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Original research paper

Establishing a reliable gait evaluation method for rodent studies☆

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HIGHLIGHTS

- We developed a systematic method to reliably generate results with CatWalk, one of the most popular gait analysis tools.
- We overcame many problems such as heel walking and poor compliance, which significantly compromised validity of results.
- We manually corrected automation errors and isolated consistent stretches of walk cycles to generate more reliable results.
- Stand Time, Duty Cycle, and Swing Speed were identified as reliable metrics for recovery evaluation after manual processing.
- Print Area and Intensity parameters were found to be not trustable and should be used with extreme caution.

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ABSTRACT

Background: CatWalk is one of the most popular tools for evaluating gait recovery in preclinical research, however, there is currently no consensus on which of the many gait parameters captured by CatWalk can reliably model recovery. There are conflicting interpretations of results, along with many common but seldom reported problems such as heel walking and poor compliance.
New method: We developed a systematic manual classification method that overcomes common problems such as heel walking and poor compliance. By correcting automation errors and removing inconsistent gait cycles, we isolated stretches of recordings that are more reliable for analysis. Recovery outcome was also assessed by quantitative histomorphometric analysis of myelinated axons.
Results: While 40–60% of runs were erroneously classified without manual intervention, we corrected all errors with our new method, and showed that Stand Time, Duty Cycle, and Swing Speed are able to track significant differences over time and between experimental groups (all $p < 0.05$). The usability of print area and intensity parameters requires further validation beyond the capabilities of CatWalk.
Comparison with existing method(s): There is currently no strategy that addresses problems such as heel walking and poor compliance, and therefore no standard set of parameters that researchers can rely on to report their findings.
Conclusion: Manual classification is a crucial step to generate reliable CatWalk data, and Stand Time, Duty Cycle, and Swing Speed are suitable parameters for evaluating gait recovery. Static parameters such as print area and intensity should be used with extreme caution.

1. Introduction

Peripheral nerve injury is a major burden to healthcare systems worldwide, affects 1.4 million patients, and costs over $150 billion dollars every year (Jia et al., 2014; Jiang et al., 2017; Jones et al., 2016; Taylor et al., 2008). Preclinical rodent studies are particularly valuable due to low cost and high translational potential (Kizilay et al., 2016; Wang et al., 2016), however, researchers must rely on postmortem pathological evaluation with only limited ways to evaluate functional recovery (Bervar, 2000; de Medinaceli

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et al., 1982). CatWalk has become a popular tool in rodent preclinical studies for evaluating functional recovery due to its ability to acquire a wide variety of gait parameters with its automatic "Auto Classify" function, and it is now widely used in mainstream research protocols in a wide variety of fields including peripheral nerve injury (Bozkurt et al., 2008; Deumens et al., 2007, 2014; Johnson and Jia, 2016), spinal cord injury (Kjell et al., 2015; Salewski et al., 2015), traumatic brain injury (Kizilay et al., 2016), neurodegenerative diseases (Neckel, 2015) etc. Google Scholar generated 1520 search results with the search term "CatWalk Gait" and 490 results with "CatWalk Sciatic" since 2006.

While CatWalk is now a widely used tool, it has significant drawbacks. Researchers often choose from wide variety of gait parameters to report their findings without justification of selection preference (Freria et al., 2016; Fujimaki et al., 2016; Hausner et al., 2014; Huang et al., 2012), and there are conflicting interpretations of results across different injury models (Bozkurt et al., 2008; Hamers et al., 2006). The lack of a standard set of parameters and interpretations may be due to common but seldom mentioned problems following rat sciatic nerve injury such as preferential heel walking (Deumens et al., 2007, 2014) and poor compliance (Neckel, 2015). While these problems significantly impair the ability of CatWalk to reliably automatically collect and analyze data, current literature has yet to propose any strategies on how to address them. Therefore, a standard method that effectively addresses these problems must first be established in order to properly identify a standard set of reliable CatWalk gait parameters.

The goal of this study is to establish a standard data processing method to account for and address inherent problems such as heel walking and poor compliance, and to identify a set of CatWalk parameters that is capable of consistently evaluating injury and recovery. Current literature evaluating CatWalk only employ a single injury group and a sham group, so to emulate current research practices, we employed three experimental groups, each receiving nerve resection injury followed by different interventions leading to different levels of recovery: one with an autologous nerve graft (clinical gold standard) to serve as positive control, one with an empty nerve conduit to serve as negative control, and one with human neural crest stem cell (NCSC) implantation to serve as the study target (Bozkurt et al., 2008), which leads to improvements in recovery that is superior to empty nerve conduits but not as effective as autologous nerve grafts (Georgiou et al., 2015; Ni et al., 2013; Wang et al., 2015).

2. Methods and materials

2.1. Animals

All animals were maintained according to NIH guidelines, and experimental protocols were approved by the IACUC of the University of Maryland School of Medicine. Every attempt was made to minimize the total number of animals used and their discomfort and pain. In this study, 36 athymic nude rats were used. Rats were individually housed in a controlled temperature ($21 \pm 1\,^{\circ}C$) and humidity ($50 \pm 15\%$) throughout this study with a 12:12 h light/dark cycle to reduce stress. All rats had free access to food and water throughout this study except the pre-surgery training period.

2.2. CatWalk training and recordings

Research protocols involving CatWalk were described in our previous studies (Johnson et al., 2015; Zhen et al., 2013). The CatWalk XT (Noldus Information Technology, The Netherlands) system includes a 1.0 m enclosed walkway on which rats to traverse from side to side and recordings are made. For our experiment, one week prior to surgery, animals were trained daily on the CatWalk system until they were able to consistently make uninterrupted runs. Animals were placed under food-restriction 12 h before the training, and food pellet rewards were used to motivate animals to cross the CatWalk runway during training. Training was considered complete when animals were able to make five consecutive uninterrupted runs within 1.0s and 2.0s. One day prior to surgery, five baseline runs were performed and recorded for each rat. Post-surgery measurements were taken at 2, 6, and 12 weeks. Each rat was placed on the CatWalk system and repeatedly performed runs until they were able to complete five uninterrupted runs.

2.3. Surgical procedure, euthanasia, histomorphometric analysis, and wet muscle weight measurements

Surgical procedures have been described in our previous studies (Johnson et al., 2015; Lewitus et al., 2011). In brief, Athymic nude rats (200–250 g) were anesthetized with isoflurane, and a 15 mm segment of the sciatic nerve was removed and repaired with one of three implants: A) inverted autologous nerve graft, B) empty nerve guide conduit, and C) human neural crest stem cell (NCSC) filled nerve guide conduit (12 rats each). NCSCs were derived from embryonic stem cells (ESCs, H9 line from WiCell, Madison, WI), and for each conduit, we injected 2×10^6 NCSCs suspended in 15 μl mixture of growth medium and Collagen I Rat Tail (Life Technologies, NY). For each group, half was euthanized at 6 weeks, and the other at 12 weeks. We used postmortem histomorphometric analysis as a reference to justify results derived from the CatWalk system since histomorphometric analysis is widely used in current studies as a gold standard for evaluating recovery following sciatic nerve injury (Gan et al., 2016; Kabiri et al., 2015; Lin et al., 2013). We have described the methods of histomorphometric analysis in past studies (Johnson et al., 2015). In brief, rats were perfused, and the middle section of the repair site was harvested and mounted in embedding resin. Sets of 0.5 μm thick specimens were sectioned on an Ultracut E microtome, stained with toluidine blue, and imaged via light microscopy at 40 x magnification. The number of myelinated axon fibers were counted with ImageJ (National Institutes of Health, Bethesda, MD). After euthanization, rat gastrocnemius muscles were retrieved from the injured limbs and compared between groups at different time points.

2.4. Automated data collection

Print area (the total print area (cm^2) for given paw), mean intensity (average pressure of print for given paw), stand time (time spent bearing weight per step for given paw), duty cycle (percentage of time spent bearing weight in each walk cycle for given paw), swing speed (the speed (cm/s) of the limb between steps for given paw), and stride length (the distance (cm) between steps for given paw) for both hindlimbs were collected from the CatWalk XT system after performing the "Auto Classify" function. Then, the Right Hindlimb (RH, injured) to Left Hindlimb (LH, healthy) ratios were calculated, and standardized against the baseline RH/LH ratio. Standardization with the contralateral hindlimb accounts for the variance due to weight and run calibration, and standardization with baseline ratios accounts for the natural tendency of each rat to bear weight on a particular side (Boyd et al., 2007). These standardized ratios were then averaged for a given rat at a given time point. These parameters were chosen due to their prevalence in recent studies. Due to the arithmetic involved in the standardization process, results are represented as percentage values. The number of runs with erroneous classifications was recorded for each group after injury at the 2-week, 6-week and 12-week time points. The