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Title: A Low-Cost Markerless DeepLabCut-Based Workflow for Spontaneous Gait and Locomotion Analysis in Freely Moving Mice

Authors and Affiliations:

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **YES**

3. Filming location: Will the filming need to take place in multiple locations? **NO**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **NO**

Current Protocol Length

Number of Steps: 48

Number of Shots: 64 (48 SCs)

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Karen Arcia:** Our research focuses on low-cost, markerless methods to quantify gait and locomotion in freely moving mice.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B roll: 3.3.1*

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Karen Arcia:** This protocol enables natural, unrestricted assessment of movement and gait across diverse preclinical research applications.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Marcos Rojas:** This protocol allows researchers to quantify spontaneous locomotion, gait dynamics, and limb coordination under natural, unconstrained conditions.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Marcos Rojas:** The most important challenge when performing this protocol is that it cannot directly measure biomechanical variables such as ground reaction forces or plantar pressure.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Marcos Rojas:** Future studies can expand this workflow with 3D motion analysis and validation across diverse movement disorder models.

- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Bioethics Committee of the Institute of Neurobiology, National Autonomous University of Mexico

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Open Field Arena and Camera Preparation

Demonstrators: Karen Arcia, Marcos Villegas

2.1. Acclimate both the triple-transgenic Alzheimer's disease mice and non-transgenic control mice in the behavioral testing room for 60 minutes before the experiment [1].

2.1.1. Establishing shot of the talent with the mouse cages in the testing room.
***Videographer:** Please obtain a CU shot as well for this shot*

2.2. Clean the acrylic open field arena thoroughly with 70% ethanol to remove residual odors and scent cues and allow the surface to dry completely [1].

2.2.1. Talent cleaning the acrylic open field arena with 70% volume per volume ethanol.

2.3. Handle 70% ethanol in a well-ventilated area, away from heat or ignition sources. Allow the arena to dry completely before use [1].

2.3.1. Shot of 70% ethanol positioned away from heat or ignition sources.

2.4. Place the open field arena securely on the wooden platform and confirm that the structure is stable and free of vibrations [1]. Inspect the transparent acrylic walls and the matte floor surface to confirm they are free of dust, moisture, and reflective residues [2].

2.4.1. Talent places the open field arena on the wooden platform and inspects the arena setup for stability.

2.4.2. Talent inspecting the walls and floor for dust, moisture, or reflective residues.

2.5. Position the external lateral white lamps on both sides of the arena at an approximate angle of 30 to 45 degrees relative to the arena plane to provide uniform illumination [1]. Adjust the lamp placement before the recording session [2] and verify that illumination at the arena level remains within the target range of 120 to 150 lux [3].

2.5.1. Talent positioning the external lateral white light lamps on both sides of the arena at an approximate angle of 30 to 45 degrees relative to the arena plane.

2.5.2. Talent adjusting the placement of the light lamps.

2.5.3. Talent checking illumination at the arena level.

2.6. Position the camera below the arena in a bottom view configuration at a fixed distance of approximately 80 centimeters from the arena plane, ensuring complete visibility of the recording area [1].

- 2.6.1. Talent positioning the camera below the arena in a bottom view configuration approximately 80 centimeters from the arena plane.
- 2.7. Connect the camera to the computer system [1] and initiate the video acquisition software before introducing the animal [2].
 - 2.7.1. Talent connecting the camera to the computer system.
 - 2.7.2. SCREEN: The video acquisition software being launched on the computer. **NOTE: Filmed by the videographer**
- 2.8. Adjust the camera focus and field of view to ensure that the arena surface is visible and sharply resolved [1]. Acquire a reference image for spatial calibration by placing the checkerboard grid or calibration pattern with known dimensions on the arena plane [2].
 - 2.8.1. SCREEN: The camera focus and field of view is being adjusted for a sharp image of the arena surface. **NOTE: Filmed by the videographer**
 - 2.8.2. Talent placing the checkerboard grid or calibration pattern with known dimensions on the arena.

3. Recording Spontaneous Locomotor Behavior

Demonstrators: Alejandro Hernandez, Valeria Saldivar

- 3.1. Before placing the mouse into the arena, display the animal identifier in the video frame to ensure direct visual association between the recording and the corresponding experimental session [1].
 - 3.1.1. Talent displaying the animal identifier within the video frame.
- 3.2. Initiate video recording before placing the mouse in the center of the open field arena to capture the initial exploratory behavior from the beginning of the session [1]. Gently place the mouse in the center of the open field arena [2].
 - 3.2.1. SCREEN: The recording is being initiated in the video acquisition. **NOTE: Filmed by the videographer**
 - 3.2.2. Talent gently placing the mouse in the center of the open field arena.
- 3.3. Continuously record spontaneous locomotion for 3 minutes without any visual, auditory, or tactile disturbances. Limit recording sessions to one per mouse [1]. Monitor the live video feed on the computer screen to confirm uninterrupted recording and proper capture of the animal's movements [2].
 - 3.3.1. SCREEN: Shot of the mouse moving freely in the open field arena during recording. **NOTE: Filmed by the videographer**
 - 3.3.2. SCREEN: The live video feed is being monitored during recording.
- 3.4. Verify camera alignment and illumination uniformity between recording sessions to ensure consistent recording conditions across animals [1].

- 3.4.1. Talent checking camera alignment between recording sessions.
- 3.5. At the end of the recording period, stop video acquisition [1] and carefully remove the animal from the arena using the same handling procedure used previously [2].
 - 3.5.1. SCREEN: The recording is being stopped.
 - 3.5.2. Talent carefully removes the mouse from the open field arena.
- 3.6. Return the animal to its home cage and allow the animal to rest before any further procedures [1].
 - 3.6.1. Talent returning the mouse to its home cage.
- 3.7. Clean the open-field arena with 70% ethanol after the session and allow it to dry completely before introducing the next animal [1]. Confirm that no residual ethanol or odor remains on the arena surface before introducing the next animal [2].
 - 3.7.1. Talent cleaning the open field arena with 70% volume per volume ethanol.
 - 3.7.2. Talent inspecting the arena surface to confirm the absence of residual ethanol or odor.
- 3.8. Document the animal identifier, session date and time, experimental condition, and the operator's name immediately after recording [1].
 - 3.8.1. Talent recording the animal identifier, session date and time, experimental condition and operator name.
- 3.9. Save the video file in a dedicated folder using a standardized naming convention [1].
 - 3.9.1. SCREEN: The video file is being saved in a dedicated folder.
- 3.10. At the end of the experimental day, shut down the camera system and the lighting equipment to prevent drift in illumination settings across sessions [1].
 - 3.10.1. Talent shutting down the camera system and lighting equipment.

4. Video Processing and Kinematic Analysis

- 4.1. Acquire the raw videos in MOV (*M-O-V*) format [1]. Convert the videos to the AVI format while maintaining a constant frame rate and resolution to ensure compatibility with DeepLabCut (*Deep-Lab-Cut*) [2].
 - 4.1.1. SCREEN: 70825_screenshot_1.mp4: 00:03-00:06
 - 4.1.2. SCREEN: 70825_screenshot_1.mp4: 00:24-00:40
- 4.2. Back up all raw video files immediately after acquisition to a dedicated folder on the analysis computer and apply a standardized naming convention [2].
 - 4.2.1. SCREEN: 70825_screenshot_1.mp4: 00:48-01:09.
- 4.3. Review each video in its entirety to confirm that the recording is complete and free of

interruptions, dropped frames, or corruption. Exclude videos with acquisition errors from analysis [1].

4.3.1. SCREEN: 70825_screenshot_2.mp4: 00:03-00:12

4.4. Convert the videos to MP4 or uncompressed AVI format if the original format is not compatible with DeepLabCut [1].

4.4.1. SCREEN: 70825_screenshot_2.mp4: 00:12-00:24

4.5. Optionally, perform temporal trimming of the video footage to eliminate non-experimental intervals at the beginning and end of the session [1].

4.5.1. SCREEN: 70825_screenshot_2.mp4: 00:24-00:36

4.6. Confirm that the resolution and frame rate match the acquisition parameters used during recording and remain consistent across all sessions [1].

4.6.1. SCREEN: 70825_screenshot_2.mp4: 00:36-00:48

4.7. Verify that the entire arena surface is visible within the video frame and no body parts are persistently occluded during locomotion [1]. ~~Place all verified and processed videos into the designated project directory for DeepLabCut analysis [2].~~ **NOTE: The VO is edited for the deleted shot**

4.7.1. SCREEN: 70825_screenshot_2.mp4: 00:57-01:00

4.7.2. ~~The verified and processed video files are being organized into the DeepLabCut project directory.~~ **NOTE: Not filmed**

4.8. Designate a video library for the project using either the videos intended for analysis or a representative subset for network training [1].

4.8.1. SCREEN: 70825_screenshot_3.mp4: 00:00-00:18

4.9. Next, launch the Python environment and import the DeepLabCut package [1]. Create a new DeepLabCut project using the suitable command [1].

4.9.1. SCREEN: 70825_screenshot_3.mp4: 00:18-00:36

4.9.2. SCREEN: 70825_screenshot_3.mp4: 00:46-00:46

4.10. Then, define the anatomical landmarks or body parts of interest in the config.yaml (*config-Y-A-M-L*) file while ensuring that the definitions are clear and anatomically consistent [1]. Optimize the body-part definitions for the experimental question and maintain identical body-part definitions across all animals and sessions [2].

4.10.1. SCREEN: 70825_screenshot_4.mp4: 00:00-00:06

4.10.2. SCREEN: 70825_screenshot_4.mp4: 00:06-00:12.

5. Frame Extraction and Body Part Labeling

5.1. Specify the start, stop, *numframes2extract* (*Num-Frames-Two-Extract*), and *cropping_parameters* in the config.yaml file according to the video length and spatial layout [1].

5.1.1. SCREEN: 70825_screenshot_5.mp4: 00:06-00:17

5.2. Using the appropriate command, extract representative frames [1]. Review the extracted frames to confirm the inclusion of variation in body posture, orientation, limb position, and gait-phase coverage across the recording [2].

5.2.1. SCREEN: 70825_screenshot_5.mp4: 00:20-00:34

5.2.2. SCREEN: 70825_screenshot_5.mp4: 00:40-00:52

5.3. Extract frames manually using the *suitable* command when specific gait cycle phases are required [1].

5.3.1. SCREEN: 70825_screenshot_6.mp4: 00:03-00:05, 00:23-00:28

5.4. Next, launch the graphical labeling interface [1].

5.4.1. SCREEN: 70825_screenshot_7.mp4: 00:05-00:08

5.5. Identify all predefined anatomical landmarks, including the representative video frame captured during acquisition [1] and the automated body point detection superimposed on the original frame [2]. Indicate the defined body landmarks and arena reference points used for analysis [3].

5.5.1. SCREEN: 70825_screenshot_7.mp4: 00:14-00:28

5.5.2. SCREEN: 70825_screenshot_7.mp4: 00:28-00:42

5.5.3. SCREEN: 70825_screenshot_7.mp4: 00:42-00:56

5.6. Label all four limbs consistently by placing each landmark at the center of the paw over the plantar pads at ground contact [1].

5.6.1. SCREEN: 70825_screenshot_7.mp4: 00:56-01:10

5.7. Use the *suitable* command to save and inspect the labels and correct inaccurate annotations if needed [1].

5.7.1. SCREEN: 70825_screenshot_7.mp4: 01:10-01:27

~~5.8. When feasible, have a second trained evaluator label a subset of frames to assess inter-rater consistency [1].~~

~~5.8.1. SCREEN: A subset of frames are being labeled by a second trained evaluator.~~

NOTE: Not filmed

6. Video Analysis and Gait Quantification

6.1. Use the appropriate command to run inference on the experimental videos [1].

Generate labeled videos to visually confirm tracking quality [2].

6.1.1. SCREEN: 70825_screenshot_8.mp4: 00:03-00:06, 01:43-01:48

6.1.2. SCREEN: 70825_screenshot_8.mp4: 02:30-02:40, 03:17-03:20

6.2. Set the pcutoff (*P-Cut-Off*) parameter in the config.yaml file to 0.6 to apply a confidence threshold during tracking visualization and evaluation [1].

6.2.1. SCREEN: 70825_screenshot_9.mp4: 00:00-00:13

6.3. Import the output CSV files into the statistical computing environment [1] along with the corresponding analysis package and the custom scripts required for behavioral quantification [2].

6.3.1. SCREEN: 70825_screenshot_10.mp4: 00:00-00:13

6.3.2. SCREEN: 70825_screenshot_10.mp4: 00:13-00:26

6.4. Convert frame indices to time using the original video sampling rate [1].

6.4.1. SCREEN: 70825_screenshot_10.mp4: 00:26-00:39

6.5. To calibrate spatial dimensions, transform pixel coordinates into physical units using the known arena dimensions and the tracked arena corner landmarks [1].

6.5.1. SCREEN: 70825_screenshot_10.mp4: 00:42-00:52

6.6. Perform quality control on the tracked coordinates before metric extraction [1]. Consider points with a likelihood value below 0.3 as missing and replace missing points with linear interpolation when appropriate [2].

6.6.1. SCREEN: 70825_screenshot_10.mp4: 00:56-01:03

6.6.2. SCREEN: 70825_screenshot_10.mp4: 01:05-01:18

6.7. Identify outlier trajectories during visual inspection and correct or exclude them when necessary [1].

6.7.1. SCREEN: 70825_screenshot_10.mp4: 01:18-01:31

6.8. Define gait segments using synchronized kinematic criteria based on body center displacement and hind limb motion [1]. Exclude non-locomotor behaviors such as rearing, turning, and grooming [2].

6.8.1. SCREEN: 70825_screenshot_10.mp4: 01:36-01:44

6.8.2. SCREEN: 70825_screenshot_10.mp4: 01:44-01:57

6.9. Manually calibrate and refine the definition thresholds according to the experimental conditions and animal model to retain only robust, continuous locomotor bouts for analysis [1].

6.9.1. SCREEN: 70825_screenshot_10.mp4: 01:57-02:10

6.10. Using the filtered continuous locomotor segments, compute gait metrics derived from geometric and temporal relationships among the tracked landmarks [1].

6.10.1. SCREEN: 70825_screenshot_10.mp4: 02:10-02:23

6.11. Extract open field metrics such as total distance traveled, average speed, time spent moving, percentage of time spent moving, number and duration of walking bouts, and cumulative distance covered in the center, periphery, and corner zones [1].

6.11.1. SCREEN: 70825_screenshot_10.mp4: 02:23-02:36

6.12. Compile all outputs into a structured master file for downstream statistical analysis [1].

6.12.1. SCREEN: 70825_screenshot_10.mp4: 02:36-02:49

6.13. Generate visual representations using the appropriate computational environments and the previously described plotting scripts [1].

6.13.1. SCREEN: 70825_screenshot_10.mp4: 02:49-03:05

Results

7. Results

- 7.1. In a representative non-transgenic mouse recording, the reconstructed body center trajectory showed continuous displacement across the arena [1]. The time-density and speed-coded visualizations highlighted regions of prolonged occupancy [2] and fluctuations in movement velocity during exploration [3].
 - 7.1.1. LAB MEDIA: Figure 3A.
 - 7.1.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the bright occupancy region represented at the corners.*
 - 7.1.3. LAB MEDIA: Figure 3B. *Video editor: Highlight the tracks shown in the grey color range*
- 7.2. A representative triple transgenic-Alzheimer mouse recording showed more spatially constrained trajectories and reduced arena coverage [1], accompanied by a more localized occupancy distribution and extended periods of lower movement speed [2].
 - 7.2.1. LAB MEDIA: Figure 3C.
 - 7.2.2. LAB MEDIA: Figure 3D.
- 7.3. The reconstructed limb pattern of the non-transgenic mouse recording showed a regular alternation between forelimbs and hindlimbs, with a consistent temporal sequence of stance and swing phases across the recording period [1].
 - 7.3.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the alternating colored bars for forelimbs and hindlimbs.*
- 7.4. The gait map showed aligned and parallel limb trajectories reflecting a relatively homogeneous spatial organization of limb movement [1].
 - 7.4.1. LAB MEDIA: Figure 4B.
- 7.5. A representative triple transgenic-Alzheimer mouse recording showed greater temporal variability in stance phase duration and limb engagement [1]. The limb trajectories appeared more dispersed and less parallel, indicating greater spatial variability during stepping [2].
 - 7.5.1. LAB MEDIA: Figure 4C.
 - 7.5.2. LAB MEDIA: Figure 4D.

1. Acclimate
Pronunciation link: <https://www.merriam-webster.com/dictionary/acclimate>
IPA: /əˈklaɪ.mɛt/
Phonetic Spelling: uh·kly·mayt
2. Triple-transgenic
Pronunciation link: <https://www.merriam-webster.com/dictionary/transgenic>
IPA: /ˌtrɪp.əl trænˈsɜːn.ɪk/
Phonetic Spelling: trip·uhl tranz·jen·ik
3. Behavioral
Pronunciation link: <https://www.merriam-webster.com/dictionary/behavioral>
IPA: /bɪˈheɪ.vjə.əl/
Phonetic Spelling: bih·hayv·yer·uhl
4. Acrylic
Pronunciation link: <https://www.merriam-webster.com/dictionary/acrylic>
IPA: /əˈkrɪl.ɪk/
Phonetic Spelling: uh·kril·ik
5. Illumination
Pronunciation link: <https://www.merriam-webster.com/dictionary/illumination>
IPA: /ɪˌluː.məˈneɪ.ʃən/
Phonetic Spelling: ih·loo·muh·nay·shuhn
6. Calibration
Pronunciation link: <https://www.merriam-webster.com/dictionary/calibration>
IPA: /ˌkæl.əˈbreɪ.ʃən/
Phonetic Spelling: kal·uh·bray·shuhn
7. Locomotor
Pronunciation link: <https://www.merriam-webster.com/dictionary/locomotor>
IPA: /ˌloʊ.kəˈmoʊ.tər/
Phonetic Spelling: loh·kuh·moh·ter
8. DeepLabCut
Pronunciation link: <https://deeplabcut.github.io/DeepLabCut/>
IPA: /diːp læb kʌt/
Phonetic Spelling: deep·lab·kut
9. Anatomical
Pronunciation link: <https://www.merriam-webster.com/dictionary/anatomical>
IPA: /ˌæn.əˈtɑː.mɪ.kəl/
Phonetic Spelling: an·uh·tom·i·kuhl
10. config.yaml
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: kuh·fig yak·muhl
11. Graphical
Pronunciation link: <https://www.merriam-webster.com/dictionary/graphical>
IPA: /ˈɡræf.ɪ.kəl/
Phonetic Spelling: graf·i·kuhl

12. Inter-rater

Pronunciation link: <https://www.merriam-webster.com/dictionary/interrater>

IPA: /,ɪn.t̬əˈreɪ.t̬ə/

Phonetic Spelling: in·ter·ray·ter

13. Inference

Pronunciation link: <https://www.merriam-webster.com/dictionary/inference>

IPA: /'ɪn.fə.əns/

Phonetic Spelling: in·fer·uhns

14. Kinematic

Pronunciation link: <https://www.merriam-webster.com/dictionary/kinematic>

IPA: /,kɪn.əˈmæt.ɪk/

Phonetic Spelling: kin·uh·mat·ik

15. Interpolation

Pronunciation link: <https://www.merriam-webster.com/dictionary/interpolation>

IPA: /ɪn.t̬ɜː.pəˈleɪ.fən/

Phonetic Spelling: in·ter·puh·lay·shuhn

16. Locomotion

Pronunciation link: <https://www.merriam-webster.com/dictionary/locomotion>

IPA: /,ləʊ.kəˈmoʊ.fən/

Phonetic Spelling: loh·kuh·moh·shuhn

17. Geometric

Pronunciation link: <https://www.merriam-webster.com/dictionary/geometric>

IPA: /,dʒiː.əˈmet.rɪk/

Phonetic Spelling: jee·uh·met·rik

18. Trajectory

Pronunciation link: <https://www.merriam-webster.com/dictionary/trajectory>

IPA: /trəˈdʒek.t̬ə.i/

Phonetic Spelling: truh·jek·ter·ee

19. Forelimbs

Pronunciation link: <https://www.merriam-webster.com/dictionary/forelimb>

IPA: /'fɔː.lɪm/

Phonetic Spelling: for·lim

20. Hindlimbs

Pronunciation link: <https://www.merriam-webster.com/dictionary/hindlimb>

IPA: /'haɪnd.lɪm/

Phonetic Spelling: hynd·lim