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Automated Gait Analysis Through Hues and Areas (AGATHA): a method to characterize the spatiotemporal pattern of rat gait

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Abstract

While rodent gait analysis can quantify the behavioral consequences of disease, significant methodological differences exist between analysis platforms and little validation has been performed to understand or mitigate these sources of variance. By providing the algorithms used to quantify gait, open-source gait analysis software can be validated and used to explore methodological differences. Our group is introducing, for the first time, a fully-automated, open-source method for the characterization of rodent spatiotemporal gait patterns, termed **Automated Gait Analysis Through Hues and Areas (AGATHA)**. This study describes how AGATHA identifies gait events, validates AGATHA relative to manual digitization methods, and utilizes AGATHA to detect gait compensations in orthopaedic and spinal cord injury models. To validate AGATHA against manual digitization, results from videos of rodent gait, recorded at 1000 frames per second (fps), were compared. To assess one common source of variance (the effects of video frame rate), these 1000 fps videos were re-sampled to mimic several lower fps and compared again. While spatial variables were indistinguishable between AGATHA and manual digitization, low video frame rates resulted in temporal errors for both methods. At frame rates over 125 fps, AGATHA achieved a comparable accuracy and precision to manual digitization for all gait variables. Moreover, AGATHA detected unique gait changes in each injury model. These data demonstrate AGATHA is an accurate and precise platform for the analysis of rodent spatiotemporal gait patterns.

Key terms

automated gait analysis; rodent; behavioral analysis; osteoarthritis; spinal cord injury; meniscus; spinal cord contusion; spatiotemporal gait patterns

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Author Contributions

KDA conceptualized the AGATHA algorithms, which were implemented and troubleshot by HEK and TP. HEK and KDA designed and conducted the studies to evaluate AGATHA's accuracy and precision and the study to evaluate AGATHA in an orthopaedic injury model. ST KS, EG-R, and DF designed the spinal cord injury studies, with gait data collected and analyzed by HEK. HEK and KDA conducted the statistical models to evaluate the data sets. Finally, HEK and KDA drafted the manuscript with all authors providing edits and commentary where needed. All authors have read and approved the final version of the manuscript.

Introduction

Locomotion is a natural behavior and contains similar elements across species²¹; thus, gait analysis has been widely used to explore the behavioral consequences of diseases^{8,17,27,30,44}. Moreover, because gait analysis is non-invasive, techniques have been developed for both clinical and preclinical research. When combined with other comprehensive biological assays, gait analysis can describe the behavioral phenotype of a disease model, thereby providing a more thorough understanding of the functional and symptomatic consequences of different disease states^{35,41,42}.

For many researchers who wish to add gait analysis to their behavioral repertoire, a commercial gait system is purchased, such as the CatWalk, Digigait, or Treadscan platforms^{6,7,17}. These systems can be used immediately and have automated software to calculate gait variables^{6,7,14,16,32,36,47}. However, commercial gait analysis systems are relatively expensive and their analysis software is typically proprietary and unalterable. Furthermore, these systems use different detection techniques which may not be suitable for all rodent models^{10,23}. Moreover, many of these gait systems have not been validated to manual calculation of the gait pattern, hence the methodological errors in these techniques are not necessarily well understood. Alternatively, if purchasing a gait system is not possible, building a gait arena from scratch can be cost effective, but analysis is often performed manually. Adaptable, open-source gait analysis software can provide a third option for researchers to build their own arena and tailor an open-source gait analysis specifically to their field and disease model. Moreover, by releasing the digitization code to the user base, methodological errors can be assessed in the recording method, lighting balance, and digitization parameters.

The goal of this study was to develop an adaptable, open-source, gait analysis method that can also be used to study rodent gait patterns. An open-source, automated gait analysis method is presented, for the first time, for the assessment of rodent spatiotemporal gait patterns, called Automated Gait Analysis Through Hues and Areas (AGATHA, available for free download at <https://github.com/OrthoBME/AGATHA>). This manuscript describes the AGATHA algorithm, validates AGATHA's results to manual digitization, and uses AGATHA to explore methodological error associated with video sampling rate and assess gait patterns in different injury models. Overall, AGATHA required less effort and produced nearly identical results to manual digitization at high video frame rates. Furthermore, AGATHA was able to detect and differentiate the gait profile of each injury model. Combined, these findings validate AGATHA relative to manual digitization and demonstrate its ability to detect spatial and temporal gait changes in rodents.

Material and Methods

Automated Gait Analysis Through Hues and Areas (AGATHA)

A custom MATLAB code, named Automated Gait Analysis Through Hues and Areas (AGATHA), was created to identify the time and positional coordinates of foot-strike and toe-off events in videos of walking rodents. This code is available for free download at

<https://github.com/OrthoBME/AGATHA>. With these time and position coordinates identified, AGATHA then calculates several spatiotemporal gait parameters explained in detail below.

AGATHA first isolates the sagittal view of the animal and locates the silhouette of the animal in each frame of the video (Figure 1a). This is achieved by subtracting a background image where the animal is not present in the arena, transforming the frame into a HSV (Hues, Saturation, Value) image, and using the hue value to convert the HSV image into a binomial silhouette (Figure 1b). Next, AGATHA locates the row of pixels representing the interface between the rat and the floor. In the binomial silhouette, the single row of pixels representing the floor interface is composed of background pixels (indicated by a value of 0, black) and rat contact points with the floor (indicated by a value of 1, white)(Figure 1c). Because a walking rat always has at least two limbs in ground contact during locomotion, this interface can be identified in every frame as the lowest row of repeated pixels in the binomial silhouette; however, please note that AGATHA may not accurately locate the rat-floor interface if the animal moves with a gait pattern containing a completely aerial phase (running trot or bounding). Second, AGATHA excludes the majority of nose and tail contacts with the floor from the analysis by comparing the contact point (white pixels) to the animal's center of area in the sagittal view, eliminating contacts that are proportionally too far from the center of area to be considered a paw strike.

By stacking the rat/floor interface across multiple frames, foot contact with the ground can be visualized over time (Figure 1d). AGATHA uses these stacked pixel rows of the rat-floor interface to determine the earliest frame associated with the paw entering the rat-floor interface (foot-strike) and last frame associated with the paw leaving the rat-floor interface pixel row (toe-off). Within the paw contact object seen in Figure 1d, foot-strike is defined as the earliest frame of the paw contact object (blue triangles and circles), while the toe-off is defined as the latest frame of the paw contact object (red triangles and circles). These temporal events can then be used to calculate temporal symmetry of the hind limbs (the percentage of the gait cycle representing when the right foot-strike occurs between two sequential left foot-strikes), duty factor (the percentage of the gait cycle that the limb is in contact with the ground) of the hind limbs, duty factor of the fore limbs, duty factor imbalance of the hind limb (left hind limb duty factor minus right hind limb duty factor), step frequency, and limb phase between the fore and hind limbs (variables explained in detail later in the manuscript). In this manuscript, data analysis is focused solely on the hind limb parameters.

In addition to the frame associated with foot-strike and toe-off, the sagittal image provides an estimation of the spatial foot-strike location along the axis of travel (Figure 2a). Using this approximate location, the precise spatial location of foot-strike can be determined in the ventral view of the animal (Figure 2b). To achieve this for white rodents, each paw print is evaluated on a frame just after foot-strike and on a second frame just prior to toe-off. In these two images, the pixels representing paw contact with the ground remain nearly constant while other pixels change, allowing the paw print to be isolated using iterative color thresholds that examine pixel change between the two video frames (Figure 2c). The centroid of the paw print is then identified and used to represent the paw's spatial location

during stance (Figure 2d). These spatial events are used to calculate stride length, step widths, and step length symmetry. In addition, the centroid of the rat in the ventral view is determined for every frame and used to calculate velocity.

As with any automated digitization package, AGATHA has limitations. To ensure accurate animal visualization at high video collection speeds, the arena should be well lit to provide contrast between the animal and background. Insufficient lighting may result in poor animal silhouette identification and affect accuracy of spatiotemporal gait measures. Furthermore, the camera should be level and at the height of the floor. Incorrect camera position can block the visualization of the animal's feet or pick up reflection artifacts from the floor; this can result in incorrect identification of the rat-floor interface. To a certain degree, AGATHA can compensate for a range of lighting and camera conditions, but the quality of data calculated will ultimately suffer if recording conditions are poor.

In total, AGATHA calculates and exports the following gait variables: trial velocity, temporal symmetry of the hind limbs (when in time a right foot-strike occurs relative to two left foot-strikes), duty factor of the hind limbs, duty factor of the fore limbs, duty factor imbalance of the hind limb, stride length, hind limb step width, step frequency, limb phase between the fore and hind limbs, and number of steps in the video^{20,21}. These parameters are based on the classic Hildebrand gait diagram to describe quadrupedal gait sequences^{19–21}. While AGATHA is able to acquire fore limb data, we have elected to compare AGATHA to manual digitization using only hind limbs; this was selected to reduce the cost and time of manual digitization.

Testing Approval

All methods and testing were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC) and conform to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) recommendations on animal research and National Institutes of Health (NIH) guidelines.

Gait Testing

Rats voluntarily explored an overground arena while videos were collected using a high-speed camera (RedLake, M3), as previously described^{2–4}. Briefly, animals were placed into a clear acrylic arena (6"×12"×72") with a mirror mounted below the floor set at a 45° angle, such that the sagittal and ventral planes of the animal could be viewed simultaneously with a single camera placed approximately 2 meters from the arena. Though not required for AGATHA to function, the back wall and ceiling in the central 48" of the arena were covered with green vinyl to provide a contrasting background for the camera views of the animal. Only white rats have been used in this study, though AGATHA routines are available to describe gait parameters of white, black, and nude rodents (both mice and rats) against a range of background colors. However, these routine have not been validated against manual digitization. The remaining 12" at both ends of the arena were covered with black vinyl to provide a "safe zone" for the animal. Halogen lights were positioned to provide indirect lighting from below and from the side of the animal. Prior to each recording session, the arena was leveled and images of a calibration grid were acquired. During recording, animals

were allowed to voluntarily explore the arena without experimenter interaction or prompting of locomotion until 5 gait trials were obtained. While 5 trials could generally be collected in 5–8 minutes, animals occasionally stayed in the arena for up to 20 minutes due to acclimation to the environment. Walking trials with overall constant velocity (linear fit of rodent centroid position relative to time with $R^2 > 0.95$ and a coefficient of variation on the slope term $< 5\%$) and a minimum of three full gait cycles (minimum of 4 foot-strike events per foot) were recorded at 1000 fps when the animal passed through the center of the arena, with a minimum of five trials per animal collected. The calculation of each gait variable conforms with the standard definitions of the Hildebrand gait diagram^{20,21}; these equations can be found in prior work or in our methodological review²³.

Validating AGATHA to Manual Digitization and Exploring the Effect of Video Frame Rate

To validate the accuracy and precision of AGATHA and to explore the effects of video frame rate, 25 videos of four naïve Lewis rats were collected at 1000 fps as described above. These 1000 fps videos were then re-sampled every 33 frames (30 fps), 17 frames (60 fps), 8 frames (125 fps), 4 frames (250 fps), and 2 frames (500 fps) to create lower frame rate doppelganger videos. Then, the 25 root videos and 150 doppelganger videos were digitized with AGATHA or via manual digitization using the DLTdataviewer subroutine for MATLAB¹⁸. Briefly, for manual digitization, each video was loaded into the DLTdataviewer subroutine and displayed frame by frame, with a person marking the hind limb foot-strike or toe-off event on the ventral view of the rat in the frame where the event first occurs. DLTdataviewer then logs the pixel coordinate and frame number of the event. Because animal centroid cannot be accurately located manually, velocity was calculated by tracking the nose position every 0.2 seconds, equating to every 100th frame for 500 fps, every 50th frame for 250 fps, every 25th frame for 125 fps, every 10th frame for 60 fps, and every 5th frame for 30 fps. Using these data, trial velocity, temporal symmetry, duty factor, duty factor imbalance (left duty factor-right duty factor), stride length, and step width are calculated for AGATHA and manual digitization.

To compare AGATHA to manual digitization, the mean of each gait parameter was compared using a paired t-test at each frame rate. Similarly, to compare the precision of the digitization methods, the standard deviation of each gait measure was compared using an F-test at each frame rate. For the paired t-tests and F-tests, corrections for compounding type 1 error were not applied because the null hypothesis aligns with the desired outcome (AGATHA is equivalent to manual digitization). To evaluate the effect of frame rate on the digitization results, nested analysis of variance (ANOVA) were conducted with post-hoc Tukey's HSD tests, when indicated. Finally, based upon the variability observed within our naïve data set and effect sizes reflecting the sensitivity required to discern subtle differences between experimental groups in previous publications^{3,4,23}, a power analysis was conducted to identify the number of animals needed for each frame rate (beta=0.80, alpha=0.05, assuming normalization to a weight and velocity matched standard and a minimum of 5 trials per animal).

Validating AGATHA Using an Orthopaedic Injury Model

To evaluate AGATHA's ability to detect gait compensations in an orthopaedic injury model, 18 Lewis rats (3 months, 200–250 grams, male) were obtained from Charles Rivers Laboratories and allowed to acclimate to the University of Florida housing facilities for two weeks prior to the investigation. After acclimation, 8 rats received a surgical medial meniscus transection injury (MMT) and 5 rats received a medial collateral ligament transection injury (MCLT sham), as previously described^{3,24}. The 5 remaining rats were left as naïve controls.

Briefly, animals were anesthetized in a 4% isoflurane sleep box, prepped for aseptic surgery, and transferred to a sterile field with anesthesia maintained by mask inhalation of 2% isoflurane. A medial midline skin incision was made on the right hind limb, and the medial collateral ligament was exposed via blunt dissection and transected. At this point, the wounds of animals receiving MCLT alone were closed. For animals receiving MMT, the joint was then placed in a valgus orientation to expose the central portion of the medial meniscus; then, a complete radial transection was performed in the central portion of the medial meniscus using an 11 blade scalpel. Surgical sites were closed with 9 mm wound clips, which were removed 10–14 days after surgery. Upon surgical recovery, rats received 48 hours of buprenorphine (0.03 mg/kg) to reduce post-surgical discomfort.

Animals were gait tested at four weeks after surgery, as described above. Because duty factor, stride length, and step width are dependent on the animal's walking velocity, data from the naïve animals were used to calculate the residual distance for each trial (measured gait parameter – predicted value based on naïve cohort), as previously described²⁵. Data over multiple trials for a given animal were averaged, with the animal averages used in a 1-way ANOVA and a post-hoc Tukey's HSD test, when indicated.

At the conclusion of the experiment, animals were transferred to an unrelated IACUC protocol.

Validating AGATHA Using a Cervical Spinal Cord Hemisection Injury Model

The ability of the AGATHA method to detect changes in gait following a unilateral high cervical SCI was examined using a spinal cord hemisection model^{15,38}. Adult male Sprague-Dawley rats (N=9) were obtained from Harlan Scientific and housed at the McKnight Brain Institute Animal Care Facility at the University of Florida. Anesthesia and injury methods have been previously described^{7,9,11,13}. Briefly, five rats were anesthetized by isoflurane, induced in a closed chamber at 3–4% in O₂ and maintained in a surgical plane at 1–2% in O₂ via nose cone for the duration of the surgery. A 1-inch dorsal midline incision was made from the base of the skull extending caudally to approximately the fourth cervical segment (C4). A laminectomy was performed at the second cervical segment (C2) to expose the cervical spinal cord. A small incision was made in the dura and a lateral hemisection performed on the left side of the spinal cord using a microscalpel, followed by gentle aspiration. Using this approach, the completeness of the lesion was readily visible and the extent of the lesion was reproducible. After lesion extent was visually confirmed, the dura was closed with interrupted 9-0 sutures, the overlying muscle sutured (with 4-0 sutures), and

the skin closed with stainless steel surgical wound clips. Following completion of the surgical procedure, rats were placed on supplemental O₂ via nose cone until they recovered from anesthesia and were awake and moving. Post-surgical care included administration of buprenorphine at 12 hr intervals (0.03 mg/kg, s.q.) for the initial 48 hours post-injury and delivery of lactated Ringers solution (5ml/12 hr, s.q.) and oral Nutri-cal supplements (1–3 ml, Webster Veterinary, MA, USA) until adequate volitional drinking and eating resumed. Four naïve (spinal intact), gender matched rats were used as controls.

Injured animals were gait tested nine weeks after surgery, as described above. Again, because duty factor, stride length, and step width are dependent on the animal's walking velocity, the gender-matched naïve animals were used to form a control line; then, the residual distance from the control line was calculated for each trial (measured gait parameter – predicted value based on naïve cohort), as previously described²⁵. Data over multiple trials for a given animal were averaged, with the animal averages used in a 1-way ANOVA and a post-hoc Tukey's HSD test, when indicated.

Validating AGATHA Using a Cervical Spinal Cord Contusion Injury Model

Further validation of the AGATHA method was accomplished using a spinal cord contusion model that more closely mimics the most common SCIs occurring in humans²⁸. For these studies, 12 adult female Sprague–Dawley rats were obtained from Harlan Scientific and housed at the McKnight Brain Institute Animal Care Facility at the University of Florida. Three females received no surgical treatments, and were studied as a control group for comparison with the contused animals.

Six animals served as a sham surgery control group and received cervical laminectomy but not contusion injury. Rats were anesthetized by isoflurane, induced in a closed chamber at 3–4% in O₂ and maintained in a surgical plane at 1–2% in O₂ via nose cone for the duration of the surgery. A 1-inch dorsal midline incision was made from approximately the second to fifth cervical segment (C2–5). A laminectomy was performed at the third and fourth cervical segments (C3–4) and the overlying muscles were closed with sterile 4-0 Vicryl suture. Skin incision was closed using sterile wound clips. Following completion of the surgical procedure, rats were placed on supplemental O₂ via nose cone until they had recovered from anesthesia and were awake and moving. The same post-surgical care was administered as described above.

Three animals received a lateralized C3/C4 contusion. Rats were anesthetized by isoflurane, induced in a closed chamber at 3–4% in O₂ and maintained in a surgical plane at 1–2% in O₂ via nose cone for the duration of the surgery. A dorsal midline incision was made from approximately the second to fifth cervical segment (C2–5). Following laminectomy at the C3/4 level, a left lateralized spinal contusion was made using the Infinite Horizon pneumatic impactor (Precision Systems & Instrumentation, Lexington, KY)³⁹. Once the spinal cord was exposed, the impactor probe (2.5 mm diameter tip) was positioned between midline and the left lateral edge of the spinal cord at C3/C4, and was raised approximately 5 mm above the intact dura. The cord was contused at a pre-set nominal force of 200 kilodynes (dwell time = 0). Upon completion of the injury procedure, overlying muscles were closed in layers with sterile 4–0 Vicryl suture and the skin incision was closed using sterile wound clips.

Following completion of the surgical procedure, rats were placed on supplemental O₂ via nose cone until they had recovered from anesthesia and were awake and moving. Post-surgical care included administration of buprenorphine at 12 hr intervals (0.03 mg/kg, s.q.) for the initial 48 hours post-injury and delivery of lactated Ringers solution (5ml/12 hr, s.q.) and oral Nutri-cal supplements (1–3 ml, Webster Veterinary, MA, USA) until adequate volitional drinking and eating resumed.

Laminectomy animals were gait tested 10 days after surgery and the contused animals were gait tested 4 weeks after surgery, as described above. Again, because duty factor, stride length, and step width are dependent on the animal's walking velocity, the gender-matched naïve animals were used to form a control line; then, the residual distance from the control line calculated for each trial (measured gait parameter – predicted value based on naïve cohort), as previously described²⁵. Data over multiple trials for a given animal were averaged, with the animal averages used in a 1-way ANOVA and a post-hoc Tukey's HSD test, when indicated.

Results

AGATHA Comparison to Manual Digitization

In the 1000 fps root videos, AGATHA reproduced manual digitization results for temporal symmetry, duty factor imbalance, and stride length; however, AGATHA showed differences relative to manual digitization for hind limb duty factor, velocity, and step width (Figure 3). On average, velocities were 8.5 cm/sec faster, hind limb duty factors were 1.5% lower, and step widths were 0.2 cm wider with AGATHA relative to manual digitization of the same trial. However, as can be observed in Figure 3, the inter-trial variability was much greater than the variability introduced by these methodological variables, and neither method had an advantage in the precision of the gait measurements at 1000 fps.

Video frame affected the measurement of hind limb duty factors (Figure 4 a–b), but did not affect the average measurement of any other gait parameters (Figure 4 c–f). For hind limb duty factors, frame rates below 125 fps were significantly different from frame rates above 125 fps for both digitization methods ($p < 0.03$). To further investigate this effect, the time associated with foot-strike and toe-off events was examined relative to the 1000 fps video (Figure 5). Here, the video frame rate affected the accuracy associated with the identification of both the foot-strike and toe-off events relative to the 1000 fps video for both digitization methods ($p < 0.001$). This finding is shown in Figure 5 using foot-strike and toe-off time residuals (time of a gait event in the doppelganger minus the time of the same gait event in the 1000 fps root video). At 30 fps, both foot-strike and toe-off time residuals were lower with AGATHA than for manual digitization ($p < 0.001$). At 60 fps, only the foot-strike time residual was significantly different between AGATHA and manual digitization ($p < 0.001$). However, these errors converged to near-zero values at recording rates over 125 fps.

Increasing the frame rate also reduced the variability of imbalance and symmetry ($p < 0.03$, Figure 4). In addition, for both AGATHA and manual digitization methods, the identification of foot-strike and toe-off events became more precise with increasing frame rate ($p < 0.002$,

Figure 5). In general, the precision of foot-strike and toe-off events in AGATHA was similar to manual digitization at half the video frame rate.

Finally, a power analysis was conducted for a non-repeated measures 1-way ANOVA to assess the number of animals per group (n) required for the detection of a given effect for each digitization method at each video frame rate (Table 1). At low video frame rates, a larger number of animals are required to achieve similar detection sensitivities to higher frame rates, regardless of the digitization method. Since the variability of imbalance and symmetry reduces with frame rate, this result was expected. However, the n required for other gait parameters also decreased with sampling rate, indicating that, while the increased precision with frame rate may not be statistically significant, the increased frame rates may still markedly affect the measurable effect size and animal numbers in an animal experiment.

Orthopaedic Injury Model

AGATHA detected gait abnormalities in MMT animals, including increased left and right hind limb duty factors and decreased stride lengths (Figure 6, bottom). Fore limb differences were not examined in this hindlimb injury model. The shifts are represented in the top of Figure 6 via a velocity-corrected image of the footprint pattern and a velocity-corrected Hildebrand plot of the foot-strike and toe-off events. Please note that the sizes of the ellipses representing the footprint are representative of 1 standard deviation of the stride length residual (width) and 1 standard deviation of the step width residual (height). This “shuffle step” gait pattern (higher stance times with shorter stride lengths) is representative of a parallel gait compensation, where the periods of the gait cycle when a hind limb must support weight without contralateral support are reduced by increasing the relative stance time on both limbs (white bars lower in the Hildebrand plot are smaller in MMT animals relative to other groups).

Spinal Cord Injury Model

AGATHA also detected gait abnormalities in models of cervical spinal cord injury models (Figure 7). For animals with cervical contusion, significant gait compensations were limited to the reduced stride lengths; however, the gait sequences also tended to be asymmetric (symmetry < 0.5, $p=0.093$). For limb data could not be analyzed in this animal due to the frequent lack of a left forelimb foot-strike (injured limb). For animals with a C2 hemisection, significant imbalances were observed in the hind limb and fore limb duty factors, with less relative time spent on the right hind limb and more relative time on the right forelimb (imbalance > 0, right duty factor residual = 0). Moreover, stride lengths were markedly shortened by approximately 4 cm, hind limb step widths were narrowed by approximately 1 cm, and forelimb step widths increased by over 1.5 cm. This “limp-like” gait pattern was present in both spinal cord injury models, and is likely representative of an antalgic gait compensation in which one limb of a limb pair tends to preferentially compensate for an injured limb.

Discussion

This study introduces, for the first time, a new digitization method for the characterization of rodent spatiotemporal gait patterns, AGATHA. Using AGATHA, detectable rodent gait compensations were quantified in an orthopaedic injury model and in two spinal cord injury models. Moreover, AGATHA's accuracy and precision were validated relative to manual digitization of the same videos, with AGATHA yielding a similar calculation to manual digitization for videos recorded above 125 fps. Using AGATHA, spatiotemporal gait profiles can be acquired in lieu of the laborious effort of manual digitization in significantly less time. As an open-source gait analysis platform, AGATHA can assess rodent gait using a simple arena that is adaptable to different video recording speeds, multiple colors of animals, and has been optimized for both mice and rats.

Prior work has shown that significant methodological and material differences in gait analysis systems can yield inconsistent results when applied to identical rodent videos¹⁰. To be clear, methodological advantages in our approach were not examined relative to commercial systems, nor do we claim any advantage over these packages. AGATHA, however, does provide an open-source platform which has been validated to manual digitization, and through which these methodological sources of variability can be examined. Unfortunately, little literature is devoted to assessing these methodological sources of error in rodent gait analysis platforms^{10,23,32,46}. The limited number of system comparison studies is partly due to lack of gait system access, proprietary software protection, and testing bias. Because commercial gait platforms are relatively expensive, it is unlikely for multiple platforms to exist within a single laboratory or university. Even if access were not a problem, it is difficult to directly compare gait results because gait platforms contain different hardware (such as different video frame capture rates) and commercial software packages tend to protect the algorithms for calculating gait variables as proprietary.

We have designed AGATHA to calculate the fundamental gait descriptors based on Hildebrand's descriptors of the quadrupedal spatiotemporal gait sequence²¹. While these are also the most common parameters reported by commercial gait analysis systems, many variables reported in other gait systems could be calculated. Since AGATHA is free and open source, users are allowed to calculate variables relevant to their animal model or other gait analysis packages.

Currently, few free gait analysis software are available. MouseWalker is freeware that can analyze gait collected on a light-refracting, overground arena, similar to the set-up of the commercial Catwalk system^{14,33}. MouseWalker is fully automated and can process both color and grayscale videos, though the equations used to calculate gait variables have been released as a MATLAB executable; as such, these equations are not easily manipulated to test methodological changes in this script. Nonetheless, the MouseWalker method may be scaled to larger animals and different fur colors, though it has currently only been used to describe gait for mice.

Another open-source analysis package is Locomouse²⁹. Using simultaneous sagittal and ventral views, Locomouse is a MATLAB program that automatically tracks body and limb movement of black mice from grayscale videos. While Locomouse is automated and supports full access to the MATLAB code, it currently calculates only few gait variables used by other systems. However, Locomouse uses a manually-defined reference library to choose how to track paws. While this is not a formal validation to manual digitization, the software attempts to match human results directly rather than relying upon a completely independent, un-validated algorithm. With more open-source options available, researchers may combine the strengths of each method specifically to their needs. Moreover, because different digitization methods use different markers to calculate gait variables, gait variables can have slightly different values when calculated by different methods. Freely providing the code used to generate the gait data will help disambiguate gait variables within the field and improve comparison across studies.

AGATHA did tend to calculate a lower trial velocity than manual digitization. This finding is most likely driven by differences in the measurement from each digitization method. Recall, AGATHA measures velocity by estimating the animal's centroid in every frame within a video, while historically, our lab has estimated velocity by noting the nose location in frames separated by 0.2 secs intervals^{3,4,25}. According to the central limit theorem, AGATHA's approach should be more accurate due to a higher sampling rate. Moreover, AGATHA is likely more consistent due to the variability of the animal's head position during walking, and this increase in head sway may cause an over estimate of velocity during manual digitization.

For temporal measures, AGATHA tended to under estimate hind limb duty factor relative to manual digitization. This under estimation is most likely driven by the identification of toe-off events. AGATHA identifies toe-off as the frame where the paw is no longer in contact with the rat-floor interface. However, at toe-off, the rodent's toe and foot rotate slightly, such that the toe can clear the floor. Manual digitizers tend to click a frame that is slightly after this toe rotation. As such, AGATHA may provide a less biased estimation of toe-off, though this effect would need to be verified in a model where altered toe-clearance motions could be expected.

While manual digitization was generally more precise, AGATHA was able to achieve comparable precision at frame rates over 125 fps. As expected, human digitizers are generally more precise than digitization algorithms^{22,37}, especially for experimental designs where fatigue of the digitizer does not play a significant role. This is well known for radiological scans and segmentation algorithms³⁴. Thus, it is critical to validate any digitization algorithm to manual methods, a validation criterion that has not necessarily been presented for some commercially available systems. Validating AGATHA against manual digitization, the estimated sample size at 1000 fps differed by generally 1 or 2 animals; at 125 fps, the estimated sample size differed by 2 to 3 animals.

Please note, the power analysis reported in this paper was performed using interanimal variance estimates in naïve animals, assuming variance in gait parameters is relatively constant across walking speed. Variance of several gait variables can be affected by velocity.

Moreover, interanimal variance is likely to increase in an experimental group relative to naïve controls. As such, the sample size estimate in Table 1 is likely lower than the sample size needed to detect a given effect size for an experiment. Of course, actual sample size for a given experiment will depend on the experimental design, variability of the experimental and control groups, and the desired detectable effect size. Nonetheless, the power analysis is intended to demonstrate the beneficial effects of controlling potential sources of variance (video sampling rate), even when those sources are lower than the interanimal variability.

The apparent transition in accuracy and precision around 125 fps may be explained by sampling frequency theory. To optimize sensitivity and minimize error, the Nyquist-Shannon rule states an effective sampling frequency should be greater than twice the duration of the fastest factor being measured⁴⁵. When applied to gait analysis systems, the fastest factor to be measured is the anticipated gait abnormality, which has been shown to range between 0.001 and 0.025 seconds in rats⁴. Sampling theory posits the smallest, accurately measurable gait change in a video taken at 125 fps is 62.5 Hz (0.016 seconds), which is near the mid-point of reported gait abnormalities. As such, any changes shorter than 0.016 seconds cannot be confidently detected at 125 fps, and this detection limit will only increase at lower video frame rates (e.g. at 30 fps, detectable changes must be larger than 0.067 seconds). Again, since AGATHA can be used across multiple frame rates, this flexibility allows the user to adjust the sampling rate to the preclinical model, while potentially achieving a sensitivity that is greater than most commercial systems that record at 150 fps or less.

It should also be noted that, while this study found manual digitization was more precise than AGATHA, manual digitization is also a tedious, labor-intensive process. It is not uncommon for weeks or months of post-processing to occur for studies employing manual digitization^{1,3,25}. As such, gait event criteria during manual digitization may shift due to fatigue or altered selection criteria across digitization days. One advantage of automated gait analysis is the consistency of the measure across trials. While these fatigue effects were not studied in this work, automated systems have been found to produce more repeatable results than manual digitization methods in some other methodologies⁴⁶. Moreover, when a single video is digitized multiple times, AGATHA will choose the same frames each time unless the video is altered, while manual digitization has the potential to choose a different frame for that same gait event when asked to digitize the same video more than once^{5,40,43,46}. Thus, for larger studies, automated digitization may prove more repeatable relative to manual methods, though these sources of methodological error have not yet been examined for rodent gait analysis.

Importantly, AGATHA was able to detect both antalgic and shuffling gait abnormalities in rodents. In our prior work, shuffling changes were observed in both the MMT and MCLT sham at 4 weeks²⁵; here, only the MMT animals exhibited the shuffling compensation. However, it should be noted that, in our past study, the four week time point was the first time point to exhibit the shuffling compensation. Thus, it is possible this particular cohort of MCLT animals had not yet transitioned to a shuffling compensation. Nonetheless, AGATHA was able to detect the expected shuffling compensation in the MMT cohort, confirming AGATHA's utility when applied to this model^{3,25}. AGATHA also detected gait changes in the spinal cord injury models. Interestingly, though the injuries were restricted to the

cervical portion of the spine, hind limb gait changes were measurable and significant. Moreover, unlike the orthopaedic injury model, the C2 hemisection model exhibited graded, antalgic gait compensations in which the left side was favored. Similar spatial gait changes have been observed in comparable hemisection and contusion injury models^{12,17,26,31}, though only one publication was found that accounted for velocity covariates⁴. Nonetheless, these data indicate high-speed gait analysis via AGATHA was able to provide a detailed gait pattern analysis indicative of spinal cord injury.

In conclusion, AGATHA is a robust method for automatically analyzing and detecting small changes in rodent gait, which has been validated to manual digitization. Moreover, frame rate is an important factor affecting the accuracy of gait analysis, whether employing AGATHA or a manual digitization method. While differences were found between AGATHA and manual digitization for some spatiotemporal variables, these errors were far smaller than the inter-trial variability. More importantly, these errors did not prevent the identification of shuffling gait compensations in an orthopaedic injury model or antalgic compensations in a spinal cord injury model. Combined, these data indicate AGATHA can be used to explore some methodological differences and characterize the spatiotemporal gait patterns in rodent injury models.

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Figure 1. Deriving Temporal Variables using Automated Gait Analysis Through Hues and Areas (AGATHA)

a) Original video frame. b) The transformed image frame in which the animal is isolated and shows as white, while the background is negated and shows as black. c) Graphical representation of the row of pixels identified as the floor in the transformed image. Within this row of pixels, portions of the animal in contact with the floor show up as white. d) Consecutive pixel rows representing the floor are stacked and the paw print objects can be represented 2-dimensionally for calculation of foot-strike and toe-off. Red shapes identify

foot-strike and blue shapes represent toe-off. Triangles are used for fore-limbs and circles are used for hind-limbs.

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Figure 2. Deriving Spatial Variables using AGATHA

a) Original video frame. b) AGATHA's isolated paw image from the ventral view. c) AGATHA's filtered representation of the paw print. d) A sequence of hind paw prints from a full trial.

Figure 3. Validating AGATHA Against Manual Digitization

Paired scatterplots of raw data from the 1000 fps root videos of the naïve Lewis rat cohort. AGATHA reproduces data digitized manually for symmetry, duty factor imbalance, and stride length, but was slightly different for velocity, hind limb duty factor, and step width. Still, differences between manual digitization and AGATHA were much smaller than inter-trial variability.

Figure 4. Effects of Video Frame Rate on Gait Variable Calculation for AGATHA and Manual Digitization

Raw data scatter plots of each gait variable for each digitization method at all video frame rates. Low video frame rate appears to affect AGATHA's accuracy more than manual digitization, especially for temporal variables. Spatial variables seem largely unaffected by video frame rate.

Figure 5. Effects of Video Frame Rate on Estimation of Foot-Strike and Toe-off

The temporal residual of foot-strike and toe-off are summarized in panel a) and b), respectively. The foot-strike and toe-off residuals are in reference to the matching gait event from each video's respective 1000 fps root video. To directly compare each video frame rate, the residuals are also represented in raw time (seconds) instead of frames.

Figure 6. AGATHA's Results for the Orthopaedic Model

a) Overall, MCLT sham animals walked faster than naive animals. b) A graphical representation of spatial variable residuals by surgical group. Normalized to the left hind foot placement, each ellipse center is located at its group's average stride length and step width. The major and minor axes of each ellipse correspond with one standard deviation of the respective residual (in this case, horizontal axes represent stride length residual deviations while vertical axes represent step width residual deviations). c) A Hildebrand graph summarizing the residualized temporal differences between groups during a step cycle. The left side of each colored box indicates foot-strike, the horizontal length of each colored box indicates duration of stance, and the right side of each colored box indicates toe-off. The white space in between represents the duration and temporal location during the step cycle of the swing phase for that limb. d)–i) Animal average scatter plots of gait results. *: indicates $p < 0.05$.

Figure 7. AGATHA's Results for the Spinal Cord Injury Models

a) Overall, there were no velocity differences between laminectomy animals, cervical contusion animals, and their respective naive group, but the C2-hemisection animals walked significantly slower than their respective naive group. b) A graphical representation of spatial variable residuals by surgical group for each spinal cord injury model. Normalized to the left hind foot placement, each ellipse center is located at its group's average stride length and step width. The major and minor axes of each ellipse correspond with one standard deviation of the respective residual (in this case, horizontal axes represent stride length

residual deviations while vertical axes represent step width residual deviations). For clarity, fore paw data are not presented in the C2 hemisection model. For paw data could not be obtained for cervical contusion due to the lack of foot-strike in the injured paw of this model. c) Hildebrand graphs summarizing the residualized temporal differences between spinal cord injury groups during a step cycle. The left side of each colored box indicates foot-strike, the horizontal length of each colored box indicates duration of stance, and the right side of each colored box indicates toe-off. The white space in between represents the duration and temporal location during the step cycle of the swing phase for that limb. d)–i) Animal average scatter plots of gait results. *: indicates $p < 0.05$.

Table 1
Detection Sensitivity of AGATHA and Manual Digitization Methods

The data represented in this table derives from the 1000 fps root videos of the Lewis naïve rat cohort. The detection sensitivity was set at a value comparable to the smallest detectable difference in means between two groups reported in the literature^{3,25}. This detection sensitivity was used to calculate the required animals per group (n) to achieve this sensitivity at a statistical power of 0.8 and a significant level of 0.05. The red and green colors in the boxes are a qualitative scale highlighting larger n (red) and lower n (green).

Data Range	Detection Sensitivity	Digitization Method	Required n per group:									
			30 fps	60 fps	125 fps	250 fps	500 fps	1000 fps	1000 fps	1000 fps	1000 fps	1000 fps
Velocity (cm/sec)	11.3 – 62.4	AGATHA	9	7	7	7	7	7	6			
		Manual	5	5	5	5	5	5	5	5	5	5
Temporal Symmetry (%)	44.4 – 56.7	AGATHA	25	10	8	8	6	4				
		Manual	7	4	3	3	3	3	3	3	3	3
Left Limb Duty Factor Residual (%)	-16.9 – 4.9	AGATHA	8	7	6	6	5	5	5	5	5	5
		Manual	8	6	5	4	4	4	4	4	4	4
Right Limb Duty Factor Residual (%)	-18.6 – 10.8	AGATHA	12	12	9	8	8	8	8	8	8	8
		Manual	10	6	6	7	6	6	6	6	6	6
Duty Factor Imbalance (%)	-10.3 – 14.6	AGATHA	38	13	10	6	4	4	4	4	4	4
		Manual	43	11	9	8	8	8	8	8	8	8
Stride Length Residual (cm)	-1.7 – 3.4	AGATHA	17	17	20	6	6	6	5	5	5	5
		Manual	22	17	16	13	13	13	9	9	9	9
Step Width Residual (cm)	-0.57 – 0.72	AGATHA	10	7	7	5	5	5	4	4	4	4
		Manual	10	6	7	7	6	6	4	4	4	4