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Low-Cost Gait Analysis for Behavioral Phenotyping of Mouse Models of Neuromuscular Disease

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DUKE UNIVERSITY MEDICAL CENTER
Department of Neurology

April 4th, 2019

Dear Phillip,

Attached to this correspondence find the revised version of our manuscript entitled '*Low-Cost Gait Analysis for Behavioral Phenotyping of Mouse Models of Neuromuscular Disease*', by Wertman et al, for review at your journal. This manuscript describes a detailed, low-cost, non-invasive procedure to perform gait assessments on mouse models of human disease.

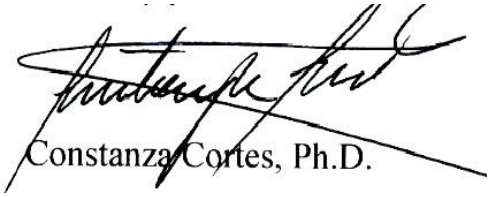
Gait abnormalities are a feature of a variety of mouse models, including neuromuscular and neurodegenerative diseases, stroke and muscle atrophy. We demonstrate the utility and sensitivity of this method to detect post-symptomatic gait deficits in a mouse model of the polyglutamine disease Spino-Bulbar Muscular Atrophy.

We thank the reviewers and editor for their suggestions. We have addressed all editorial and reviewer concerns, including formatting modifications, addition of requested references and clarifications in protocol instructions.

Thank you for your consideration for publication in your journal.

Please do not hesitate to contact me if there is any additional information you need.

Best regards,



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TITLE:

Low-Cost Gait Analysis for Behavioral Phenotyping of Mouse Models of Neuromuscular Disease

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KEYWORDS:

gait analysis, stride length, footprint analysis, phenotyping, behavior, neurodegenerative disease, neuromuscular disease, mouse models

SUMMARY:

Footprint analysis is a low-cost alternative to digitized gait analysis programs for researchers quantifying movement abnormalities in mice. Because of its speed, simplicity, and longitudinal potential, it is ideal for behavioral phenotyping of mouse models.

ABSTRACT:

Measurement of animal locomotion is a common behavioral tool used to describe the phenotype of a given disease, injury, or drug model. The low-cost method of gait analysis demonstrated here is a simple but effective measure of gait abnormalities in murine models. Footprints are analyzed by painting a mouse's feet with non-toxic washable paint and allowing the subject to walk through a tunnel on a sheet of paper. The design of the testing tunnel takes advantage of natural mouse behavior and their affinity for small dark places. The stride length, stride width, and toe spread of each mouse is easily measured using a ruler and a pencil. This is a well-established and reliable method, and it generates several metrics that are analogous to digital systems. This approach is sensitive enough to detect changes in stride early in phenotype presentation, and due to its non-invasive approach, it allows for testing of groups across life-span or phenotypic presentation.

INTRODUCTION:

Locomotion requires complex neurological and musculoskeletal coordination, and deficits in a single aspect of motor pathways can produce observable gait abnormalities[1,2]. Gait analysis is a critical tool for researchers testing mouse models because it provides quantifiable behavioral data on how a given disease, injury, or drug impacts an animal's movement[3]. However, digitized gait analysis requires the purchase of a treadmill, a camera, and associated software, which can be prohibitively expensive for researchers. Gait analysis is often used intermittently to track longitudinal changes in motor function, hence it may be difficult to justify the expenditure if sporadically used[4]. Although digitized analyses may provide more detailed gait metrics than simple footprint analysis, these more complex measures are not always necessary or relevant for the characterization of a behavioral phenotype[5].

Here we present a low-cost manual footprint analysis method as a quick and sensitive alternative to digitized gait analysis programs[6,7]. Manual footprint analysis has been demonstrated to detect significant gait differences in a multitude of murine disease models[4,7-17], and in at least one case, this low-cost method identified changes in gait that were not detected by a common digitized gait analysis program[12]. The total cost of materials is nominal, and it can be easily adapted to other rodent research models.

While there are many different gait metrics from which data can be drawn, the method we describe focuses on three specific metrics: stride length, stride width (a.k.a. "track width"), and toe spread. It is important to note that the parameters to be assessed should be determined on a model-by-model basis. This method of gait analysis is not designed to measure cognitive function, and it is not recommended for studies that require complex biomechanical measurements of gait[16].

We present behavioral data from a cohort of pre- and post-symptomatic mice modeling X-linked Spinal and Bulbar Muscular Atrophy (SBMA), a neuromuscular disease characterized by motor neuron degeneration and muscle atrophy. These mice develop progressive deficits in gait that coincide with the onset of other disease-specific phenotypes. This demonstrates the validity and specificity of this method, and confirms that it can reliably discriminate between affected and non-affected animals.

The experimental mice in this study were 2.5 (pre-symptomatic) and 9-month-old (post-symptomatic) BAC fxAR121 transgenic mice on a C57BL/6 background (n_{expt}=12). This model was generated in our lab and has been fully characterized as a powerful mouse model of SBMA[9]. Non-transgenic littermates were used as controls (n_{ctrl}=8). SBMA is a sex-limited disease which fully manifests in males only, so male mice were used exclusively for this study. During planning stages, researchers must take into account the National Institutes of Health's considerations of sex as a biological variable to determine group sizes and composition[18].

PROTOCOL:

All testing conducted with mice was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Duke University. Personnel responsible for testing and scoring

must be blinded to animal genotype or experimental condition until gait analysis and scoring of papers has been completed for the entire cohort.

1. Testing material preparation

1.1. Conduct testing with a tunnel built from 3 pre-cut clear acrylic panels that are 0.375 inches thick. Assemble tunnel by gluing panels together with a sealant that specifically bonds acrylic and will not emit odors when dried.

1.1.1. For standard C57BL/6 mice, use the following tunnel measurements: 2.5 in. wide, 3 in. high, and 13 in. long. Mice must be able to comfortably walk through the tunnel and take enough steps (>4) so that gait can be measured.

1.2. Construct the goal chamber with pre-cut gray acrylic panels 0.375 inches thick, glued together with the same sealant as used on the tunnel. The interior measurements of the chamber are 4 in. wide, 4 in. long, and 3 in. tall. Match the opening of this chamber to the opening of the tunnel (2.5 in. wide x 3.0 in. tall). Because the mice naturally prefer darkened spaces to well-lit spaces, use material that is opaque and dark in color.

1.3. Use paper for tracking steps that is thick and smooth (watercolor paper works well). Cut individual papers strips to be slightly wider and longer than the width and length of the tunnel. If using the tunnel dimensions described here, cut papers to 15 in. long by 3.5 in. wide.

1.4. Use two contrasting colors (e.g., green and purple) of non-toxic washable water-based paint. Assign one color for hind-limbs, the second one for forelimbs. Mice will lick the remaining paint from their feet after testing, so the selected paint must be completely non-toxic.

1.5. Use two round barrel paintbrushes, one for each paint color (~0.5 cm in diameter, tapered/pointed brush tip).

1.6. Select a ruler with markings down to millimeters, and a caliper with measurements down to 0.1 mm. Pencil is recommended to write on the scoring papers.

1.7. **Optional**: For animals with high anxiety or low motivation, provide a behavioral incentive in the goal chamber. This can include small amounts of sterilized sunflower seeds (placed in the home cage 2 days prior to testing to allow habituation). On the day of testing, place sunflower seeds inside the goal chamber to encourage mice to walk through without stopping.

2. Data collection

2.1. If testing is performed in a separate room, acclimate the mice to the new room for 30 minutes and then start the behavioral assays. Additionally, because mice are naturally nocturnal, ensure all mice are fully awake and alert for at least 5 minutes before testing.

2.2. Prepare the testing setup by positioning the tunnel over the paper and marking the paper with mouse ID and testing date. Position the goal chamber at the end of the tunnel, connecting both open ends. Add sunflower seeds at the end of the tunnel (inside the goal chamber) for motivation if needed.

2.3. Remove the mouse to be tested from its cage and grip it firmly by its scruff, making sure to grip the tail to stabilize movement of its hind limbs.

2.4. Paint forepaws so the entire underside of all toes and the center of the foot are fully covered in paint. Repeat this with a contrasting color of paint on its hind paws. Wipe off any paint that the mouse gets on other parts of its body with a clean damp cloth to prevent smudges that may interfere with data collection.

NOTE: Mouse handling must be performed by experienced researchers to minimize animal stress.

2.5. Place the mouse at the start of the tunnel and allow it to walk all the way into the goal chamber, and then retrieve the mouse, gently wipe off its feet with a water-dampened cloth, and return it to its home cage.

2.6. Allow paper with footprints to dry fully before scoring. Wipe down the testing area and tunnel with ethanol or an equivalent cleaning solution in between each animal.

3. Scoring criteria

3.1. Use steps that are consistently spaced with clear, non-smudged footprints for scoring. **Figure 1B** is a good example of a footprint sequence that can be scored. In order to generate sufficient scoring data, there must be at least 2 consecutive steps from each foot, but 4-6 steps per foot is recommended. Do not include the first and last footprints on the paper, as they are unlikely to represent normal gait because the mouse is changing its walking speed.

3.2. Use stride length, stride width, and toe spread as three different measures of gait that can be analyzed using this method.

NOTE: Stride length and width require clear sequential prints where the forefoot region is well defined in paint. Toe spread does not require sequential prints for scoring, only clear prints of the first and last toes on a single foot. However, if a given footprint is not included in measurements of stride length or width, it cannot not be scored for toe spread. All three measures are assessed in centimeters.

3.2.1. Define stride length as the distance between two sequential footprints created by the same foot (i.e., one stride) (**Figure 1A, 1B**).

3.2.1.1. With a pencil, draw a 2-4 mm circle around the fore-foot region of both forelimb

footprints (identified by assigned color above) in a single stride and draw a line between them using a ruler.

3.2.1.2. Record the distance between two prints from the middle of each circle (i.e. center of each foot pad) as Right-Fore 1 (RF1) or Left-Fore 1 (LF1).

3.2.1.3. Repeat for all steps that can be scored (RF2, LF2, RF3, LF3 and so on).

3.2.1.4. Repeat for right and left hind-limb footprints.

3.2.1.5. Average all individual recorded stride distances for each limb. For statistical analysis, individual cohort members can be averaged together.

3.2.2. Define stride width as the measure of distance between left and right forelimbs or hind-limbs (**Figure 1A, 1B**).

3.2.2.1. To assess this distance, draw and measure a line from the circled forefoot region of one hind-limb that intersects perpendicularly with the line for stride length on the contralateral hind-limb.

3.2.2.2. Repeat this for all hind-limb prints that can be scored, and then average the measurements. The method of calculation for stride width is the same for fore- and hind-limbs.

3.2.3. Define toe spread as the distance between the first and last toes on a single fore- or hind-limb footprint (**Figure 1A, 1B**).

3.2.3.1. Use calipers to measure the distance between the tip of the first toe print and the tip of the last toe print.

3.2.3.2. Repeat for all hind-limb prints that can be scored and average the measurements. The method of calculation for toe spread is the same for fore- and hind-limbs.

3.3. If the paper cannot be scored, allow the animal to rest for 10 minutes before trying again.

REPRESENTATIVE RESULTS:

With sufficient numbers of animals, this procedure is capable of detecting gait differences between mouse genotypes, within the same strain over time. **Figure 1B** shows representative traces of footprint images collected in our lab, using a mouse model of X-linked Spinal and Bulbar Muscular Atrophy (SBMA), a neurodegenerative disorder affecting lower motor neurons and skeletal muscle. We have previously reported that male BAC fxAR121 transgenic mice develop significant weight loss, impairments in grip strength, and shortened stride length at post-symptomatic ages when compared to non-transgenic littermate controls[9].

Here we present gait analysis results from a cohort of pre-symptomatic (2.5 months of age) and

post-symptomatic (9 months of age) BAC fxAR121 transgenic and littermate control male mice (**Figure 2**). Prior to disease onset, BAC fxAR121 transgenic mice display similar stride length, stride width, and toe spread compared to their littermate non-transgenic controls. After disease onset, BAC fxAR121 transgenic mice display significantly shorter stride length ($p_{forelimb}$= 0.001, $p_{hind\text{-}limb}$= 0.009) (**Figure 2A**). Similar longitudinal analysis revealed no differences in stride width at either age tested ($p_{2.5months}$=0.709, $p_{9\ months}$=0.204) (**Figure 2B**). Post-symptomatic BAC fxAR121 transgenic mice also have significantly narrower hind toe spread (p=0.01) than age-matched littermate controls (**Figure 2C**). BAC fxAR121 mice model a neuromuscular disease that primarily affects hind-limbs, so detailed measures of forelimb gait were not collected. We encourage researchers using this gait analysis method to consider the phenotype of their mouse models and choose forelimb or hind-limb gait metrics accordingly.

FIGURE AND TABLE LEGENDS:

Figure 1: Gait Analysis Measures and Troubleshooting. A. Schematic representation of gait analysis on mice, depicting stride length, stride width, and toe spread information. **B**. Representative example of a gait analysis footprint sequence that can be scored, depicting measurement of all three parameters. **C**. Representative examples of problematic gait analysis footprint sequences that cannot be scored.

Figure 2: SBMA BAC fxAR121 transgenic mice exhibit a progressive, neurodegenerative gait phenotype that can be detected via gait analysis. A. Despite no differences at pre-symptomatic ages (2.5 months, n_{ctl}=11, n_{expt}=12), BAC fxAR121 mice develop significantly reduced stride length compared to their non-transgenic littermate controls at post-symptomatic stages (9 months, n_{ctl}=8, n_{expt}=12). **B**. No changes were detected in stride width at either age. **C**. Symptomatic SBMA BAC fxAR121 transgenic mice display significantly reduced hind limb toe spread compared to non-transgenic littermate controls. N= 8-12/group. ANOVA with post-hoc Tukey test * $p < 0.05$, ** $p < 0.01$. Error bars represent SEM.

DISCUSSION:

Using the low-cost gait analysis method described above, we show successful identification of several parameters of gait dysfunction at post-symptomatic ages in the BAC fxAR121 mouse model of SBMA. Decreases in stride length are consistent with prior SBMA studies of mouse models and human patients[9]. We also show for the first time that there are significant differences in hind-limb toe spread in symptomatic SBMA mice compared to non-transgenic littermate controls. Interestingly, decreases in hind toe spreading can be caused by weakness in paw extensor muscles, tightness in paw flexor muscles, or poor nerve innervation[2,19], which is also consistent with the etiology of SBMA.

The mice should readily run to the goal chamber due to their natural behavioral preference for small dark spaces, but some mice may not continuously move through the tunnel. If a mouse jumps, stops, or turns around within the tunnel (see examples in **Figure 1C**), repeat the assay after a rest period on a new scoring paper. The results may be salvageable if a mouse stops at the very beginning of the tunnel since it can often be gently prodded into running to the goal box.

Applying too much or too little paint to a mouse's feet can produce unusable results. Excess paint can lead to smudged or distorted prints, while insufficient paint can produce faint or unidentifiable prints (**Figure 1C**). In either case, repeat the assay on a clean scoring paper to prevent inaccurate measurements.

Very young mice (<3 months old) are more likely to jump forward in the tunnel, whereas older (>8 months old) or very phenotypic mice are more likely to stop or resist forward movement entirely. Adding a behavioral incentive (sunflower seeds) in the goal chamber can help decrease the frequency of problematic behaviors by encouraging unmotivated mice to traverse the tunnel without stopping.

Tunnel dimensions should reflect the dimensions of the subject; if using mice that are significantly larger or smaller than an average lab mouse (due to age, diet, or genetic mutations), we recommend changing the tunnel and goal chamber dimensions to match the animal's size. In the tunnel, the mice should be able to walk comfortably in a straight line, but should have some difficulty turning around to discourage this behavior. The goal chamber should match the height of the tunnel and mice should fit comfortably inside the chamber.

Researchers who use the toe-clipping method of identification for their mice may not be able to collect data on toe spread, but other measures of gait like stride length and stride width can still be collected. Toe-clipping does not significantly impact gait in mice as long as no more than two toes are clipped per mouse[20].

This gait analysis method does not reflect cognitive function, so it should not be used as a measure of cognition. Others intending to use this method should consider the neuromuscular groups affected in their mouse model, and then choose fore- or hind limb metrics accordingly. This method of gait analysis is not recommended for researchers who study pain responses requiring footpad injections, or for studies requiring biomechanical measures of locomotion that cannot be described by footprints alone, like temporal measurements of limb motion or joint rotation[21].

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DISCLOSURES:

The authors have nothing to disclose.

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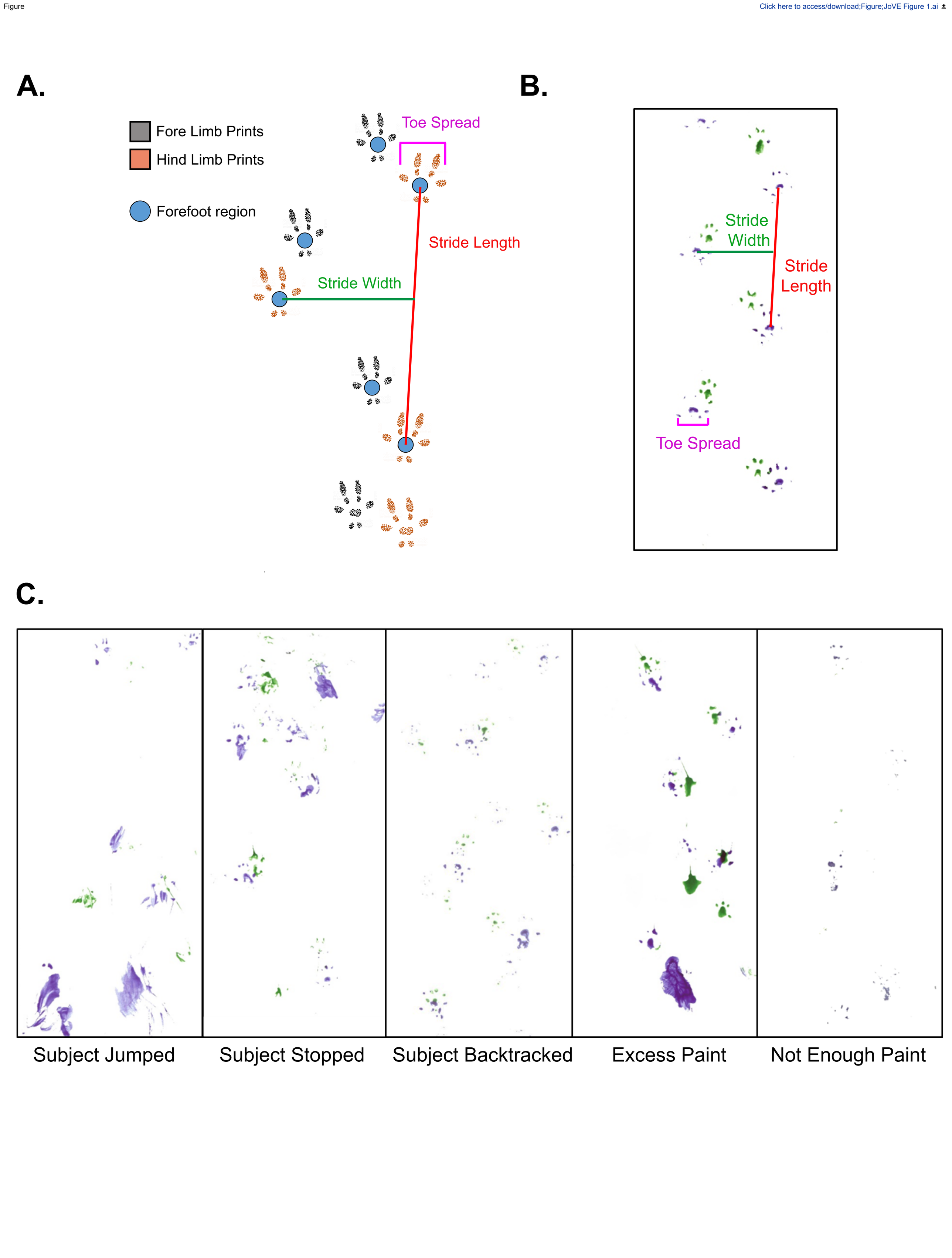

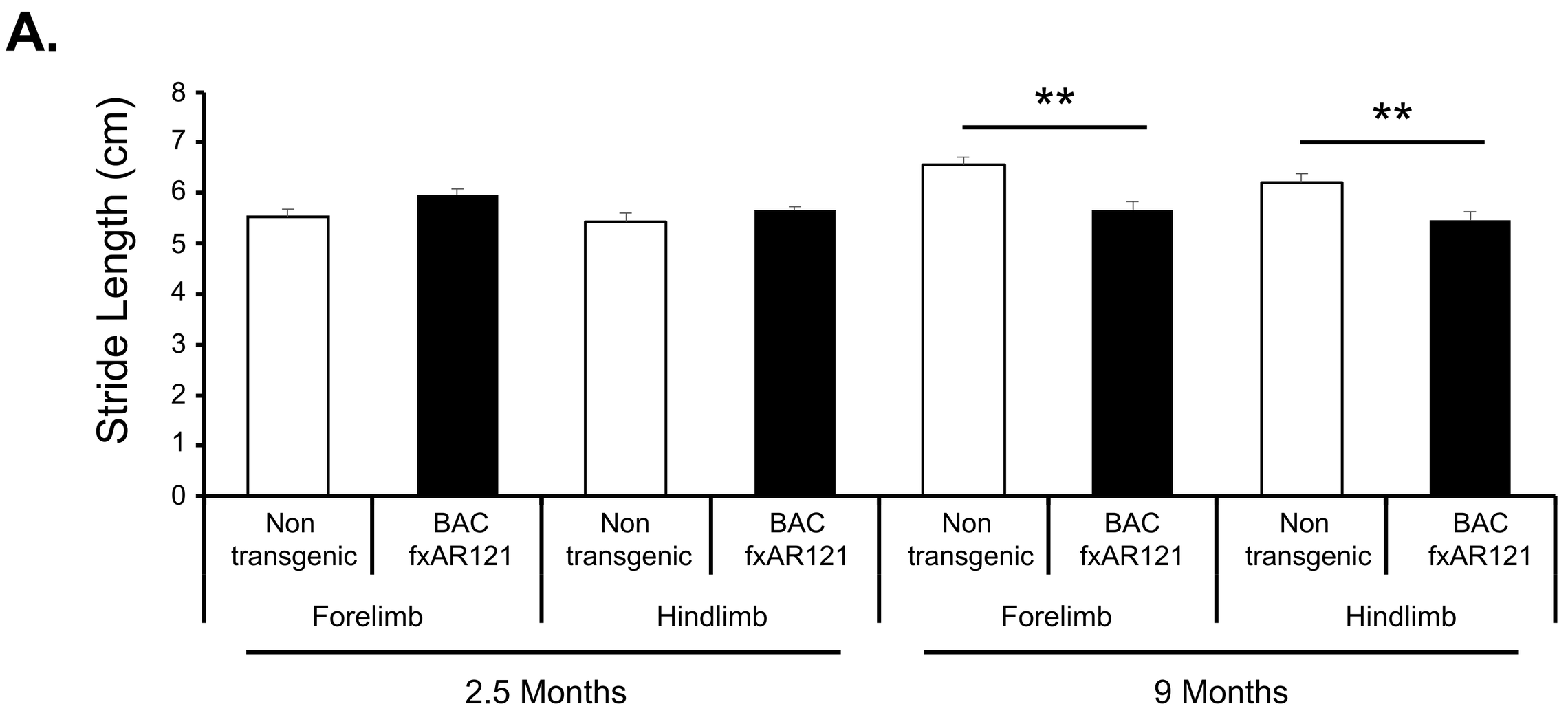

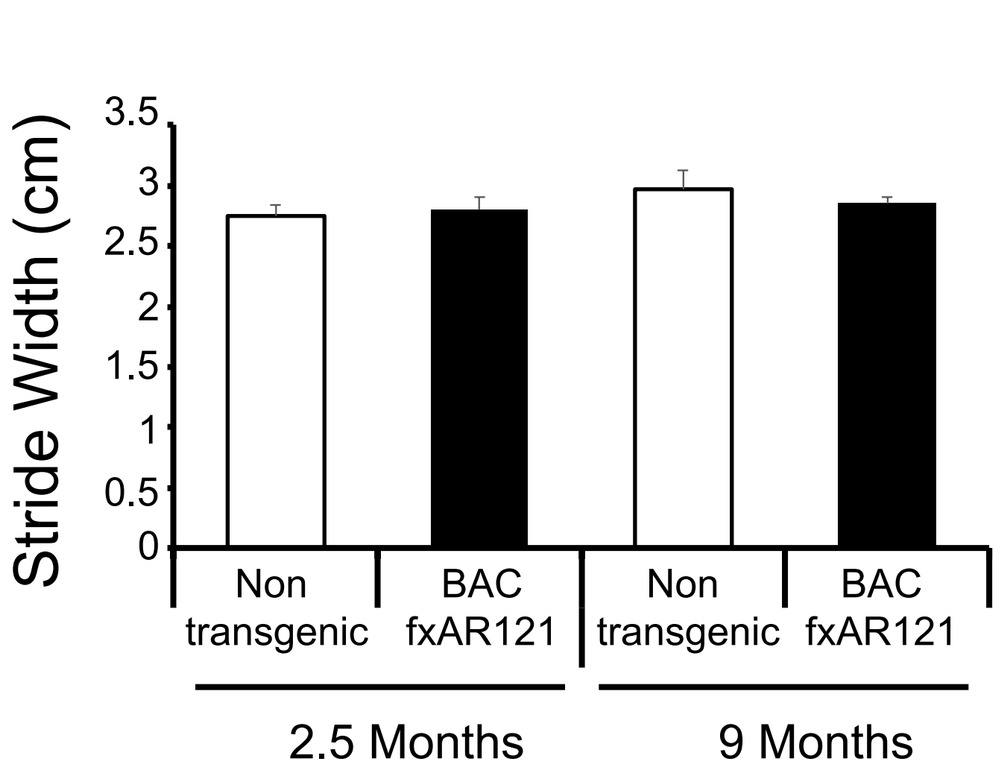

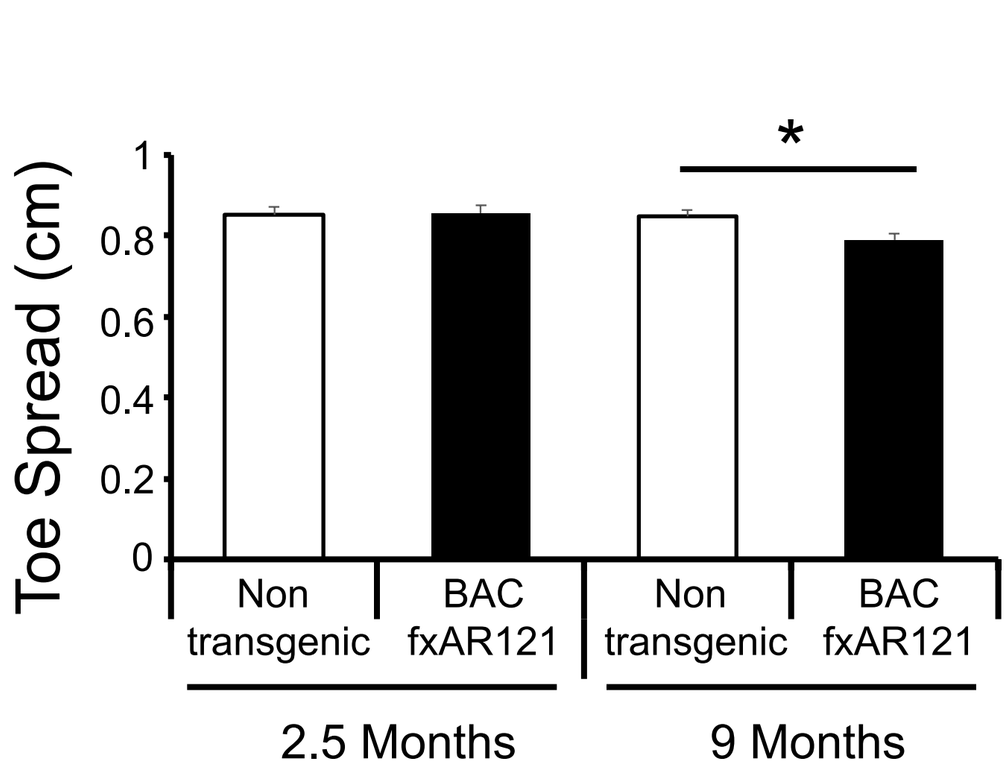

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Caliper	n/a	n/a	must have markings down to 0.1 mm
Craft Glue	E6000	n/a	
Footprint Paint (Tempera Paint)	Artmind	n/a	must be non-toxic
Round Barrel Paintbrushes	Symply Simmons	n/a	0.5 cm diameter
Ruler	n/a	n/a	must have markings down to millimeters
Scoring Paper (Watercolor Pads)	Canson	n/a	cut to size
Tunnel and Goal Chamber	Interstate Plastics	n/a	cut to size

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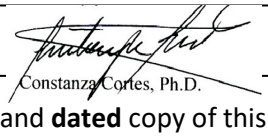
Signature:	 Constanza Cortes, Ph.D.
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Reviewer Rebuttal: Wertman et al, 2019

Ms #: JoVE59878

Title: "Low-Cost Gait Analysis for Behavioral Phenotyping of Mouse Models of Neuromuscular Disease"

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Completed.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Completed.

3. Please include email addresses of all authors in the manuscript.

Added.

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Corrected

Protocol:
1. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

Edit completed.

2. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. If revisions cause the highlighted portion to be more than 2.75 pages, please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Protocol is currently under 2.75 pages.

3. For each step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Confirmed.

Specific Protocol steps:
1. 2.1: Where did these mice come from? Please include a reference here and/or include in the Table of Materials.

A reference to the original publication describing these mice has been added.

Figures:
2. Figure 2C: 'Toe spread', not 'Toes spread'.

Graph label on Figure 2C has been fixed.

References:
1. Please do not abbreviate journal titles.

We are using the provided JoVE Endnote Style add-on. Please advise on how to further modify if necessary.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Confirmed.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This paper describes a modification of the footprint test as a low cost gait analysis system for phenotyping mice.The modifications to the system are ingenious, and would make a good JOVE video. Expensive gait analysis systems do not always give the right answers.

Minor Corrections needed:

Some references need adding

Line 52 Add reference for Pallier, Brain Res Bull. (2009) 78, 347-55. He found this by comparing footprints and an automated system.

Line 54 Add a reference. The footprint analysis was first described in a paper by Carter et al, J Neurosci (2009) 19(8):3248-57.

L55. Add this reference. Sugimoto H, Kawakami K. Low-cost Protocol of Footprint Analysis and Hanging
Box Test for Mice Applied the Chronic Restraint Stress. J Vis Exp. 2019 Jan 23;(143)

Line 83 The line of mice needs a reference.

All these references have been added in the requested sections.

Line 84. You can't have > or equal to when you describe a number of mice. How many mice exactly were used?

Exact number of mice has been added (n=8, n=12, etc)

Line 86. NIH should be in full, and a reference given

Corrected, and reference added.

Line 125. It should be made clear that the aim is to get a print of the footpad and all toes. This should be clearer in the figure as well.

We have edited the wording for clarity.

Figure 1A. You should show a mouse-like footprint, with foot- and toe-pads in the diagram. The cartoon shown in A looks like a monkey footprint, not a mouse footprint.

We have changed the diagram, and now use a textbook mouse-like footprint.

Throughout, don't use GA as an abbreviation, it is not necessary.

Corrected.

Reviewer #2:
Manuscript Summary:
There is not disagreement on the significance of the foot print gait analysis and its specificity in detecting changes in locomotion. The cost-effectiveness is not a not purchasing the CatWalk system is also not disputed. Utilizing a tunnel system may help control the environment around the mice. The addition of the goal chamber with food will increase the likelihood the mice will complete the task of walking to the chamber. Stride length, with and paw spreading are important gait markers used to monitor locomotor changes. Alternate colors between left and right paw and segregating the toes from the heel helps to identify specific each paw. The acrylic clear tunnel will also allow researcher to monitor mouse activity. In total this analysis is a cost effective and with careful researcher execution can generate good data for gait analysis.

Major Concerns:
In describing the construction of the tunnel, (Line 89, paragraph) enough description must be given to describe the tunnel. Dimension of the acrylic panels (length and width) would help in explaining the ambiguity.

We have modified the description for clarity.

Minor Concerns:
Using paint brush to paint the toes may prove to be difficult in that size and scale of the mice paw. Also, addition of paint with a paintbrush may excite animals those are hyper or sensitive to extrasensory input. The difficulty as presented in Fig 1C in regard to the pitfall of this model, it may be a concern that additional trials to get the proper step response would lead to increased handling and excessive activation of the paw resulting in modified data. The promising outcome using the SBMA mice does add confidence that this model with careful testing can be a cost-effective method.

Mouse handling by definition is stressful. We have added wording to further clarify experienced researchers must perform the testing, and paw over-stimulation as a potential side effect.