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Evaluating Gait and Locomotion in Rodents with the CatWalk

Jacqueline M. Garrick,[1,4] Lucio G. Costa,[1,2] Toby B. Cole,[1,3] and Judit Marsillach[1]

[1]Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, Washington
[2]Deptartment of Medicine and Surgery, University of Parma, Parma, Italy
[3]Center on Human Development and Disabilities, University of Washington, Seattle, Washington
[4]Corresponding author: *jacqueg@uw.edu*

Motor deficits can significantly affect the completion of daily life activities and have a negative impact on quality of life. Consequently, motor function is an important behavioral endpoint to measure for in vivo pathophysiologic studies in a variety of research areas, such as toxicant exposure, drug development, disease characterization, and transgenic phenotyping. Evaluation of motor function is also critical to the interpretation of cognitive behavioral assays, as many rely on intact motor abilities to derive meaningful data. As such, gait analysis is an important component of behavioral research and can be achieved by manual or video-assisted methods. Manual gait analysis methods, however, are prone to observer bias and are unable to capture many critical parameters. In contrast, automated video-assisted gait analysis can quickly and reliably assess gait and locomotor abnormalities that were previously difficult to collect manually. Here, we describe the evaluation of gait and locomotion in rodents using the automated Noldus CatWalk XT system. We include a step-by-step guide for running an experiment using the CatWalk XT system and discuss theory and considerations when evaluating rodent gait. The protocol and discussion provided here act as a supplemental resource to the manual for this commercially available system and can assist CatWalk users in their experimental design and implementation. © 2021 Wiley Periodicals LLC.

Keywords: CatWalk • gait • locomotion • motor deficits • phenotyping • rodents

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BASIC PROTOCOL

The act of walking is a complex behavior, requiring the coordination of limb movement, balance, postural control, proper speed adjustment, and cadence, among other factors. Motor deficits can arise from dysfunction in a variety of systems, such as the cardiovascular (Clark et al., 2019, Hattori, Kitamura, Tsuji, Nagatsuka, & Ihara, 2014), musculoskeletal (Sayed-Zahid et al., 2019), and peripheral (PNS) and central nervous systems (CNS; Carter et al., 1999, Neumann et al., 2009, Pan et al., 2005, Yin et al., 2017). Arthritis and physical injury can cause limping and protective posturing of the limb (Lakes & Allen, 2016), while gait disturbances due to specific CNS injury or affected circuitry can be recognized by their characteristic deficit, such as ataxia (in cerebellar disorders; Cendelin, 2014) and shuffling gait (in Parkinson disease; Broom et al., 2017). For toxicologic studies, evaluating motor function impacts is critical when investigating drug toxicity and

potential side effects, as drug-induced movement disorders significantly affect the quality of life of patients and can reduce drug adherence while increasing the risk for adverse outcomes by inhibiting daily life activities (Demler, 2014).

Rodents are a common model used for behavioral studies, but they present unique challenges when evaluating gait. As prey animals, it has been proposed that they are evolutionarily conditioned to hide disability and pain (Mogil, 2015) and can compensate for injury with their quadrupedal stature in ways bipedal humans cannot. For example, quadrupeds can shift their weight to their forelimbs in the case of a hindlimb injury, where bipeds are unable to do so (Saunders et al., 2017). As such, some motor deficits may not be as easily detected in rodents as they are in humans. To assess these subtle deficits, the ability to sensitively collect gait parameters in an environment suited to the animal is crucial. A variety of gait evaluation methods for rodents have been developed over the years, many relying on manual scoring by eye, making them unable to capture subtle effects or measure multiple parameters at once owing to the combinatory limitations of the human eye and the speed of rodent locomotion.

High-speed video gait analysis solves many of the problems of manual scoring by eye and allows researchers to collect a variety of gait parameters with high sensitivity, providing quantitative measurement for spatiotemporal and kinetic parameters that were previously difficult to collect. Furthermore, these parameters can be measured in a passive environment such that the animal is free to walk within the analysis arena with minimal handling or intervention of the researcher, limiting the stress of the animal during study. Both open-source systems (Jacobs et al., 2018; Mendes et al., 2015; Zhang, Li, & Han, 2020) and commercial products, such as the CatWalk XT system (Noldus Information Technology) and MotoRater (TSE Systems), are available for camera-assisted gait tracking with immobile walkways. Active environment analysis with the use of a treadmill can also be employed using both open-source (Williams et al., 2020) or commercial products (such as the DigiGait [Mouse Specifics, Inc.] and TreadScan [CleverSys, Inc.]).

In this article we describe details of the CatWalk XT system for rodents, with many of the core concepts discussed being applicable to other open-source and commercial systems. The CatWalk uses a glass walkway placed above a high-resolution camera, where light is internally reflected within the walkway and only released when the animal's paw contacts the glass, illuminating the paw upon contact. As the rodent travels across the platform, the camera records a video, and the CatWalk XT software measures the light intensity of the illuminated paw, providing a host of paw statistics and gait metrics for researchers to analyze. A schematic of the system is shown in Figure 1. Unlike treadmill systems, where researchers set the speed of the walkway belt and force the animal to meet a specified speed, the non-moving walkway of the CatWalk is better suited to analyze natural gait and walk speed. This system can capture numerous independent variables for gait analysis—such as walk and swing speed, cadence, and stride, among others—and a variety of paw metrics. These parameters can be used to identify a motor phenotype in transgenic animals or evaluate motor toxicity of chemicals and drugs.

This article provides a step-by-step guide for setting up and conducting an optimal experiment using the CatWalk XT system. The protocol is designed and intended to be used as a supplemental resource to the CatWalk XT system manual, providing users with tips throughout the setup process and a discussion of common practices when conducting CatWalk experiments.

The CatWalk XT system has been widely used to evaluate rodent gait and locomotion. Here, users will follow the steps provided to set up an experiment within the software and record test animals as they traverse the walkway. Each traverse of the walkway is termed
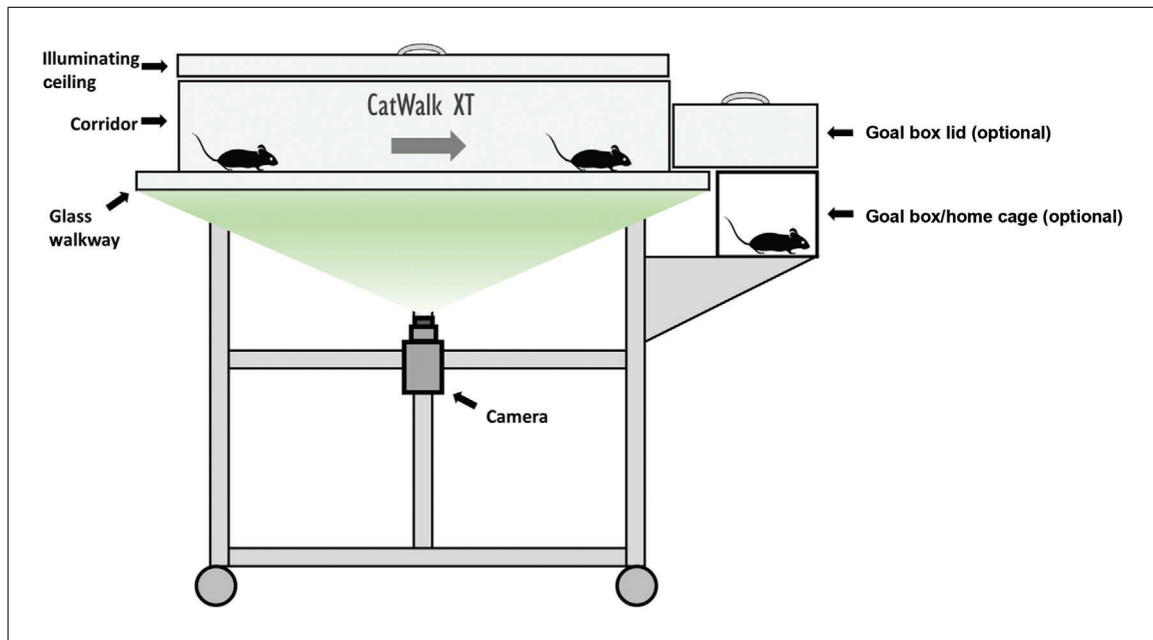
Figure 1 CatWalk XT system. Illustration of the CatWalk XT system. Rodents are placed on one end of the apparatus and walk across a walkway while a camera, from below, records paws to calculate various gait parameters.

a "run," while all runs for a given animal are termed a "trial." Once all test animals have been measured, gait parameters are exported as an Excel file for further analysis. As the protocol expands on points made in the manual, it serves to assist first time and relatively new users in understanding how the CatWalk works and important considerations of specific settings.

Here, we describe implementation of the system using CatWalk XT version 9.1. While users may have access to a different version, which may exhibit some minor software differences, the theory of gait analysis and other basic concerns discussed in this protocol are applicable to all versions.

STRATEGIC PLANNING

Determining variables of interest

Upward of 100 variables are produced by the CatWalk XT software and are available for researchers to evaluate. Not all variables are independent, and researchers run the risk of type 1 error by analyzing every variable provided by the software without forethought as to what variables are of interest for a given experiment. Care should be taken to determine a priori what parameters are important for the experiment at hand and how they contribute to the description of a motor phenotype in the context of other collected data, such as any mechanistic studies or additional behavioral endpoints. For example, when studying traumatic brain injury (TBI), the location of the induced TBI may be factored in when considering what parameters to evaluate.

Acclimating animals to the walkway and testing room

Acclimating the animals to the walkway for 1 to 2 min per day for 1 week prior to testing until they are consistently walking across the test area will provide a more even baseline between the experimental groups. Variation can also be minimized by running the test at the same time of day for each experimental group and acclimating the mice to the testing room 30 min prior to testing. Further discussion on minimizing variability can be found in Critical Parameters.

Room settings

Use of the CatWalk should take place in a dark room (≤1 lux) or under red light conditions. If desired, the walkway and all components can be sequestered in a sealable enclosure that blocks out ambient light, allowing the room to be illuminated while testing.

Configuration of the detection settings

Detection settings for paw prints should be set according to the manufacturer's instructions. A representative control animal is used to determine the optimal paw detection settings to help the software distinguish between paw prints and background noise; this can be an animal planned for experimentation. When the control animal is placed in the walkway, the camera gain and intensity threshold can be adjusted so the paw print is clearly visualized. An ideal print should have the paw pad and toes clearly visible and separated. If the toes are not visible, or if there is no definition between the toes and/or paw pad, adjust the camera gain and intensity threshold. These settings should be revisited between experiments if there are significant changes in the animals to be used—such as species, strain, age, or weight—or if other physical parameters of the animals change, which may influence paw impact. Sex may also impact these parameters, and representative male and female animals should be compared to determine if different settings are required. If physical parameters vary greatly within an experiment (e.g., one experimental group is obese and the other is underweight), take a representative rodent from each experimental group, and determine if they require different threshold settings for optimal print visualization. If so, two different files should be created, each with different detection settings optimized to the representative rodents. Some parameters rely on clearly visualized paws with separated toes to be calculated, such as print length (measured as the distance from the third toe to the heel of the paw), toe spread (measured as the distance between the first and fifth toes), and intermediate toe spread (measured as the distance between the second and fourth toes). These measurements can be particularly important for the study of specific deficits, such as recovery from sciatic nerve damage (Baptista et al., 2007). The creation, in this example, of two files with optimized settings ensures that these parameters can be measured appropriately in both groups.

Materials

> 70% ethanol
> Glass cleaning solution
> Mice (≥6 weeks old) or rats (≥8 weeks old)
>
> Noldus CatWalk XT walkway and components:
> > Adjustable plastic corridor
> > Removable metal grills for each end of corridor
> > Goal box for end of corridor (*optional*)
> > Camera
> > 15 × 10–cm calibration sheet
> > Lightbox
> Attractant for goal box (*optional*)
> Low-lint wipes
> Computer (Windows XP Professional or higher, 2.60 GHz CPU, 6 GB RAM)
> > running Noldus CatWalk XT software, version 9.1

NOTE: All protocols involving live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Prepare walkway

1. Clean walkway and interior corridor walls with 70% ethanol, following up with glass cleaning solution using low-lint wipes on the walkway surface to minimize streaking.

   The walkway should be cleaned thoroughly between each animal, as insufficient cleaning may leave residual odors from the previous animal and cause pauses in the run for sniffing or other investigatory behavior in the test area, ultimately leading to an invalid run. Additionally, urine and feces on the walkway can obscure paw prints and cause unnecessary background noise and software read issues. Ensure that the walkway is dry and free of any residual glass cleaning solution before placing an animal inside.

2. Place corridor in the middle of the walkway (Fig. 1), and put grills in place on each end, ensuring the corridor is properly seated on its poles and contacts the walkway.

   The width of the corridor will depend on the size of the animals and should generally be narrow enough for the rodent to walk forward freely and limit turning around; a corridor that is too wide may promote excessive turning behavior. If the rodent turns around during a run within the acquisition window, the run is invalid and will need to be repeated.

3. *Optional:* Set up goal box at the end of the walkway with preferred attractant (Fig. 1).

   The goal box may be used at the end of the corridor to create incentive to cross the field, where entrance to the home cage or other attractant (e.g., food pellet, rodent candy) may be used.

Define walkway

Defining and calibrating the walkway should be done before each experiment but is not necessary to repeat between animals unless changes are made to the walkway during the experiment. If adjustments are made to the corridor width, camera height, or any other components mid-experiment, then the walkway will need to be redefined to accommodate these changes. Refer to the manual for screenshots of the software.

4. Define layout of the walkway by selecting the "Application Startup Tasks" window in the software, clicking on "Setup the walkway," and then clicking on "Define the layout of the walkway." From "Setup," click on "Define the walkway."

   An adjustable white rectangular box should be visible on the runway area. From this window, you should now see the image of the walkway.

5. Adjust camera if you are unable to visualize the walkway. To achieve five to six step cycles (ideal for a run with rodents), set camera 45 cm below the walkway for mice and 70 cm for rats. Verify camera height is appropriate for your experiment and walkway is in focus.

6. Adjust white rectangular box (step 4) to select the area of interest for the walkway (the walkable space within the corridor).

Calibrate walkway

7. Select "Application Startup Tasks" window, and click on "Setup the walkway." Then click on "Calibrate the walkway." From "Setup," click on "Calibrate walkway."

8. Place 15 × 10–cm calibration sheet onto the walkway in the previously defined area.

9. Resize white rectangle on the screen to fit the rectangle on the calibration sheet.

Create an experiment

10. From the "File" menu, select "New Experiment."

You may select a previously used experiment profile under "Setup Profile" if you have any saved at this step.

11. Name and select desired save location for the experiment.

12. To set the experiment settings, navigate to the "New Experiment Tasks" window and select "Experiment Settings."

13. Under "Time Points," add a time point if desired for the experiment.

    Time points are useful for repeated testing over time if gait effects by treatment are to be evaluated at multiple time points. These may be omitted if they are not of experimental interest.

14. Select animal type (rat or mouse).

    Proper animal selection is critical, as various software components calculate parameters differently for rats and mice.

15. Define experimental groups in "Treatment Groups," and click "Add Group" to add a treatment group. Adjust names of the experimental groups accordingly.

    The experimental group names will carry through to the exported data files, so it is useful to change the name of the treatment groups. Additionally, you can input further information in the "Description" window, which is exported with the data output and can be analyzed as an independent variable.

Define experiment settings

16. Set desired "Run Criteria" by adjusting the "Minimum run duration," "Maximum run duration," and "Minimum number of compliant runs to acquire" and by checking/setting the "Use maximum allowed speed variation."

    Note that once runs have been acquired, the run settings cannot be changed for a given experiment. If you find that the run settings were not appropriately set for the experiment after acquiring a few trials, you will need to start a new experiment and repeat the trials with the adjusted settings.

Set trial list

17. Navigate to the "Experiment Tasks" window, and select "Open Trial List."

18. Select "Add Trial(s)" to add as many trials (animals) as your experiment requires, and assign them to the appropriate treatment group. Edit trial name to your choosing, keeping in mind that all trial names must be unique. Adjust animal ID, time point (if selected; see "Create an experiment," steps 10 to 15), and an optional description.

    Too many trials within an experiment may cause slowdowns and crash the software. If you have >500 trials for an experiment, you should break the experiment up into multiple files.

Define acquisition settings

19. Under the "Setup" menu, select "Acquisition Settings."

20. Select criteria for recorded runs under "Run Settings."

    These settings are not what decide if a trial is compliant or not; that, instead, is done by editing the "Experiment Settings" (step 16). Under "Run Settings," you may select a time threshold for which the run will be aborted and the acquisition will stop. Further, you may select to have the aborted runs discarded and not recorded in the acquisition window. These settings may be useful to save program memory and disc space in situations where the animals routinely make noncompliant runs and when you have many trials in an experiment.

Acquire a trial

21. Navigate to the acquisition window by selecting "Open Acquisition" in the "Experiment Tasks" window.

22. Select trial to be acquired in the drop-down menu.

23. With an empty and clean walkway, retrieve a background image by clicking "Snap Background."

    A background image should be collected prior to each trial, as the cleaning between animals can lead to different background noise and minor positional changes of the corridor.

24. Place animal at the end of the corridor.

25. Lower illuminated ceiling by grabbing the handle and gently lowering it onto the walkway.

26. On the "Acquisition" window, click "Start Acquisition."

    Start acquisition as quickly as possible upon placing the animal inside corridor to ensure you do not miss the first crossing of the test window.

27. Allow animal to cross the test field for as many compliant runs desired for your experiment, and visualize each run to verify compliance.

    A minimum of three compliant runs per trial per animal are recommended for data analysis. The software will automatically cease recording once all compliant trials have been acquired. If one or more trials were recorded as complaint based on time but cannot be used for analysis purposes (e.g., mouse turned around mid-run, stopped mid-run, etc.), you can restart the acquisition by clicking "Start Acquisition" to collect additional runs. For this reason, you should visualize each run on the screen as they occur to verify compliancy based on the chosen experimental parameters and should not rely on the software's determination based on the time gate alone.

28. Remove animal from the walkway, and return to home cage.

29. Clean walkway with 70% ethanol and glass cleaner, and proceed with the next animal.

30. Repeat steps 22 to 29 until all animals have been tested.

Review and classify runs

31. Navigate to the "Video" section, and select a run from the "View" list.

32. Play run, adjusting playback speed as desired.

33. If a run is non-compliant and you would like to delete it, right click run and select "Delete."

    Runs that are deleted are permanently deleted and cannot be restored.

34. To classify runs, navigate to the "Experiment Tasks" window, and select "Classify Runs."

35. Use the Automatic Footprint Classification (AFC) module to automatically detect paw prints, or manually select prints if this module is not available or if manual selection is preferred.

    The AFC module functions well to label paw prints in situations of normal gait. In situations where the tail, nose, or other parts of the animal's body are repeatedly contacting the glass, the AFC does not identify these contacts well, and manual selection will be necessary. For all runs, you should first automatically classify the paw prints (if the module is available), and then go through the run and manually assign any non-paw print contact, as well as verify the assignment made by the AFC for each paw print,

reassigning any erroneously labeled paw prints. Users can manually assign prints by drawing a box with their cursor over the print and assigning the appropriate label to it from the pop-up menu. In cases where the glass has significant background or paw prints are close together, the AFC will have a higher rate of mislabeled or unlabeled paw prints. Refer to the manual for additional usage recommendations of the AFC module, as well as detailed manual labeling instructions.

Exporting data

36. Under the "Analyze" menu, select "View Run Statistics."

37. Under the "File" menu, select "Export." Of the three export options available in the drop-down menu ("Run Data," "Run Statistics," and "Trial Statistics"), select the preferred exportation.

   Run data will export the frame-by-frame data of a single run. Run statistics compiles the run data into a single value per parameter per run. Trial statistics compiles the run statistics into a single value per parameter per trial/animal. See "Understanding Results" for additional information.

COMMENTARY

Background Information

Locomotion and gait have been studied for decades, with recent technological advancements providing wider access to gait analysis. One of the earliest and simplest methods for evaluating locomotion is observation in an open field environment. While this method is simple, with researchers recording gait measurements by eye or with the assistance of video tracking software for later manual evaluation, observational methods are limited by the capabilities of the human eye and can generally only detect gross locomotor and gait abnormalities (Lakes & Allen, 2016). Treadmills can also be used for observational gait studies, which can be useful in models where motivation to cross an open field is a challenge or when researchers desire to control the speed of the animal (Pereira et al., 2006). Results from observational studies can vary widely between observers, and protocols are difficult to standardize between laboratories. Further, lack of detection of subtle changes poses significant challenges to such observational methods, highlighting that these methods alone provide only an incomplete picture of locomotion and gait.

A more complex and quantitative test, footprint analysis, can be conducted by dipping a rodent's paw into ink or photo developer solution and having them walk across a sheet of paper or film, respectively (Carter et al., 1999; van der Zee, Schuurman, Traber, & Gispen, 1987). From this, calculations based on footfall, print size, and various other parameters can be measured. While more quantitative in nature than strict observational methods, this test has its own limitations, such

as the ink itself altering the gait of the animals due to the feel on the paw, smearing of the ink obscuring paw measurements, and the inability to measure desired parameters like velocity (Jacobs, Kloefkorn, & Allen, 2014).

The CatWalk gait analysis method addresses many of the limitations of observational and ink-based footprint analysis. Developed by Frank Hamers, this method was originally designed to assess front limb–hind limb (FL–HL) coordination in spinal cord injury, a metric historically difficult to assess in open field observation (Hamers, Koopmans, & Joosten, 2006). Using a special glass walkway, developed by Betts and Duckworth (Betts, Duckworth, Austin, Crocker, & Moore, 1980), that illuminates paw prints and a camera from below to record, a wealth of parameters were found to be measurable with this method, beyond FL–HL coordination (Hamers, Lankhorst, van Laar, Veldhuis, & Gispen, 2001). By employing a computer program to analyze the paw prints, intra-observer variability is removed, and detailed metrics unable to be caught by the human eye are now able to be recorded. Passive, automated systems such as the Noldus CatWalk XT based on this method have greatly simplified gait analysis in rodents and allowed for collection of dynamic parameters that were previously difficult to collect, such as swing duration, stance duration, and interlimb coordination (Kappos et al., 2017). These parameters are important for the characterization of nerve injuries to both the PNS (Chiang et al., 2014; Deumens, Jaken, Marcus, & Joosten, 2007) and CNS (Hamers et al., 2006; Koopmans et al., 2005; Zheng et al., 2021). Further, the CatWalk

system has been used to evaluate motor phenotypes of transgenic animals (de Haas, Russel, & Smeitink, 2016; Robinson et al., 2012) and rodent models of disease, such as multiple sclerosis (Bernardes & Oliveira 2017), Huntington disease (Abada, Nguyen, Schreiber, & Ellenbroek, 2013), Parkinson disease (Wang et al., 2012; Zhou et al., 2015), and stroke (Hetze, Römer, Teufelhart, Meisel, & Engel, 2012). Modeling of the impacts of mechanical injury on gait have also been extensively studied with the CatWalk, such as those that occur with TBI (Chen et al., 2012; Walter et al., 2020) and limb injury (Gabriel, Marcus, Honig, Walenkamp, & Joosten, 2007).

Despite its undeniable importance, it should be mentioned that motor phenotyping is only one piece of the picture when considering a behavioral phenotype and should be part of a larger assessment battery when evaluating, for instance, the effects of toxicants or characterizing a transgenic line. Cognition and motor function are deeply intertwined and overlap in the frontal lobes, cerebellum, and basal ganglia, where the executive function for movement intent and prediction of the movement of others are controlled (Leisman, Moustafa, & Shafir, 2016). Given the intertwining of these functions, motor and cognitive deficits often coexist in specific CNS injuries, and both should be investigated for a robust phenotypic analysis. In addition to including cognitive tests when conducting behavioral phenotype analysis, the translational application of the rodent model for gait abnormalities should be considered. There are fundamental differences between quadruped and biped gait that can complicate direct translation of rodent gait abnormalities to humans. For example, rodents and other quadrupeds show an increased frequency of gait patterns at higher speeds, such as trotting and galloping, which are generally not seen in bipedal humans (Broom et al., 2017). These differences will have varying impacts on data interpretation depending on the experimental model and questions being addressed but should be considered nonetheless.

Critical Parameters and Troubleshooting

For the CatWalk, there are no concerns with maintaining a novel environment, as it is designed to test inherent locomotor ability and gait function and as there is no learning component being assessed. Therefore, repeated testing of the same animal is acceptable with no waiting period required between tests. In cases where the mice are tested for long periods or appear to be fatigued by the testing regime, researchers should use their best judgement in applying a wait period between testing to ensure mice are not fatigued when tested, as this could influence their performance.

Reducing stress as much as possible is important for most behavioral assessments. With locomotion and gait evaluation, stress can influence the way in which the animals move and can make subtle treatment effects more difficult to detect due to increased variability. A stressful or fearful rodent in the CatWalk may be less inclined to cross the testing field, may alter their speeds erratically during the test, or may exhibit other undesired behaviors that can impact data analysis. Be mindful to minimize stress for the animals when placing them inside and removing them from the apparatus, as to cause as little stress as possible and to not trigger negative associations with the apparatus itself. Training the animals to travel the walkway beforehand, for example, can familiarize them with the environment, lessening fear and anxiety and ultimately reducing variability due to run differences by providing more consistently compliant runs. Frequent handling of the animals, in general, can acclimate them to being picked up and moved by researchers, alleviating stress when being placed into and removed from the walkway.

In certain experimental circumstances, there may be justification for studying gait while the animal is under stress. While there may be no spontaneous motor phenotype at baseline, a motor deficit may arise under stress conditions and provide further information for the behavioral phenotype (Sugimoto & Kawakami, 2019). Stress as a modulating factor should be performed in a controlled manner, first following the abovementioned guidelines to allow for each animal to be at a similar baseline, after which stress loading may be applied by the desired technique.

Disruptions to cognitive ability may also present with a motor deficit, depending on the affected brain region. For example, mechanical injury to the cortex by controlled cortical impact causes both cognitive (Hamm et al., 1992; Thompson et al., 2006) and motor impairment (Chen et al., 2012; Neumann et al., 2009). Furthermore, phenotypic evaluation of cognition and memory, using classic tests such as the Morris water maze, T-maze, and novel object test require the animal to move through a testing field to appropriately conduct the test (Rodriguiz & Wetsel, 2006). Motor deficits,

even in the absence of cognitive deficit, may impact the output of these tests and erroneously display as cognitive deficits if the animal cannot move through the test field. For this reason, motor testing and gait evaluation with the CatWalk or other tests should be conducted before testing cognitive ability, and any determined motor deficit should be considered when interpreting cognitive tests that require locomotion.

A minimum of three compliant runs per trial are recommended for analytic purposes; more may be included, particularly if you suspect that there may be variation in the runs. Runs that fall below the minimum and above the maximum set run duration (step 16) will be labeled as non-compliant. All non-compliant runs will still be acquired and recorded in the acquisition window unless otherwise specified in "Acquisition Settings." The minimum and maximum run settings will vary based on experimental conditions. When deciding on an appropriate time window, you may use the acclimation days to narrow down this window or refer to previously described literature for a given model. For an ideal compliant run, the rodent should freely and calmly cross the test window without interruption. Some treatments may cause fear or anxiety that modify the animal's run behavior (e.g., rodents may dart across the test window), and the minimum time will thus need to be adjusted to reflect faster run speeds. Some of this behavior may be mitigated by acclimation to the testing apparatus, but the outcome of acclimation may vary by duration or frequency of the acclimation and experimental treatment conditions. Conversely, some treatments may significantly impact locomotion and cause the treatment groups to move slower compared with the control. In this case, the maximum will need to be adjusted to include longer run times so that all runs by treated animals are not erroneously labeled as non-compliant.

The selection of "Use maximum allowed speed variation" is another layer of compliancy based on speed consistency. Some rodents may start the run fast and slow down or may start slower and speed up mid-run, causing significant variation in their run speed. Speed variations can be due to a variety of factors, such as a startle mid-run or a legitimate effect of treatment. If this option is unselected, only the minimum and maximum times will be used to determine if a run is compliant. If you desire to analyze speed variation as a variable, you should leave this unselected (as is the program default), since determining compliant runs based on speed variation will artificially gate the runs within a specific variation window and may mask differences in this parameter.

For software troubleshooting, refer to the manual provided with the system.

Understanding Results

The parameters measured by the CatWalk fall into eight general categories: (1) paw statistics, (2) step sequence, (3) base of support, (4) print positions, (5) support, (6) phase dispersions, (7) couplings, and (8) other. These parameters are automatically calculated by the CatWalk XT software and can be exported on a per-run basis ("Run Statistics") or, alternatively, on a trial basis ("Trial Statistics") in which all the collected runs are averaged together to output a single value for each parameter per animal. In general, exporting the trial data to obtain one value per parameter per animal is the preferred exportation method. In cases where you are interested in the inter-run variation of a single animal, exporting on a run basis would be beneficial to examine this variation before averaging the runs to obtain a single trial value. Refer to the user manual for detailed information on how the parameters in each category are calculated within the software and what total parameters are available. Parameters of interest will vary depending on experimental goals and should be determined a priori for appropriate data analysis (see Fig. 2 for graphical representations of select parameters). Some parameters for basic gait and locomotor evaluation include:

Average speed: walk speed measured as distance over time, expressed in centimeters/second.

Cadence: frequency of steps during the trial, expressed as number of steps/second.

Swing: time of paw spent in the air between two consecutive steps, expressed in seconds (Fig. 2A).

Swing speed: parameter calculated using swing and stride length, expressed in centimeters/second.

Stride length: distance between paw placement in two consecutive steps of the same paw, expressed in centimeters (Fig. 2A).

Stance: duration of paw contact on glass, expressed in seconds (Fig. 2A).

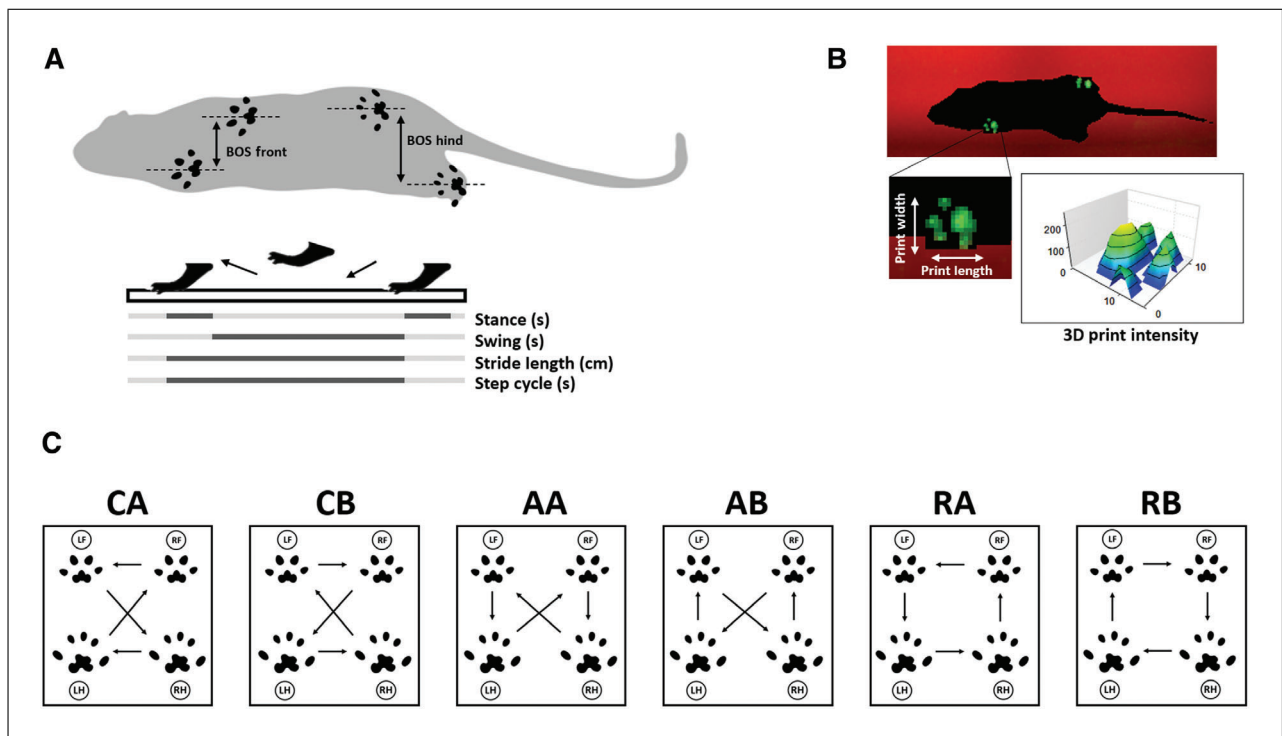Step cycle: duration of the stride, expressed in seconds (Fig. 2A).

Figure 2 Selected CatWalk parameters. (**A**) Illustration showing base of support (BOS) measurement in front and hind limbs, as well as a graphical representation of stance (s), swing (s), stride length (cm), and step cycle (s). (**B**) Screenshot from a CatWalk XT video demonstrating paw print visualization and intensity output from 3D Footprint Intensities provided in software, along with a graphical representation of paw print statistics for print length and width. (**C**) Common step patterns in rodents, with arrows indicating step directions of left front (LF), left hind (LH), right front (RF), and right hind (RH) paws. Patterns fall into three categories: cruciate (CA, CB), alternate (AA, AB), and rotate (RA, RB).

Print intensity: average pressure exerted by a single paw on floor contact, expressed in arbitrary units (Fig. 2B).

Print area: measurement of the complete paw print including all frames that makes up a stance, expressed in square centimeters.

Print length: measured length of the print area, expressed in centimeters (Fig. 2B).

Print width: measured width of the print area, expressed in centimeters (Fig. 2B).

Base of support: distance between either two front paws or two hind paws, expressed in centimeters (Fig. 2A).

Sequence regularity index (SRI): percentage index measuring interlimb coordination, calculated based on the number of normal step sequence patterns (NSSP), number of paws, and paw placements, SRI = 100% × (NSSP × no. of paws) / no. of paw placements.

Step sequence: measures percentage spent in the six NSSPs noted in rodents (Fig. 2C): cruciate (CA: RF – LF – RH – LH, CB: LF – RF – LH – RH), alternate (AA: RF – RH – LF – LH, AB: LF – RH – RF – LH), and rotate (RA: RF – LF – LH – RH, RB: LF – RF – RH – LH).

Support time: relative duration spent supporting weight on all combinations of paws. Combinations include zero paws, single paw, girdle two paws (LF and RF or LH and RH), lateral two paws (LF and LH or RF and RH), diagonal two paws (LF and RH or RF and LH), three paws, or four paws.

Rodents typically have a symmetrical gait pattern, with their left and right limb foot strikes being spaced equidistant, timed at around 50% of the gait cycle, and the front and hind foot strikes for a given side overlapping in space. An example of this typical gait pattern in a control mouse, obtained by following the Basic Protocol, can be found in Figure 3. An example video of a control mouse in the walkway can be viewed in Video 1. Additionally, rodents tend to be balanced and spend an equal amount of time on their left and right limbs (Jacobs et al., 2014). While symmetry in the gait pattern is generally expected across all control animals, size, weight, and other factors may influence other parameters of interest, such as swing and stride length. Comparing the treatment group to a proper control group on an identical background,
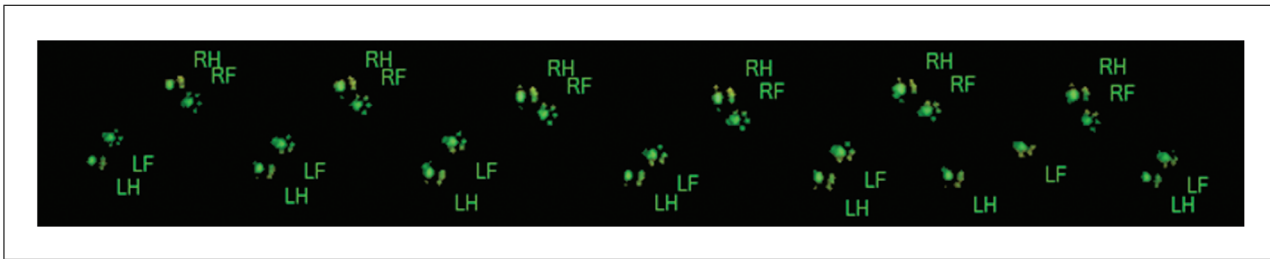
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Figure 3 Basic rodent gait pattern. Representative run from CatWalk demonstrating a normal gait pattern, viewed from below, with the animal walking from left to right. Left front (LF), left hind (LH), right front (RF), and right hind (RH) paw prints are labeled. Animal is a 4-month-old male wildtype C57BL/6 mouse. The foot strikes are equally spaced between the left and right sides, with the front and hind limbs overlapping in their step.

Video 1 Example of normal rodent step cycle in CatWalk XT Software. A compliant run with a 4-month-old C57BL/6 male mouse played at one-fifth normal speed, visualized from below, with the animal walking from left to right. Paw print results are displayed on lower half of the video.

with as many physical variables held constant as possible, will provide the best interpretation of what parameters are deviating from normal within a given experiment. In cases where physical parameters cannot be held constant (e.g., treated animals have a smaller stature compared with controls), these factors should be considered when interpreting the data.

A selection of example parameters, generated by following the Basic Protocol, can be viewed in Figure 4. Here, control and transgenic mice on a C57BL/6 background (N = 16 per group) were compared to determine baseline differences in their gait and locomotor function. No differences in male and female mice were found for any parameters (data not shown), and both sexes were combined

for analysis. Metrics measured include speed of the mice (Fig. 4A), cadence (Fig. 4B), regularity index of their gait (Fig. 4C), base of support (Fig. 4D), and the percentage of time the mice spent bearing weight in specific limb configurations (Fig. 4E). In this experiment, transgenic mice did not vary from controls on most metrics measured. However, transgenic mice were found to spend significantly less time bearing weight in the "girdle" support position, which is the placement of weight on the two front limbs or two hind limbs simultaneously. These mice have also shown deficits when tested on the rotarod, where transgenic animals had a shorter latency to fall off a spinning rod than controls. We anticipate that the reduction in girdle support observed in the
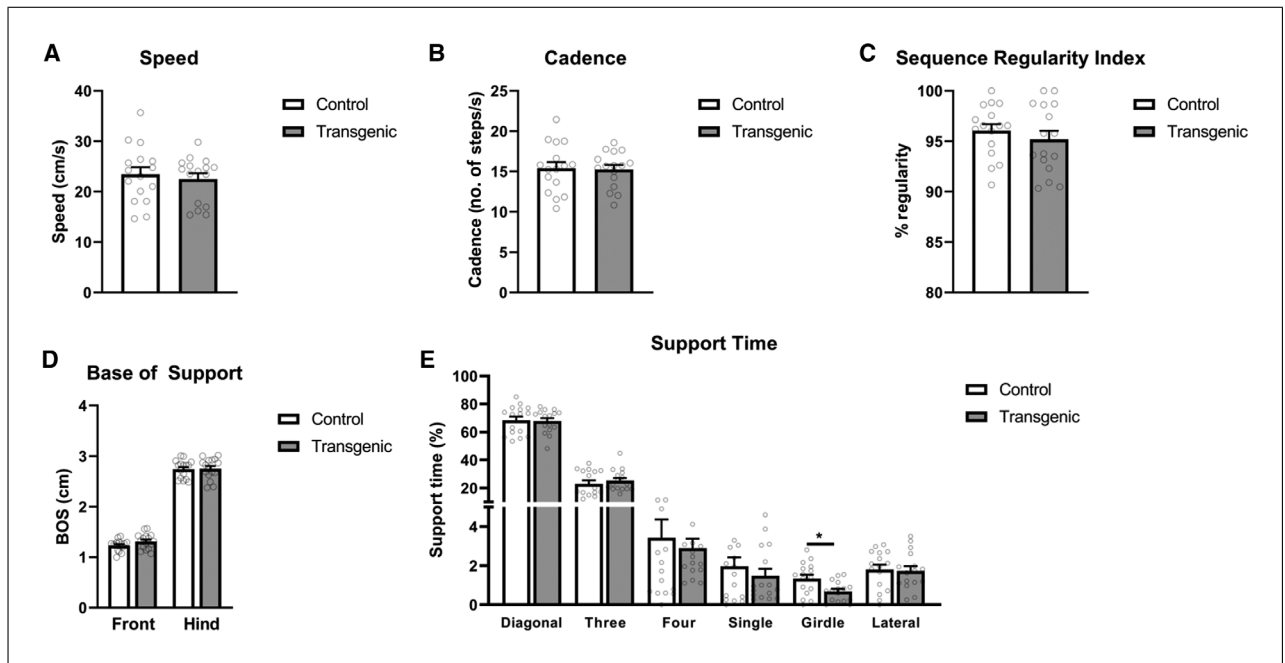
Figure 4 Example CatWalk data. (**A**) Average speed in cm/s; N = 16 per group. (**B**) Cadence (no. steps/s); N = 16 per group. (**C**) Sequence regularity index; N = 16 per group. (**D**) Base of support (cm); N = 16 per group. (**E**) Percent support time; N = 16 per group; *$p < 0.05$, one-way ANOVA.

CatWalk is related to their decreased rotarod success, as bearing weight on the front limbs simultaneously is advantageous for staying on the rod longer. While further analysis of this transgenic line is ongoing to fully understand the associated motor deficits, these data demonstrate that the CatWalk can be an important tool in motor phenotyping to pinpoint deficits and to provide plausible explanation for observed deficits in other tests. Data from the CatWalk can be presented in bar graphs as shown in Figure 4 and in line graphs for displaying data collected over time. Statistical analysis will depend on the experiment and the number of groups being analyzed by the user.

Time Considerations

As previously discussed, a 1- to 2-min acclimation period per day per animal for 1 week prior to testing is recommended to familiarize the animals with the testing apparatus. For testing days, if the animals are freely walking across the test area and providing compliant runs, each animal can be tested in under 10 min, with the inclusion of cleanup time. This test can also be repeated, as many times as desired, to measure the effect of treatment over time and to conduct a pre- and post-exposure analyses. Manual print assignment can be completed in 1 to 2 min per run, depending on how many prints must be categorized.

Author Contributions

Jacqueline M. Garrick: conceptualization, data curation, formal analysis, methodology, writing-original draft, writing-review and editing; **Lucio G. Costa:** funding acquisition, supervision, writing-review and editing; **Toby B. Cole:** conceptualization; **Judit Marsillach:** writing-review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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