

# CD8 $\alpha$ -CI-M6PR Particle Motility Assay to Study the Retrograde Motion of CI-M6PR Receptors in Cultured Living Cells

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## Abstract

The cation-independent mannose 6-phosphate receptors (CI-M6PR) bind newly synthesized mannose 6-phosphate (Man-6-P)-tagged enzymes in the Golgi and transport them to late endosomes/lysosomes, providing them with degradative functions. Following the cargo delivery, empty receptors are recycled via early/recycling endosomes back to the trans-Golgi network (TGN) retrogradely in a dynein-dependent motion. One of the most widely used methods for studying the retrograde trafficking of CI-M6PR involves employing the CD8 $\alpha$ -CI-M6PR chimera. This chimera, comprising a CD8 ectodomain fused with the cytoplasmic tail of the CI-M6PR receptor, allows for labeling at the plasma membrane, followed by trafficking only in a retrograde direction. Previous studies utilizing the CD8 $\alpha$ -CI-M6PR chimera have focused mainly on colocalization studies with various endocytic markers under steady-state conditions. This protocol extends the application of the CD8 $\alpha$ -CI-M6PR chimera to live cell imaging, followed by a quantitative analysis of its motion towards the Golgi. Additionally, we present an approach to quantify parameters such as speed and track lengths associated with the motility of CD8 $\alpha$ -CI-M6PR endosomes using the Fiji plugin TrackMate.

## Key features

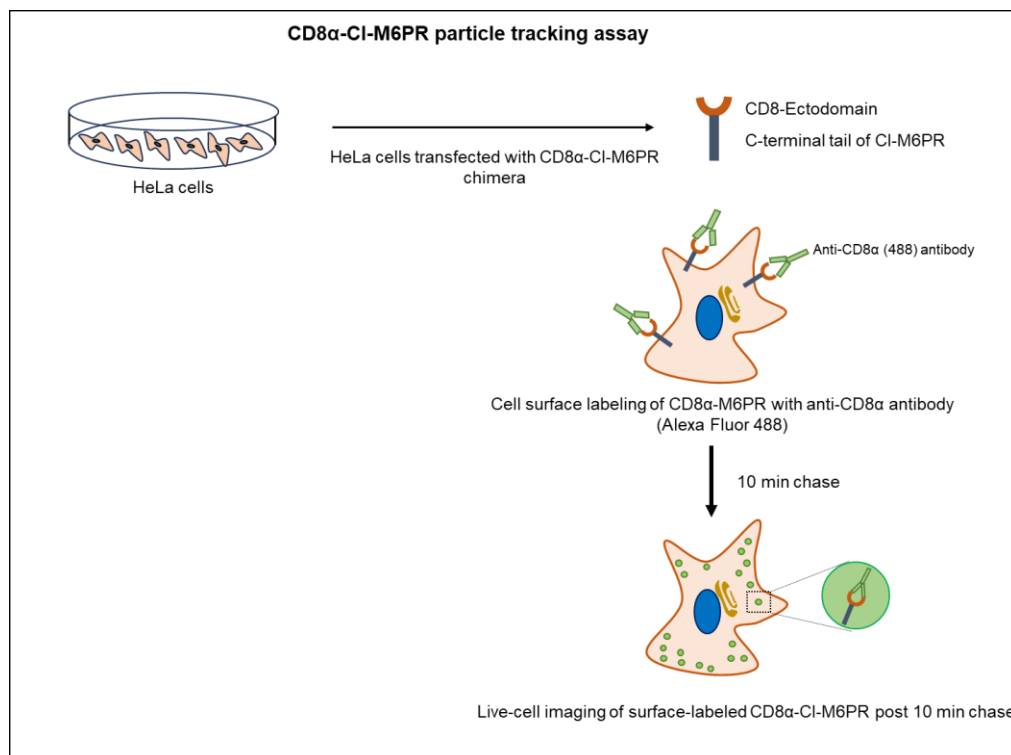
- This assay is adapted from the methodology by Prof. Matthew Seaman for studying the retrograde trafficking of CI-M6PR by expressing CD8 $\alpha$ -CI-M6PR chimera in HeLa cells.
- The experiments include live-cell imaging of surface-labeled CD8 $\alpha$ -CI-M6PR molecules, followed by a chase in cells.
- Allows the monitoring of real-time motion of CD8 $\alpha$ -CI-M6PR endosomes and facilitates calculation of kinetic parameters associated with endosome trajectories, e.g., speed and distance (run lengths).

**Keywords:** Trafficking, Endosome motility, CI-M6PR endosomes, Trans-Golgi network, Run lengths, Retrograde motion

**This protocol is used in:** J Cell Biol (2023) <https://doi.org/10.1083/jcb.202108001>

## Background

At steady state, the cation-independent mannose 6-phosphate receptors (CI-M6PR) are primarily localized to the trans-Golgi network (TGN), with a perinuclear endosomal population and a minor fraction on the plasma membrane. Post cargo delivery to endolysosomal compartments, CI-M6PR receptors undergo trafficking from all the cellular locations to early/recycling endosomes; subsequently, they are retrieved back to TGN in a dynein-dependent manner. This endosome-to-TGN retrieval of CI-M6PR is crucial for maintaining lysosomal activity and is regulated by various proteins involved in sorting and packaging into carrier vesicles followed by their motility towards TGN. Dysregulation in CI-M6PR receptor trafficking is linked to neurodegenerative and lysosomal storage disorders such as Batten's disease and Parkinson's disease [1,2]. Initial studies on CI-M6PR trafficking either required radiolabeling newly synthesized CI-M6PR and tracking their journey from Golgi or predominantly relied on measuring the redistribution of CI-M6PR receptors from TGN to other cellular compartments [3]. However, these methodologies only provide information on the localization of the cargo upon its impaired trafficking without revealing the exact direction of trafficking defect. Recently, several studies have employed the CD8 $\alpha$ -CI-M6PR chimera (developed by Matthew Seaman; [4]) to study retrograde trafficking of CI-M6PR, involving colocalization of CD8 $\alpha$ -CI-M6PR with different endocytic markers at steady-state. This chimera, a fusion of the ectodomain of CD8 and the cytoplasmic tail of CI-M6PR, allows trafficking only in the retrograde direction towards the Golgi [5]. Here, we present a protocol that extends the use of CD8 $\alpha$ -CI-M6PR chimera in a live-cell imaging setup, where surface receptors are labeled and then chased in cells as they move towards the Golgi (Figure 1). We also provide an approach to perform the quantitative analysis of CD8 $\alpha$ -CI-M6PR motion as they move from early/recycling endosome to Golgi, including the calculation of kinetic parameters such as speed and distance associated with their motion.



**Figure 1. Schematic illustrating the CD8 $\alpha$ -CI-M6PR trafficking assay to track motility of CD8 $\alpha$ -CI-M6PR endosomes internalized from the cell surface.** HeLa cells expressing CD8 $\alpha$ -CI-M6PR were incubated with primary (anti-CD8 $\alpha$ ) and secondary (Alexa Fluor 488-conjugated dye) antibodies on ice to specifically label surface receptors, followed by live-cell imaging in warm media at 37 °C after 10 min of chase.

## Materials and reagents

### Biological materials

1. 70%–80% confluent HeLa cells (ATCC)

*Note: We have used HeLa cells for this analysis because they are easy to transfect and considerably flat for 2D analysis of endosome motility; however, other cell lines can also be used.*

### Reagents

1. Plasmid CD8 $\alpha$ -CI-M6PR-pIRES Neo2 (a kind gift from Prof. Matthew Seaman)
2. Gibco™ Dulbecco's modified Eagle medium (DMEM), high glucose, with GlutaMAX™, sodium pyruvate (Thermo Fisher Scientific, catalog number: 10569-010), storage: 2–8 °C
3. Dulbecco's phosphate buffered saline (DPBS), without calcium and magnesium (Lonza Bioscience, catalog number: 17-512F), storage: 15–30 °C
4. Opti-MEM® reduced serum media (Thermo Fisher Scientific, Gibco™, catalog number: 11058021), storage: 2–8 °C
5. Heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10082147), storage: -20 °C
6. Antibiotic-antimycotic (Thermo Fisher Scientific, Gibco™, catalog number: 15240-062), storage: -20 °C
7. Gibco™ 1 M HEPES (Thermo Fisher Scientific, catalog number: 15630-080), storage: 2–8 °C
8. Gibco™ 100× MEM non-essential amino acid solution (Thermo Fisher Scientific, catalog number: 11140050)
9. Gibco™ DMEM, high glucose, HEPES, no phenol red (Thermo Fisher Scientific, catalog number: 21063029), storage: 2–8 °C
10. X-tremeGENE™ HD transfection reagent (Roche® Life Science Products, catalog number: 6366236001)
11. siRNA transfection reagent Dharmafect-1 (GE Healthcare, catalog number: T-2001-03)
12. Mouse anti-human CD8 monoclonal antibody (BD Pharmingen™, catalog number: 555631), storage: 4 °C
13. Alexa Fluor 488-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, catalog number: A11029), storage: 4 °C
14. Citrate anhydrous (HiMedia, catalog number: GRM-1023)
15. Sodium citrate tribasic dihydrate (Tri sodium citrate dihydrate) (Sigma, catalog number: S4641)

### Solutions

1. Citric acid buffer, pH 4.5 (see Recipes)

### Recipes

#### 1. Citric acid buffer, pH 4.5

Reagent	Volume
Citric acid anhydrous	100 mL
Tri-sodium citrate dihydrate	100 mL
Total	200 mL

#### 0.1 M Citric acid anhydrous

Reagent	Quantity
Citric acid anhydrous	2.626 g
Total	125 mL

### 0.1 M Tri-Sodium Citrate dihydrate

Reagent	Quantity
Tri-sodium citrate dihydrate	3.676 g
Total	125 mL

Adjust pH to 4.5 with sodium citrate.

## 2. Complete medium

Dulbecco's modified Eagle medium (DMEM), high glucose, with GlutaMAX™, sodium pyruvate supplemented with 10% FBS, 10 mM HEPES, 1× antibiotic-antimycotic, and 1× MEM non-essential amino acid solution

## Laboratory supplies

1. 35 mm  $\mu$ -dish, high glass-bottom live-imaging dishes (ibidi, catalog number: 81158)
2. BD Falcon 15 mL centrifuge tubes (Corning, Falcon®, catalog number: 352096)
3. BD Falcon 35 mm cell culture dish (Corning, Falcon®, catalog number: 353001)

## Equipment

1. Cell culture incubator (Eppendorf, New Brunswick™, model: Galaxy® 170 R)
2. Confocal microscope (ZEISS, model: LSM 710)
3. HulaMixer™ sample mixer (Thermo Fisher Scientific, catalog number: 15920D)

## Software and datasets

1. Fiji software (<https://imagej.net/software/fiji/>) 2.15.1
2. ZEN software, ZEN 2012 v. 8.0.1.273 (ZEISS)

## Procedure

### A. Experimental setup

*Note: This protocol has been modified from CD8 $\alpha$ -CI-M6PR trafficking assay [4], aiming to assess the motility of surface-labeled CD8 $\alpha$ -CI-M6PR towards the Golgi in HeLa cells treated with siRNAs against a specific gene of interest.*

1. Seed approximately  $0.14 \times 10^6$  HeLa cells in a 35 mm glass-bottom live-imaging dish and incubate overnight in a cell culture incubator.  
*Note: For transfection, seed  $0.25 \times 10^6$  HeLa cells and incubate overnight in a cell culture incubator.*
2. On the following day, prepare siRNA transfection mix by combining 100 nM siRNA oligos and transfection reagent Dharmafect-1 (Dharmacon) in 200  $\mu$ L of Opti-MEM and incubate the mixture at room temperature for 30 min. After incubation, add siRNA transfection mix dropwise over cells and incubate for 48 h.
3. After 48 h of siRNA treatment, transfect the cells with CD8 $\alpha$ -CI-M6PR. To prepare the transfection mix, add 2  $\mu$ g of CD8 $\alpha$ -CI-M6PR-pIRES Neo2 plasmid DNA in 200  $\mu$ L of Opti-MEM and X-tremeGENE™ HD transfection reagent in a ratio of 1:1 (1  $\mu$ g DNA: 1  $\mu$ L transfection reagent). Incubate the transfection mix at room temperature for 30 min, then carefully add it dropwise over cells.

4. To fluorescently label the primary antibody, incubate the mouse anti-CD8 antibody along with Alexa Fluor 488-conjugated anti-mouse IgG in 1 mL of serum-free DMEM media at 4 °C for 30 min in a tube rotator.
5. Following 12–14 h of transfection, incubate the complex of anti-CD8 $\alpha$  with Alexa Fluor 488-conjugated IgG with cells in a live-cell imaging dish on ice for 30 min to label the surface CD8-CI-M6PR receptors.  
*Note: As a control for the non-specific attachment of Alexa Fluor 488-conjugated antibodies to the plasma membrane, execute the antibody labeling and subsequent steps using untransfected cells.*
6. After incubation, carefully aspirate the DMEM media containing anti-CD8 $\alpha$ /Alexa Fluor 488-conjugated antibody complex.
7. To remove any unbound or non-specifically attached antibodies, wash the cells once with DPBS followed by two washes with ice-cold citric acid buffer, pH 4.5. Add approximately 1 mL of citric acid buffer to cells and incubate on ice for 5 min. Discard the buffer and repeat the washing step twice with citric acid buffer. Finally, add 1 mL of DPBS to remove any residual citric acid buffer from the cells.  
*Note: Prepare fresh citric acid buffer and use it ice-cold for washes.*
8. After washing, add 2 mL of prewarmed phenol red-free DMEM media supplemented with 10% FBS on cells and incubate it for 10 min.
9. After incubation, transfer the dish to the humidified live-cell imaging chamber maintained at 37 °C with 5% CO<sub>2</sub> and perform live-cell imaging of the transfected cells showing labeled CD8 $\alpha$ -CI-M6PR receptors. Post 10 min chase, labeled CD8 $\alpha$ -CI-M6PR receptors present on plasma membrane traffic to TGN due to the retrograde sorting signals present on the cytoplasmic tail of CI-M6PR.

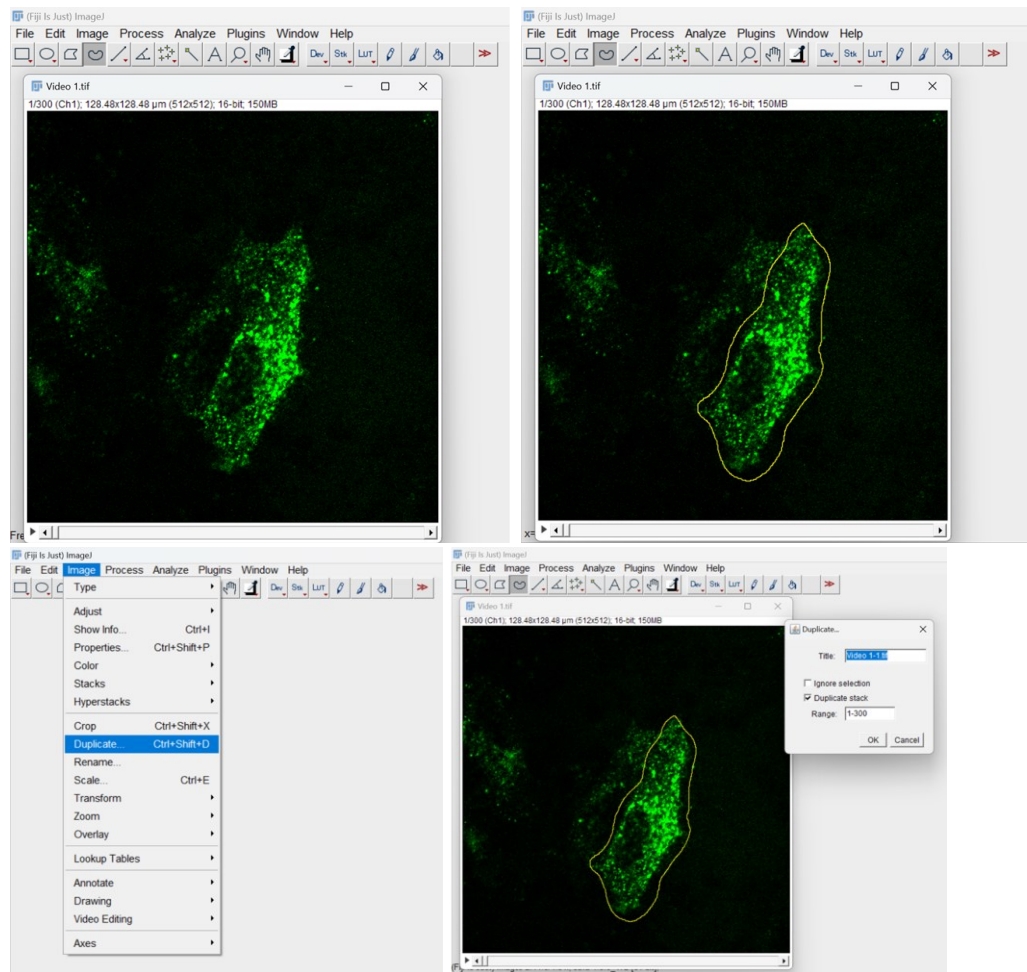
## B. Live-cell imaging and video acquisition

1. Use 512 × 512 frame size for higher acquisition speed and acquire a timelapse video of cells for approximately 200–300 frames minimum without any time interval.
2. Record the video of the labeled CD8 $\alpha$ -CI-M6PR receptors for a duration not exceeding 15 min, following a 10 min chase, during which they are expected to reach TGN.

## C. Image analysis and quantification using ImageJ/Fiji

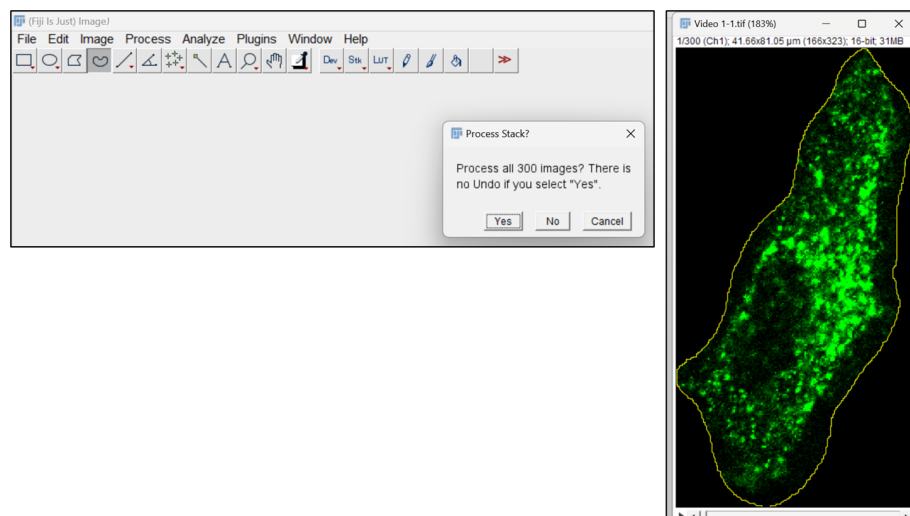
*Note: In the manuscript [6], we conducted particle tracking analysis using a semi-automated custom-written program and a MATLAB script [7]. However, in this protocol, we have presented an alternative protocol employing the TrackMate plugin for the analysis of CD8 $\alpha$ -CI-M6PR particles.*

1. Open the video file using Fiji. If there are multiple cells in a field, use the free-hand tool from the Fiji tool panel to select a single cell. Duplicate the selection by either using Ctrl+shift+D or go to *Image* menu and choose the *Duplicate* option. A pop-up window will appear; select the *Duplicate stack* option (Figure 2).



**Figure 2. Duplicate video stack and select the cell for analysis**

2. Remove the background fluorescence using the *Edit* menu. Select the *Clear outside* option (Figure 3). Choose *yes* to the process stack option allowing it to remove the background from all frames.



**Figure 3. Clear outside from all frames of video**

- Subtract cytoplasmic background fluorescence from the cell to improve the endosome tracking process. Select *Subtract background* option in the *Process* menu in Fiji toolbar. Input the value of *Rolling ball radius* appropriately according to your background fluorescence in the video file (Figure 4). *Note: The value of Rolling ball radius should be higher than the largest object in the image.*

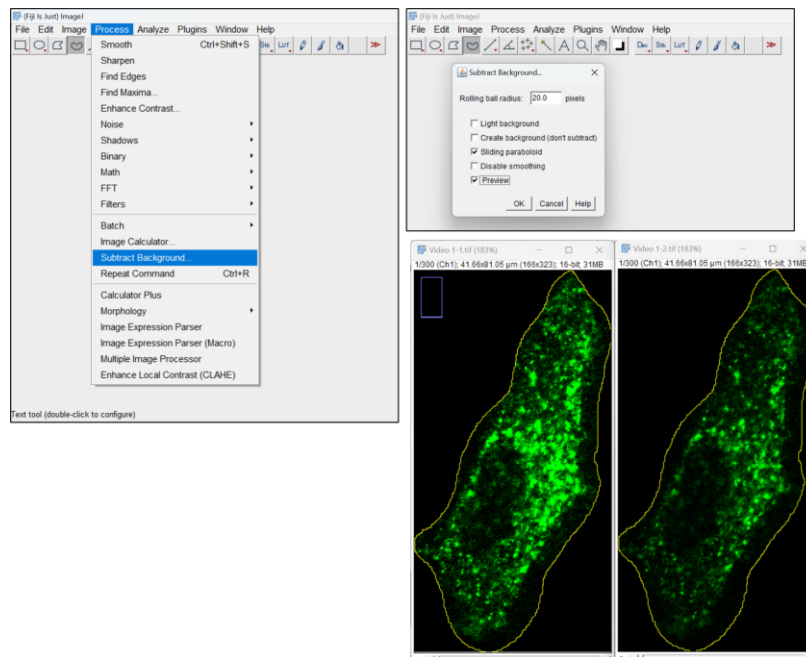


Figure 4. Background subtraction in cell

- Perform particle tracking on video file. Select *Plugins > Tracking > TrackMate* option. A dialog box will open with spatial and temporal information of the data. It provides information on pixel dimensions, number of frames, and also the dimensionality of the video file (2D or 3D lapse video). To proceed with tracking, press the *Next* button (Figure 5).

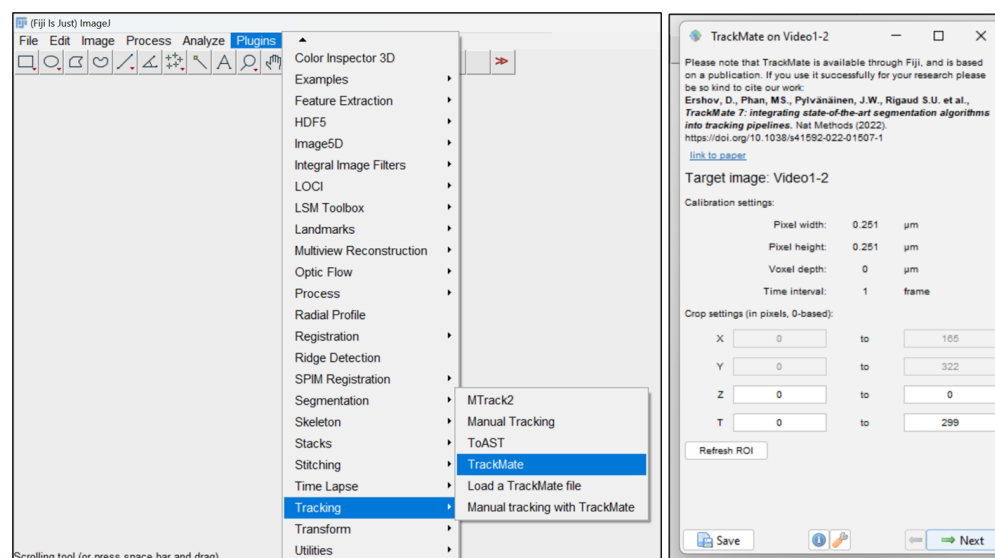


Figure 5. Particle tracking using TrackMate plugin

- Next, a window will open with three options as object detectors. Choose the *Difference of Gaussian (DoG)* detector, designed to identify round particles like endosomes and being particularly effective for particle sizes below ~5 pixels. Proceed by clicking the *Next* button (Figure 6).

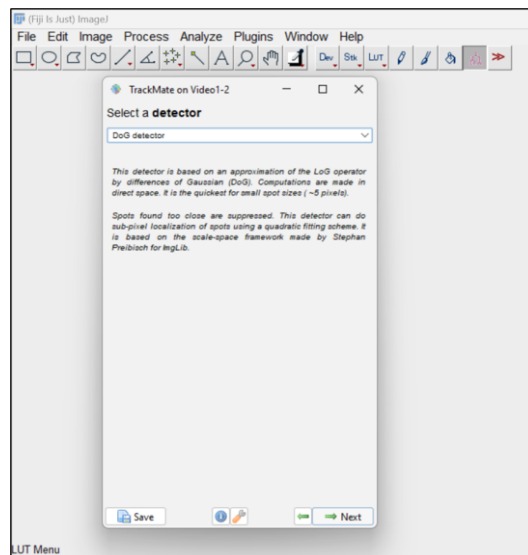


Figure 6. Selection of *difference of Gaussian (DoG)* detector

- In the subsequent DoG detector window, input the values for a few parameters associated with particles to be analyzed, such as *Estimated Blob diameter* and *Quality threshold*. For *Estimated Blob Diameter*, enter the approximate diameter of the endosomes or measure it using the line tool in Fiji. *Quality threshold* allows you to limit your analysis to a specific number of endosomes, excluding spots/endosomes with quality below the threshold. Click the preview button to see the selected endosomes and assess how well the parameters fit the data. Adjust the *Quality* threshold to eliminate any false spot intensities from the analysis. Click on *Preview* to see the selected spots and accordingly adjust the threshold to choose the desired endosomes. You can also see the number of endosomes that were selected for the selected threshold (highlighted here in red outline) (Figure 7). Go to the next window once adjustments have been made.

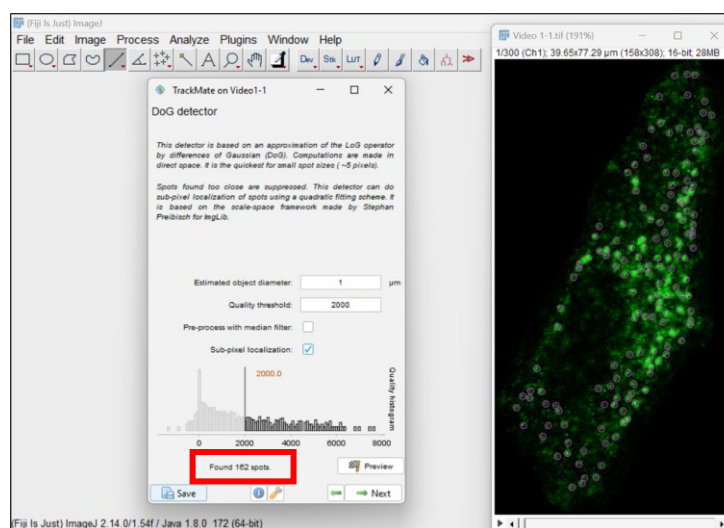


Figure 7. *Difference of Gaussian (DoG)* detector parameters for particles



- Next, choose the HyperStack displayer, which allows you to overlay the end results over the image (Figure 8).

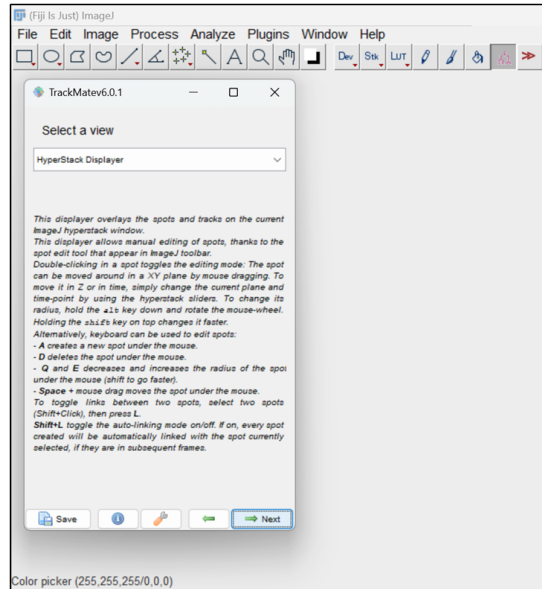


Figure 8. Choose the HyperStack displayer

- This window allows you to do auto or manual thresholding to select the particles. Choose *Auto* here. Click *Next* (Figure 9).

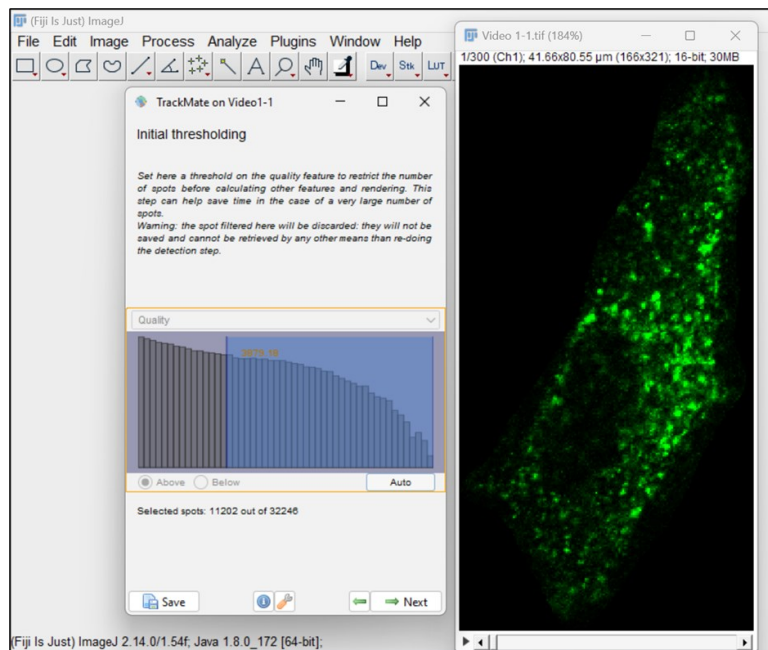
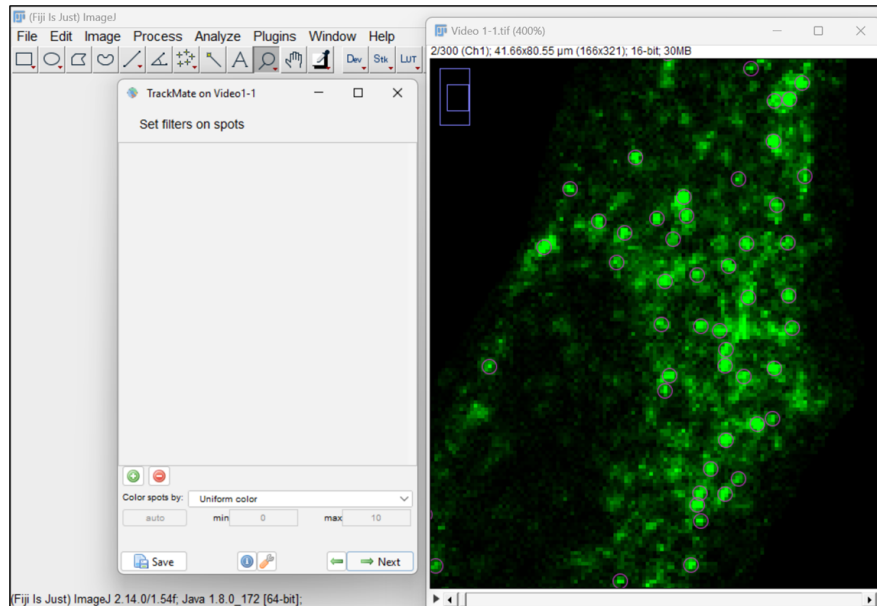


Figure 9. Thresholding on particles/spots

- At this point, we can see the spots that are selected for analysis. We can run the video and observe the spots moving through all frames. In this window, you can set filters on the spots; however, for now, we will

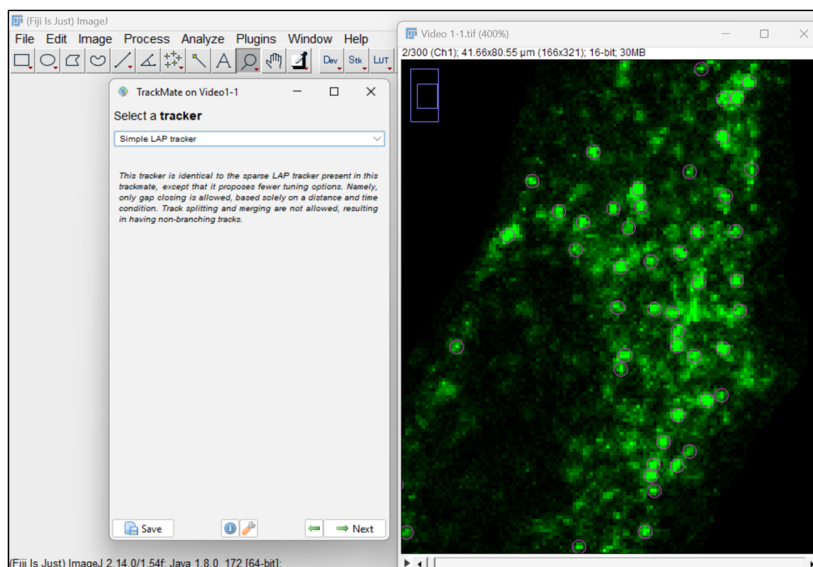
proceed with default settings and click *Next* (Figure 10).

*Note: You can also filter spots based on parameters like intensity, quality, radius of the particles, frame, or positions (X, Y) and do a comparative analysis of choices.*



**Figure 10. Set filters and choose particles or spots for analysis**

- Now, select the simple LAP (linear assignment problem) tracker, ideal for particles undergoing Brownian motion. This tracker creates tracks by linking particles across different frames in the video (Figure 11).



**Figure 11. Selection of simple linear assignment problem (LAP) tracker**

- Next, a LAP tracker window will open, allowing you to input parameters for particle-to-particle linking. Enter 2  $\mu\text{m}$  in the option for *Linking max distance*, which defines the maximum distance for two spots to be linked between two frames. Put the value of *Gap-closing max distance* as 2  $\mu\text{m}$ , meaning that it will not link any two spots that are more than two frames apart. Set *Gap-closing max frame gap* to 1, which is

the maximum frame difference allowed for a spot to be missed for linking (Figure 12).

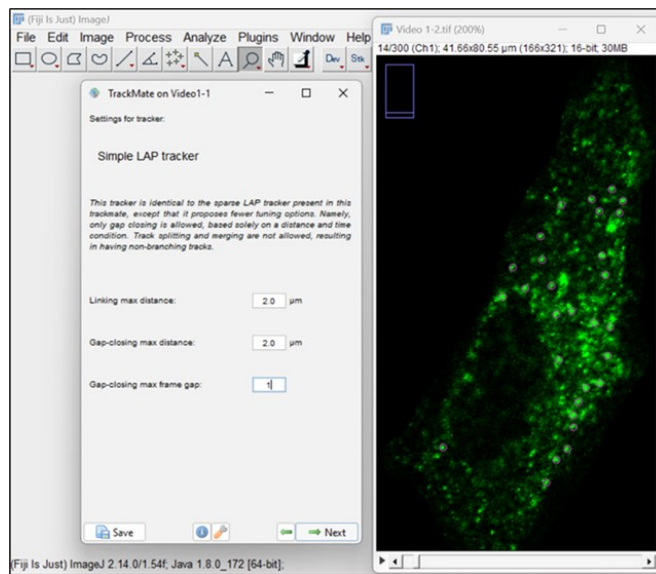


Figure 12. Configuration for linking particles by simple LAP tracker

12. The next window allows the visualization of the tracks generated by LAP tracker. Click *Next* (Figure 13).

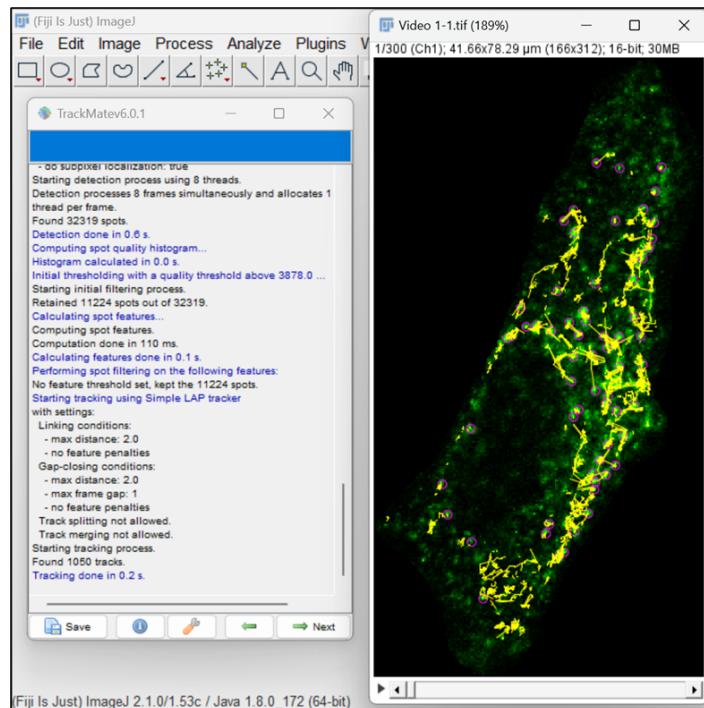


Figure 13. Tracks generated by LAP tracker

13. This window allows you to set filters on tracks. You can choose any option for viewing tracks, but here we have opted to set the color of the tracks based on maximal velocity. Click *Next* (Figure 14).

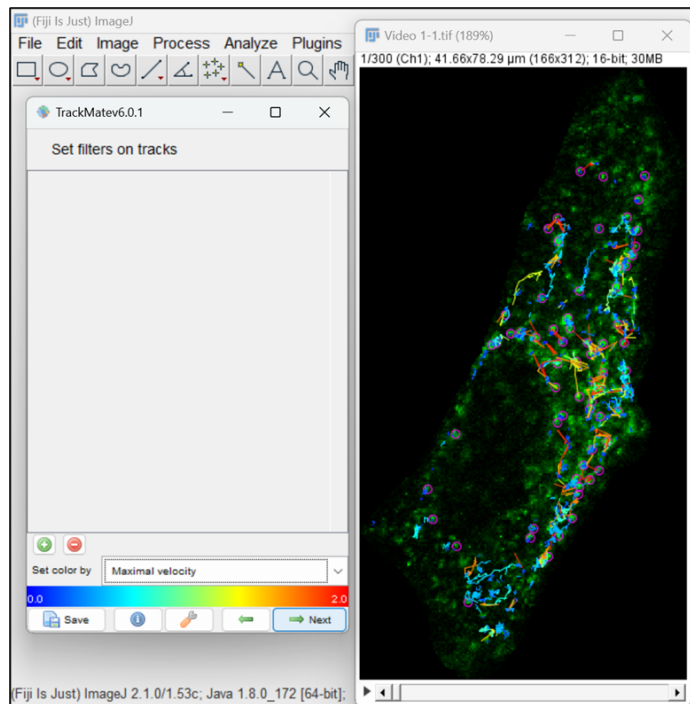


Figure 14. Color filter for visualization of tracks

- The next window allows to further change the display options. Here, you can increase or decrease the size of the spot by changing its radius. Furthermore, you can also change the display of the tracks. Click on *Next* button. A new panel will open with options to plot different features associated with particle motility as a function of another. To continue, click on *Next* (Figure 15).

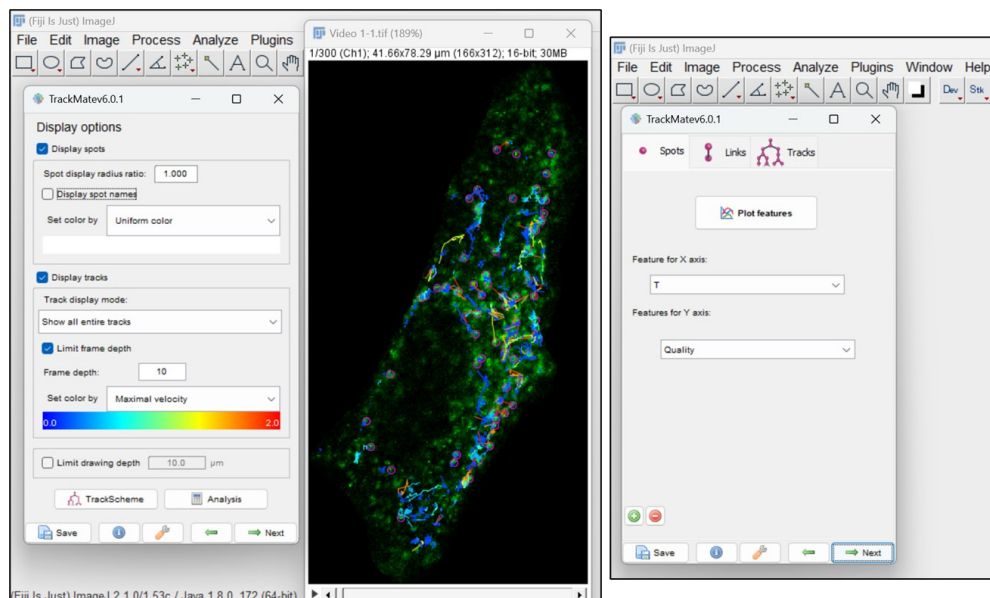


Figure 15. Display options for particles and tracks

- Select *Overlay* option in the next window and click on *Execute*. A pop-up window will open, showing the total number of frames. Click on *ok*. You can save the video with all the captured tracks (Figure 16).

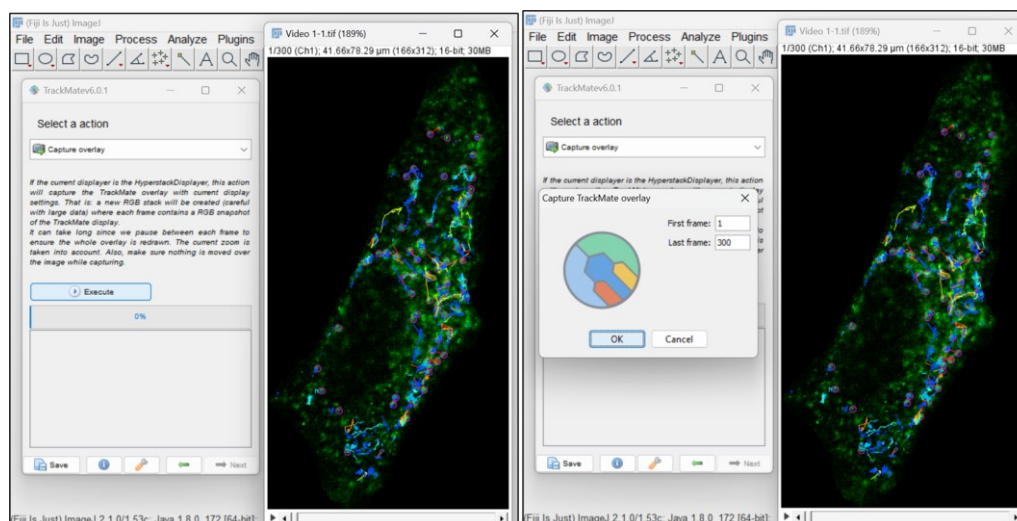


Figure 16. TrackMate features and analysis

- To obtain measurements associated with particle/spot's motion such as distance, displacement, maximum speed, and duration of a track, go back to the *Display options* window (shown in Figure 15) and click on *Analysis*. Three different windows will open, providing information on quantitative parameters for all the tracks associated with the particle motion (Figure 17).

Spots in tracks statistics			Links in tracks statistics			Track statistics						
File	Edit	Font	File	Edit	Font	File	Edit	Font				
Label	ID	TRACK_ID	Label	TFLabel	TRACK_ID	NUMBER_SPOTS	NUMBER_GAPS	LONGEST_GAP	NUMBER_SPLITS			
ID109953	109953	0	(ID109953 : ID110112)	0	Track_2112	12	21	0	0	0		
ID110112	110112	0	(ID110112 : ID109785)	0	Track_2113	13	2	0	0	0		
ID109785	109785	0	(ID109785 : ID110272)	0	Track_2114	14	25	0	0	0		
ID110272	110272	0	(ID110272 : ID110693)	0	Track_2115	15	26	0	0	0		
ID110693	110693	0	(ID109952 : ID110105)	1	Track_2116	16	33	0	0	0		
ID109952	109952	1	(ID110105 : ID109775)	1	Track_2117	17	2	0	0	0		
ID110105	110105	1	(ID109775 : ID110261)	1	Track_2118	18	6	0	0	0		
ID109775	109775	1	(ID110261 : ID110661)	1	Track_2119	19	26	0	0	0		
ID110261	110261	1	(ID110661 : ID110636)	1	Track_2120	20	15	0	0	0		
ID110661	110661	1	(ID110636 : ID110418)	1	Track_2121	21	7	0	0	0		
ID110636	110636	1	(ID110418 : ID110888)	1	Track_2122	22	6	0	0	0		
ID110418	110418	1	(ID110888 : ID111044)	1	Track_2123	23	12	0	0	0		
ID110888	110888	1	(ID111044 : ID111649)	1	Track_2124	24	2	0	0	0		
ID111044	111044	1	(ID111649 : ID111350)	1	Track_2125	25	28	0	0	0		
ID111649	111649	1	(ID111350 : ID111200)	1	Track_2126	26	7	0	0	0		
ID111350	111350	1	(ID111200 : ID111498)	1	Track_2127	27	21	0	0	0		
ID111200	111200	1	(ID111498 : ID111970)	1	Track_2128	28	24	0	0	0		
ID111498	111498	1	(ID111970 : ID111809)	1	Track_2129	29	58	0	0	0		
ID111970	111970	1	(ID111809 : ID112259)	1	Track_2130	30	5	0	0	0		

Figure 17. Track analysis tables showing different kinetic parameters associated with particle motion

## Data analysis

From the data extracted from the track statistics file or table, we can acquire values such as track duration, displacement, mean velocity, and maximum speed for all the tracks identified by the LAP tracker. In the video, we have detected roughly ~1,000 tracks by linking two spots over time. Each track encompasses values for displacement, duration, and velocity. Displacement signifies the distance between spots in consecutive frames, while duration

indicates the time span. Velocity values denote the link velocity defined for each link between two spots. Subsequently, we can compute and plot the median, maximum, minimum, and average velocities from link velocities under different conditions or treatments. Analyze the tracks from approximately 7–10 cells and plot the values for each quantitative parameter against various treatments. Further, calculate the statistical significance of differences in mean values between two or more conditions by utilizing either an unpaired two-tailed Student's t-test or analysis of variance (ANOVA), respectively. These tests are applicable when the data approximately follows a normal distribution and satisfies assumptions such as homogeneity of variances. For non-normal distributions, which lack symmetry, statistical comparisons are done between the medians of the two groups using Mann-Whitney U test.

## Validation of Protocol

This protocol has been used and published in the following research article:

- Rawat, S. et al. (2022). RUFY1 binds Arl8b and mediates endosome-to-TGN CI-M6PR retrieval for cargo sorting to lysosomes. *J. Cell Biol.* (Figure 10, panel D)

*Note: This article has used a semi-automated custom-written program and a MATLAB script [7], but here we have employed an alternative approach using TrackMate plugin for the particle analysis.*

## Acknowledgments

The authors adapted and modified this protocol from a previously published study [4] and validated it in a recently described study [6]. The authors would like to thank Prof. Matthew Seaman (University of Cambridge, UK) for sharing the CD8 $\alpha$ -CI-M6PR construct. S. Rawat acknowledges fellowship support from University Grants Commission. M. Sharma would like to acknowledge funding support from the Department of Biotechnology (DBT)/Wellcome Trust India Alliance Senior Fellowship (IA/S/19/1/504270), SERB-POWER Grant (SPG/2021/002790), and Janaki Ammal-National Women Bioscientist Award (BT/HRD/NWBA/39/01/2018-19). M. Sharma also acknowledges the infrastructure and financial support from IISER Mohali.

## Competing interests

The authors declare no competing financial interests.

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