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3D Kinematic Gait Analysis for Pre-Clinical Studies in Rodents

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Subject: Manuscript Number JoVE59612, Re-submission to *Journal of Visualized Experiments (JoVE)*

Dear Editor,

We thank you for your time and giving us an opportunity to re-submit our manuscript titled "3D Kinematic Gait Analysis for Pre-Clinical Studies in Rodents" to JoVE. We greatly appreciate the Reviewers' comments, as well as their time and effort in pointing out ways to better our work.

We have responded to each point raised by each Reviewer in a 'response to reviewer' document and have also made the relevant change in our revised manuscript narrative (all changes are shown in RED).

Thank you for your time. We look forward to your decision.

Sincerely,
The Authors

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TITLE:

3D Kinematic Gait Analysis for Preclinical Studies in Rodents

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KEYWORDS:

3D kinematics, treadmill locomotion, rodents, gait analysis, quadrupedal locomotion, task performance

SUMMARY:

Presented here is a protocol to collect and analyze three-dimensional kinematics of quadrupedal locomotion in rodents for preclinical studies.

ABSTRACT:

The utility of three-dimensional (3D) kinematic motion analysis systems is limited in rodents. Part of the reason for this inadequacy is the use of complex algorithms and mathematical modeling that accompany 3D data collection and analysis procedures. This work provides a simple, user-friendly, step-by-step detailed methodology for 3D kinematic gait analysis during treadmill locomotion in healthy and neurotraumatic rats using a six-camera motion capture system. Also provided are details on 1) calibration of the system in an experimental set-up customized for quadrupedal locomotion, 2) data collection for treadmill locomotion in adult rats using markers positioned on all four limbs, 3) options available for video tracking and processing, and 4) basic 3D kinematic data generation and visualization and quantification of data using the built-in data collection software. Finally, it is suggested that the utility of this motion capture system be expanded to studying a variety of motor behaviors before and after neurotrauma.

INTRODUCTION:

In rodents, forelimb and hindlimb locomotor deficits after neurological disorders are commonly assessed using subjective scoring systems[1]. Automated systems[2-5] have been adopted for gait analysis but suffer from disadvantages, because the primary outcomes are based on footprint analysis and fail to capture crucial segmental and joint kinematic variables that can otherwise reveal true kinematics of limb movements[2]. Since most gait parameters are correlated, a

collection of gait parameters is needed to understand compensations adopted by rats to completely assess motor deficits.

In the past decade, several 3D motion analysis systems[6] have been developed for biomedical research in humans. These systems have been successful and proven to be effective in capturing deficits in locomotion in healthy human adults as well as altered kinematics of walking[6,7]. In rodents, currently available 3D kinematic motion systems adopt complex algorithms and modeling for the movement behavior and utilize sophisticated data analysis techniques[8-11], which eventually limit their versatility. Moreover, the methods used for collecting data with most 3D motion capture systems are not adequately explained in the literature. The details on data collection and analysis procedures, limitations, and techniques involved in effectively using the system are lacking.

Consequently, one of the common notions among researchers is that 3D motion tracking kinematic assessments are rather effortful and time-consuming procedures that need technical expertise and elaborate data analysis. The intent of this work is to break down the data collection and analysis protocols and describe the methodology through a step-by-step process so that it is objective, easy-to-learn, and can be systematically approached. Today, there is an emerging emphasis on assessing functional motor behavior in a more comprehensive and systematic manner following neurological injuries and interventions in preclinical studies.

In the realm of quadrupedal locomotion, presented here is the use of a 3D motion tracking system that can provide additional information such as body posture, paw rotation with respect to body axes, inter-relationships of joints, and more accurate information regarding coordination, all while concurrently visualizing the whole animal from all planes. This, in turn, can reveal critical differences in motor behavior within and between healthy and injured rats through multiple outcomes. With a more refined kinematic analysis that is accurate and objective, the risk of wrongly inferring effects of an intervention is minimized. The generated data from this motion capture software is visualized frame-by-frame for the quality of movement and can be automatically tracked, and data collection or quantification does not require any additional algorithms or modeling. The aim of this work is to provide methodological details and considerations involved in data collection and analysis of 3D gait kinematics during treadmill locomotion in healthy and spinal cord-injured rats. This protocol is intended for use by preclinical researchers who utilize neurological rat models in experiments.

PROTOCOL:

This study was carried out in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Stony Brook University Chancellor's Animal Research Committee.

1. Set-up of motion capture system

1.1. Set-up

1.1.1. Mount six cameras on the wall (or tripods) using finely adjustable geared heads. Position three cameras above on each side of the treadmill, with each camera angled 20°–45° below the horizon, approximately 2.0 m away from the treadmill and approximately 0.5 m away from adjacent cameras for maximum coverage of markers (**Figure 1**).

1.1.2. Equip each camera with a ring light for the visualization of retroreflective markers.

1.2. Start the motion capture system.

1.2.1. Under **Project | Specification**, define the desired markers for the experiment.

NOTE: For demonstration purposes, a total of 22 markers is used for both the forelimbs and hindlimbs (11 markers on each side) to assess bilateral quadrupedal locomotion. Alternatively, the **Import marker set with specific marker ID** option allows for preset calculations within the software.

2. Calibration of motion capture system

2.1. Capturing the calibration video

2.1.1. Place the L-shaped calibration frame (hereafter referred to as the "L-frame") orthogonally on the treadmill, with the long leg of the L-frame pointing in the rat's walking direction (**Figure 2**).

2.1.2. Open the motion capture software and select **Record** to capture the calibration video.

2.1.3. Move the trident shaped calibration frame (hereafter referred to as the "wand") throughout the treadmill area in space so as to cover all areas that the rat will be walking in.

NOTE: The L-frame contains four markers that establish the global coordinate system and the wand contains three markers that will calibrate the 3D walking space of the rat.

2.1.4. Record a minimum of 1 min of footage to ensure adequate wand data points are present for proper calibration at 120 frames/s.

2.1.5. Save the videos as 3D calibration files.

2.2. L-Frame tracking

2.2.1. Right click the camera group and select **3D tracking**. Under the drop-down window, select **3D calibration videos**, then **All calibration cameras**.

2.2.2. Using the fixed point function, track the L-frame origin, L-frame short, L-frame mid, and L-

frame long on each of the six calibration videos. Define all points and select the **Search Automatically** button.

NOTE: L-frame position must remain consistent relative to the treadmill during the whole experiment to prevent the coordinate system shifting.

2.3. Wand tracking

2.3.1. Right-click on the camera group and select **Automatic 3D-Wand Tracking**.

2.3.2. Select all cameras in the camera selection. On the bottom right of the window, select **Options**, de-select **Detect L frame,** and select **Start Tracking**.

2.3.3. After automatic tracking, select **Assign Wand Short, Wand Mid, and Wand Long markers** for all six cameras.

2.3.4. On the **3D tracking** window, select **Export to raw data**, then select the **Overwrite existing wand data** option to save the tracking.

2.3.5. Select **Yes** in the window that appears after the last step to save the most accurate data.

NOTE: This new tracking will be saved as **Tracking** under **Automatic tracking** tab in the left sidebar. Saved tracking can be accessed and edited later.

2.4. Calculating calibration

2.4.1. Right-click **Camera calibration groups** and select **New wand calibration group**.

2.4.2. Select **All** cameras and hold down the **Ctrl** (control) key while selecting **Ok**.

2.4.3. Change wand length to 100.00 mm, L-frame floor offset to 7.00 mm, iterations for outlier-detection to 4, and allowed wand length-deviation to 0.300.

2.4.4. Change the **Camera that the wand must be visible in** option to 4, and turn on the following: fix aspect ratio, fix skew parameter, and fix principal point (**Table 1**).

NOTE: These settings were experimentally determined to be optimum for this set-up.

2.4.5. Accept calibrations with standard deviation of wand length less than 3 mm and residual value of less than 0.004.

NOTE: If the cameras or associated equipment is moved beyond this point, one will be required to recalibrate the system.

3. Training and preparing the animal for treadmill locomotion

3.1. Training rats for treadmill locomotion

3.1.1. Acclimatize rats to the treadmill for 5 min prior to a training session[12].

3.1.2. Train the rats to walk with full weight bearing on their limbs at 13 cm/s for 15 min/session in the mornings for 1 week. Train all rats until they are capable of consistently walking (minimum of 10 continuous steps) on the treadmill[12].

3.1.3. Complete training sessions around the same time of the day for each rat.

3.2.1. Prior to data collection, anesthetize the rat by placing the rat in an incubation chamber.

3.2.2. Deliver Isoflurane gas (1.0%–2.5%) and 0.4 L of oxygen for approximately 5 min. Pinch the foot of the rat to check for the depth of anesthesia.

3.2.3. Proceed when the rat does not respond to the paw pinch (negative paw withdrawal reflex).

3.3.1. Shave the rat in regions where markers will be placed to avoid the fur from interfering with marker tracking (**Figure 1**).

3.3.2. Palpate the skin for the bony landmark to place the markers. Use pen markers for joints distal to the elbow and the knee (**Figure 3**).

NOTE: The retroreflective markers are plastic hemispheres 0.5 cm in diameter covered with retroreflective tape.

3.3.3. Place the markers as desired on bony landmarks prior to data collection (**Figure 3**).

NOTE: In healthy, non-neurotraumatic rats, the retroreflective markers placed over distal joints are often removed by rats. For demonstration purposes, retroreflective markers are placed over the proximal bony landmarks (iliac crest, hip, knee joint for the hindlimbs, shoulder and elbow joints for the forelimb), and pen markers are used for the more distal landmarks. In our hands, this has yielded reproducible results between and within rats (unpublished data).

4. Motion capture

4.1. Select the red camera button on the top bar of the motion capture software to record a trial. Designate the saving location on the computer and select **Begin recording** to record at 120 frames/s.

4.2. Set a user-defined treadmill speed and allow the rat to walk for approximately 30 s, or a

minimum of 10 continuous steps.

4.3. Stop the recording and check that the footage contains at least ~10 continuous steps before continuing.

4.4. Create a new camera group for each trial after saving the recorded video.

5. Motion tracking

5.1. Select the "**+**" sign adjacent to the saved camera group. This will display a list of all six cameras.

5.2. Assign 3D calibration to the camera file

5.2.1. Go to **Camera calibration group** and right-click on **Calculated calibration**. Select **Assign 3D camera parameters**. Assign the calibration file to the appropriate camera files in the saved camera group. Hover over the camera file to verify calibration (reads as **3D calibration valid**).

5.3. Individual camera 2D tracking

5.3.1. Right-click the camera group for motion tracking. Select **2D tracking**.

5.3.2. Select seven to ten best continuous and consistent steps for tracking. Record the frame number at the first contact of the reference limb on the treadmill for each step.

NOTE: For demonstration purposes, the left hind limb was selected as the reference limb.

5.4. Various tracking options are available.

5.4.1. Right click the marker of interest and select **Automatic Tracking**, which will detect bright circular spots created by retroreflective markers (**Figure 4**).

5.4.2. Alternatively, track markers using **Pattern matching**, which will use an algorithm built into the software to track markers based on size and color (**Figure 4**).

5.4.3. Manually track and correct undetectable markers or errors in tracking.

5.5. Use black markers when distal joints retroreflective markers are not possible.

5.5.1. Track black markers using advanced image processing by inverting black markers to bright spots for automatic tracking.

NOTE: The **Image processing** option can be used to help track markers that are difficult to detect or see.

5.5.2. Right-click on the rat video in the 2D tracking window. Select **Image processing**.

5.5.3. Select **Advanced view** and add a combination of the four filters (brightness, contrast, gamma) to make the black marker as dark as possible in comparison to its surroundings. Lastly, add **Invert,** and the black marker will become a bright marker that can be tracked automatically (**Figure 5**).

5.6. Marker placement correction

5.6.1. Manually track and correct undetectable markers or errors in tracking.

5.6.2. To manually track the video, select the desired marker on the right sidebar. Right-click and select **Manual tracking**. Begin tracking the selected marker on the rat video that appears frame-by-frame.

5.6.3. To rectify any errors, go to the frame where the tracking error occurred. Right-click on the specific marker tab in the right sidebar and click **Delete point**. Retrack the point manually at the accurate position.

5.7. Using the above method, complete 2D tracking for all cameras used for all desired markers in the frame range of continuous steps.

5.8 Click on **Save** throughout the tracking process.

6. Kinematic analysis

6.1. Phase assignments

6.1.1. Right-click **Phases** and select **Edit phase model**.

6.1.2. Customize gait cycle phases for each limb according to the deficits one chooses to study (e.g., addition of drag phases, toe curl phases, etc.) within the traditional stance and swing phases of a step cycle. Define the phases for the experiment (**Figure 6**).

NOTE: For demonstration purposes, three phases are shown here, and the left hindlimb is used as the reference limb for the seven to ten gait cycles.

6.1.3. Right-click the camera in the camera group and select **show video.**

6.1.4. Assign phases of the gait cycle for each limb within the software using the add phase button or F11 shortcut key.

6.1.5. Select the appropriate limb under analysis and designate the first frame of tracking as the

start of the **Stance phase**.

6.1.6. Progress the video to the frame where stance phase ends and swing phase starts. Designate this frame as the start of **Swing phase**.

6.1.7. Progress the video to the first frame where foot begins to descend. Designate this frame as the start of **Mid swing phase**.

NOTE: Herein, the stance phase of a step cycle for each limb is defined as the first frame in which the limb contacts the treadmill surface. Swing phase is defined as the first frame in which the limb leaves the treadmill surface. Mid swing phase is the frame where the limb achieved maximum clearance and begins to descend. A complete gait cycle is defined from the onset of initial stance to the stance phase assignment of the next gait cycle.

6.1.8. Repeat these steps until the limb phases assignments are complete for each step. Repeat for the other three limbs.

6.2. 3D coordinate calculations

6.2.1. Perform 3D calculations after tracking all six cameras.

6.2.2. Right click on the camera group and select **New 3D calculation**, click **All** for camera selection and then select **OK**.

NOTE: A new folder will appear. This folder contains all the 3D coordinate data for all the markers tracked. To view and/or edit phases, right-click on **3D coordinates** in the left sidebar and select **Edit Phases** (**Figure 7**).

6.2.3. Generate data of interest such as joint height or velocity diagrams with data points by dragging out marker of interest to view side by side with the assigned gait phases. (e.g., joint kinematics in **Figure 8**).

6.3. 3D diagram

6.3.1. Click **3D diagram** to generate a 3D figure of the trial.

6.4. Export raw data by right-clicking **3D coordinates/Export**.

6.4.1. Right-click the **3D coordinate file** and select **Export**.

6.4.2. Open the file in a spreadsheet software and import the data into MATLAB.

6.4.3. Create a program to create plots of limb step height coordination.

NOTE: 3D coordinate data can be exported into analysis softwares or custom defined macro scripts to generate more data, beyond what the software features offers.

REPRESENTATIVE RESULTS:

This protocol demonstrates a methodology for quantitative 3D kinematic data collection and analysis for treadmill locomotion in rodents using a simple, built-in software. The results show that the protocol is feasible in collecting and analyzing quadrupedal locomotion kinematics in healthy and spinal cord-injured rats. Researchers with rat handling experience must place markers on rats, then calibrate and use the motion capture system without any critical issues. Data are easily generated without the use of complex algorithms.

Here, the protocol was implemented in healthy and spinal cord injured (C5 right hemisection) rats. For the purpose of this manuscript, only representative results are shown. Overall, various kinematics of joint and limb segment movement were easily obtainable from the 3D coordinates of each marker. Critical differences between abnormal gait and healthy gait cycles were easily detected with multiple outcomes, including (but not limited to) step height measures, joint velocity, joint angle (**Figure 9**), step cycle phase durations for all four limbs, and limb coordination (**Figure 10**). Analysis of qualitative data in the form of plots and stick diagrams can guide determination of the quantitative tools used to implement for the final outcomes of this analysis approach (**Figure 11)**.

In a representative healthy rat, the elbow angle profile demonstrated smooth, single peaks with consistent adjacent gait cycles that displayed a complete range of motion (**Figure 9**). The alternating stance phase and swing phase durations of the traces suggested consistent intralimb coordination. In contrast, the elbow angle profile of a representative spinal cord injured rat demonstrated multiple distorted peaks, which were less consistent and of smaller ranges of motion. In addition to alterations in the lengthened stance phase and shortened swing phase durations, there was a deficiency in intralimb coordination for the RFL.

In the presented representative data plotted for coordination, it was found that coordination plots (**Figure 10**) generated from representative healthy rats showed well-defined, alternating rhythmic coordination in ipsilateral limbs during gait cycles (L-shaped pattern) and in-phase D-shaped pattern with contralateral limbs. In comparison, a representative spinal cord-injured (C5 right hemisection) rat showed poor non-alternating and non-rhythmic coordination in ipsilateral limbs and an unusual alternating rhythmic coordination (L-shaped pattern) in one of the contralateral limb pairings (**Figure 10**). Given the observable deficit in the right forelimb in the recorded motion video, this suggests that the RFL and LHL were unable to bear full weight without the support of either LFL or RHL at any given time. This trend suggests a compensatory mechanism to cope with the forced walking speed on a treadmill.

Quantifiable data were easily generated from use of the 3D system, but this involved accessing separate tabs and selecting from a multitude of options available within the software. There is current work on developing an automated template that will generate quantitative and

qualitative data into a single report for the most obvious outcomes of interest (without the need to individually generate different outcomes), as is routinely done with kinematics system utilized for clinical studies. Thus, several endpoints can be compiled and exported in a report format that can be easily visualized immediately after a trial.

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental set-up. (**A**) Schematic of a six-camera kinematic data collection set-up for a treadmill walking task. A set of three cameras is placed on both sides of the treadmill to capture (frame-by-frame) left and right marker motions during locomotion. (**B**) Diagram depicting marker placement over bony landmarks on the rodent's forelimbs and hindlimbs to capture quadrupedal locomotion kinematics. A total of 11 markers is placed on each side of the rat. The shaded regions show the area where the rat is shaved.

Figure 2: Markers for calibration. (**A**) Marker set assignments for the wand calibration system, using two calibration frames: L-Frame and wand (**B**) The coordinate system is defined by the L-Frame, where the L-Frame origin (intersection of two legs of the frame) is defined as (0,0). The two limbs of the L-Frame, L-Frame short, and L-Frame long define the x- and y-axes, respectively, and the z-axis is defined as perpendicular to the XY plane.

Figure 3: Marker types. (**A**) Photograph of the rat's lower trunk showing the two types of markers used. (**B**) Photograph demonstrates a retroreflective marker placed on the iliac crest with double-sided tape that is designed to adhere to the skin (circled red). (**C**) Photograph demonstrates the placement of a pen marker over the right metatarsophalangeal joint (circled red).

Figure 4: Motion tracking. (**A**) Image shows the motion tracking interface where multiple markers can be tracked simultaneously using the "Track automatically" and "Track using pattern matching" functions. (**B**) Magnified view of a retroreflective marker is detected as a bright white circular spot in the "Track automatically" function during marker assignment. The software recognizes this spot as a bright blue circular spot. The red dot is eventually recognized as the center of the prescribed marker. A centered red spot within the circular marker decreases the chances of experimental errors during data tracking. Deviation from the center suggests that subsequent measurement and analyses are likely to be inaccurate. (**C**) Magnified view of a retroreflective marker selected for pattern matching. Based on the size, shape, and color of the selected marker, the software automatically identifies markers matching the description in the subsequent video frames.

Figure 5: Basic and advanced tracking options. (**A**) Videos can be processed within the software by right clicking the video during analysis so that unclear or blurry markers are clearly visualized to allow automatic tracking. For demonstration purposes, two types of image processing settings are shown that are adjusted to accommodate different ambient lighting conditions during data collection for easy tracking. (**B**) A representative video frame prior to image processing. (**C**) For basic image processing (processing type I), brightness, and contrast settings

are adjusted for a clearer view. (**D**) Using the advanced image processing settings (processing type II), the right metatarsophalangeal joint marker (black marker) is inverted and can then be tracked automatically.

Figure 6: Phase assignments. Gait cycle for each limb can be divided into discrete phases according to the experimental design. For demonstration purposes, three gait cycle phases are shown. (**A**) Stance phase is defined as the first frame in which the limb contacts the treadmill surface. (**B**) Swing phase is defined as the first frame in which the limb leaves the treadmill surface (**C**) Mid swing phase is the first frame after limb clearance where the paw begins to descend. In (**D**), a complete gait cycle is defined from the onset of the initial stance to the stance phase assignment of the next gait cycle.

Figure 7: Advanced options for step phase analysis. This option allows an in-depth view of tracking and phase assignments, as well as the ability to alter phase assignments. (**A**) Marker selection box to view and select the desired marker. (**B**) Coordinate selection window: highlighting the coordinate of interest (in this case, z-coordinates) will show up as red in the main diagram window. (**C**) Phase selection window: assigned phases for a limb can be viewed with respect to markers and coordinates selected in (A) and (B). Phases can also be edited through this window. (**D**) Diagram window: coordinates for a specific marker can be compared simultaneously during individual gait cycle phases. The green and yellow represent the stance and swing phases respectively for the right hindlimb during quadrupedal locomotion.

Figure 8: Sample of 3D kinematic data. (**A**) Various outcomes from each joint marker can be pictorially visualized from the 3D coordinate dataset after video tracking (**B**) Representative data generated for the stance and swing phase cycles for each forelimb and hindlimb during quadrupedal walking in a rat. Colors represent stance and swing phases of consecutive step cycles. Red and green correspond to the right forelimb and hindlimb stance phases respectively. Blue and teal correspond to the left forelimb and hindlimb stance phases respectively. Yellow corresponds to the swing phase of each limb. (**C**) Multiple data groups (discrete markers or outcomes) can be simultaneously compared with ease. The z-coordinate velocity data of the left and right knee joint markers are arbitrarily selected to demonstrate the vertical velocity of the knee joint marker from the treadmill surface.

Figure 9: Representative joint angular kinematics data from healthy and cervical spinal cord-injured rats during treadmill locomotion. (**A**) Elbow joint angle profile in a healthy representative rat demonstrates smooth, single peaks of joint angle traces with consistent adjacent gait cycles that display complete range of motion. Red and yellow bars denote the stance and swing phases respectively of the forelimb step cycle. (**B**) In contrast, the traces in a representative spinal cord injured rat are relatively more distorted and show inconsistent multiple peaks with an overall smaller range of joint motion.

Figure 10: Representative limb coordination data from a healthy and cervical spinal cord injured rat during treadmill locomotion. (**A**) The z-coordinate values of the metacarpophalangeal (MCP) and metatarsophalangeal (MTP) markers depicting step height

measures are plotted in six different combinations between limbs from approximately 10 steps during quadrupedal walking. Shown are representative demonstration of all the six possible limb coordination pairs. (**B**) A healthy rat shows clear alternating rhythmic coordination (L-shaped pattern) for all pairs (i, ii, iii, iv). When limbs are in phase (v, vi), the coordination pairs follow a D-shaped pattern. (**C**) In a cervical spinal cord injured rat, note (i) the poor coordination between the two forelimbs, (iii) right ipsilateral pairing and (iv) unusual coordination for one of the contralateral limb pairs. Note that coordination plots (B,C) do not share the same scale.

Figure 11: Animated 3D stick figure. Example of a 3D stick figure generated from tracked data.

Table 1: Advanced settings for calibration. The table summarizes the parameters we used to accurately calibrate the six-camera set-up. These settings were experimentally tested and found optimal for our set-up.

DISCUSSION:

This protocol article demonstrates the use of a motion tracking system for the collection and analysis of 3D kinematic data during quadrupedal treadmill locomotion in rodents. Important features of the 3D kinematic motion analysis system include detailed quantification of kinematics of joint movement (step phase kinematics, joint angles, range of motion, step velocities) from multiple joints and limbs simultaneously, detection of motor deficits unmeasurable by the naked eye, elimination of subjective bias in data collection and analysis, and easy visualization of the whole limb and postural kinematics that can be compared with simultaneous juxtaposition of the entire rat in motion. Without the need for added algorithms and modeling, the methods show the software's capability to quantitatively analyze kinematics of gait with great detail, efficiency, ease, precision, and reproducibility.

In rodents, forelimb and hindlimb locomotor deficits after a spinal cord injury (SCI) are commonly assessed using subjective and standardized locomotor scoring systems such as the BBB scoring system[1]. Subjective scoring systems generally introduce tester bias because different researchers may assign different scores for the same motor deficit or same score for different motor deficits, resulting in reduced reproducibility and sensitivity of the test[13]. Moreover, the inability to detect subtle deficits wears the risk of wrongly inferring on effects of an intervention.

To combat these problems, automated systems[2-5] and systems with or without the use of complex algorithms[14,15] have been adopted by some investigators. These tests perform step cycle analysis that reveal weight bearing profiles and step sequence patterns derived from paw placement movements of rats walking across a runway. However, a major disadvantage of ventral plane video gait analysis is that the subject's body itself is not directly visible[2,3]. These data become limited to information obtained from impressions of feet and paw movements, questioning the selection and interpretation of gait parameters in assessing deficits (see Chen et al.[16]). Details of movement that reveal dynamic segmental kinematics of limb movement and positioning are not accurately obtainable for locomotion analysis[3,5]. Critical deviations of joint

angle data (i.e., range of motions, velocity of movements, etc.), relationship of joints with respect to each other within a limb and between limbs, and underlying body mechanics that alter gait patterns are impossible to obtain. As such, whether any observable gait impairments are consequent to alterations in single and/or multiple segmental joint movements (i.e., proximal-distal intralimb coordination, postural relationships of the trunk with respect to the position and gait cycle of the limbs, etc.) remain masked.

Few currently available systems capture gait kinematics and assess motor dysfunction qualitatively and quantitatively but are less widely used. A full-body high speed video-tracking gait analysis system films spontaneous gait cycles from three sides (one ventral and two lateral planes) and track bony landmarks to output a number of gait outcomes[17,18]. Sagittal plane kinematic gait analysis is employed by some researchers to obtain 2D motion data of the interested hindlimb[19,20]. However, a third dimension of movement that occurs orthogonal to the viewing plane (lateral or sagittal) is undetectable in the analyses[11,18,19].

Other more sophisticated, 3D, three body-segment rodent body postural data collection systems utilize 3D mathematical modeling of data collection as well as analysis system to track and quantify the 3D motion of the rodent's body-segments while including head motion patterns[8]. Madete et al.[9] have developed a marker based optoelectronic motion capture system to quantify 3D body postural kinematics during overground locomotion on walking beams using a seven camera system. The main outcomes studied in the latter two works primarily focus on the overall posture of the rodent rather than gait analysis. 3D motion capture systems provide high throughput 3D kinematic gait data using multiple cameras and elaborate software systems, as reviewed by Bhimani et al.[21]. Older versions of the presented 3D motion analysis system have also been used in previous work in rats with and without neurotrauma[12,22,23].

Despite the availability of 3D motion collection and analysis systems for research, preclinical use of this technique in rodents remains relatively limited. Part of the reason for this problem is that the data collection and analysis protocols rely heavily on the use of building kinematic models and complicated algorithms that fit a kinematic model of the rat's hindlimb during walking to generate fine, high-resolution gait kinematics[8,9,11,22]. The detailed methodology presented here provides details of the procedure involved throughout the experimental process including animal handling, training, experimental set-up, data collection, and analysis steps.

Also provided are details on calibration of the system, which is the fundamental part of the protocol, that will assure reproducibility between adjacent trials within and between subjects. The described step-by-step techniques introduce objectivity into the data collection procedure and make it highly reproducible. The generated data from this motion capture software can be visualized frame-by-frame for quality of movement and automatically tracked. Further described is how this data collection or quantification does not require any additional algorithms or modeling. Students, staff, and researchers can utilize simple statistical software to generate basic kinematic output without reliance on specific technical expertise.

This system can also be used for overground locomotion, reaching and grasping, and other experimental set-ups to suit the experimental goal. The number and type of markers can also be adjusted for the tail, back, trunk, or ears, as needed. A bigger advantage of the presented software in comparison to systems is its ability to collect high-resolution video data of the subject. As such, complex sets of calculations (i.e., angular motions, stick lines connecting multiple joints, etc.) can be superimposed onto the recorded video. Marker placement and the generated 3D data can be verified with actual movements of a rat in motion. In contrast, with the other 3D motion capture system, only the markers are captured, and any re-analysis must be done on the stick diagrams (skeletal framework) instead of the video of the actual subject. Consequently, verification of marker placement on actual subject movement is lacking.

Based on experience with this system, calibration plays a crucial role in the success of data collection. The calibration of the system is highly sensitive to change. and slight movement of any one camera can compromise the entire 3D coordinate data collection and analysis process. Only two cameras on each side of a viewing plane are required for data collection, but the third camera is highly recommended to provide more accuracy by cross-referencing the locations of each marker with other cameras. As the number of tracking cameras increase, the accuracy of the 3D coordinate for a specific marker will also increase. During occasions in which markers become obscured due to gait deficits (such as toe curling or drag in the case of gait after neurotrauma), these conditions may demand extensive manual tracking. Nevertheless, the amount of data eventually generated from the tracking is worthy of the time invested in manually tracking the markers, making it an invaluable tool in detecting subtle motor deficits.

In our experience, any tediousness associated with use of the system lies beyond use of the equipment and technology itself. Similar to other protocols for assessment of motor behaviors, the method with which rats are handled and trained for the task greatly affects outcomes. For example, isolating rats from their cohort is critical during testing; otherwise, rats that are not tested but are still present during testing show eventual deterioration of task performance. Optimal room temperatures, lighting, and noise levels are other determinants. Fouad et al. published other challenges that accompany functional motor testing in rats[24]. Indeed, blinded users from this laboratory who followed the methodology correctly did not experience any major hurdles with data collection, motion tracking, and data analysis.

In this paper, a 3D motion capture system to collect and analyze locomotor data effectively is described so that researchers can gather enormous amounts of in-depth locomotor data quickly from multiple rats. We are currently working on creating an automated data analysis template that can be built into the software and become capable of generating a report of pre-determined outcomes within few seconds for treadmill locomotion in rodents, similar to what is done in human studies using motion capture and analysis systems[6,25]. The development of this template will permit preclinical researchers to obtain detailed rodent locomotor data at the convenience of few clicks of a mouse button. It is hoped that the methods provided in this work will prove useful to preclinical researchers to assess rodent motor behavior more objectively. We are now finessing the use of this system to collect high-throughput 3D kinematic data

during common, skilled forelimb behaviors such as reaching and grasping. Importantly, the usefulness of this method can be expanded to rats with a variety of neurotraumatic and non-neurotraumatic injuries.

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DISCLOSURES:

The authors have nothing to disclose.

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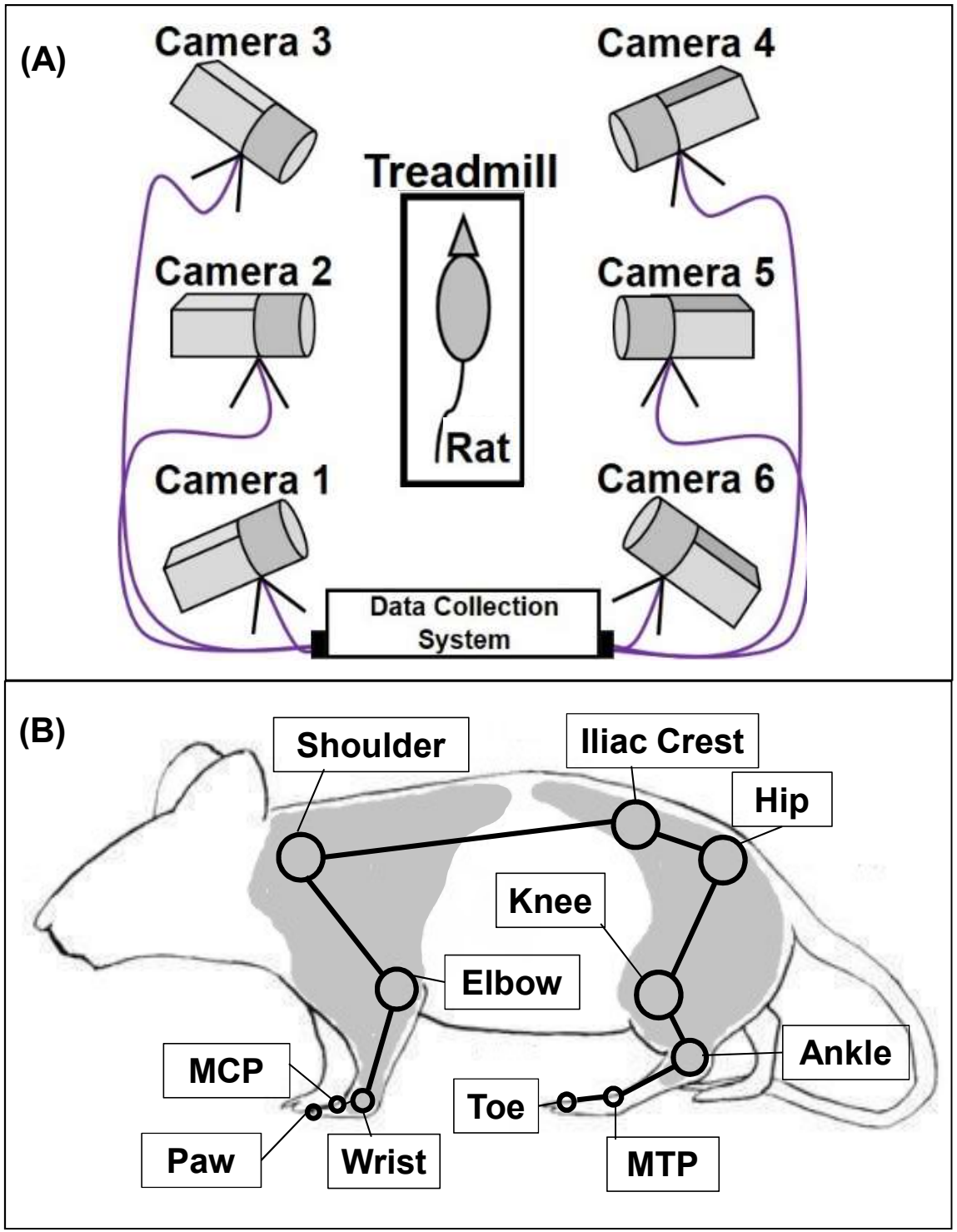

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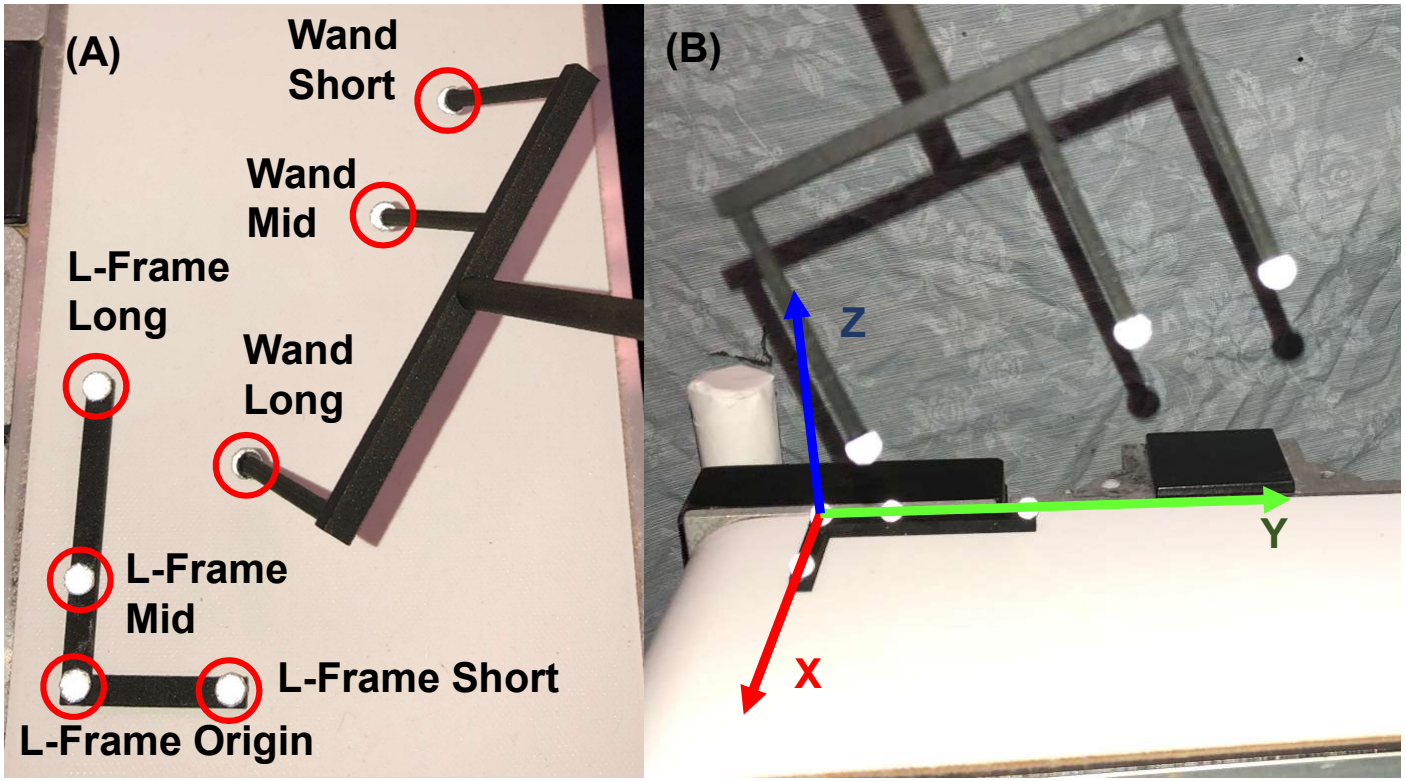

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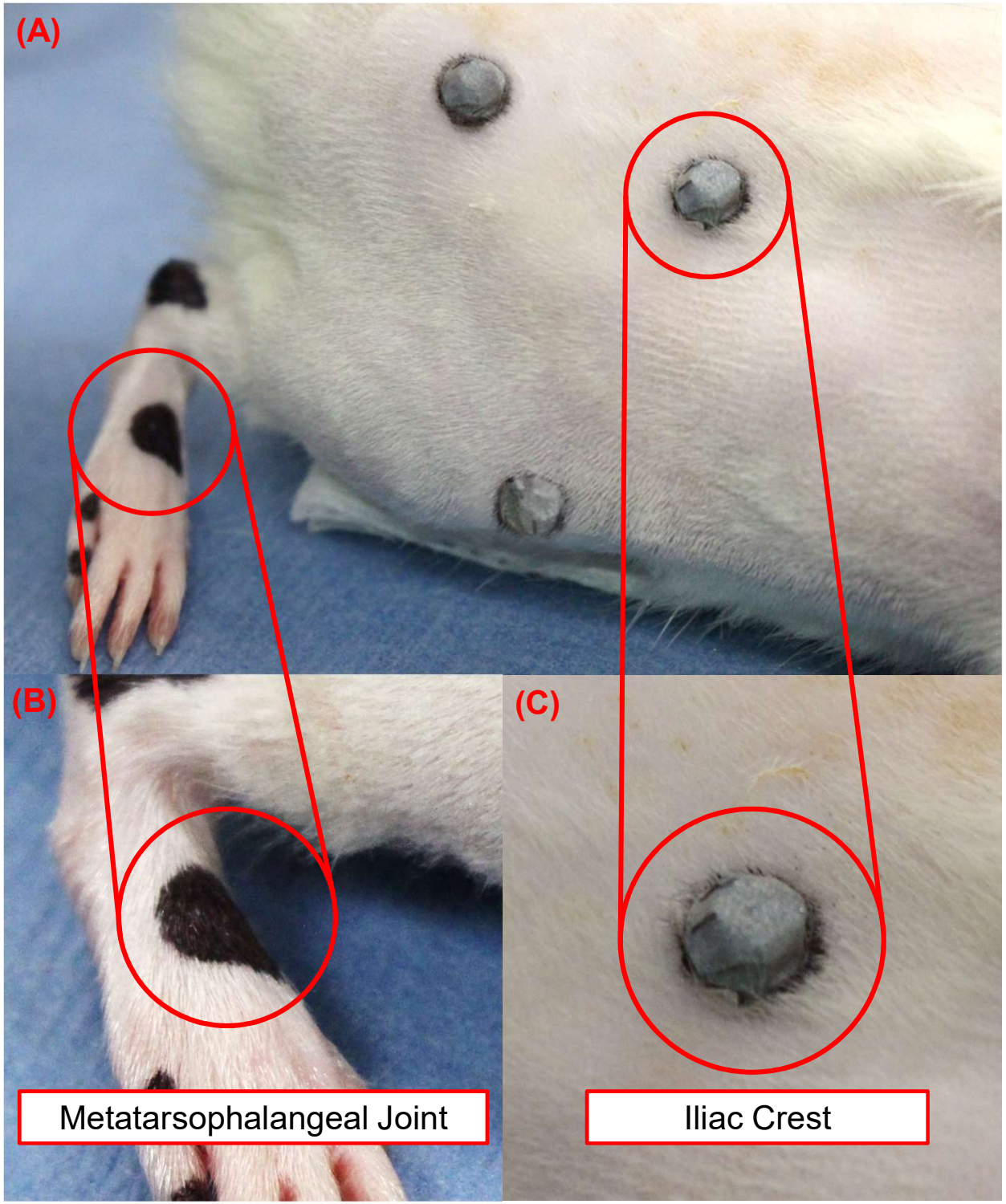

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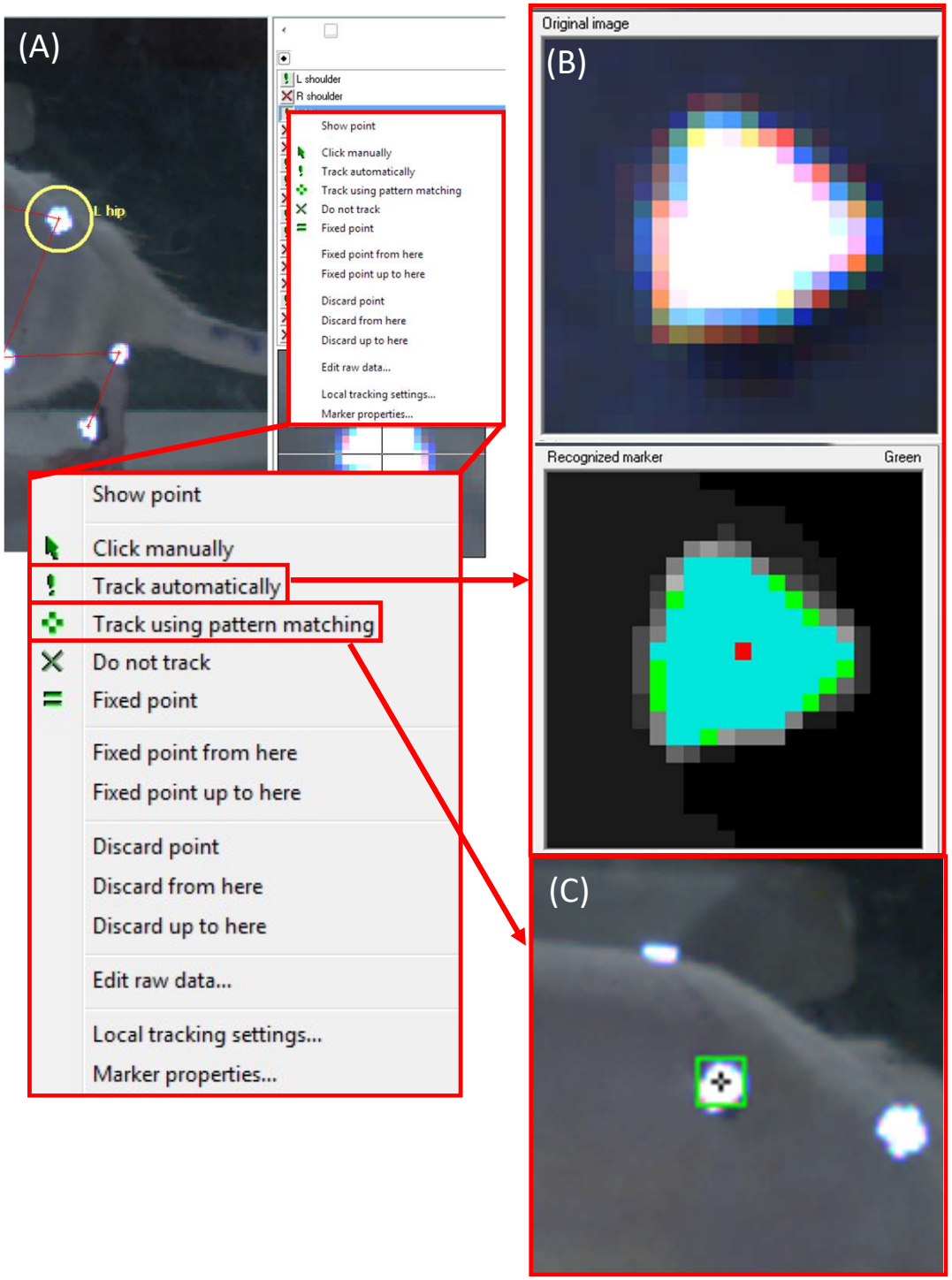

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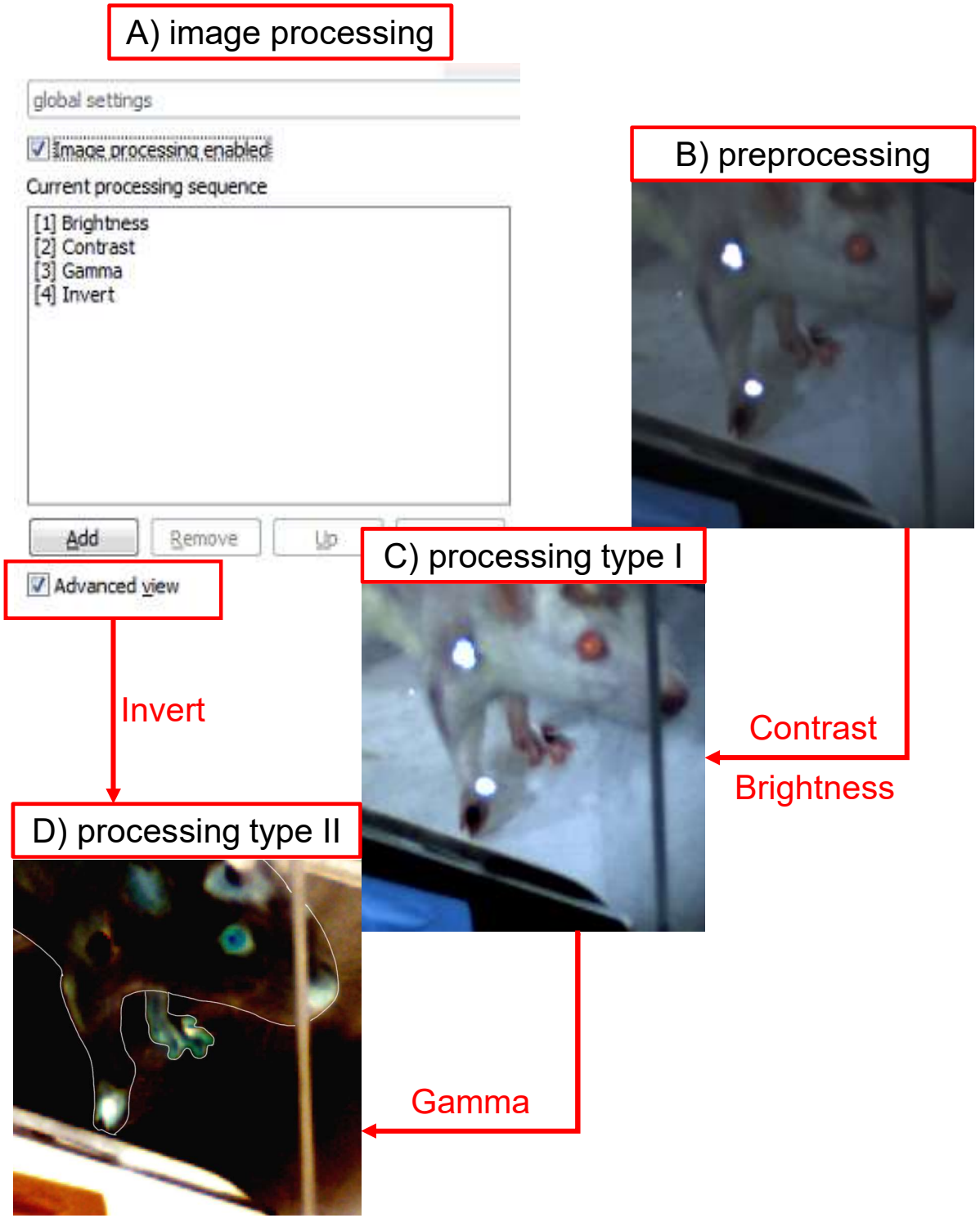

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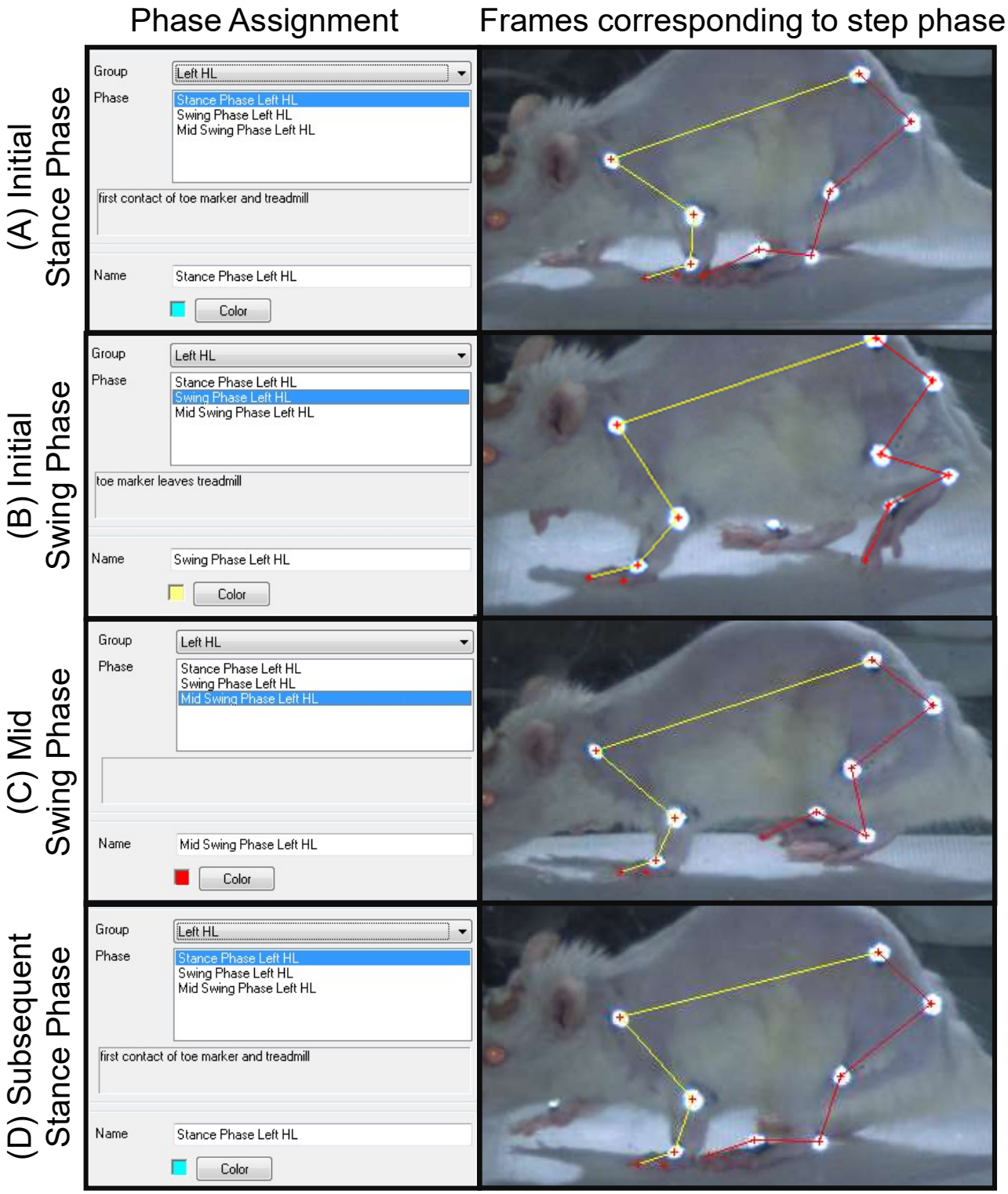

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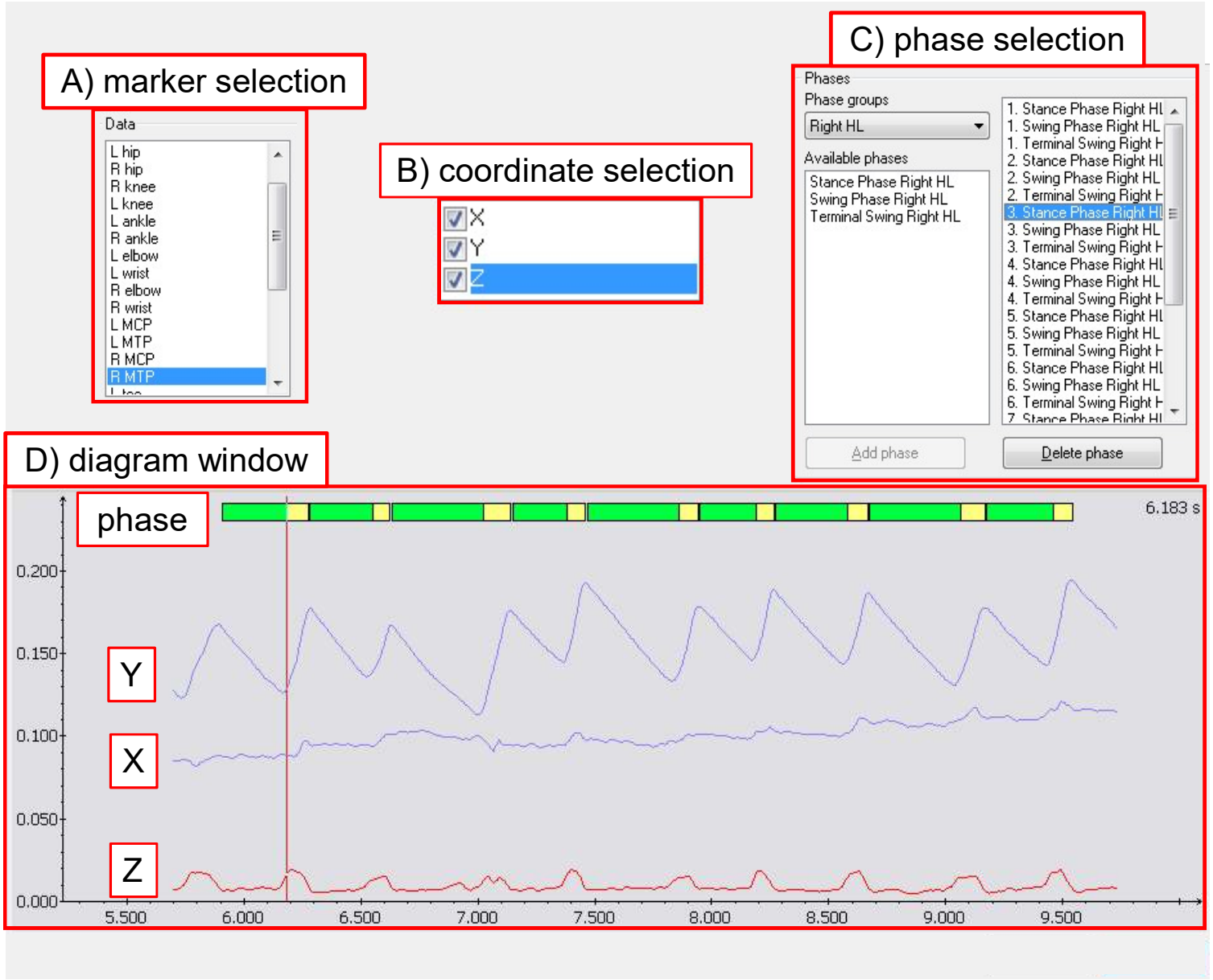

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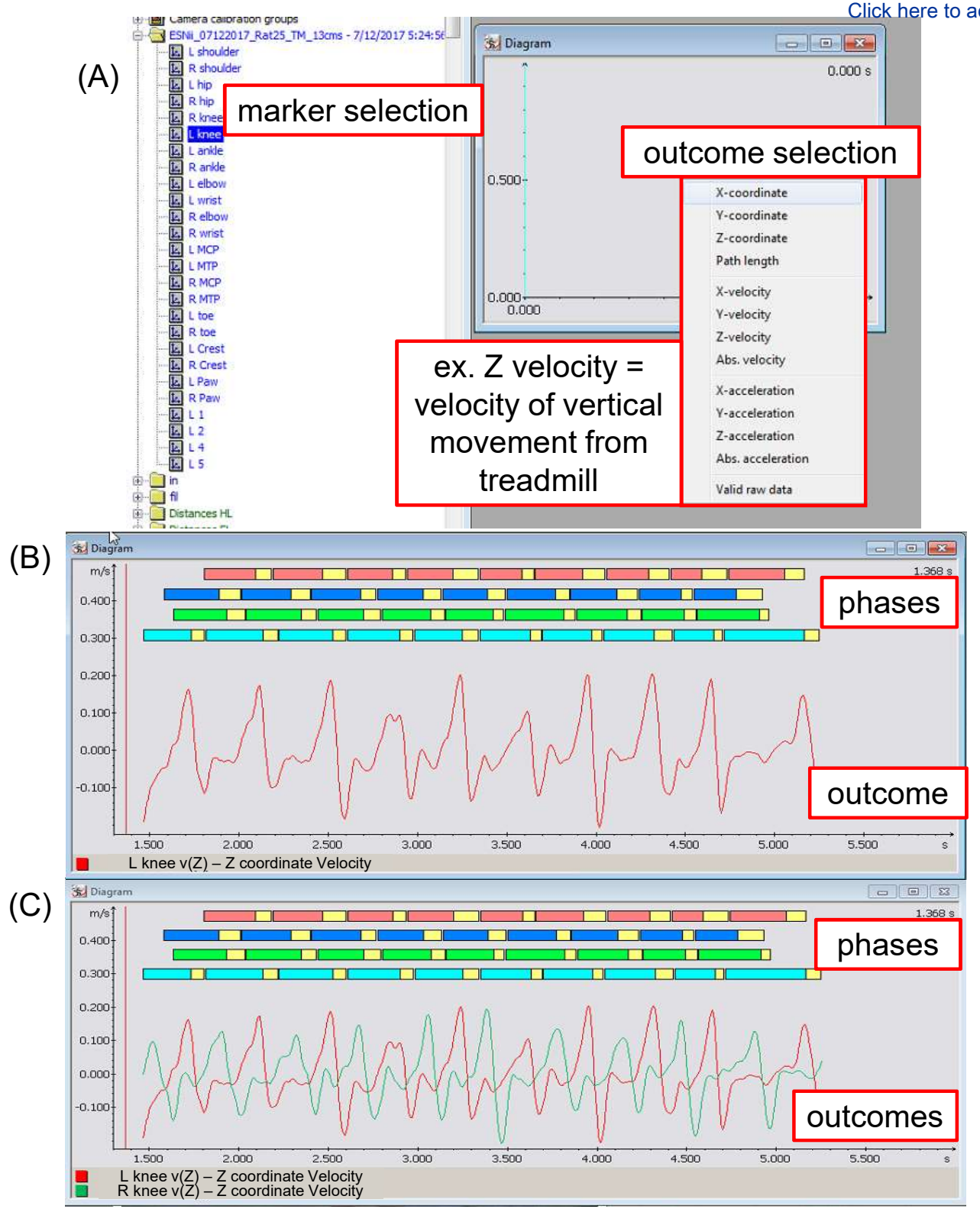

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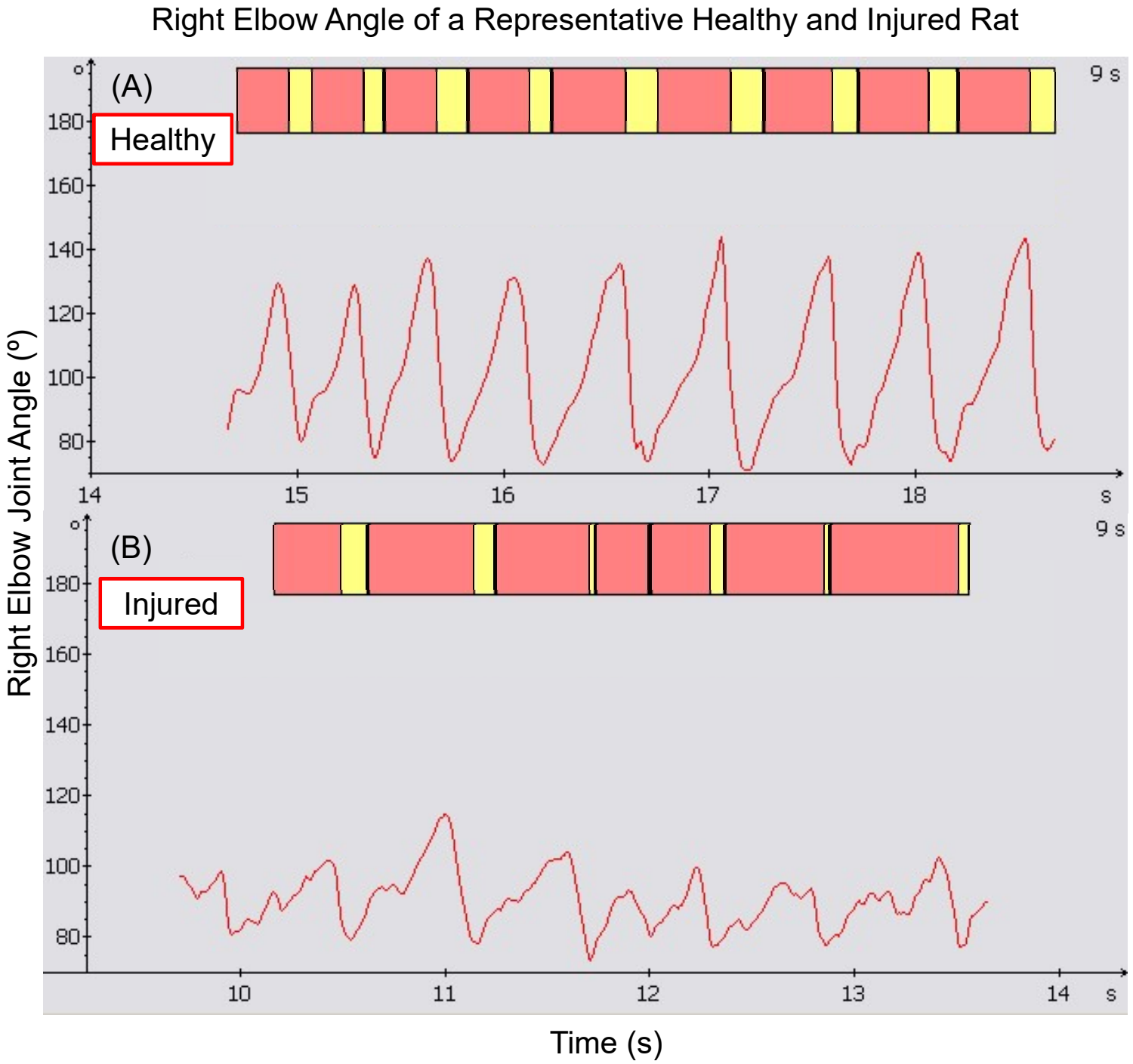

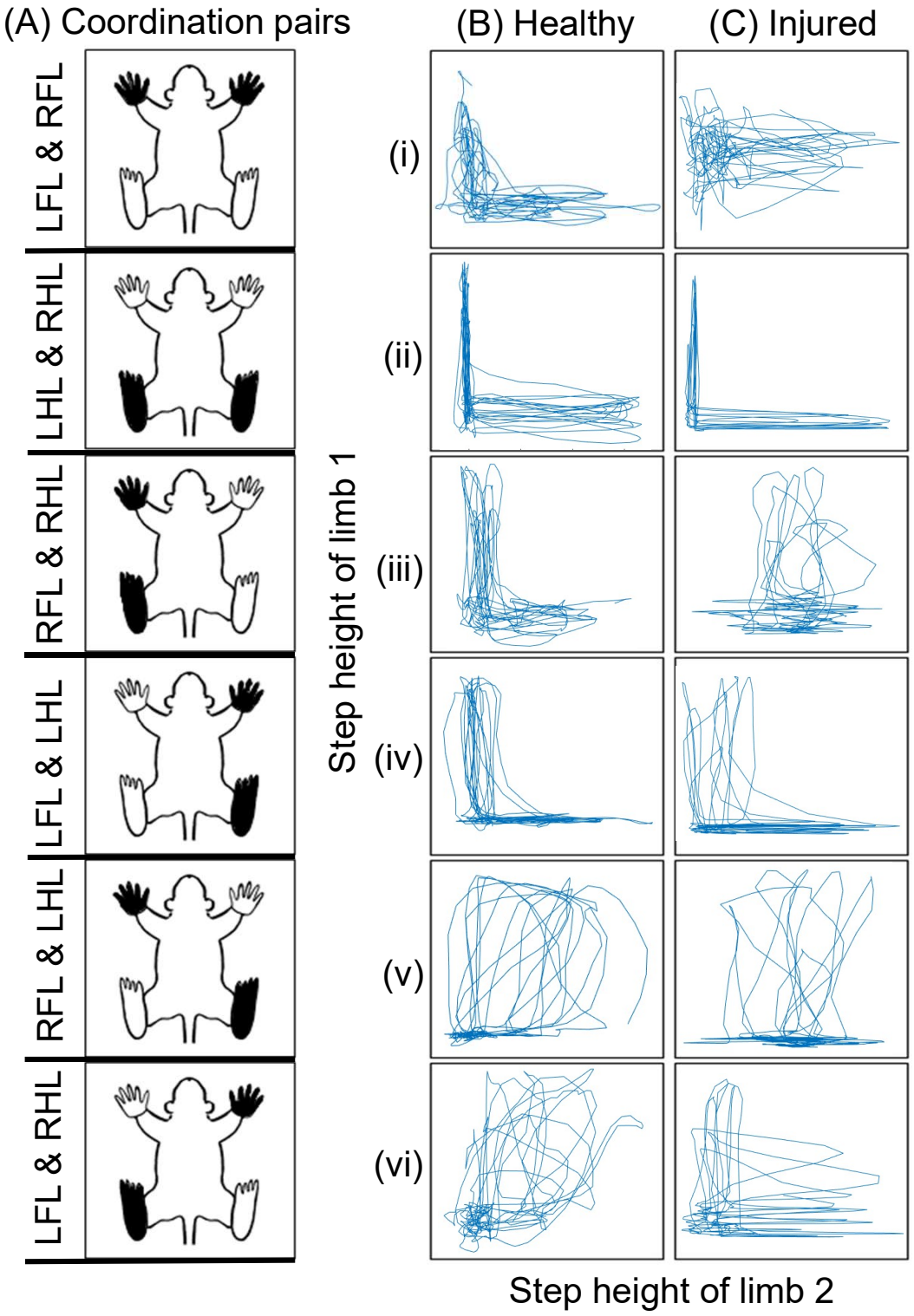

Animated Figure 11

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Video or Animated Figure

Figure 11 Animated Figure.mp4

Calibration Parameter	Calibration Inputs
Wand Length [mm]	100
L-Frame Floor offset [mm]	7
Iterations for outlier-detection	4
Allowed wand-length-deviation	0.3
Wand must be visible in at least __ cameras	4
Fix aspect ratio	Checked
Fix skew parameter	Checked
Fix principal point	Checked

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
6 camera Basler (Scout scA640-120gu) motion capture system.	Simi Reality Motion Systems GmbH Max-Planck-Str. 11 85716 Unterschleibheim, Germany	N/A	Recording device for motion analytics.
Calibration Frame and Wand	Simi Reality Motion Systems GmbH Max-Planck-Str. 11 85716 Unterschleibheim, Germany	N/A	L-shaped calibration defining the global coordinate sysetem, and the
Markers	Shah Lab	N/A	Recording device for motion analytics. Markers are Custom made in o
Matlab	Mathworks, Inc, Natic, Ca	N/A	Data analysis software

Rodent Cage	Custom Made within Stony Brook.	N/A	Clear plexiglass cage used to keep the rodent on the treadmill.
Simi Reality Motion Systems	Simi Reality Motion Systems GmbH Max-Planck-Str. 11 85716 Unterschleibheim, Germany	N/A	3D tracking Software.
Treadmill	Mk Automation Inc., Bloomfield, CT 06002	N/A	Treadmill used for rodent locomotion.

trident shape wand (100 mm)
ur lab from 0.5cm googly-eyes covered with reflective tape.

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1. **(3) Email address for each author has been provided on Page 1 of the main narrative draft in lines 17-18.**
2. **(4) Short Abstract has been rephrased to include "Here, we present a protocol to …" in lines 27-28.**
3. **(5) The introduction has been revised to include all of the following:**
    a. **This has been included in line 82 – 85.**
    b. **This has been included in line 59 – 69.**
    c. **This has been included in line 59 – 69.**
    d. **This has been included in lines 69 – 71.**
    e. **This has been included in lines 85 – 86.**
4. **(11) The step 1.1.2 (now 3.1.2) refers to the training process that was involved in the protocol, which was done without markers attached to the rats.**
5. **(12) (13) The protocol scripts are not special codes, but basic mathematical scripts that can be created in Excel or MATLAB and can be done by a beginner.**
6. **(16) All figures were original. No figures were reused from a previous publication.**

Reviewers' comments:

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Reviewer #1:

3D Kinematic Gait Analysis for Pre-Clinical Studies in Rodents - JoVE59612

Manuscript Summary:

The aim of the paper is to provide a methodology for 3D kinematic gait analysis. The authors claim that 3D kinematic is more powerful than other existing methods to evaluate locomotor deficits in rodent, but it needs complex algorithms for data analysis. The paper combines protocol for limb motion recording as well as data analysis with a new built-in software. It describes the animal training and preparation, then the motion capture system and the calibration procedure, and finally motion tracking and analysis by means of the software. The description of the successive steps is adequate and all steps seem to be listed in the procedure. However, it is quite difficult to evaluate the use of the software without having it. The title and abstract are appropriate. To my opinion, this paper is suitable for publication. However, the study could be more persuasive if the authors consider the following remarks.

We thank Reviewer 1 for his/her valuable comments and are glad that he/she thinks that this work is suitable for publication. We address below concerns raised.

Minor Concerns:

Markers. The authors should provide more details about markers. They look like half-spheres on Fig. 3. Is the shape important? Which diameter is recommended? References are not clear on the Table, I did not find such marker on the Simi's website. For pre/post analysis, it is recommended to tattoo the anatomic

landmarks position with permanent ink in order to position the markers at the same place on the successive recording sessions (precise joint localization can be done under light anesthesia).

We thank the reviewer for pointing out this detail. The material section in Table 1 has been edited to provide more details on markers used in this work. In Fig. 3, the markers used are plastic hemispheres (0.5 cm diameter) that are covered with retroreflective tape. This is now included in the protocol (Lines 200-201). We agree with the Reviewer that use of tattoos can be more reliable, but in our hands, this has proven to be a rather ineffective procedure (especially after a spinal cord injury, where rats seem to chew on the tattooed area and cause severe skin scabbing).

Cameras. The position is unclear. Are really camera below the treadmill or slightly above (I assume it is above)? Are cameras at the same height? With my own system, the light emitted by cameras often generates reflections on the plexiglass cage. Do the authors have the same problem and if applicable, how do they process? Sampling rate should be mentioned.

All cameras are slightly above the treadmill. Cameras are not at the same height, and they are not required to be at the same height. As long as the cameras are not moved post-calibration, the software will be able to provide accurate 3D data. The light emitted by cameras does generate reflections on the plexiglass cage. However, these reflections and obscurity of markers are overcome with cleaning of the plexiglass with alcohol and adjustments of camera and room lights. The sampling rate of the camera was 120 frames per second and has been included in the protocol (lines 128-129 and 215-216).

What is the area covered by cameras? Is it possible to study rats walking on a beam, on a runway on even on a ladder? "The system can be expanded to behaviors including reaching and grasping function". Is the system sensitive enough for grasping? Can very tiny movements be detected with accuracy? Although video shows a rat walking on a treadmill, the title suggests that the protocol is applicable in rodents. Do the authors have tested the protocol in mice?

The area covered by the cameras depends on how cameras are positioned and can be adjusted to various experimental setups. It is possible to study rats walking on a beam, on a runway and a ladder. This system has been used in humans and is capable of covering a large area. However, the limitation is the marker size and camera resolution. If the field is large (>~3meters) and the markers very small, the cameras do not adequately detect the retroreflective markers.

We are currently using the same software and the set-up has been modified for reaching and grasping function, with indications that the system is sensitive enough for grasping where small movements can be detected with accuracy. This methodology and its findings will be presented in a future manuscript. However, we have not tested this protocol in mice with great detail, but have preliminary evidence that the change in the setup is minor, limited to change in marker size and camera zoom levels.

Software. What are the benefits of the new software with respect to others, such as Vicon? Can the present software normalize step duration, to provide statistical analysis?

Note that a bigger advantage of the presented software in comparison to systems such as the VICON is the ability to collect high resolution video data of the subject. As such, complex sets of calculations (such

as angular motions, stick lines connecting multiple joints, etc) can be superimposed onto the recorded video. Marker placement and the generated 3D data can be verified with actual movements of a rat in motion. In contrast, with the VICON system, only the markers are captured and any re-analysis must be done on the stick diagrams (skeletal framework) instead of the actual subject. Consequently, verification of marker placement on actual subject movement is lacking.

These advantages also stand out in comparison to other commercially available systems such as the CatWalk, which is based on footprint analysis and fails to capture crucial segmental and joint kinematics.

This is now better clarified in the text (Lines 555-561).

Reviewer #2:

Major Concerns:

This manuscript explains about the 3D motion capture system for rodent gait analysis. The paper is generally well written. However, I do not see any innovativeness of the present study. The methodologies used here such as marker tracking, calibration and 3d visualization are all already-existing, and have been incorporated in conventional commercial and non-commercial motion capture systems. Motion capture system for human gait analyses can surely be used for smaller animals like rodents! What is the efficacy of the present protocol compared to the conventional systems that are also "simple, user-friendly, largely automated, efficient and reproducible" and "objective"? This should be better explained in the manuscript.

We do not disagree with this Reviewer that 3D motion capture systems for rodents already exists. We however would like to emphasize that the methods employed in collecting data with use of most 3D motion capture systems are not adequately explained in the literature. The details of the data collection and analysis procedures, limitations and techniques involved to most effectively use the system are not spelled out either. As an example, we spent 3-4 months finessing the technique presented and the presented protocol can help decrease time and resources that other labs can expend in attempting to figure out how the system functions even though it is commercially available. Our intention of this protocol's paper therefore is to provide details of methods so that many more pre-clinical researchers will use 3D motion capture in their labs for more objective assessments of rodent locomotor behavior (beyond the use of subjective assessment scales). See Lines 555 to 561 in Discussion.

Moreover, this protocol is a preamble to publishing an 'automatic data report' for rat locomotion that will contain more than 40 outcome measures within few seconds after a video is tracked. Today, such reports are possible with Human motion analysis systems, but are currently lacking in pre-clinical studies. Refer to the lines 606-609.

General advantages of this system over other systems are laid out in Lines 507-513; and Lines 551-576.

Reviewer #3:

C1: The authors summarize different approaches to analyze the locomotion in humans and rodents which is very interesting and important. Nevertheless, it is very advisable to improve the gap between the bench and the clinical situation. In order to avoid this break, further data and analysis about this "jump" are mandatory during the research article.

We agree with the Reviewer that motion analysis systems used in human and preclinical studies need to be matched. We think that this paper is the first step towards this goal. Once this protocol is approved in

the form of a manuscript, it is our hope that researchers will use this to generate data and compare/contrast with what is reported in the human literature.

C2: Why did you choose this time? In this reviewer opinion´s is a very little time. Did you acclimatize the rats in the room before the experiment?

In our hands, this time is adequate to get rats to begin to step on the treadmill. Our previous works support our existing experiments (Shah et al. 2012, Shah et al 2013)

C3: What time did you choose morning or afternoon? Please, justify this selection.

We trained our rats in the mornings when they are relatively more active. We now mention this in the protocol (Line 189).

C4: Please, justify why one minute will ensure proper calibration.

We thank the Reviewer for pointing this out. The protocol has been edited to justify the one minute of calibration (Line 128-129).

C5: Do the authors mean during the whole experiment, during a single run, etc. Please specify.

We thank the Reviewer for pointing this out. The protocol has been edited to clarify the statement (Lines 141-142).

C6: Nowadays several methods and techniques are feasible in detecting deficiencies in quadrupedal locomotion. Nevertheless, the technique described here is a powerful tool to do that. Please, describe in deep details the advantages of your technique.

We thank the Reviewer for this comment and agree with him/her that this technique is powerful. The benefits of this software include being able to perform complex sets of calculations that can be visualized on the recorded video in the form of a stick diagram. This software is capable of displaying the rat and allows users to retrack the markers. In other systems, users are stuck with the marker placement and unable to correct any errors in marker placement. In addition, this system allows users to better visualize the overall walking behavior by having the image of the rodent superimposed or juxtaposed to the generated data. This software is also very versatile, where it could be used for multiple experimental setups (example: beam, overground walking, reaching-grasping behavior, etc.).

C7: A brief description of several parameters is not enough to point out this technique as a useful 3d technique. It will very useful if the authors summarize and quantify al the data in a matrix. It has been done in several research articles.

We thank the Reviewer for this point. However, we believe that a matrix of all the data is beyond the scope of this manuscript. The main goal is the protocol itself. However, in support of the Reviewer's comment, we are currently working on creating an 'automatic data report' for rat locomotion that will contain more than 40 outcome measures within few seconds after a video is tracked. This protocol serves as the preamble to publishing this template of data analysis in rats. Please see Figure 1 below, which is a snapshot of a very small portion of the template, created exclusively for this Reviewer.

C8: In this reviewer´s opinion will be much more useful if the authors elaborate a separate section about future lines of 3D kinematic research.

We thank the Reviewer for this comment. We highlight in Lines 606-611 in the Discussions section that we are attempting to establish a template where researchers can generate detailed rodent locomotor data (figure 1) within a few clicks. In addition, we are also finessing this system to collect high throughput 3D kinematic data during common skilled forelimb behaviors such as reaching and grasping.

C9: How? please include more details on the article.

We thank the Reviewer for this point. More details have been included in the figure legends. Please refer to lines 429 – 430.

C10: The lack of standardization is one of the biggest problems in kinematic analysis. Since you achieve a 3D reproducible technique please go in further details about how you will help to standardize this technique.

Calibration is the fundamental part of this technique which assures reproducibility between adjacent trials, within and between subjects. Thus, this technique is made very objective and greatly reduces subjective bias. We now include this important point raised by the Reviewer in the Discussion section in Lines 555-561.

C11: This is a very important point. In this reviewer´s opinion not enough details are provided in this article. More details about the handling are needed.

We thank the Reviewer for raising this important point. However, details of further elaboration of this point are beyond the scope of this protocol's paper. It is well known that various environmental and inherent variables contribute to differences in rodent motor behavior. We refer the interested reader to this article (Fouad et al, 2013, Frontiers in Integrative Neuroscience) in the literature, now mentioned in lines 595 in the discussion.

C12: This is a very interesting research article and it will be very useful to homogenize the research in different pathologies and animal models.

Nevertheless, it will not completely be done unless the authors include a short section about the translation between the animal model and clinical situation. This gap has been present in the kinematic research as well as in different areas, and it need to be jumped. Hundreds of articles in animal models have been published every year (for instance in stroke). Nonetheless, no new treatment have come up. This gap is one of the main problems, so link the article, technique and result with human kinematic research is essential if we want to overcome this limitation.

We resonate with this Reviewer's concern on bridging the translational gap between human kinematic studies and preclinical studies in rodents. The protocol presented here promises to serve as a sensitive tool in detecting deficits after an injury as well as intervention. We are hopeful that the outcomes that this system generates will prove useful to a variety of pre-clinical researchers to assess the effectiveness of their interventions. Indeed, we ourselves have begun to use the range of outcomes that the system yields for our studies and are expanding its use for upper extremity function too. These collective efforts will prove as effective steps in bridging the gap that we face today in the field. Moreover, it is critical to point out that the lack in translation to the clinic from bench side is not only because of lack of sensitive assessment measures but a variety of other variables (example: species differences, insurance policies,

the dosage of intervention, etc.). And when these are collectively dealt with in the field, we will eventually witness major and obvious progress in bridging the perceived gaps.

Height per phase

Limbs	Body region	Phase	Mean Height left	StDev. left	Mean Height right	StDev. right
Height of Forelimbs [cm]	Shoulder	Stancephase	5.30	0.68	4.64	0.20
		Swingphase	5.18	0.38	4.90	0.89
	Elbow	Stancephase	2.74	0.42	1.76	0.14
		Swingphase	2.80	0.26	2.03	0.44
	Wrist	Stancephase	0.75	0.12	0.77	0.10
		Swingphase	1.19	0.16	1.30	0.29
	Paw	Stancephase	-0.10	0.06	-0.24	0.14
		Swingphase	0.34	0.20	0.16	0.30
Height of Hindlimbs [cm]	Iliac Crest	Stancephase	6.37	0.38	6.38	0.32
		Swingphase	6.64	0.43	6.77	0.23
	Hip	Stancephase	4.71	0.56	4.49	0.58
		Swingphase	4.87	0.67	4.71	0.42
	Knee	Stancephase	2.23	0.20	2.50	0.17
		Swingphase	2.97	0.24	3.26	0.14
	Ankle	Stancephase	1.29	0.36	0.84	0.45
		Swingphase	1.63	0.37	1.47	0.27

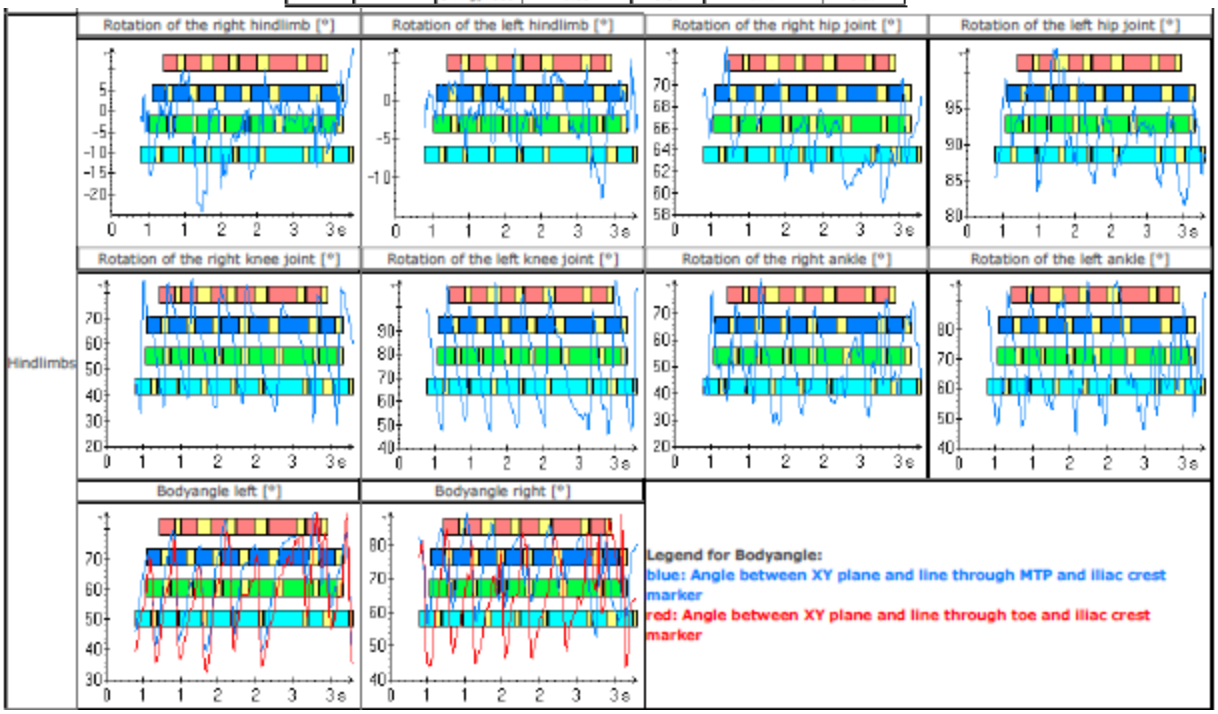

Figure 1: Sample Report. Screen capture of the sample report created from template within the software (in response to Point 7 raised by Reviewer 3).

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Cambridge, MA 02140
www.JoVE.com

Title: 3D Kinematic Gait Analysis for Pre-Clinical Studies in Rodents

URL: https://www.jove.com/video/59612/title?status=a61618k

Were animals used humanely and was the appropriate anesthesia or analgesia provided for potentially painful procedures? Yes
I don't have any animal welfare concern with this video.

Please provide additional comment, if necessary.

1. Please be specific in your comments. If possible, divide your comments into 2 categories:
   a) Absolutely not acceptable - for serious errors and deviations from the animal research standards.
   b) Improvement requires - for minor deviations, missing parts, etc....

   For each comment, please specify if the changes in video are required, or if only changes in the complementary text are necessary. **Obviously, changes in the video are more difficult so it is important to note if changes in the text are sufficient.** Please use the chart below to provide details on each issue (replace examples listed):

#	**Time in the video**	**comment**	**Change in video required Yes/No**	**Change in text is sufficient Yes/No**	**Suggested Changes**
Example	2:20 – 2:34	Name of drug used for anesthesia is not mentioned	No	Yes	
1					
2					

Author's Response:

We thank the veterinarian for reviewing our video.

JoVE59612R1

"3D Kinematic Gait Analysis for Pre-Clinical Studies in Rodents"

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

We thank the editor for formatting the manuscript.

2. Please address specific comments marked in the manuscript and perform the changes as required in the text and in the video.

The specific comments have been addressed within the manuscript document and changes have been made in the text and the video. Original editorial comment boxes have been removed in the revised version of the manuscript. Figure 10: Please refer to line 366. Figure 11: Please refer to line 368.

3. Please incorporate the anesthesia steps in the text protocol and discuss how do you measure the depth of anesthesia.

We thank the editor for pointing this out. This has now been referenced in line 187-192.

4. Please ensure that the length of the protocol section do not exceed more than 10 pages including headings and spacings.

Our protocol section length is 6 pages.

5. Figure 8 and 10 are missing from this submission. Please check.

Figure 8 and 10 are now included in the submission. We thank the editor for pointing this out.

6. Please address the vet review comments as well. This is important. Please include a point by point rebuttal for the same as well.

No concerns are raised by the Veterinarian after watching our video. We include our response in the rebuttal letter.