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## Analysis and consolidation of TrackMate outputs for measuring two-dimensional cell motility using nuclear tracking --Manuscript Draft--

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<b>Corresponding Author:</b>	Graycen Wheeler, Ph.D. University of Colorado Boulder Boulder, Colorado UNITED STATES
<b>Corresponding Author's Institution:</b>	University of Colorado Boulder
<b>Corresponding Author E-Mail:</b>	Graycen.Wheeler@colorado.edu
<b>Order of Authors:</b>	Graycen Wheeler Amrita Purkayastha Eric Bunker David Bortz Xuedong Liu
<b>Additional Information:</b>	
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**TITLE:**

Protocol for Analysis and Consolidation of TrackMate Outputs for Measuring Two-Dimensional Cell Motility Using Nuclear Tracking

**AUTHORS AND AFFILIATIONS:**

Graycen E. Wheeler<sup>1</sup> ([graycen.wheeler@colorado.edu](mailto:graycen.wheeler@colorado.edu))

Amrita Purkayastha<sup>1</sup> ([amrita.purkayastha@colorado.edu](mailto:amrita.purkayastha@colorado.edu))

Eric N. Bunker<sup>1</sup> ([eric.bunker@colorado.edu](mailto:eric.bunker@colorado.edu))

David M. Bortz<sup>2</sup> ([dmbortz@colorado.edu](mailto:dmbortz@colorado.edu))

Xuedong Liu<sup>1</sup> ([liux@colorado.edu](mailto:liux@colorado.edu))

1. Department of Biochemistry, University of Colorado Boulder, Boulder, CO

2. Department of Applied Mathematics, University of Colorado Boulder, Boulder, CO

**SUMMARY:**

This protocol for high-throughput measurement of cell motility in HaCaT keratinocytes describes methods for collecting and processing images of cell nuclei and performing particle tracking using the ImageJ plugin TrackMate.

**ABSTRACT:**

Collective cellular migration plays a key role in many fundamental biological processes including development, wound healing, and cancer metastasis. To understand the regulation of cell motility, we must be able to measure it easily and consistently under different conditions. Here we describe a method for measuring and quantifying single-cell and bulk motility of HaCaT keratinocytes using a nuclear stain. This method includes a MATLAB script for analyzing TrackMate output files to calculate displacements, motility rates, and trajectory angles in single cells and in bulk for an imaging site. This motility analysis script allows for quick, straightforward, and scalable analysis of cell motility rates from TrackMate data and could be broadly used to identify and study the regulation of motility in epithelial cells. We also provide a MATLAB script for reorganizing microscopy videos collected on a microscope and converting them to TIF stacks, which can be analyzed using the ImageJ TrackMate plugin in bulk. Using this methodology to explore the roles of adherens junctions and actin cytoskeletal dynamics in regulating cell motility in HaCaT keratinocytes, we demonstrate evidence that Arp2/3 activity is required for the elevated motility seen after  $\alpha$ -catenin depletion in HaCaT keratinocytes.

**INTRODUCTION:**

Precise, responsive regulation of cellular motility in epithelial cells is crucial for wound healing and for replenishing the epithelial layer. Failure to sustain motility can lead to problems with embryonic development and wound healing<sup>1</sup> and overactive motility signaling is a key contributor to cancer metastasis<sup>2</sup>.

Understanding cellular control of motility in HaCaT keratinocytes offers important insights into these processes. The procedures outlined here provide consistent measurements and calculations of the average magnitude of cellular motility on a single-cell or population level. We

have used this method to measure motility in HaCaT keratinocytes after either genetic perturbation or treatment with small molecule inhibitors to understand the cell signaling that controls rates of motility. While the described experimental setup measures average speed of cellular motility without accounting for directionality of movement, the MATLAB code used to analyze tracking data could be altered to calculate both magnitude and direction of motility across each timepoint.

Collective cell motility is crucial for epithelial-mesenchymal transition both in development and in cancer metastasis<sup>3</sup>. Cells achieve motility through coordination of adhesion, polarization, protrusion, and retraction<sup>4</sup>. These processes rely heavily on coordinated regulation of the dynamic actin cytoskeleton. Activation of Rac1 GTPase downstream of PI3K or various receptor tyrosine kinases polarizes the cell, resulting in actin polymerization<sup>5</sup>. Actin cytoskeletal dynamics are regulated by this and other Rho-mediated GTPases, which are activated by downstream of various growth factors. These Rho-mediated GTPases then activate the Arp2/3 complex that stimulates actin branching<sup>6</sup>. These actin cytoskeleton dynamics are closely related to another key regulator of motility: intercellular junctions. We are particularly interested in adherens junctions, which form strong adhesions between cells, and the cytosolic components coordinate with the actin cytoskeleton to maintain tissue integrity<sup>7</sup>.

We have previously shown that HaCaT keratinocytes expressing an shRNA-mediated  $\alpha$ -catenin knockdown demonstrate higher motility rates than those expressing a non-targeting shRNA control<sup>8</sup>. We wish to use the motility analysis tools we developed to further understand the mechanism of this elevated motility upon depletion of  $\alpha$ -catenin.  $\alpha$ -Catenin is a required cytosolic component of adherens junctions<sup>9</sup>. It participates in adherens junctions through its interaction with  $\beta$ -catenin, which it binds as a monomer, but it can also dissociate from adherens junctions to form a homodimer that binds and bundles actin filaments, which inhibits Arp2/3 activity<sup>10, 11</sup>. While  $\alpha$ -catenin does not interact directly with actin while it is bound to  $\beta$ -catenin, it may still facilitate or strengthen the coordination between adherens junctions and the cytoskeleton through binding to vinculin, which stabilizes actin filaments<sup>12</sup>.

While the TrackMate plugin for ImageJ is a well-established method for particle tracking that can be used to track cell nuclei, we found that the available tools for parsing, analyzing, and consolidating TrackMate outputs to calculate cell motility for multiple imaging sites were not integrated and were challenging to use for those without expertise in multiple programming languages. Tinevez and Herbert offer a primer on using TrackMate, but the mean square displacement analysis section requires considerable MATLAB skill<sup>13</sup>. We have identified existing methods for extracting motility rates from time-lapse microscopy videos offer more complex three-dimensional analyses and require more programming expertise<sup>14, 15</sup>. Pathfinder, a cell tracking and motility analysis software previously developed in our lab, measures cell migration speed and direction but is only executable on Windows machines and requires a limiting Java Runtime environment<sup>16</sup>. Because TrackMate is such a reliable and well-documented tool, we developed a straightforward method for generating, analyzing, and organizing large two-dimensional TrackMate datasets using MATLAB. Our script also removes the repeated integer datapoints that sometimes occur in TrackMate outputs after a tracked cell crosses out of the

imaging site, allowing for the inclusion of cells with high motility and/or directionality in analysis without the inclusion of these spurious datapoints. We also provide a script for reorganizing images collected on an ImageXpress Micro XL into TIFF stacks that can be analyzed in bulk using TrackMate.

In this protocol, cells are seeded in a 96-well imaging plate. After allowing enough time for the cells to adhere to the bottom of the plate, we treat them with Hoechst nuclear stain and any small molecules or other agents or conditions whose effects on motility are of interest. We collect images of the cell nuclei over the course of five or more hours, after which the image sequences are processed into background-subtracted TIFF stacks using MATLAB. These TIFF stacks are analyzed using the ImageJ TrackMate plugin, which registers and tracks each individual cell across timepoints<sup>17</sup>. After we have generated cell tracks for all imaging sites, we use a custom MATLAB script to remove spurious datapoints that occur after cells leave the imaging field and calculate average motility rates for each imaging site. The script only analyzes cells that are tracked for a user-specified minimum number of timepoints and allows the user to filter cells by overall distance and/or direction traveled. The result is the average displacement, average direction of travel, overall displacement, and overall direction of travel for each cell, which are used to calculate the bulk averages of those values for all cells in an imaging site. This protocol can be performed in bulk on large imaging experiments, which lends itself to relatively high-throughput motility analysis (**Figure 1**).

## **PROTOCOL:**

### **1 Cell culture and imaging plate preparation**

1.1 Culture HaCaT cells in cell culture medium (Dulbecco's Modified Eagle's Medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/ streptomycin, and 10% (v/v) fetal bovine serum) at 37 °C and 5% carbon dioxide in a humidified incubator.

1.2 Seed desired number of cells per well (we use 5000 – 20,000 cells/well) in a 96-well imaging plate, such as a 96-well black polystyrene microplate. To three wells, add only medium (no cells).

1.3 Rest the plate on a completely flat surface for 20 – 30 min so that cells can attach to the bottom of the plate with even spatial distribution.

1.4 Transfer plate to a humidified cell culture incubator (37 °C, 5% carbon dioxide) for 4-6 h to allow cells to adhere completely to the bottom of the plate.

1.5 Remove medium from plate and replace with cell culture medium with 2 µg/mL Hoechst dye (a 1:5000 dilution of 1 mg/mL stock solution).

1.6 Return plate to humidified cell culture incubator (37 °C, 5% carbon dioxide) for 2 h.

1.7 Gently wash plate three times with 100  $\mu$ L of phosphate buffered saline per well before adding 90  $\mu$ L of imaging medium (phenol red-free Dulbecco's Modified Eagle's Medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/ streptomycin, and 10% (v/v) fetal bovine serum) to each well.

1.8 If measuring the effects of small molecule inhibitors or other effectors, treat cells immediately before imaging.

## **2 Imaging**

2.1 Image the plate (including media-only wells) at 10x magnification in a microscope with a humidified environmental chamber at 37 °C and 5% carbon dioxide.

NOTE: The analysis scripts in this protocol are written for data collected on a Molecular Devices ImageXpress Micro XL widefield microscope.

2.2 Collect images according to the temporal resolution the experiment requires. For best results, record timepoints at most every 15 min for at least 5 h. If measuring the effects of small molecule inhibitors or other perturbances on cells, image for at least 5 h after the time expected to see an effect. For our purposes, we image for 100 8-min timepoints for a total imaging time of 13 h 20 min.

## **3 Image Processing**

3.1 Use the provided custom MATLAB script "ImageReorganizer.m" to remove thumbnail images and reorganize the image files by well rather than timepoint. The ImageXpress outputs files in this folder organization: "[Experiment Name] > [YYYY-MM-DD] > [Run number] > Timepoint\_[Timepoint] > [Image files]." Once images have been reorganized, this script performs background subtraction on images from each timepoint using the averaged and gaussian blurred images of the media-only wells from that timepoint. This script then organizes the background-subtracted images into a TIFF stack for each imaging site. Each TIFF stack is named as "[Well]\_[Site].tif."

3.1.1 If the experiment extended over more than one day, combine the contents of the [Run number] folders for all days so that all "Timepoint\_[Timepoint]" subfolders are in the same "[Run number]" folder.

3.1.2 Note that this script requires the MATLAB function `int2str`<sup>18</sup>. Input the local path to this function after "addpath" on line 2.

3.1.3 Input the rows for media-only wells as "emptyrows" on line 9 and the columns as "emptycolumns" on line 10.

3.1.4 Input the rows for experimental wells as “rows” on line 15 and the columns as “columns” on line 16. Input the number of imaging sites per well as “sites” on line 17.

3.1.5 Input the pathname for the “[Run number]” folder into the path bar in MATLAB and run the script.

## 4 Cell tracking

NOTE: TrackMate can be run on all TIFF stacks in a designated directory using a slightly modified version of the `Run_TrackMate_Headless.groovy` script from Tinevez et al.<sup>17</sup> or can be performed manually for each individual TIFF stack. We run FIJI manually on a few sample TIFF stacks to ensure that we are using the correct parameters before running the Groovy script on all TIFF stacks using those parameters.

4.1 To run the TrackMate plugin in FIJI for all TIFF stacks in a folder, download and use the [Run\\_TrackMate\\_Headless.groovy](#) script from Tinevez et al.<sup>17</sup> according to their provided protocol. We use a slightly modified version of this script, which allows us to easily batch both the input files and the output filenames and input all parameters using one graphical user interface for input. Below are detailed instructions for using this modified script, which is provided here as `TrackMate_Headless_Mod.groovy`.

4.1.1 Open the script in FIJI by selecting **File>Open** and then navigating to the location of the `TrackMate_Headless_Mod.groovy` script.

4.1.2 Select **Run**.

4.1.3 Input the directory in which the TIFF stacks are located (default output from “StackGenerator.m” should be “[Experiment Name] > [YYYY-MM-DD] >> stacks”) as “Input directory.”

4.1.4 Create an output directory and input its pathname as “Output directory.” The default for “File extension should be “.tif;” if not, input “.tif” as “File extension.”

4.1.5 To only run TrackMate on a subset of files in the directory, use the input space after “File name contains” to include those. Otherwise, leave that space blank.

4.1.6 Select “Keep directory structure when saving.”

4.1.7 Input “Spot radius,” “Quality threshold,” “Max frame gap,” “Linking max distance,” and “Gap-closing max distance” in pixels according to your desired settings. We typically use 3.5, 18, 2, 15, and 40, respectively, for HaCaT nuclei at 10x magnification.

4.1.8 Select **Okay**. This script should output an XML file for each TIFF stack in the designated output directory.

4.2 Alternatively, run the TrackMate plugin in FIJI manually for each TIFF stack according to the protocol outlined in Tinevez et al.<sup>13</sup>

4.2.1 Open a TIFF stack in FIJI and open the TrackMate plugin by selecting **Plugins >Tracking >TrackMate** in the menu bar.

4.2.2 If a popup window displays the prompt “It appears this image has 1 timepoint but [number of timepoints] slices. Do you want to swap Z and T?” select **Yes**.

4.2.3 In TrackMate v4.0.1, use default settings except for “Estimated blob diameter” (we use 7.000 pixels for HaCaT nuclei at 10x magnification) and threshold (we use 18.000, but this will vary based on the microscope used and intensity of Hoechst dye).

4.2.4 Use the **Preview** function to ensure optimized particle detection and adjust blob diameter and threshold settings as necessary.

4.2.5 For “Gap-closing max distance,” default settings often work for basal HaCaT motility, but gap-closing max distance may need to be increased to up to 40.0 pixels under conditions that enhance motility.

4.2.6 Visually confirm that tracking is optimized with minimal gaps in cell traces.

4.2.7 Under **Select a action** [sic], choose **Export tracks to XML file** and execute.

4.2.8 Repeat for all TIFF stacks.

## 5 Quantification of motility rates

NOTE: Use the provided custom MATLAB script "TrackMateAnalysis.m" to remove failed traces and quantify motility for all TrackMate output files. The script removes tracks that move out of frame (whose location traces default to integers) and tracks that are tracked for fewer than a designated number of timepoints. The script removes tracks that move out of frame (whose location traces default to integers) and tracks that are tracked for fewer than a designable number of timepoints. The output is average displacement per timepoint for individual cells and all cells in an imaging site. (Example output: 'Output.mat')

5.1 Note that this script requires the provided MATLAB function “import\_trackmate\_xml.m.” Input the local path to this function after “addpath” on line 4.

5.2 On line 7, indicate the minimum number of timepoints a particle should be tracked over to be included in your consolidated output.

5.3 On lines 12 and 13, indicate the range of distances traveled (in pixels) that should be included in the filtered analysis. To include all possible distances traveled input a range from 0 to the width of your imaging field.

5.4 On lines 14 and 15, indicate the range of trajectories (in degrees) that should be included in the filtered analysis. To include all possible trajectories, input a range from 0 to 360.

5.5 Run the script in the folder containing all TrackMate output XML files.

NOTE: The script generates and saves a MATLAB cell structure containing all data generated in this analysis. Tables{1,:} lists the names of Trackmate output XML files for each imaging site analyzed by this script. Tables{2,:} is a substructure containing the positions (pixels), displacements (pixels), and angles of travel (degrees, with 0° indicating a rightward direction of travel within the imaging site) for all single cells at each timepoint. It also contains the total distance traveled (pixels), average motility (pixels/timepoint), average angle of travel (degrees) for each cell across all timepoints, as well as the total angle of travel between the first and last timepoint for each cell. The 3<sup>rd</sup> and 4<sup>th</sup> rows of Tables respectively contain the average and standard deviation motility values (pixels/timepoint) for all cells across all timepoints in each imaging site. The 5<sup>th</sup> and 6<sup>th</sup> rows respectively contain the average and standard deviation values for total distance traveled (pixels) by all cells across all timepoints in each imaging site. The 7<sup>th</sup> and 8<sup>th</sup> rows contain the average and standard deviation values for overall angle of travel (degrees) between the first and last timepoints for all cells in an imaging site. Whereas rows 3-8 of Tables contain these values for all cells that meet the minimum timepoint tracking criteria, rows 9-14 contain the same values but only for cells that meet the user-indicated filter criteria.

#### REPRESENTATIVE RESULTS:

To ensure that our analysis script was reliable and consistent, we measured the motility of HaCaT keratinocytes in three independent experiments. We found that while the standard deviation of cell motilities was variable between experiments (possibly due to the sensitivity of HaCaT cells to confluence and mechanical stimuli), the average motility was consistently replicated across multiple experiments without statistically significant differences between replicates according to a two-tailed student's t-test (**Figure 2**).

Because of the role of the Arp2/3 complex in mediating actin dynamics, the known inhibitory effect of  $\alpha$ -catenin on Arp2/3, and our previous observation that depletion of  $\alpha$ -catenin leads to increased motility<sup>3</sup>, we investigated whether inhibiting the Arp2/3 complex using small molecule inhibitor CK-666 would abrogate enhanced motility upon  $\alpha$ -catenin depletion. Two HaCaT keratinocyte cell lines stably expressing either an shRNA knockdown targeted to  $\alpha$ -catenin or a non-targeting shRNA control, which were generated and validated as previously described<sup>8</sup>, were seeded on a 96-well imaging plate.

We used our method to compare the motility of HaCaT keratinocytes expressing an  $\alpha$ -catenin knockdown to the motility of control cells at a single confluence (**Figure 3**). Consistent with the knowledge that  $\alpha$ -catenin can inhibit actin dynamics and cell motility by competing with the



Arp2/3 complex for actin binding, we observed a significant decrease in the motility of  $\alpha$ -catenin knockdown cells but not in control cells after treatment with Arp2/3 inhibitor CK-666 when cells were at moderate to high confluence. This suggests that Arp2/3-mediated actin dynamics are required for the elevated motility we see in HaCaT keratinocytes upon depletion of  $\alpha$ -catenin but not for basal motility levels in control cells.

#### FIGURE AND TABLE LEGENDS:

**Figure 1. Flowchart for motility analysis protocol.** (A) Reorganize raw file structure and convert single-timepoint TIF images into a TIF stack of all timepoints for each imaging site. (B) Run TrackMate to generate an XML file containing cell traces for each imaging site. (C) Filter traces by minimum number of tracked timepoints and run comparative motility analysis for all imaging sites.

**Figure 2. This method provides a consistent and reliable measurement of motility in HaCaT keratinocytes.** HaCaT keratinocytes were seeded in a 96-well plate and imaged with a timepoint every 8 minutes for 100 timepoints (13 h 20 min total). Images were analyzed according to the protocol above. This plot shows average motility across sixteen replicate imaging sites from three independent experiments. Note that the y-axis begins at 3  $\mu\text{m}/\text{h}$ . In each experiment, measurements were performed in duplicate or triplicate wells with two imaging sites per well, and each data point represents the average motility of all cells in an imaging site. Error bars represent standard deviation for all four imaging sites. P-values were determined by two-tailed student's t-test.

**Figure 3. Arp2/3 activity is required for elevated motility in HaCaT cells expressing an  $\alpha$ -catenin shRNA knockdown.** Cells were treated with DMSO (vehicle control) or 54  $\mu\text{M}$  CK-666 at time 0 and then imaged with a timepoint every 8 minutes for 100 timepoints (13 h 20 min total). Images were analyzed according to the protocol above. This plot shows the average motility across sixteen replicate imaging sites from three independent experiments for each condition with cell density matched across conditions. In each experiment, measurements were performed in duplicate or triplicate wells with two imaging sites per well, and each data point represents the average motility of all cells in an imaging site. Error bars represent standard deviation for all four imaging sites. P-values were determined by two-tailed student's t-test.

#### DISCUSSION:

The methodology and analysis tools described above provide a straightforward and scalable means for measuring and quantifying the motility of HaCaT keratinocytes that uses MATLAB and requires minimal programming experience. This protocol calls for the nuclei of cells to be stained and imaged over the course of at least 5 h. While data collection can be performed on any suitable microscope, this protocol provides a script for processing images collected on a microscope into a TIFF stack for each imaging site that shows a time lapse of cell nuclei locations over all timepoints. After these TIFF stacks have been processed through the TrackMate plugin for ImageJ, this protocol allows for output tracks to be consolidated into a single MATLAB cell structure containing average motilities, standard deviations, and tracks for each imaging site and for single cells.

While other cell motility analysis tools are available, this methodology uses MATLAB to determine motility rates for cells tracked with the ImageJ TrackMate plugin in a straightforward and scalable manner. This image analysis pipeline is computationally efficient enough to be run on a personal computer using either a Windows or iOS operating system; while the scripts provided are written for use on an iOS machine, they are functional on a Windows machine once the pathnames have been updated to include backslashes rather than forward slashes. Importing XMLs for the post-TrackMate analysis and consolidation script can be time-intensive, but the result is a single MATLAB cell structure that is smaller to store and more easily accessible than XML files for all imaging sites.

The method described in this manuscript creates possibilities for broad studies on regulators of cellular motility and could allow for a more in-depth understanding of the signaling that controls motility in HaCaT keratinocytes moving forward. Because of the role of keratinocyte motility in wound healing and tumorigenesis, an increased understanding its regulation has the potential for clinical translations that improve human health outcomes.

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#### **DISCLOSURES:**

X.L. and the University of Colorado Boulder have a financial interest in development of HDAC inhibitors for therapeutics and own equity in OnKure. X.L. is a cofounder and member of the board of directors of OnKure, which has licensed proprietary HDAC inhibitors from the University of Colorado Boulder. OnKure has neither involvement in the experimental design nor funding of this study.

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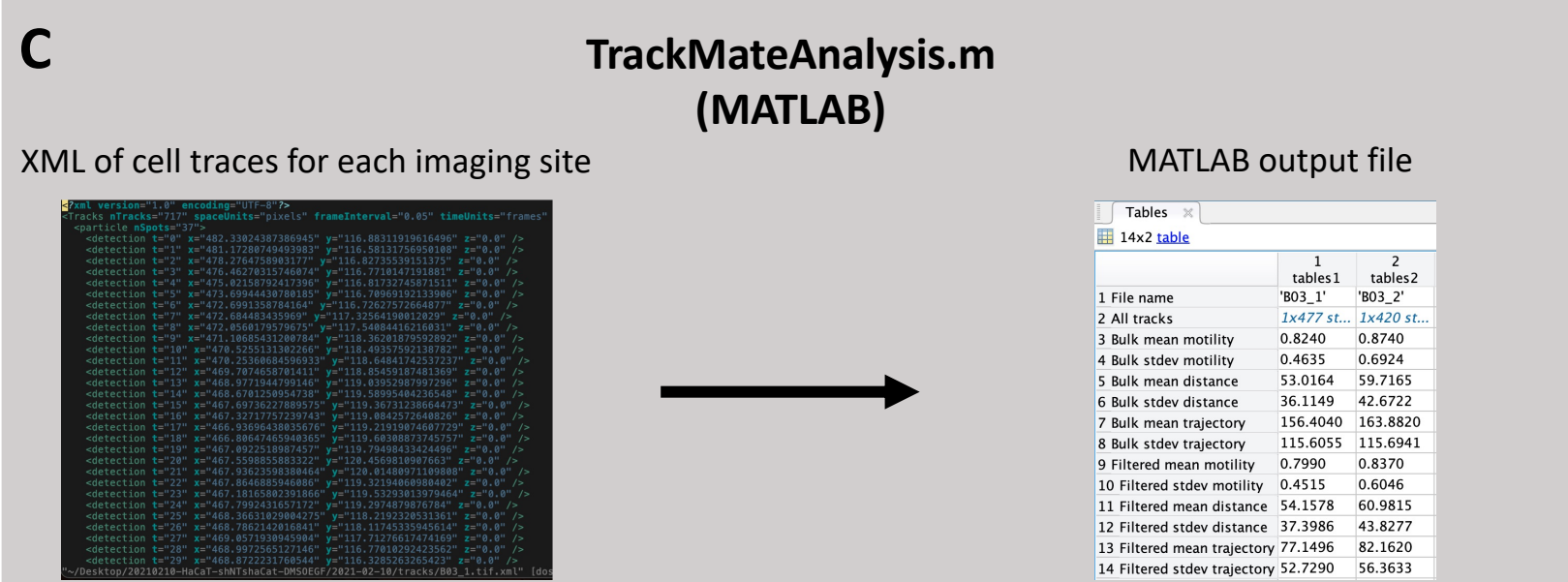
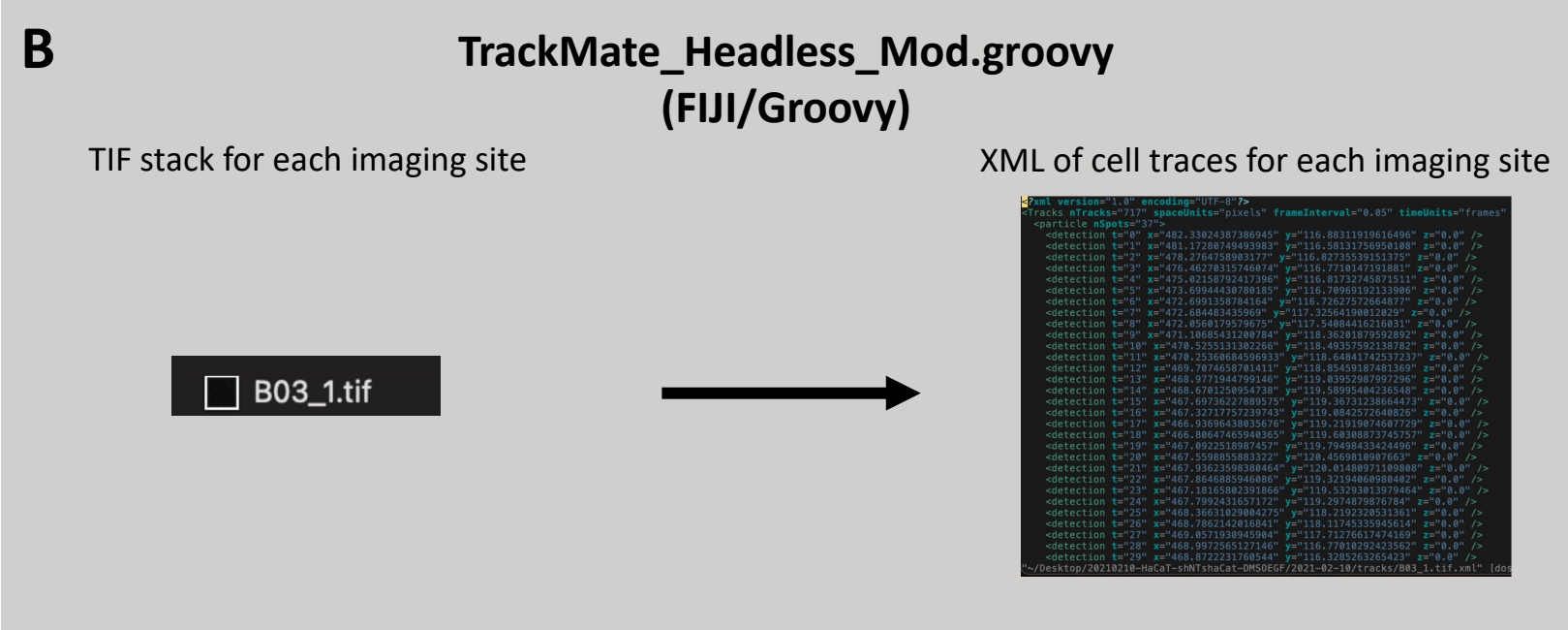
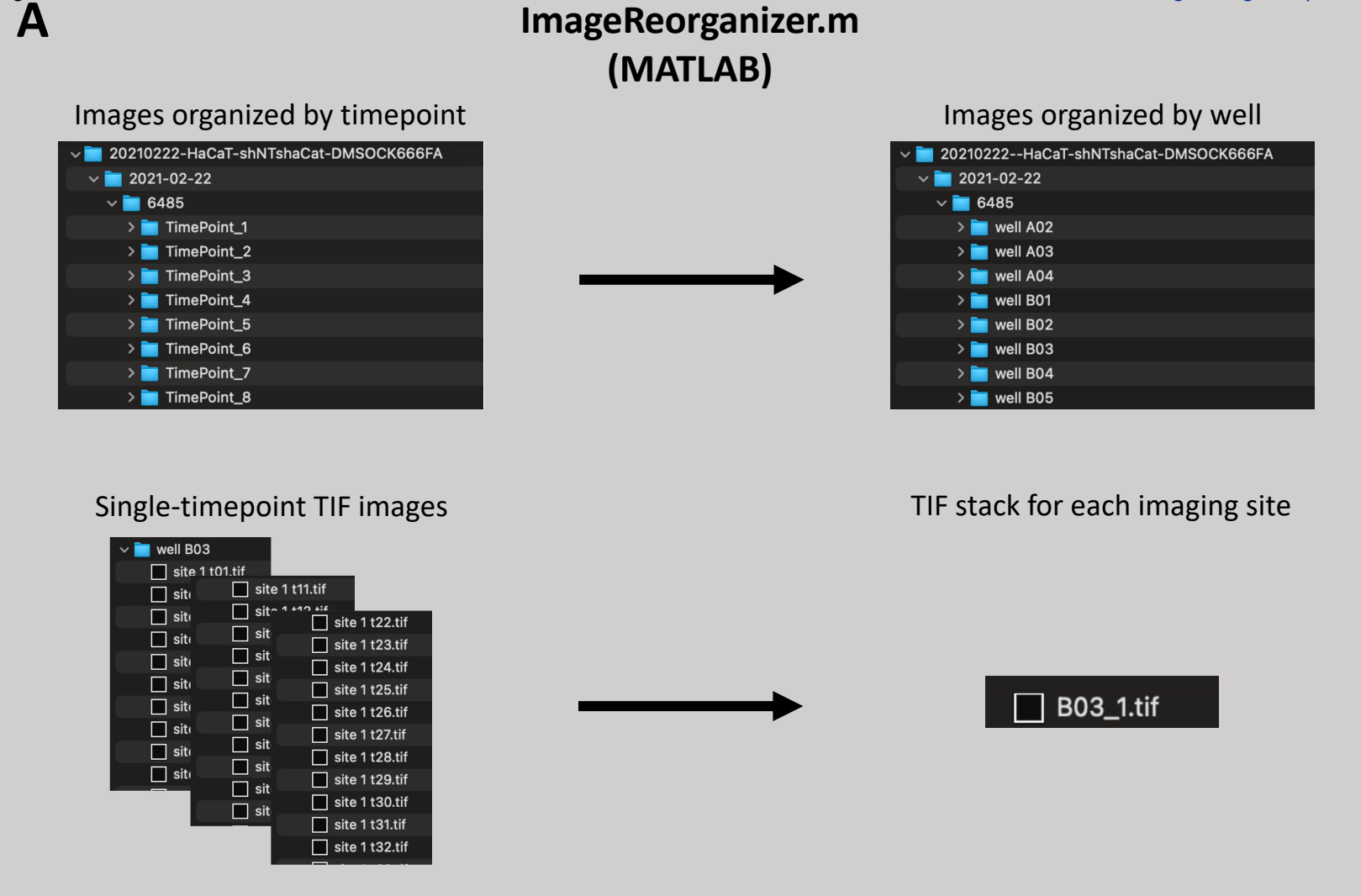


Figure 2

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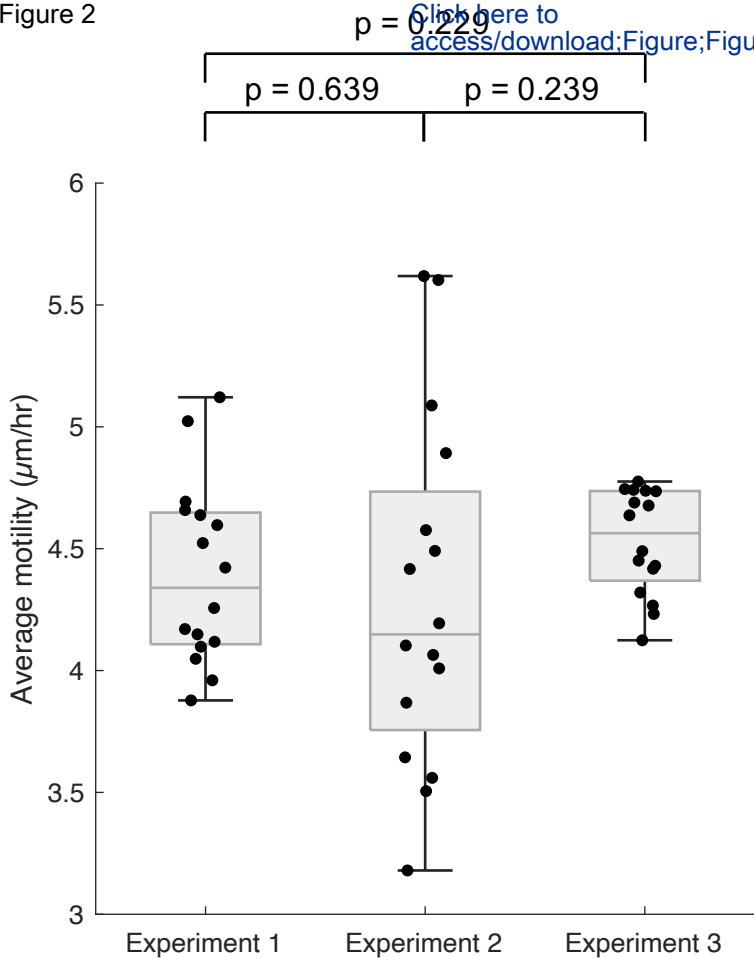
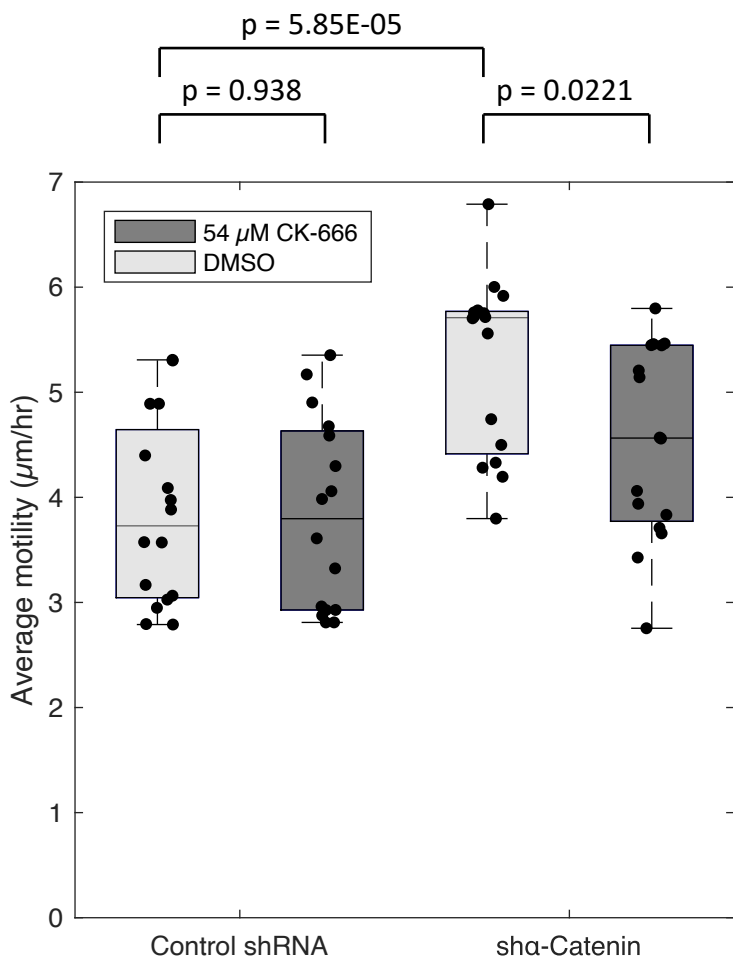


Figure 3

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**Table of Materials**  
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Thank you for all your help in shaping and improving this manuscript! Below are the itemized revisions and our responses.

*Changes to be made by the Author(s):*


*Please number the references in order of appearance. The first numbered reference should be 1. Currently, it is reference 7.*

The references have been updated to match this format.

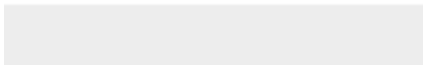
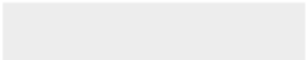
*Please send us the completed video so that it can be reviewed.*

We continue to work on producing a video and look forward to uploading it as soon as possible.



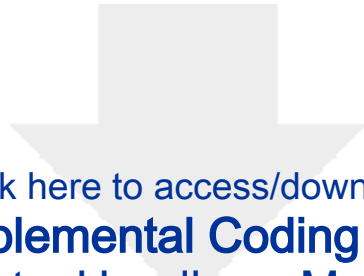


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