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Visualizing Diffusional Dynamics of Gold Nanorods on Cell Membrane Using Single Nanoparticle Darkfield Microscopy

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Dear editors,

Thank you very much for your attention and comments on our paper. We have revised the manuscript according to your kind advices and detailed suggestions using the attached file. Enclosed please find the responses. We sincerely hope this manuscript will be finally acceptable to be published on *JoVE*.

Manuscript Title: Visualizing Diffusional Dynamics of Gold Nanorods on Cell Membrane Using Single Nanoparticle Darkfield Microscopy

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Explanation of the manuscript's significance:

The dynamics of nanoparticles (NPs) on membrane is closely associated with the cellular uptake process, which is essential for the understanding of cell functions, viral or bacterial infections and the development of artificial nanomedical delivery systems. Single particle tracking (SPT) analysis could probe the position and orientation of individual nanoparticles on cell membrane, and reveal their translational and rotational states. Fluorescence-based microscopic imaging techniques have become valuable tools for observing NPs/molecules in living cell environment. However, the inherent disadvantages of single fluorophores, such as low intensity and rapid irreversible photobleaching, reduce the accuracy and duration of tracking. Therefore, non-fluorescent plasmonic NPs, replacing the fluorescent probes, have attracted more and more attention in long-term imaging studies due to their unique optical characteristics. Based on the scattering signals of plasmonic NP probes, several kinds of optical microscopic imaging technologies have been used to study the mechanism of biological processes. In addition, the time-resolved trajectories and orientational angles generated by individual NPs are normally stochastic and heterogeneous, so it is necessary to present abundant dynamic information with various analysis methods.

In this work, we propose an integrated protocol for studying the diffusion dynamics of AuNRs on live cell membrane with SPT method. The dynamic of AuNRs was monitored with single nanoparticle darkfield microscopy, extracted using ImageJ and MATLAB, and characterized by comprehensive analytical methods. CTAB@AuNRs did abnormal diffusion motion on U87 cell membrane, and its motion shows spatiotemporal heterogeneity. Our SPT methods can be potentially used to study surface or intracellular particle diffusion in different biological cells, and can become a powerful tool for investigations of complex cellular mechanisms.

Thank you very much for all your help and looking forward to hearing from you soon.
Sincerely yours,

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TITLE:

Visualizing Diffusional Dynamics of Gold Nanorods on Cell Membrane Using Single Nanoparticle Darkfield Microscopy

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KEYWORDS:

cell membrane, single particle tracking, darkfield microscopy, diffusional dynamics, gold nanorods.

SUMMARY:

Here, we show the use of traditional dark-field microscopy to monitor the dynamics of gold nanorods (AuNRs) on cell membrane. The location and orientation of single AuNRs are detected using ImageJ and MATLAB, and the diffusive states of AuNRs are characterized by single particle tracking analysis.

ABSTRACT:

Analyzing the diffusional dynamics of nanoparticles on cell membrane plays a significant role in better understanding the cellular uptake process and provides a theoretical basis for the rational design of nano-medicine delivery. Single particle tracking (SPT) analysis could probe the position and orientation of individual nanoparticles on cell membrane, and reveal their translational and rotational states. Here, we show how to use traditional dark-field microscopy to monitor the dynamics of gold nanorods (AuNRs) on live cell membrane. We also show how to extract the location and orientation of AuNRs using ImageJ and MATLAB, and how to characterize the diffusive states of AuNRs. Statistical analysis of hundreds of particles show that single AuNRs perform Brownian motion on the surface of U87 MG cell membrane. However, individual long trajectory analysis shows that AuNRs have two distinctly different types of motion states on the membrane, namely long-range transport and limited-area confinement. Our SPT methods can be potentially used to study the surface or intracellular particle diffusion in different biological cells and can become a powerful tool for investigations of complex cellular mechanisms.

INTRODUCTION:

The dynamics of nanoparticles (NPs) on the membrane is closely associated with the cellular

uptake process, which is essential for the understanding of cell functions, viral or bacterial infections and the development of artificial nanomedical delivery systems[1,2]. Single particle tracking (SPT) technique is a robust tool for characterizing the heterogeneous behaviors of NPs[3,4]. In general, cell membrane is fluidic, which means that the components such as proteins and lipids can move laterally in the plasma membrane plane[5-7]. The spatiotemporal complexity of membrane organization and structure may lead to spatiotemporal heterogeneity of the interaction between NPs and membrane. Hence, direct visualization of the movement of NPs on the membrane requires both high spatial and temporal resolution.

Single particle tracking microscopy that monitors the localization of individual particles in living cells with a spatial resolution of tens of nanometers and a time resolution of milliseconds has been well developed to study the NPs or membrane molecules dynamics[8,9]. Fluorescence-based microscopic imaging techniques have become valuable tools for observing NPs/molecules in living cell environment[9-12]. For example, total internal reflection fluorescence microscopy, which images thin layers (~100 nm) of the sample at the substrate/solution interface with a high spatiotemporal resolution has been widely used in studies of membrane molecules dynamics[13,14]. However, the inherent disadvantages of single fluorophores, such as low intensity and rapid irreversible photobleaching reduce the accuracy and duration of tracking[13]. Therefore, non-fluorescent plasmonic NPs, which replace the fluorescent probes, have attracted more and more attention in long-term imaging studies due to their unique optical characteristics[15]. Based on the scattering signals of plasmonic NP probes, several kinds of optical microscopic imaging technologies have been used to study the mechanism of biological processes, such as dark-field microscopy (DFM)[16], interferometric scattering (iSCAT) microscopy[17] and differential interference contrast microscopy (DICM)[18]. In addition, the motion and rotation dynamic of AuNRs can be obtained using DFM and DICM[18-22]. Typically, in an SPT experiment, the motion of the object is recorded by the optical microscope, and then analyzed by SPT analysis methods[3]. The time-resolved trajectories and orientational angles generated by individual NPs are normally stochastic and heterogeneous, so it is necessary to present abundant dynamic information with various analysis methods.

Here, we provide an integrated protocol that monitors the dynamics of AuNRs on cell membrane using DFM, extracts the location and orientation of AuNRs with ImageJ and MATLAB and characterizes the diffusion of AuNRs with SPT analysis methods. As a demonstration, we show here how to use the SPT protocol to visualize dynamics of unmodified AuNRs (CTAB-AuNRs, synthesized by cetyltrimethylammonium ammonium bromide molecule as protective agent) on U87 MG cell membrane. It has been demonstrated that CTAB-AuNRs can adsorb proteins in biological environment, move on cell membrane and then enter cells[2,20,22]. U87 MG cell is the most common and most malignant tumor of the central nervous system, and its membrane receptors are abnormally expressed. The membrane receptors can interact with proteins on AuNRs, which influence the dynamics of AuNRs. Our protocol is generally applicable to other SPT experiments in the field of biology.

PROTOCOL:

1. Cell culture

1.1. Prepare complete medium for U87 MG cells by adding fetal bovine serum (final concentration 10%) and penicillin-streptomycin (final concentration 1%) to the minimum essential medium (MEM). Use plastic cell culture dish for cells subculture.

1.2. Passage cells 2 to 3 times a week.

1.2.1. Remove the culture medium and rinse the cell layer with Dulbecco's phosphate-buffered saline (D-PBS) 2~3 times when confluent (80%~90%).

1.2.2. Add 1.0 to 2.0 mL of Trypsin-EDTA solution to the cell culture dish and observe cells under an inverted microscope until cells become round (3~5 min).

1.2.3. Add 3.0 mL of prepared complete medium and disperse the cells by gently pipetting.

1.2.4. Add cell suspension (1 mL) to new culture dish with fresh cell medium (3 mL) and resuspend the cells.

1.3. Maintain the cells at 37 °C and 5% CO_2 in a humidified atmosphere.

2. Microscope slide preparation

NOTE: U87 MG cells of third to tenth generation with high activity are used in SPT experiments.

2.1. Sterilize 22 mm × 22 mm coverslips already cleaned with Piranha solution by immersing in ethanol (99.9%).

2.2. Use forceps to take out the cover slip from the ethanol solution (step 2.1) and sterilize by burning ethanol on the flame. Once all ethanol is burnt, place coverslips in a plastic cell culture dish (35 x 10 mm) filled with 2 mL of cell medium (no phenol red).

2.3. Add 50 µL of the cell suspension from step 1.2.3 on the coverslip and gently push the dish back and forth and left and right to evenly distribute the cells. Place it in a humidified atmosphere.

2.4. When U87 MG cells on the coverslip reach 20%–40% confluency (~12 h), add 20 µL of CTAB-AuNRs (138 pM) into the dish and disperse. Incubate in humidified atmosphere for 5 min.

2.5. Add 100 µL of the culture medium (no phenol red) from dish in step 2.4 into the groove of the grooved glass slide (**Figure 1**) which is precleaned with Piranha solution.

2.6. Take the coverslip out from the dish and inverted on the top of the groove of the microscope glass slide (**Figure 1**). Seal with nail polish, let it dry and place it on the stage to perform SPT

experiments.

3. Performing single particle tracking experiments with darkfield microscopy (Figure 1).

3.1. Place a drop of oil on the oil-immersed darkfield condenser (NA 1.43-1.20) and turn the knob to make the condenser contact the glass slide.

3.2. Put a drop of oil on the top of the cover glass and turn the focusing knob to make the 60x oil immersion objective (NA 0.7–1.25) touch the oil.

3.3. Turn on the light source and slightly turn the focusing knob to focus the imaging plane.

NOTE: In the field of view, the background is black, the cells are bright, and the CTAB-AuNRs (aspect ratio~2:1**, Figure 2**) are small colored (red, yellow, or green) scattering spots.

3.4. Capture sample scattering light by a color CMOS camera. Click "**Camera icon**" in the software to record and export TIFF format to save images.

4. Data acquisition

4.1. Extract single long-term trajectory

4.1.1. Operate as described in **Figure 3** to convert the time-series dark-field images from "RGB color" mode to "8 bit" mode. In the Image J click **Image | Type | 8 bit.** To adjust the contrast, click **Image | Adjust | Brightness | Contrast**.

4.1.2. Select a target particle and cutoff the time-series backgrounds by boxing and deleting the background with "**Ctrl+X**".

4.1.3. Open the particle detection and particle Linking window by clicking the "**Plugins | Particle Tracker Classic | Particle Tracker**".

4.1.4. Set Radius to 6, Cutoff to 0 and Percentile to 0.01%.

NOTE: To detect the particle, adjust above three parameters with the assistance of Preview. Ensure that radius is slightly larger than the targeted particle and smaller than the smallest inter-particle separation. Percentile is the lower limit of intensity distribution that to be candidate particles.

4.1.5. Set the Link Range to 5 and Displacement to 10.

NOTE: To link the particle between consecutive adjacent frames, adjust the above two parameters. Displacement is the maximum pixels that a particle can move between two succeeding frames, and Link Range is the number of consecutive frames to consider when

determining the best corresponding match.

4.1.6 Click "**OK**" to open the ParticleTracker Results window to see the results.

4.1.7 Click "**Visualize All Trajectories**" to inspect the generated trajectories.

4.1.8 Click "**Relink Particles**" menu at the top to re-link the detected particles with different link range and percentile parameters, if the software generated trajectory does not match the moving trajectory of the AuNR.

4.1.9 Click "**Save Full Report**" to save results if the software generated trajectory and the moving trajectory of the AuNR are matched.

NOTE: An example of extracting single long-term trajectory with Image J is shown in **Figure 4**.

4.2. Extracting Multi-trajectories

NOTE: An example of extracting multi-trajectories with Fiji is shown in **Figure 5**. There are several stages, and each stage constitutes a step in the tracking process. The result of each step is displayed immediately, which allows the user to go back to readjust settings when the output is unsatisfactory.

4.2.1. Operate as described in **Figure 3** to convert the time-series dark-field images from "RGB color" mode to "8 bit" mode. In the Image J pathway click "**Image | Type | 8 bit**". To adjust their contrast, click "**Image | Adjust | Brightness | Contrast"**.

4.2.2. Open the start panel by clicking "**Plugins** | **Tracking | TrackMate**". Click "**Next**".

4.2.3. Choose the "**LoG detector**" from the drop-down menu and click "**Next**".

4.2.4. Adjust parameters of the detector configuration panel. Set Estimated blob diameter to 10, set Threshold to 0, and Select "**Do Sub-pixel Localization**".

4.2.5. Click "**Next**" to open initial spot filtering panel.

NOTE: Several parameters can be adjusted to further optimize the target points. In this example, no other parameters were adjusted.

4.2.6. Click "**Next**" and select "**HyperStack displayer**" from the drop-down menu.

4.2.7. Click "**Next**" to open Spot filtering panel. Set the Quality above 1.88, X above 38.86, Y above 56.54.

4.2.8. Click "**Next**" and select "**Simple LAP tracker**" from the drop-down menu. Configure the

simple LAP tracker by adjusting three parameters, that is, the Linking max distance = 15, Gap-closing max distance = 15 and Gap-closing max frame gap = 5.

NOTE: Linking-max distance is the maximum displacement of a point between two frames. Gap-closing max distance is the maximum displacement of two segments. Gap-closing max frame gap is the largest frame between two points to be bridged.

4.2.9. Click "**Next**" to track. When finished, continue to click "**Next**".

4.2.10. Set filters on tracks, such as set Number of spots in track above 300.

4.2.11. Keep clicking "**Next**" until the final save panel open. Select "**Export tracks to XML file**" from the drop-down menu, and then click "**Execute**" to save in csv format.

4.3. R/G values

NOTE: Scattering intensities of targeted AuNRs in the R and G channels are obtained from color darkfield images by using a code written in MATLAB (https://github.com/fenggeqd/JOVE-2020/tree/master/RGandPolarangle), and the extraction principle is presented in **Figure 6**.

4.3.1. Use the function of *xycoordination.m* to find the center pixel coordinate of the AuNR in each frame according to the x-y coordinate (extracted by ImageJ/Fiji).

4.3.2. Use the function of *RGextraction.m* to delimit a 3 x 3 pixels matrix, extract the 9 scattering-intensity values of R or G channels, and calculate an average value (μ, defined as R or G).

NOTE: The 3 x 3 pixels matrix is centered on the pixel coordinates obtained from step 4.3.1.

4.4. Polar angle

NOTE: The polar angle is the angle between the longitude of the AuNR and the optical axis (as shown in **Figure 1**), which can reflect the spatial (Z axis) rotation dynamics of the AuNR.

4.4.1. Use the function of *polarangle.m* to calculate the polar angles (θ) by the dual-channel differential method[22], $f(R-G) \propto sin^2\theta$.

5. Data analysis

NOTE: A systematic and robust data analysis framework is essential for the performance and efficiency of SPT analysis methods. The custom software written in MATLAB is used (https://github.com/fenggeqd/JOVE-2020/tree/master/Analysis_parameters). A graphing and analysis software (see **Table of Materials**) is used for drawing the plots.

5.1. Analysis parameters

5.1.1. Use scripts of *csv_data_extract_dis_vel_ss.m* and *csv_data_MSD.m* to calculate dynamic parameters according to formulas shown in **Table 1**.

NOTE: These parameters are used to analyze the dynamics of AuNRs and consist of three parts. (1) Trajectory related parameters: displacement, step size, velocity, radius of gyration (R_g), and turning angle (T_a); (2) MSD parameters: diffusion coefficient (D_t) and abnormal diffusion exponent (α); and (3) Rotation related parameters: polar angle and rotational lability (σ).

[Place **Table 1** here].

5.2. Visual analysis of trajectory

NOTE: Trajectory visualization can intuitively present the spatiotemporal heterogeneity of particle motion and the trajectory (coordinate) distribution of dynamic parameters, such as time-mapping trajectory, R_g- and D_t-mapping trajectory, and polar-angle mapping trajectory. Mapping trajectories were drawn using the graphing and analysis software.

5.2.1. Set x coordinate as X, y coordinate as Y, and time (R_g, D_t, polar angle) as Z.

5.2.2. Click "**Plot | Scatter plot | Color-mapping Plot**".

5.2.3. Add the color bar.

5.3. MSD analysis

NOTE: Motion activity and motion mode of the particles can be obtained by MSD analysis[23]. The larger the D_t, the more active the diffusion motion of the particles. when α~1, particles do normal diffusion motion, otherwise they perform abnormal diffusion motion.

5.3.1. MSD-τ figures

5.3.1.1. Set time interval (τ) as X, MSD data as Y.

5.3.1.2. Click "**Plot | Scatter plot**"

5.3.1.3. Fit data by clicking "**Analyze | Fitting | Nonlinear curve fitting (Function: Allometricl)**".

5.3.2. MSD-τ double-logarithmic figure

NOTE: MSD-τ double-logarithmic figure, whose slope is α and intercept is D_t, can intuitively present particle motion.

5.3.2.1. Set logarithmic time interval as X, and logarithmic MSD as Y.

5.3.2.2. Click "**Plot | Scatter plot**".

5.3.2.3. Fit data by clicking "**Analyze | Fitting | Linear curve fitting**".

5.3.3. D-τ figure (MSD/4t)

NOTE: D=MSD/4τ, is a function of time τ and anomaly factor α, and the D-τ figure directly shows the change of diffusion coefficient with time. When D increases with time, α is greater than 1 and particles do superdiffusion motion.

5.3.3.1. Set logarithmic time interval (τ) as X, and logarithmic D as Y.

5.3.3.2. Click "**Plot | Scatter plot**".

5.3.3.3. Fit data by clicking "**Analyze | Fitting | Nonlinear curve fitting (Function: "Allometricl")**".

NOTE: The shorter the trajectories, the higher the inaccuracy of the diffusion estimates. In general, D_t and α through MSD-τ analysis of long interval time (> 30 τ) were obtained. However, simple and rough fitting will smooth out motion details. Hence, MSD-τ analysis of short interval time (< 10 τ) should be performed to analyze the motion behavior of particles in a short time.

5.4. Statistical analysis

5.4.1. Multi-particles statistical analysis

NOTE: Multi-particles statistical analysis can reflect the motion state of particles in a spatial region, which indirectly indicates the spatial heterogeneity environment. For instance, if the histogram of D_t exhibits a large-scale distribution or multi-peaks distribution, it means that the motion activities of particles are heterogeneous.

5.4.1.1. Set dynamic parameters (such as D_t, R_g, max displacement) as Y.

5.4.1.2. Click "**Plot | Histogram**".

5.4.1.3. Double click the histogram, and set division size or the number of divisions. Click "**Apply**".

5.4.2. Single-particle statistical analysis

NOTE: The statistical analysis of single particles can show the motion behavior of individual particles, which also indirectly reflects the spatiotemporal heterogeneity of the surrounding environment.

5.4.2.1. Multiple frames

5.4.2.1.1. Calculate dynamic parameters (such as T_a, step size, polar angle) of all frames of single long trajectory, and copy to Origin table and set as Y.

5.4.2.1.2. Click "**Plot | Histogram**".

5.4.2.1.3. Double click the histogram, and set division size or the number of divisions. Click "**Apply**".

NOTE: If both T_a and step size exhibit a small value distribution, the particles do a small-step super-diffusion motion.

5.4.2.2. Moving windows

5.4.2.2.1. Calculate dynamic parameters (such as R_g, D_t) of all frames of single long trajectory through moving-window method (11 frames), and copy to Origin table and set as Y.

5.4.2.2.2. Click "**Plot | Histogram**".

5.4.2.2.3. Double click the histogram, and set division size or the number of divisions. Click "**Apply**".

5.5. Time-series analysis

NOTE: Statistical analysis can reveal the motion state of NPs, and time-series analysis can present the motion behavior as a supplement. Combining several time-series parameters, it can discriminate the motion behavior of NPs at the temporal and spatial levels.

5.5.1. Set time as X, time-series parameters as Y (such as displacement, polar angle and R_g).

5.5.2. Click "**Plot | Multi-pane diagram | Stacked plot | Line + Symbol**".

REPRESENTATIVE RESULTS:

In the protocol, the unmodified 40 x 85 nm CTAB-AuNRs were used. As shown in **Figure 2B**, its longitudinal plasmonic maximum at is ~650 nm (red region) and transverse resonance is at 520 nm (green region). Previous literatures have revealed that the optical properties (such as LSPR intensity) of plasmonic AuNRs will change significantly with their diameter[20,22]. In **Figure 2C**, the scattering intensity from CTAB-AuNRs on U87 cell membrane, showed typical Gaussian distribution with narrow width and was consistent with that of CTAB-AuNRs on glass, which indicates that CTAB-AuNRs tracked in this experiment were well monodispersed.

The dynamic of CTAB-AuNRs on membrane was monitored by DFM (12 fps). As shown in **Figure**

7, more than 500 trajectories (the trajectory length exceeds 300 frames) were obtained. Additionally, approximately 40 trajectories per cell can be generated. The R_g of all 500 trajectories showed a small-value distribution, the mean R_g was 0.5 μm (SD=0.6 μm), and max-displacement was also more distributed at small values (**Figure 8**). All 500 trajectories can be divided into two groups: long-range diffusion (R_g>0.5 μm, black trajectories) and confined diffusion (R_g<0.5 μm, red trajectories).

According to position and orientation of CTAB-AuNRs, several parameters are calculated for data analysis. There are many mays to analyze and present parameters during SPT analysis. The ensemble-time averaged MSD analysis shown in **Figure 9A** shows that CTAB-AuNRs normally diffuse with α~1. However, the density distribution of D_t-α, obtained from all trajectories, reveals that the dynamic of AuNRs showed a heterogeneous distribution with superdifusion motion, Brownian motion and subdiffusion motion (**Figure 9B**).

In addition to multi-particles statistical analysis, single-particle (multi-frames) statistical analysis was also performed. **Figure 10** shows two representative long-term trajectories: confined (R_g=0.34 μm) and moving (R_g=1.48 μm). The polar-mapping trajectory showed that both the motion range and polar angle of confined AuNRs were smaller than those of moving AuNRs, which means that stronger the interaction between AuNRs and membrane, the more serve the confinement of its translational and rotational dynamics. Correspondingly, the histograms of T_a and polar angle of the two AuNRs showed different modes.

To further study the motion behavior of AuNRs, the time-series parameters were analyzed, including trajectory, displacement, polar angle and R_g. As shown in **Figure 11**, different patterns were presented over time. Compared with the long-distance diffusion AuNR, the rotation of the confined AuNR was relatively limited, and its polar angles were more concentrated, distributed in the range of 10° ~ 40°. However, although the overall R_g of the moving AuNR was larger than the overall R_g of confined AuNR, the time-series R_g of moving AuNR was smaller. Combining the time-series distribution of trajectories points and displacement, it can be inferred that confined AuNR moves in a limited area, but its motion range in a short time is larger than that of the moving AuNR confined on the membrane. Overall, the spatiotemporal heterogeneous distribution of translational and rotational dynamics is caused by the spatial and temporal complexity of membrane organization and structure.

FIGURE AND TABLE LEGENDS:

Table 1: Three types of parameters used for analysis. These include trajectory related parameters (displacement, step size, velocity, R_g and T_a), MSD parameters (MSD, D_t and α) and rotation related parameters (polar angle and rotational lability).

Figure 1: Schematic diagram of the optical path of darkfield microscopy, microscope slide preparation, and the dual-channel difference method for calculating polar angle (θ) of AuNRs. Scale bar in image is 2 μm.

Figure 2: Characterization of CTAB-AuNRs. (**A**) TEM images of CTAB@AuNRs. (**B**) LSPR spectrums of CTAB@AuNRs. (**C**) Distribution of single particle intensity: CTAB@AuNRs on glass (left panel), CTAB@AuNRs on plasmon membrane (right panel).

Figure 3: Pretreatment of darkfield images. Time-series color images were converted to "8 bit" mode and their contrast were adjusted.

Figure 4: Extraction of single long-term trajectory with ImageJ.

Figure 5: Extraction of multi-trajectories with Fiji.

Figure 6: Extraction of R/G values of AuNRs with MATLAB.

Figure 7: All 527 trajectories of CTAB@AuNRs on U87 MG cell membrane tracked with Fiji. There were two groups: red trajectories (R_g<0.5 μm) and black trajectories (R_g>0.5 μm).

Figure 8: Multi-particles statistical analysis of all 527 trajectories. Distribution of R_g (**A**) and maximum displacement (**B**).

Figure 9: MSD analysis of all 527 trajectories. (**A**) Plot of ensemble-time averaged MSD versus τ. (**B**) Density plot in the α– log (D_t) plane. The color code represents the local density of trajectories between 0 (blue) and 1 (red).

Figure 10: Single-particle statistical analysis. (**A-B**) Two representative long-term polar-mapping trajectories which were tracked with ImageJ. The color code represents the polar angle value between 0 (green) and 90 (red). Distribution of turning angle (C-D) and polar angle (E-F).

Figure 11: Time-series analysis. (**A**,**C**) Time-mapping trajectories. The color from red to blue represents the direction of time. (**B,D**) Time-series of parameters: displacement (blue), polar angle (green) and radius of gyration (R_g, red).

DISCUSSION:

The presented protocol is used to study the dynamics of AuNRs on cell membrane. The protocol consists of four parts, including microscopic imaging, data extraction, dynamic parameters calculation and data analysis methods, and each part is flexible and universal. Therefore, there are many possible future applications, for instance, studying movement of NP-linked membrane molecules on membrane, endocytosis dynamics of NP-labeled receptors, dynamic analysis of intracellular NPs and vesicle-coated NPs transportation along microtubules, and so on.

The basic step is to use DFM to image the movement of AuNRs on the cell membrane. Currently, many imaging technologies for the analysis of cell membrane dynamics have been developed, of which fluorescence microscopy has been widely used[24]. However, the photobleaching properties of fluorescent probes lead to the inability to track particle dynamics for long periods of time. Here, light-stable plasmonic AuNRs were used. Several imaging techniques have been developed

based on the scattering characteristics of plasmonic NPs[15]. Particularly, DFM with oblique illumination mode only collects scattered light from the samples, which makes it have a high signal-to-noise ratio.

Both the translational and the rotational dynamics of AuNRs are obtained and analyzed. Most studies focus on investigating the positional fluctuations of the NPs on the membrane, and ignoring the effects of orientational changes[25]. In addition, the cell membrane plane and the imaging plane (x-y plane) are largely the same in most cases, and the difference in the interaction between the AuNR and the cell membrane is more notable in the z direction. Hence, even without azimuth angles, the polar angle is valuable for the analysis of the interaction between the AuNRs and cell membrane. Based on DFM and unique optical properties of AuNRs, dual-channel polarization DFM is used to obtain the orientation information of AuNRs ($I_1 + I_2 \propto sin^2\,\theta$)[19,26]. Nevertheless, the calculation accuracy of polar angles is affected by the maximum and minimum detection intensities which are strongly influenced by surroundings. Hence, some modifications need to be made. Here, (R-G) was used to calculate the polar angle, and the G value serving as an internal reference could efficiently decrease the systematic errors and increase the measurement accuracy.

The critical steps are data extraction and dynamic parameter calculation, which is essential for analyzing the dynamics of NP on the membrane. The trajectories of AuNRs on membrane are extracted with high spatial resolution[27,28] from sequence analysis using ImageJ/Fiji. The R\G values and polar angles are calculated with MATLAB (*https://github.com/fenggeqd/JOVE-2020*). Several parameters are similarly calculated using MATLAB according to positions and polar angles (**Table 1**). In SPT analysis, there are many ways to perform parameters analysis and display. Hence, it is necessary to use as many analysis methods and visualization tools as possible to analyze the dynamics of NP, and then to summarize and extract a novel point of view from many analysis results. Normally, there are always some trade-off to make, which is not an easy work.

The dynamic state and motion behavior of NPs can be analyzed and presented systematically from top to bottom using comprehensive analytical methods and multivariate presentation ways. Using multi-particles statistical analysis, we can obtain the ensemble motion state of NPs across the whole cell membrane plane. The dynamic studies on individual NPs/molecules based on fluorescence imaging[29] mostly use this overall statistical method because only a large number of short-term trajectories can be monitored. However, the ensemble averaging method will smooth and ignore individual details. As a supplement, the single-particle statistical analysis is used to quantitatively present the dynamic state of individual NPs. Meanwhile, it is important to perform time-series parameter analysis of individual or segment trajectories, which can provide motion behaviors of NPs as a function of time.

In summary, we propose an integrated protocol for studying the diffusion dynamics of AuNRs on live cell membrane with SPT method. The dynamic of AuNRs was monitored with single nanoparticle DFM, extracted using ImageJ and MATLAB, and characterized by comprehensive analytical methods. AuNRs did abnormal diffusion motion on U87 MG cell membrane, and its motion shows spatiotemporal heterogeneity. This protocol can be potentially used for the

studies of other type of complex biological systems.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

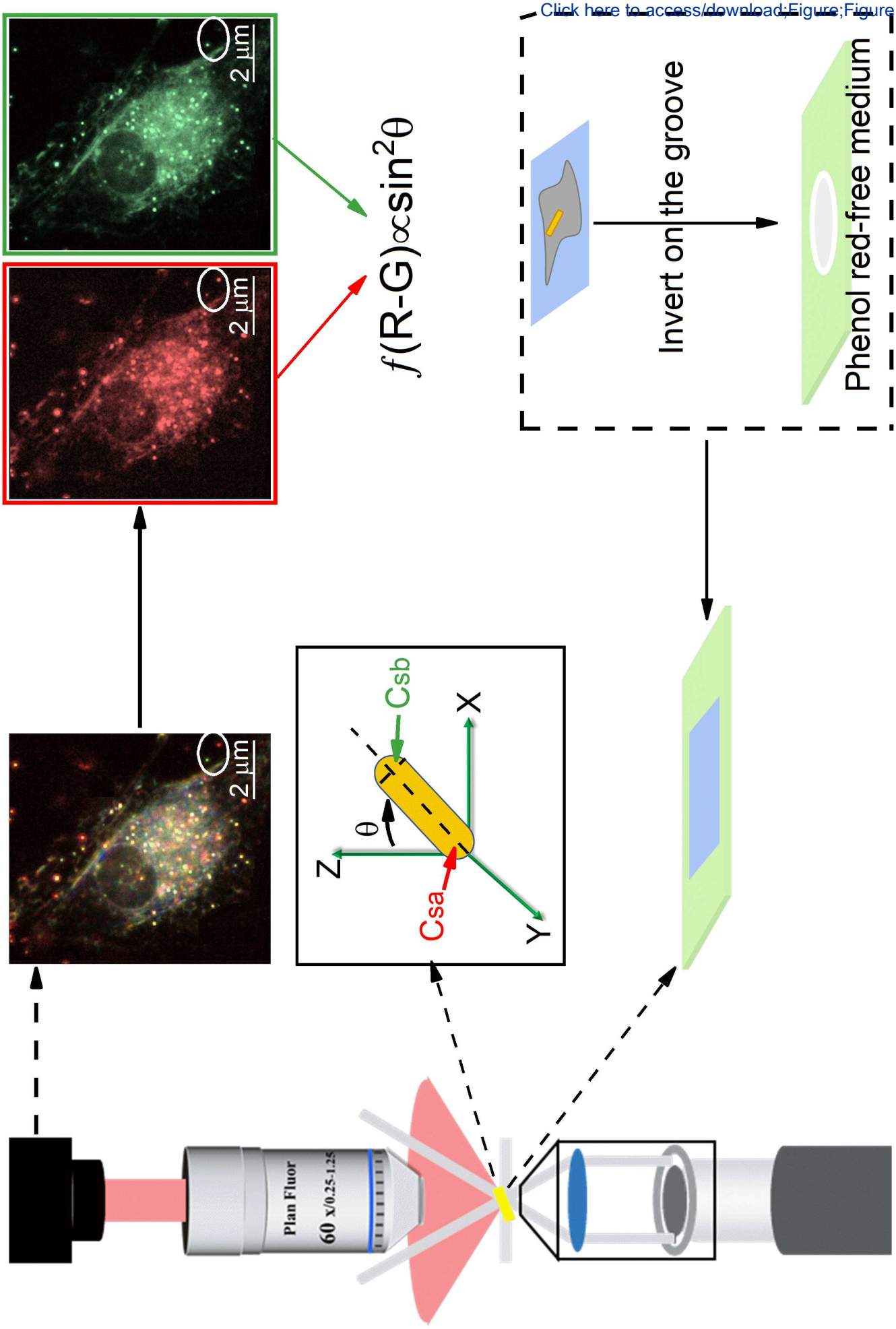

Figure 2

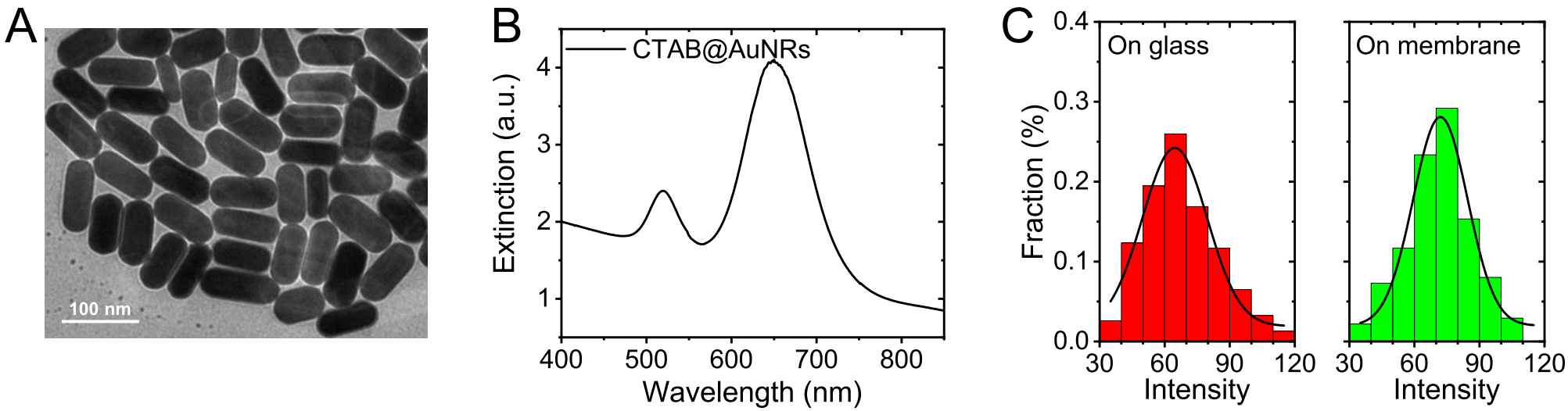

Figure 3

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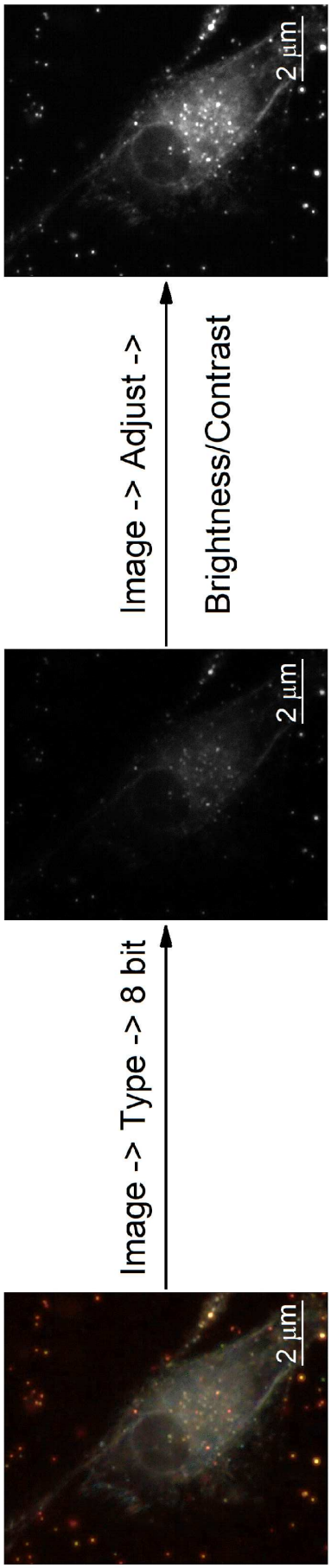

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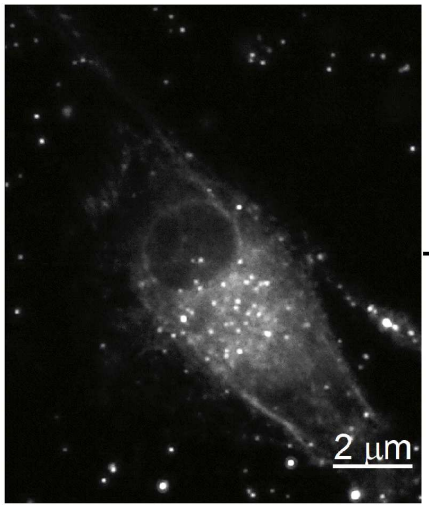

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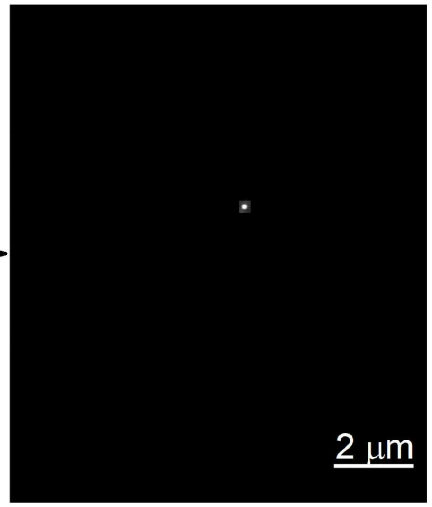
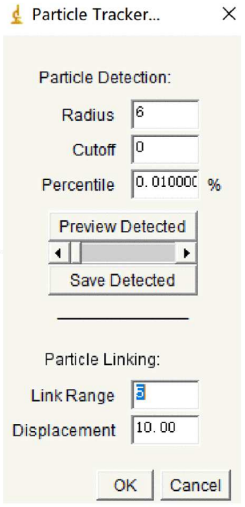
Cutoff



Plugins->

Particle Tracker Classic ->

Particle Tracker



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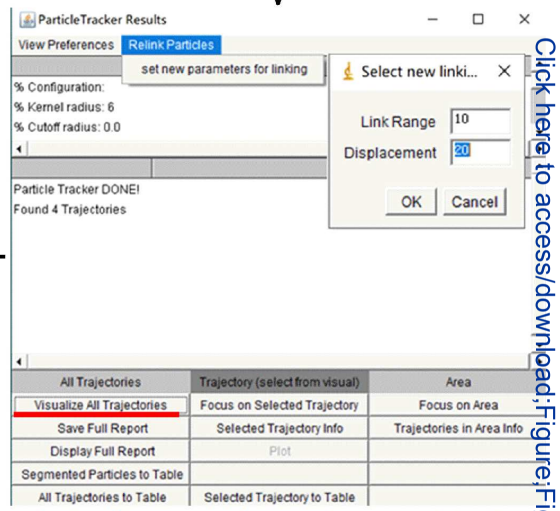

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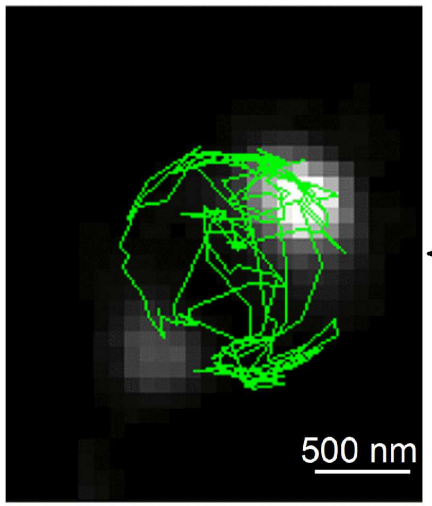

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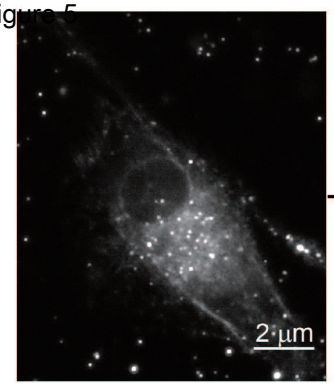
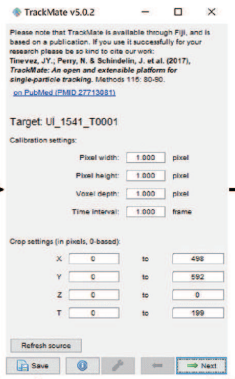


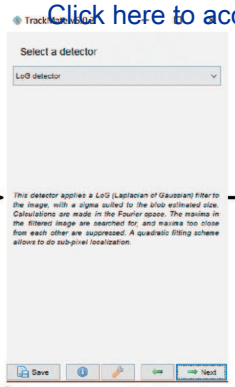
Plugins ->

Tracking ->

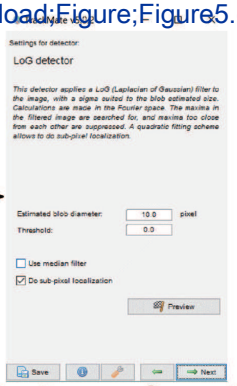
TrackMate



Select source and ROI



Choose detector



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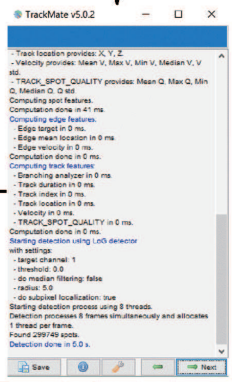

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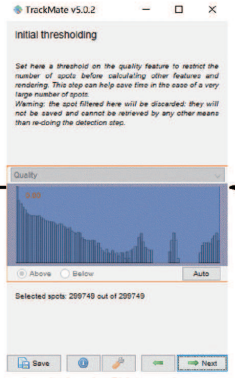

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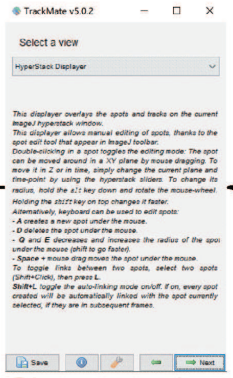
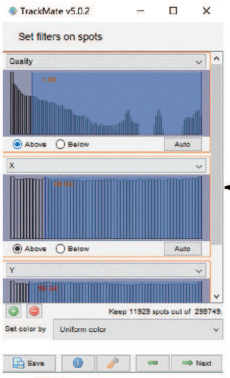
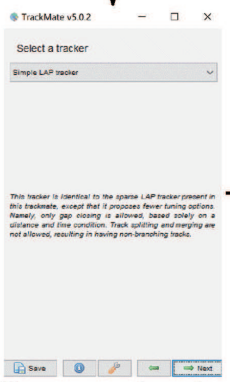


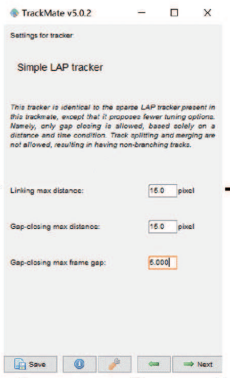
Choose a view



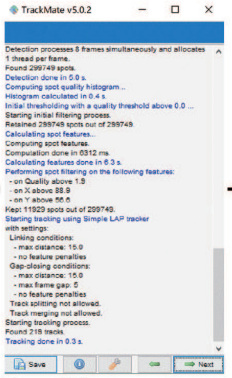
Filter spots



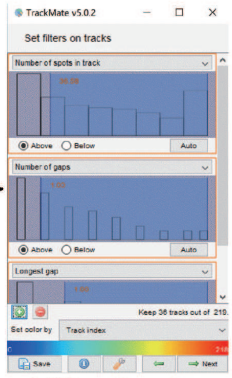
Choose a tracker



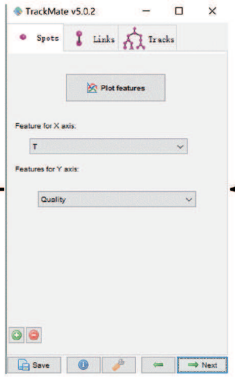
Tracker configuration

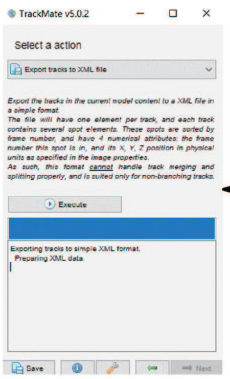


Tracking occurs



Filter tracks





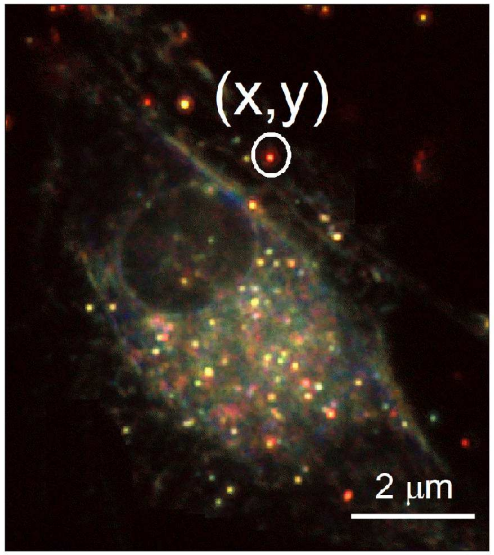


Image J

(x_i, y_i)

Matlab

3 × 3 Matrix

(x_{i-1}, y_{j-1})	(x_i, y_{j-1})	(x_{i+1}, y_{j-1})
(x_{i-1}, y_j)	(x_i, y_j)	(x_{i+1}, y_j)
(x_{i-1}, y_{j+1})	(x_i, y_{j+1})	(x_{i+1}, y_{j+1})

Matlab | 9 R or G

(R_{i-1}, G_{j-1})	(R_i, G_{j-1})	(R_{i+1}, G_{j-1})
(R_{i-1}, G_j)	(R_i, G_j)	(R_{i+1}, G_j)
(R_{i-1}, G_{j+1})	(R_i, G_{j+1})	(R_{i+1}, G_{j+1})

Gaussian fitting

(R, G)

Figure 7

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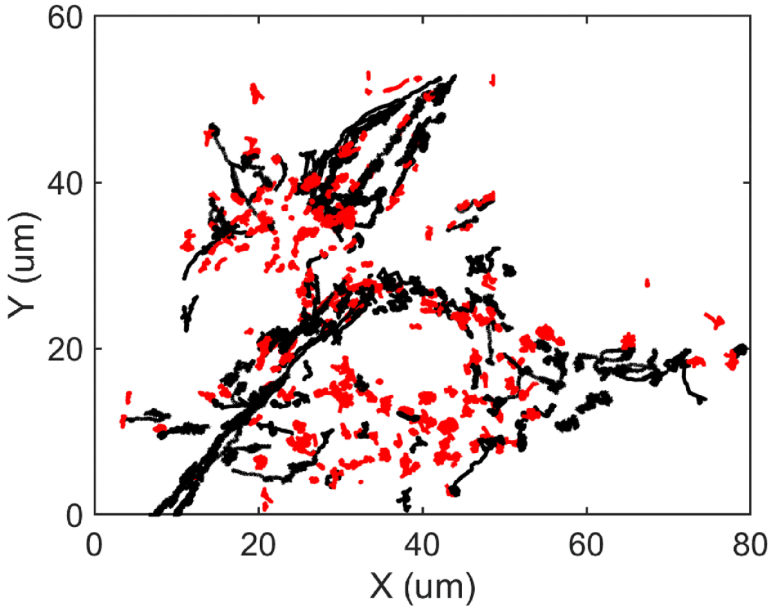

Figure 8

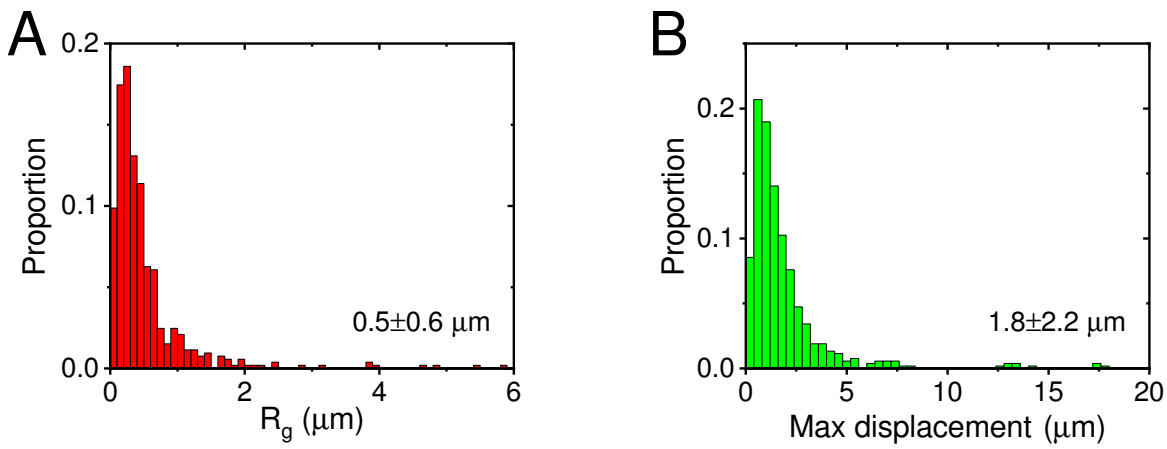

Figure 9

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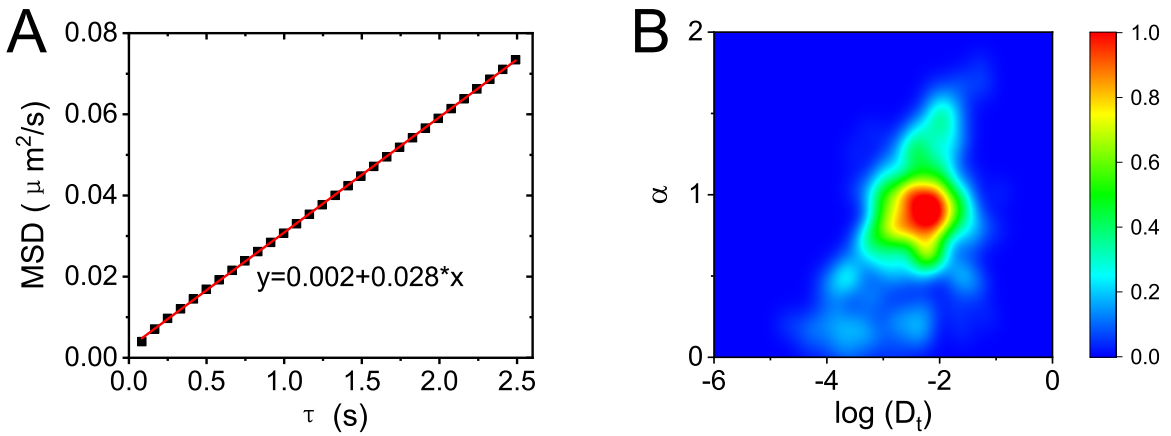
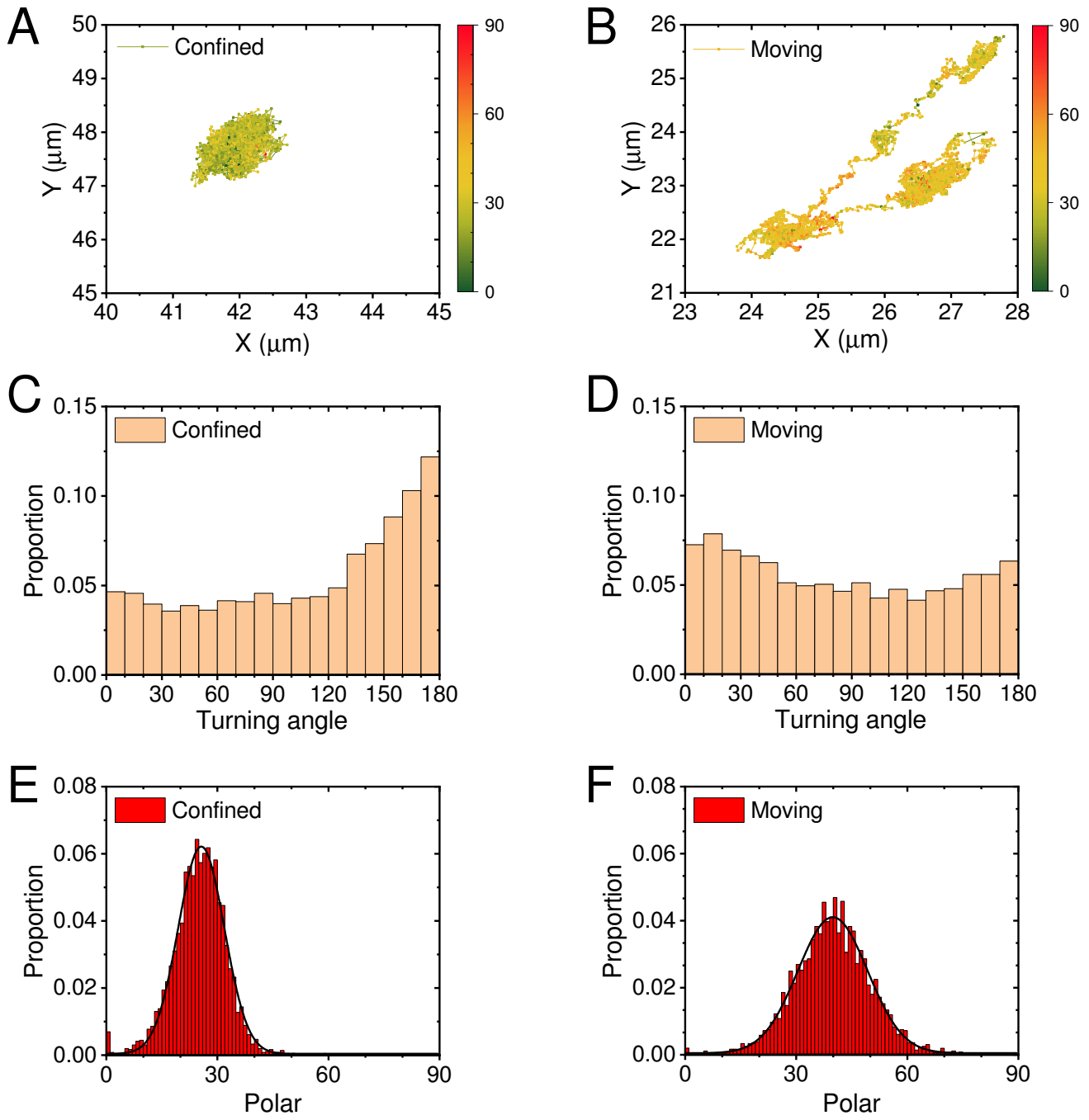
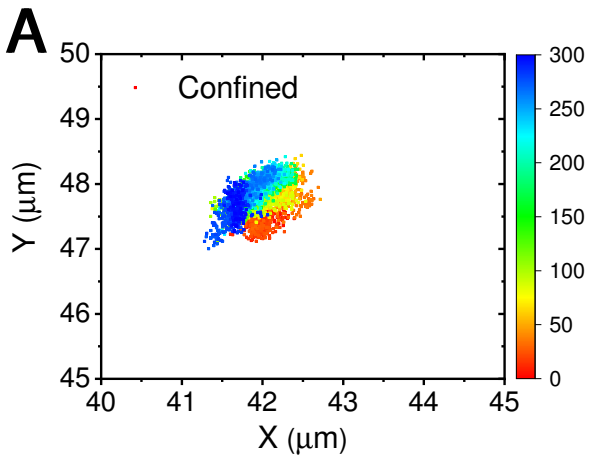
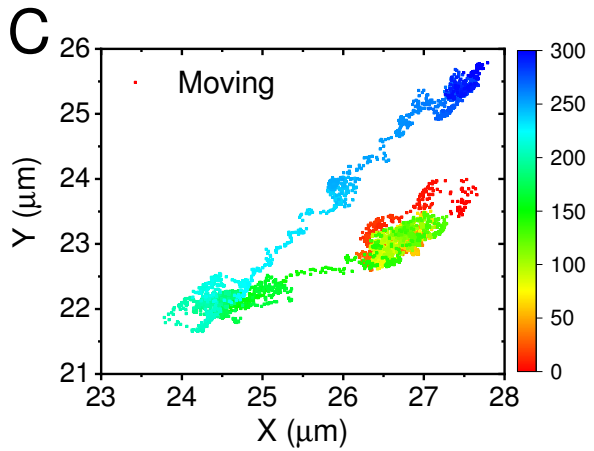
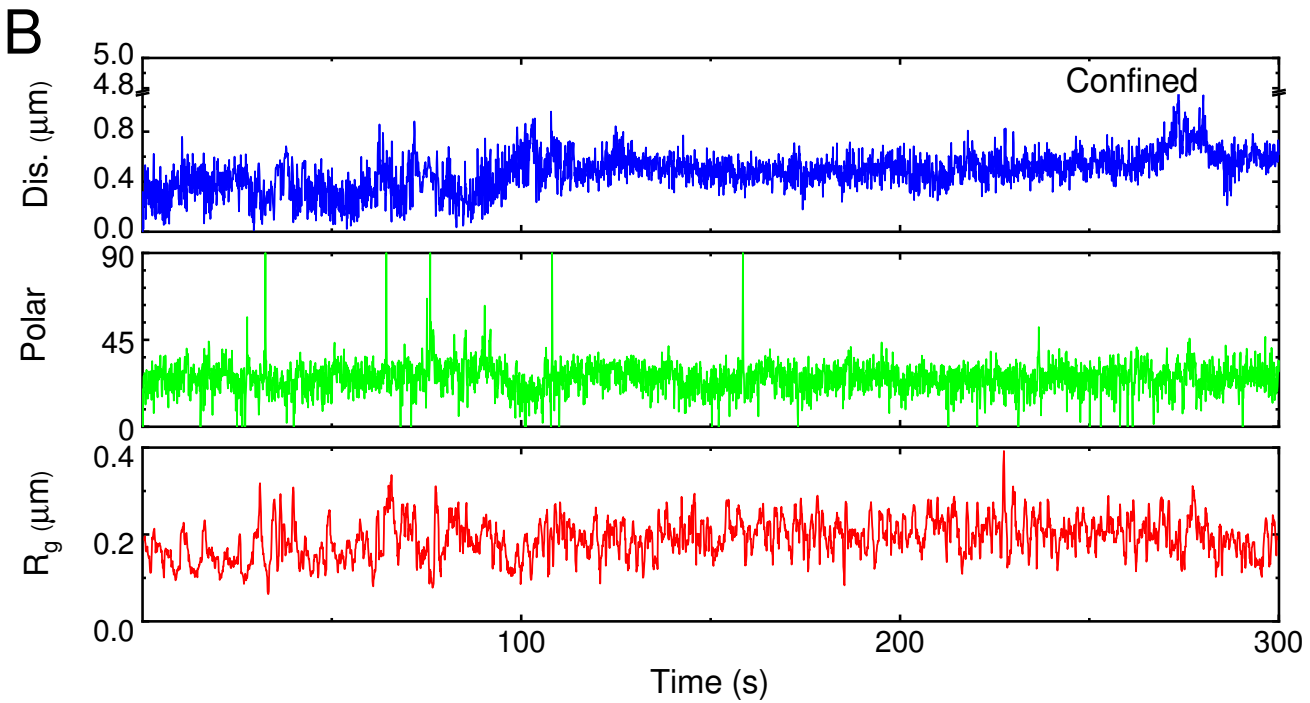


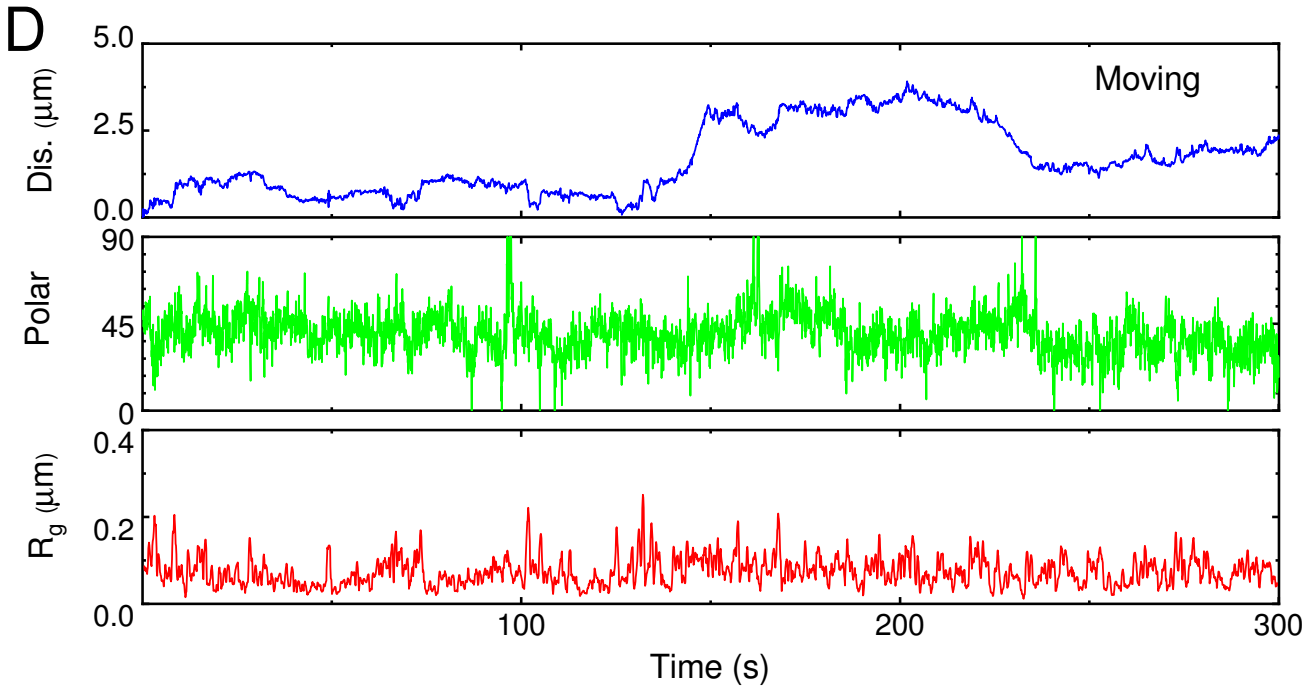
Figure 10











Measures	Definition	Physical meaning
Displacement	$d = \sqrt{(x_i - x_1)^2 + (y_i - y_1)^2}$	Changes in position of objects
Step size	$s = \sqrt{(x_i - x_{i-1})^2 + (y_i - y_{i-1})^2}$	Distance between two adjacent points
Velocity	$v = {}^{s}/_{\tau}$	Speed of objects motion
R_g	$R_g{}^2(\tau) = \frac{1}{N}\sum_i^N [(x_i - \langle x \rangle)^2 + (y_i - \langle y \rangle)^2]$	Moving range of objects in a specific time interval
T_a	$\cos T_a = \frac{A \cdot B}{(\lvert A \rvert \lvert B \rvert)}$	Motion direction of objects between two adjacent points
MSD	$MSD = \frac{1}{N-j}\sum_{i=1}^{N-j}((x_{i+j} - x_i)^2 + (y_{i+j} - y_i)^2)$	Average moving distance of objects in a specific time interval
D_t	$MSD = 4 * D_t * t^{\alpha}$	Diffusion ability of objects
α	$MSD = 4 * D_t * t^{\alpha}$	Normal diffusion (α~1)
Polar angle	$sin^2\theta = \frac{(R-G) - (R-G)_{min}}{(R-G)_{max} - (R-G)_{min}}$	3D orientation information of objects
σ	$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(\theta_i - \bar{\theta})^2}$	Degree of dispersion of the polar angle data set

Name of Material/Equipment	Company	Catalog Number	Comments/Description
CTAB coated gold nanorods(CTAB-AuNRs)	Nanoseedz	NR-40-650	85 nm * 40 nm
Color CMOS camera	Olympus	DP74	Japan
Coverslips	Citoglas	z10212222C	22*22 mm
Dark-field microscopy	Nikon	80i	upright microscope
Fetal bovine serum (FBS)	Gibco	10099141	
Fiji	National Institutes of Health	2.0.0-rc-69/1.52 p	a distribution of ImageJ
Grooved glass slide	Sail brand	7103	Single concave
Image J	National Institutes of Health	1.52 j	
MATLAB	MathWorks	R2019b	
MATLAB Code			https://github.com/fenggeqd/JOVE-2020
Minimum essential medium (MEM)	Gibco	10-010-CVR	with phenol red
Minimum essential medium (MEM)	Gibco	51200038	no phenol red
Origin	OriginLab	Origin Pro 2018C	
Penicillin-streptomycin	Gibco	15140122	
Plastic cell culture dishes	Falcon	353002	
Plastic cell culture dishes	Falcon	353001	35*10 mm
U87 MG cell	American Type Culture Collection	ATCC HTB-14	a human primary glioblastoma cell line

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   Reply: Thanks for the editor. We have finished the revision using the attached file.

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