Submission ID #: 69506
Scriptwriter Name: Pallavi Sharma
Project Page Link: https://review.jove.com/files_upload.php?src=21199378

Title: Utilizing vmTracking to Improve the Accuracy of Multi-Animal Pose Estimation in Rodent Social Behavior Studies

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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, all done**

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: 20
Number of Shots: 34

Introduction

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

INTRODUCTION:

What is the scope of your research? What questions are you trying to answer?

1.1. **Hirotsugu Azechi:** We aim to understand social interactions in freely moving mice by developing accurate multi-animal tracking methods.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera Videographer's NOTE: Take 2

What are the current experimental challenges?

1.2. **Hirotsugu Azechi:** Achieving accurate pose tracking with reliable individual identification in multi-animal conditions remains difficult.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera Videographer's NOTE: Take 2

CONCLUSION:

What research gap are you addressing with your protocol?

1.3. **Hirotsugu Azechi:** Our protocol addresses the lack of reliable identity maintenance in multi-animal pose tracking.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera Videographer's NOTE: Take 1

What advantage does your protocol offer compared to other techniques?

1.4. **Hirotsugu Azechi:** It improves pose-tracking accuracy by combining virtual markers with existing markerless tools.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera Videographer's NOTE: Take 2

What questions will future research focus on?

1.5. **Hirotsugu Azechi:** Our future research will use these data to address how group dynamics shape social behaviors in mice. Videographer's NOTE: Take 2

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

Ethics Title Card

This research has been approved by the Animal Care and Use Committee at the Doshisha University

Protocol

2. Creation of a Virtual Marker Video

Demonstrator: Hirotsugu Azechi

2.1. To begin, create a new multi-animal project by selecting **Manage Project** for markerless video tracking **[1]**. Modify the **individuals** and **body parts** fields in the config.yaml *(Config-Y-A-M-L)* file according to the analysis requirements **[2-TXT]**.

2.1.1. WIDE: Talent selecting the Manage Project option on the main menu of DLC software. Videographer's NOTE: I shot this scene twice. The first one is a wide shot, including the talent. The second is a closer up version focusing only on the screen.

2.1.2. SCREEN: 2_1_2.mp4 **TXT: Use individual1 to individual3 and bodypart1 to bodypart6. Adjust the settings as desired throughout the procedure.**

2.2. Select **Extract Frames** to extract frames from the video for labeling and training **[1-TXT]**.

2.2.1. SCREEN: 2_2_1. ~~**TXT: Adjust the settings are desired throughout the procedure**~~ **NOTE: The text is moved to step 2.1.2 as per the author's request**

2.3. Next, label each extracted frame by selecting **Label Data [1]**. Assign the appropriate body parts to each visible individual, without necessarily maintaining consistent IDs between frames **[2]**.

2.3.1. SCREEN: 2_3_1-2_3_2 00:00-00:16.

2.3.2. SCREEN: 2_3_1-2_3_2 00:17-00:25.

2.4. To create a training dataset from the extracted frames, select **Create Training Dataset [1]**. Set the network to **dlcrnet_ms5** *(D-L-C-R-net M-S-five)* in the configuration **[2]**.

2.4.1. SCREEN: 2_4_1-2_4_2.mp4 00:20-00:35

2.4.2. SCREEN: 2_4_1-2_4_2.mp4 00:36-00:45.

2.5. Select **Train** to start training the network **[1]**. Set the **Maximum iterations** parameter to **200000** for this training session. All other training parameters can remain at their default values **[2]**.

2.5.1. SCREEN: 2_5_1-2_5_2.mp4 00:00-00:05.

2.5.2. SCREEN: 2_5_1-2_5_2.mp4 00:06-00:20 .

2.6. Evaluate the trained network by selecting **Evaluate [1]**.

2.6.1. SCREEN: 2_6_1.mp4 00:06-00:20.

2.7. To run pose estimation on the selected videos, choose **Analyze videos** generating .h5 coordinate data and other output files in the videos folder inside the project folder **[1-TXT]**.

2.7.1. SCREEN: 2_7_1.mp400:06-00:20.

2.8. To perform manual verification of the tracking results, create a labeled tracking video by selecting **Create videos [1]**. Enable the **Create video with animal ID colored?** option in the DLC *(D-L-C)* GUI *(G-U-I)* to include individual IDs in the video **[2]**.

2.8.1. SCREEN: 2_8_1-2_8_2.mp4 00:04-00:14.

2.8.2. SCREEN: 2_8_1-2_8_2.mp4 00:15-00:20.

2.9. Then, inspect the generated labeled videos to check for identity switches or missing predictions. If major errors are present, prepare for retraining **[1]**.

2.9.1. SCREEN: 2_9_1.mp4 00:06-00:20.

2.10. To retrain, extract, and annotate outlier frames, select **Extract/Refine Outliers** *(Extract-Or-Refine-Outliers)* **[1]**. Once annotation is complete, click **Merge dataset** to proceed automatically to training dataset creation, and then repeat the training pipeline **[2]**.

2.10.1. SCREEN: 2_10_1.mp4 00:06-00:20.

2.10.2. SCREEN: 2_10_2.mp4 00:12-00:25.

2.11. To correct the multi-animal DLC results, open the custom GUI and retain only the required keypoints for virtual markers **[1]**. .

2.11.1. SCREEN: 2_11_1-2_11_2.mp4 00:50-01:10.

2.12. Select **Refine tracklets** to correct the .h5 file containing only the selected keypoints **[1]**. Adjust the keypoints so everyone retains a consistent ID across the video **[2]**. Save the file, and the .h5 file in the videos folder will be updated automatically **[3]**.

2.12.1. SCREEN: 2_12_1-2_12_2.mp4 00:03-00:14.

2.12.2. SCREEN: 2_12_1-2_12_2.mp4 00:16-00:25.

2.12.3. SCREEN: 2_12_3.mp4 00:08-00:20.

2.13. Next create a new labeled video from the corrected data by selecting **Create videos [1]**. In DeepLabCut 2.2, if a labeled video already exists in the videos folder, a new video will not be created. Remove, rename, or move the existing file before proceeding **[2]**. NOTE: The VO for 2.13.2 is revised

2.13.1. SCREEN: 2_13_1. **TXT: Remove, rename, or move any labeled video to prevent overwrite errors**

2.13.2. SCREEN: 2_13_2.mp4 00:30-00:40. NOTE: Screen capture video 2_13_2.mp4 to be used.

2.14. Rename the labeled virtual marker video to a short filename to prevent errors during downstream DeepLabCut processing **[1]**.

2.14.1. SCREEN: 2_14_1.mp4.

3. Perform Pose Tracking of Virtual Marker Video using Single Animal DLC (saDLC)

3.1. Create a new saDLC *(S-A-D-L-C)* project by selecting **Manage Project** for tracking the virtual marker video **[1]**. In the **config.yaml** file, list all combinations of individuals and body parts in the **bodyparts** field **[2].**

3.1.1. SCREEN: 3_1_1.mp4 00:05-00:20.

3.1.2. SCREEN: 3_1_2-3_1_3.mp4 00:40-00:50

3.2. To extract frames from the renamed virtual marker video, select **Extract Frames [1]**.

3.2.1. SCREEN: 3_2_1.mp4

3.3. Now, label each extracted frame in **Label Data,** using the virtual markers to assign consistent individual IDs and ensure both IDs and body parts match across frames **[1]**. When a body part is partially hidden but the individual's ID is known, estimate its position and apply the label accordingly **[2]**.

3.3.1. SCREEN: 3_3_1-3_3_2.mp4 00:04-00:20.

3.3.2. SCREEN: 3_3_1-3_3_2.mp4 00:55-01:10.

3.4. Create a training dataset from the extracted frames by selecting **Create Training Dataset**. Set the network to **efficientnet-b0** *(Efficient-Net-b-Zero)* for this training

session **[1]**.

3.4.1. SCREEN: 3_4_1.mp4 00:27-00:40

3.5. Begin training the network by selecting **Train [1]**. Set the **Maximum iterations** parameter to **200000** for this session and all other settings can remain at their default values **[2]**.

3.5.1. SCREEN: 3_5_1-3_5_2.mp4 00:01-00:08.

3.5.2. SCREEN: 3_5_1-3_5_2.mp4 00:09-00:15.

3.6. After evaluating the trained network, perform manual verification as demonstrated earlier **[1-TXT]**.

3.6.1. SCREEN: 3_6_1.mp4 00:20-00:35 **TXT: Perform retraining with outlier extraction, if necessary, based on the results. NOTE: The text has been rephrased**

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Results

4. Results

4.1. In crowded scenes, vmTracking achieved a significantly higher match percentage than maDLC **[1].**

4.1.1. LAB MEDIA: Figure 6A. *Video editor: Highlight the taller cyan bar labeled "vmTracking" and the shorter yellow bar labeled "maDLC" in the crowded scenes match graph.*

4.2. The number of false negatives was significantly lower with vmTracking than with maDLC **[1].** However, there was no difference between vmTracking and maDLC in false positive **[2]** and ID switch counts **[3].**

4.2.1. LAB MEDIA: Figure 6B. *Video editor: Highlight the much shorter cyan bar for "vmTracking" compared to the taller yellow bar for "maDLC" in the false negative graph.*

4.2.2. LAB MEDIA: Figure 6C

4.2.3. LAB MEDIA: Figure 6D.

4.3. In non-crowded scenes, vmTracking achieved a significantly higher match percentage than maDLC **[1].** The number of false negatives was significantly lower with vmTracking than with maDLC **[2].** There was no difference in false positive and ID switch counts between vmTracking and maDLC **[3].**

4.3.1. LAB MEDIA: Figure 6E. *Video editor: Highlight the taller magenta "vmTracking" bar*

4.3.2. LAB MEDIA: Figure 6F. *Video editor: Highlight the much shorter magenta "vmTracking" bar*

4.3.3. LAB MEDIA: Figure 6G.

4.3.4. LAB MEDIA: Figure 6H.

4.4. In crowded scenes, the match percentage in maDLC plateaued at approximately 85% after around 400 annotated frames, with no further improvement **[1],** while vmTracking showed a steady increase up to about 95% at around 1,000 frames **[2].**

4.4.1. LAB MEDIA: Figure 6I. *Video editor: In the upper plot, highlight the flat dark blue curve labeled "Match in maDLC" after the 400-frame mark.*

4.4.2. LAB MEDIA: Figure 6I. *Video editor: In the upper plot, highlight the upward light blue curve labeled "Match in vmTracking" increasing toward 1,000 frames.*

4.5. In maDLC, increasing the number of annotated frames did not reduce the number of false negatives **[1].** Scenes with a higher proportion of correctly assigned virtual markers tended to show higher match percentages **[2].**

4.5.1. LAB MEDIA: Figure 6I. *Video editor: In the lower plot, highlight the flat red curve labeled "False negatives in maDLC" across the x-axis range.*

4.5.2. LAB MEDIA: Figure 6J. *Video editor: Highlight the blue bars labeled "Both match" across scenes 1 to 20*

4.6. Some scenes with low proportions of correctly assigned virtual markers still showed high match percentages, including crowded scenes 3 and 11 [**1].**

4.6.1. LAB MEDIA: Figure 6J. *Video editor: Highlight the bars for crowded scenes 3 and 11 where the blue segments are high*

- DeepLabCut

Pronunciation link: https://www.merriam-webster.com/dictionary/DeepLabCut
IPA: /ˈdiːp læb kʌt/
Phonetic Spelling: deep·lab·kut

- markerless

Pronunciation link: https://www.merriam-webster.com/dictionary/markerless
IPA: /ˈmɑːrkər.ləs/
Phonetic Spelling: mar·ker·luhs

- config.yaml

Pronunciation link: https://www.merriam-webster.com/dictionary/YAML
IPA: /ˈjæməl/
Phonetic Spelling: yam·uhl

- Extract Frames

Pronunciation link: https://www.merriam-webster.com/dictionary/extract
IPA: /ɪkˈstrækt/
Phonetic Spelling: ik·strakt

- Label Data

Pronunciation link: https://www.merriam-webster.com/dictionary/label
IPA: /ˈleɪbəl/
Phonetic Spelling: lay·buhl

- dlcrnet_ms5

Pronunciation link: No confirmed link found

IPA: /ˌdiː el siː ɑːr nɛt ɛm ɛs faɪv/
Phonetic Spelling: dee·el·see·ar·net·em·es·five

- Train

Pronunciation link: https://www.merriam-webster.com/dictionary/train
IPA: /treɪn/
Phonetic Spelling: trayn

- Evaluate

Pronunciation link: https://www.merriam-webster.com/dictionary/evaluate
IPA: /ɪˈvælјuˌeɪt/
Phonetic Spelling: ih·val·yoo·ayt

- Analyze

Pronunciation link: https://www.merriam-webster.com/dictionary/analyze
IPA: /ˈænəˌlaɪz/
Phonetic Spelling: a·nuh·lyze

- GUI

Pronunciation link: https://www.merriam-webster.com/dictionary/GUI
IPA: /ˈguːi/
Phonetic Spelling: goo·ee

- .h5

Pronunciation link: https://www.merriam-webster.com/dictionary/HDF5
IPA: /ˌeɪtʃ diː ɛf faɪv/
Phonetic Spelling: aych·dee·ef·five

- Extract/Refine Outliers

Pronunciation link: https://www.merriam-webster.com/dictionary/outlier
IPA: /ˈaʊtˌlaɪər/
Phonetic Spelling: out·lyer

- tracklets

Pronunciation link: https://www.merriam-webster.com/dictionary/tracklet
IPA: /ˈtræk.lɪt/
Phonetic Spelling: trak·lit

- saDLC

Pronunciation link: No confirmed link found
IPA: /ˌɛs eɪ diː el siː/
Phonetic Spelling: ess·ay·dee·el·see

- efficientnet-b0

Pronunciation link: No confirmed link found
IPA: /ɪˈfɪʃənt nɛt biː zɪroʊ/
Phonetic Spelling: ih·fish·uhnt·net·bee·zee·roh

- iterations

Pronunciation link: https://www.merriam-webster.com/dictionary/iteration
IPA: /ˌɪtəˈreɪʃən/
Phonetic Spelling: ih·tuh·ray·shuhn

- virtual markers

Pronunciation link: https://www.merriam-webster.com/dictionary/virtual
IPA: /ˈvɝːtʃuəl/
Phonetic Spelling: ver·choo·uhl

- vmTracking

Pronunciation link: No confirmed link found
IPA: /ˌviː ɛm ˈtrækɪŋ/
Phonetic Spelling: vee·em·trak·ing

- maDLC

Pronunciation link: No confirmed link found
IPA: /ˌɛm eɪ diː el siː/
Phonetic Spelling: em·ay·dee·el·see

- false negatives

Pronunciation link: https://www.merriam-webster.com/dictionary/false%20negative
IPA: /fɔːls ˈnɛgətɪv/
Phonetic Spelling: fawls·neg·uh·tiv

- false positives

Pronunciation link: https://www.merriam-webster.com/dictionary/false%20positive
IPA: /fɔːls ˈpɑːzətɪv/
Phonetic Spelling: fawls·poz·uh·tiv

- ID switch

Pronunciation link: https://www.merriam-webster.com/dictionary/ID
IPA: /ˌaɪˈdiː/
Phonetic Spelling: eye·dee

- annotated

Pronunciation link: https://www.merriam-webster.com/dictionary/annotated
IPA: /ˈænəˌteɪtɪd/
Phonetic Spelling: a·nuh·tay·tid

- plateaued

Pronunciation link: https://www.merriam-webster.com/dictionary/plateaued
IPA: /plæˈtoʊd/
Phonetic Spelling: pla·tohd

- retraining

Pronunciation link: https://www.merriam-webster.com/dictionary/retraining
IPA: /ˌriːˈtreɪnɪŋ/
Phonetic Spelling: ree·tray·ning