choice of LED color and the choice of not having a background color. The camera used in the setup (GoPro Hero8 Black) provides two user-selectable resolutions; 1920·1080 and 3840·2160 (4K) and both of these were tested, where the lower resolution resulted in video recordings where the paw and paw part detection was difficult to execute. Therefore, a resolution higher than 4K may result in video recordings enhancing the paw and paw part detection. This may lead to increased accuracy in paw and paw part area values and it may also lead to a more correct representation of the paw and paw parts shapes, which in turn could be a possible entry to differentiate false paw prints arising from for instance the rat's head. However, an increase in

image resolution would come at the cost of increased computational demand.

The CatWalk XT system uses a red light as a background light. The alternative of having a red background light was also tested in this project's setup, but this setup resulted in video recordings where detection of the body was impracticable in frames where the rat was close to the walls of the box. In these frames the region of the body and the region of the walls got the same pixel value (0), making the body detection and therefore the paw classification impossible. However, in the frames where the rat was not close to the walls, the contours of the rat's body were more distinct, leading to more correct detection of the body's position. This can lead to a more correct paw classification since the region of the rat's body may get a more correct centroid value and orientation value. It could also lead to a less insufficient paw and paw part detection and therefore a better estimation of their respective areas since the pixel value of the region of the rat's body versus the regions of the paws and paw parts differs more using red background light. To get around the problem regarding when the rat's body is close to the walls a solution may be to choose another color than black on the walls.

To enhance the correctness of the paws and paw parts areas and possibly other parameters the program written in MATLAB can be changed and optimized in several ways. The program written for this project uses the same method of detection (although on three different levels) for all of the frames. Assuming this method is optimal for the classification of the paws, another detection method is optimal for determining the paw's areas. A possible approach would therefore be to use different kinds of detection methods throughout the video recording and thereafter use one of them to classify the paws, another one to determine the paw areas, a third one to determine differences in intensity inside the paw areas and so on.

Another approach would be to add one or more cameras to record the rat from different directions, such as from above, like in the open field method described in section 2.4 and possibly from the sides. This would give the opportunity of generating describing and detailed data, possibly enhancing the analysis regardless of whether the rat is standing, sitting still or moving in a gait. However, this might make the optimization of the open field setup more challenging, since it would need to be optimized with respect to filming both from above and underneath.

5.2.7 Kalman filter

The initial attempt to detect the region of the rat's body included Kalman filtering. This was done by writing a program in MATLAB. Although this approach was fairly successful, it later on in the process was found not necessary since the program including blob detection was sufficient to follow and detect the rat's body. Nevertheless, if the setup was to be changed to having a red background light, the problem regarding the region of the body and the region of the walls getting the same pixel value may be solved by using a Kalman filter. This approach could be a possible way of differentiating between the walls and the rat's body and it could also result in even higher correctness of the rat's position and orientation.

5.3 Deep learning based segmentation & object tracking

The method of classifying the paw prints using a combination of DeepLabCut (DLC) and MATLAB is including the rat's body's direction being determined by data generated from deep learning based segmentation and object tracking in DLC and the body's position and the paw's location relative to the body is determined by data generated from image analysis in MATLAB. This method generated a significantly lower accuracy than the method where image analysis in MATLAB was used alone. However, this combined method has several ways to be improved. When combining the methods, it would be preferred to combine the body's direction and position, gathered from DLC, with the positions of the paws, gathered from MATLAB and from this, compute the relative positions between the body and the paws. This approach was tested, but generated a lower accuracy, due to the relatively low detection rate of the rat's belly, when using DLC.

To increase the detection rate of the belly and also the rear end and head of the rat an alternative would be to add further brightness to the video recording and possibly other image processing parameters. Another alternative would be to optimize parameters when labeling and training the video recording, using DLC. This has not been tested and evaluated due to time limits in combination with the training procedure being time consuming and also due to technical problems along the way. The most effective way to optimize the prerequisites to enhance the detection rate of DLC would probably be to change the setup for the video recording. The optimal lighting to facilitate detection of the rat's body is presumably daylight or similar. A setup under these preferences may therefore result in a higher detection rate, leading to higher accuracy when determining the body's position, which further can lead to higher classification accuracy. However, a setup in daylight will most likely impair the FTIR-enabled detection of the paws. Therefore, the optimal setting may be with red background light, as discussed in section 5.1.6, due to the more distinct reproduction of the rat's body using this setup.

5.4 Deep learning based segmentation, object tracking & classification

The classification method where only DLC was used to classify the paws resulted in a low accuracy compared to the other two classification methods. This is most likely due to DLC not being designed to track objects having a high tendency of not being present in the frames, which is the case concerning the paws. The classification accuracy would most likely be improved with other setups, such as a setup in daylight and no LED light, which would make the paws visible and detectable at all times. However, this would only generate information regarding the classification of the paws and no information regarding if a paw is pressure loaded or not.

5.5 Ethics

The '3R' principle, mentioned in section 2.7 was taken into consideration throughout the project. The experimental animals, i.e. the rats, were replaced by other subjects during the construction of the setup when for instance the FTIR-enabled detection of the camera was carried out. During the data collection, four rats were video recorded since three different constellations were tested (setup including black background recorded with lower resolution as well as higher resolution as well as setup including red background). Also, an additional recording was carried out to enable comparison between any differences between two different individuals. Except to enable such comparison, the choice of using more than one rat was based on the fact that a rat becomes less active after spending some time in the box. However, this choice is also following the aim of refining the experiment since the individual rat spent less time in the box than they would if a smaller number of rats had been used. It should also be taken into account that the rats were not subject to any physical harm.

6

Conclusion

The aim of this thesis was to implement a system allowing analysis of motor behavior, such as gait pattern, of rats in an open field setup by means of video recording and subsequent image analysis. Further, the aim was to evaluate different image analysis methods; blob detection and classification using MATLAB as well as deep learning based segmentation using DeepLabCut.

The open field setup worked and generated data as intended. The rat was able to move unhindered and video recordings of high image quality were generated. However, this setup was found to be inapplicable with the image analysis method using DeepLabCut including deep learning based segmentation, object tracking and classification of the paws. This method requires a different type of setup when the aim is to detect, track and classify the paws.

The image analysis method where deep learning based segmentation, object tracking and classification of the rat's head, belly and rear end was done using DeepLabCut to determine the direction of the rat, was combined with detection and classification of the paws, using MATLAB and blob detection based image analysis was found to be insufficient. However, the deep learning based segmentation, object tracking and classification of the rat's head, belly and rear end generated acceptable results and therefore, this combination of image analysis methods has potential for development. Image analysis based on blob detection, using MATLAB was found to be the method best suited to the setup. This method had an insufficiency in detecting the rat's paw prints, in particular the front paw prints, which complicates analysis regarding detailed pressure load of the paws. However, the method resulted in relatively high accuracy in classifying the paws detected, suggesting the system is being a possible implement to enhance gait pattern and body movement analysis as well as body posture analysis. This thesis is presenting a number of possible parameters, such as stance linearity, footprint clustering, stance time and swing time, suitable for this purpose. Further, suggestions on how to optimize detection of the paw prints and thereby enable detailed pressure load analysis are presented. Depending on the motive for the experiment one want to perform and what characteristics of the rat one want to analyze both deep learning image analysis using DeepLabCut and blob detection based image analysis using MATLAB can be valuable tools. The methods can, with the presented setup, be used alone, as well as combined with each other to get a more complete assessment when evaluating potential treatments methods for Parkinson's disease and chronic pain conditions.

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