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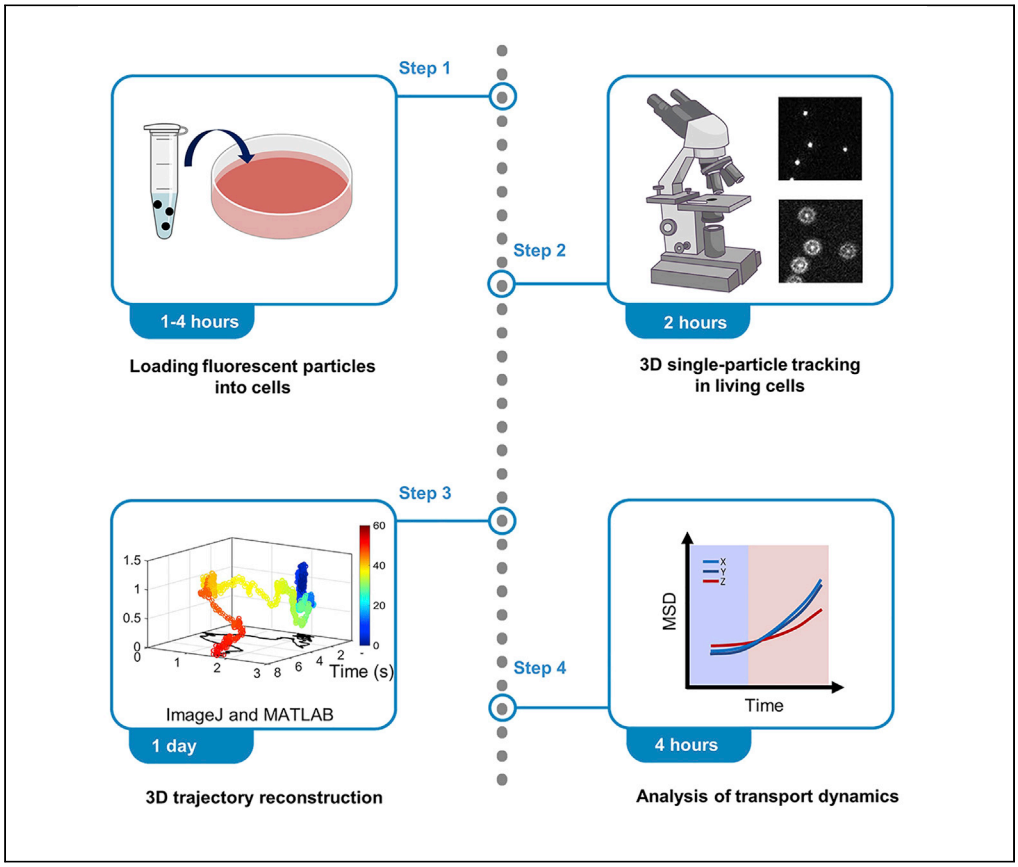
Protocol

Quantifying transport dynamics with three-dimensional single-particle tracking in adherent cells



Intracellular transport plays an important role in maintaining the physiological functions of cells. Here, we describe a protocol for 3D single-particle tracking within living cells. We detail the use of a two-focal imaging system and the analytical steps for quantifying 3D transport dynamics. This protocol can be used to characterize the intracellular diffusion and trafficking of macromolecules, nanoparticles, and endocytic vesicles in adherent cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Three ways to deliver fluorescent probes into living cells

Description of set up for 3D single-particle tracking microscopy

Imaging and quantitative analysis of 3D intracellular transport

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Quantifying transport dynamics with three-dimensional single-particle tracking in adherent cells

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SUMMARY

Intracellular transport plays an important role in maintaining the physiological functions of cells. Here, we describe a protocol for 3D single-particle tracking within living cells. We detail the use of a two-focal imaging system and the analytical steps for quantifying 3D transport dynamics. This protocol can be used to characterize the intracellular diffusion and trafficking of macromolecules, nanoparticles, and endocytic vesicles in adherent cells.

For complete details on the use and execution of this protocol, please refer to Jiang et al. (2022).

BEFORE YOU BEGIN

The protocol below describes the specific steps for using A549 cells. However, we also used this protocol in other adherent cells.

Cell culture

Timing: 1 week

1. Prepare the following materials and equipment for cell culture.
   a. Fetal bovine serum (FBS) (50 mL).
   b. Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L-glucose, L-glutamine & sodium pyruvate (450 mL).
   c. Penicillin–streptomycin (6 mL).
   d. Phosphate buffered saline without calcium and magnesium (PBS) (500 mL).
   e. Trypsin-EDTA (0.05%, 20 mL).
   f. Flasks (25 cm^2).
   g. Water bath kettle.
   h. CO_2 incubator.
   i. Serological pipettes (2 mL and 5 mL).
   j. Electronic pipette.
   k. Centrifuge.
2. Prepare complete culture medium.

a. Thaw a bottle of FBS (50 mL) at 4°C.
b. Add 50 mL FBS and 5 mL penicillin–streptomycin to 445 mL DMEM to make the complete DMEM.

3. Culture A549 cells.
   a. Place the cryogenic vials in a 37°C water bath to rapidly thaw cryopreserved A549 cells.
   b. Transfer the vial content to a 15 mL centrifuge tube with 7 mL complete culture medium and spin at 150 × *g* for 5 min with the centrifuge. Discard supernatant, resuspend cells with 4 mL complete medium. Then transfer the medium to a 25 cm^2 cell culture flask.

⚠ CRITICAL: The thawed liquid contains DMSO, which is cytotoxic.

   c. Culture the cells in a CO_2 incubator at 37°C. The culture medium should be changed every 2–3 days.
   d. When the cells reach 80%–90% confluency, subculture the cells. First discard the culture medium and rinse the cells with 1 mL of prewarmed PBS.
   e. Remove PBS, then add 1 mL 0.05% trypsin-EDTA solution to the cells and incubate at 37°C for approximately 2 min until the cells become round-shaped.
   f. Gently remove and discard the trypsin-EDTA solution, add 4 mL fresh complete culture medium and disassociate cells from the flask by gently pipetting.
   g. Transfer the cells to a 15 mL sterile centrifuge tube using an electronic pipette. Add 3 mL of fresh complete culture medium and 1 mL of liquid containing the cells to the flask (subculture cells at a ratio of 1:4).

⚠ CRITICAL: The flask should be changed at most every 2 weeks.

Note: Prewarm the culture medium, PBS, and the trypsin-EDTA solution to 37°C before use and maintain aseptic operation to avoid contamination.

Rough alignment of the dual view system

Timing: 1 h

Note: This part refers to the alignment procedure for the Photomerics® DV2 imaging system.

4. Prepare the following equipment.
   a. A used Petri dish.
   b. The beam splitter cube (Photomerics® DV2, #127462 70/30) that splits the beam into two beams with a 30:70 intensity ratio. A lens (f = 400 mm) is inserted into the path with 70% intensity.
   c. 0.05″ hex screws.
5. Turn on the brightfield light on the microscope. Select the 60× objective.
6. Adjust the lens to focus on the bottom of the dish. The microscope is set to direct the image to the camara. Use IQ software to display a live preview image.
7. Install the beam splitter cube on the dual channel system. Put the cube half-way in the DV2 tube for bypass mode.

Note: In bypasss mode, the light will directly pass through the tube. In dual view mode, the light passing through the tube will be split into two beams by the beam splitter.

8. Move the DV2 slider to dual-view mode.

Note: On the screen, we can see an image with one bright area between two black areas. The black areas indicate where light is blocked from reaching the CCD. The bright area is the imaging area.

9. Adjust the location of the image edges by rotating the aperture screws on the tube with hex screws. This operation should be used to adjust the left edge of the image to be located at 1/4 of the width and the right edge to be located at 3/4 width.
10. Keep the slider lever in dual-view mode. Push the splitter cube all the way into the DV2 tube.
11. Adjust the knobs on the tube so that the imaging windows are separated and occupy one-half of the CCD camera without overlapping.
12. Adjust the knobs on the tube to image the sample approximately in the center of both windows.

Note: These steps are based on the DV2 alignment procedure. Because we are imaging a bead simultaneously in the focused and defocused planes using a single CCD camera, we only perform a rough imaging window calibration. However, for dual-color imaging or FRET (fluorescence resonance energy transfer) experiments, we need to perform the calibration strictly using a standard Petri dish containing fluorescent particles (such as F8811 with a diameter of 200 nm) covering both wavelength ranges.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle medium	Corning	10-013-CVR
FluoSpheres™ Carboxylate-Modified Microspheres	Thermo Fisher Scientific	F8803
Influx™ pinocytic cell-loading reagent	Thermo Fisher Scientific	I-14402
Qdot™ 655 Streptavidin Conjugate	Thermo Fisher Scientific	Q10123MP
Epidermal growth factor with biotin conjugate	Thermo Fisher Scientific	E3477
Fetal bovine serum	Gibco	10099141c
Nocodazole	Sigma-Aldrich	M1404
Latrunculin A	Sigma-Aldrich	L5163
Penicillin-streptomycin	Gibco	14150122
0.25% Tripsin-EDTA	Gibco	25200-056
Experimental models: Cell lines		
A549	ATCC	N/A
Software and algorithms		
ImageJ version 1.48k	National Institutes of Health, USA	https://imagej.nih.gov/ij
MATLAB 2018a	MathWorks	https://www.mathworks.com/
Origin 2018	OriginLab	https://www.originlab.com/
IQ	Andor	https://andor.oxinst.com/products/iq-live-cell-imaging-software/
Code and testing dataset for 3D tracking	This paper	https://github.com/chaojiang1901/3D-particle-tracking-MATLAB
Other		
Corning flask (25 cm2)	Corning	430168
Serological pipette	Biologix	07-5005
Glass bottom dish	Cellvis	210622
Centrifuge tube (15 mL)	Corning	430790
Inverted microscope	Olympus	IX73
TIRF objective lens	Olympus	60×, oil
EMCCD	Andor	Ultra DU897
CCD	N/A	N/A
Piezoelectric device	piezosystem jena	N/A
405 nm laser	Coherent	OBIS 405, 100 mW

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
488 nm laser	Coherent	Sapphire 488, 200 mW
561 nm laser	Coherent	Sapphire 561, 200 mW
940 nm laser	Thorlabs	N/A
Optical fiber	N/A	N/A
Shutter	Vincent Associates	Uniblitz LS6T2
Attenuator	Mfopt	N/A
Lens (f =400 mm)	Daheng Optics	GCL-010167
Dual view system	Photometrics	DV2

MATERIALS AND EQUIPMENT

3D single-particle tracking platform

The 3D single-tracking system is built on an inverted Olympus IX73 microscope, which is equipped with a 60 × 1.45 N.A. total internal reflection fluorescence (TIRF) objective lens and an ANDOR EMCCD (Ultra DU897). To realize 3D single-particle imaging, we have added focus locking and two focal imaging apparatuses (Figure 1). In the focal-locking part, the incident far-infrared laser (940 nm) is imaged as two spots on the CCD after total reflection at the interface between the specimen and cover glass, in which the distance between the two spots reflects the offset of the objective lens in the axial direction. Then, the piezoelectric device uses the distance as feedback control to compensate for the axial offset of the lens, thus realizing the focus-locking function. In the two-focal imaging part, we use the dual channel simultaneous-imaging system (DV2, Photometrics) in the emission path. A beam splitter is installed to divide the emission signals into two beams with a 30:70 intensity ratio, and each beam is sent to one-half of the EMCCD (Jiang et al., 2020; Toprak et al., 2007). A lens (f = 400 mm) is inserted into the path of the beam with 70% intensity to generate diffraction rings. In the excitation path, different wavelengths are used: a 405 nm laser (OBIS 405−100 mW, Coherent), a 488 nm laser (Sapphire 488−200 mW, Coherent), and a 561 nm laser (Sapphire 561−200 mW, Coherent). Mechanical shutters (Uniblitz LS6T2, Vincent Associates) are applied to control the on−off state of these lasers. The laser intensities are controlled by the laser power supplies and attenuator. All laser beams are combined by dichroic mirrors and coupled into an optical fiber, and then the output light is focused at the back focal plane of the objective. By adjusting the incident angle of the focused laser, different excitation modes can be selected: highly inclined and laminated optical sheet (HILO) and epifluorescence (Figure 1). HILO imaging, which provides a deeper penetration depth compared with the TIRF imaging, is generally used to image fluorescence probes inside cells at a high signal-to-noise ratio (Li et al., 2015; Tokunaga et al., 2008). EMCCD parameter setting, image sequence acquisition and shutter control are all performed by the IQ software or Micro-Manager.

STEP-BY-STEP METHOD DETAILS

Cell preparation

Timing: 1 day

Prepare cells in glass-bottom Petri dishes for 3D single-particle tracking experiments.

1. Repeat steps 3d–3g in the cell culture section to disassociate the A549 cells.
2. Plate cells in glass-bottom dishes (35 mm dish with 10 mm bottom well) with 2 mL complete DMEM and incubate in a humidified 37°C, 5% CO2 incubator for at least 24 h.

⚠ CRITICAL: The bottom of the Petri dish is a coverslip, which is suitable for an oil-immersion objective.

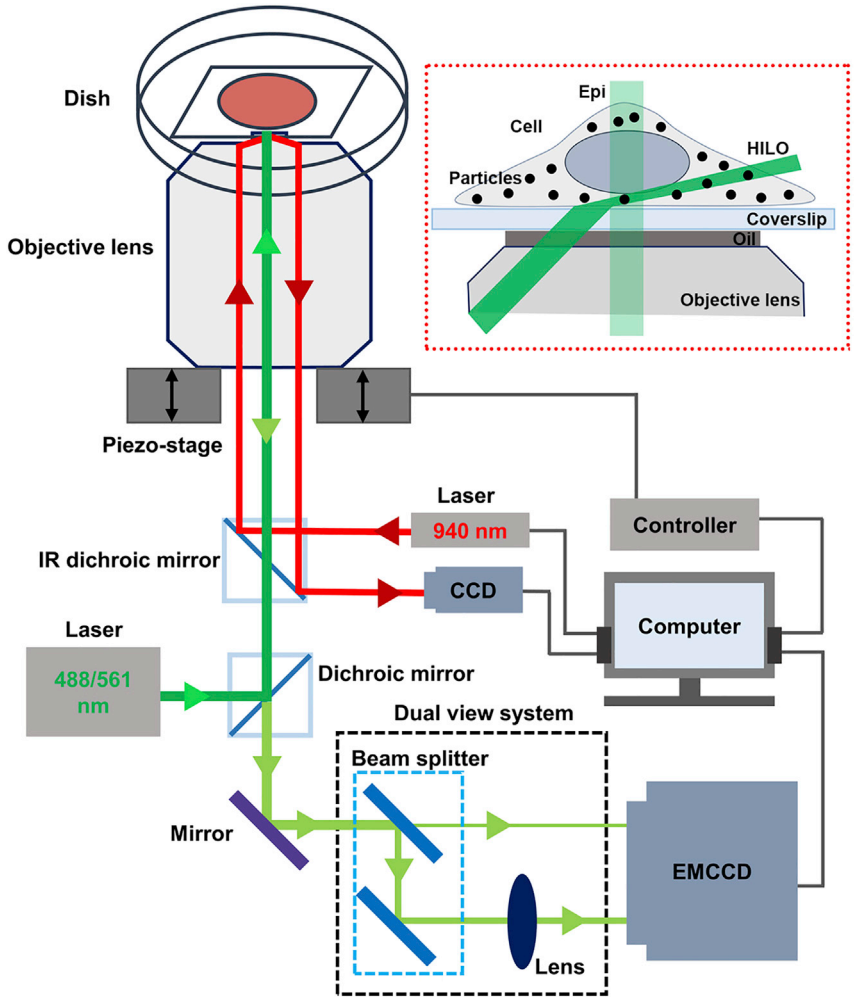


Figure 1. Schematic of the 3D single-particle tracking platform

A schematic of exciting fluorescent particles using HILO and Epi is shown in the red dotted box. The red line represents 940 nm infrared light in the focus-locking part, and the green line represents the fluorescent light in the two-focal imaging part. Arrows indicate the direction of the light. CCD, charge-coupled device; EMCCD, electron-multiplying CCD; Epi, epifluorescence; HILO, highly inclined and laminated optical sheet.

3. Cells grown to 50%–60% confluency as observed by light microscopy are appropriate for the experiments.

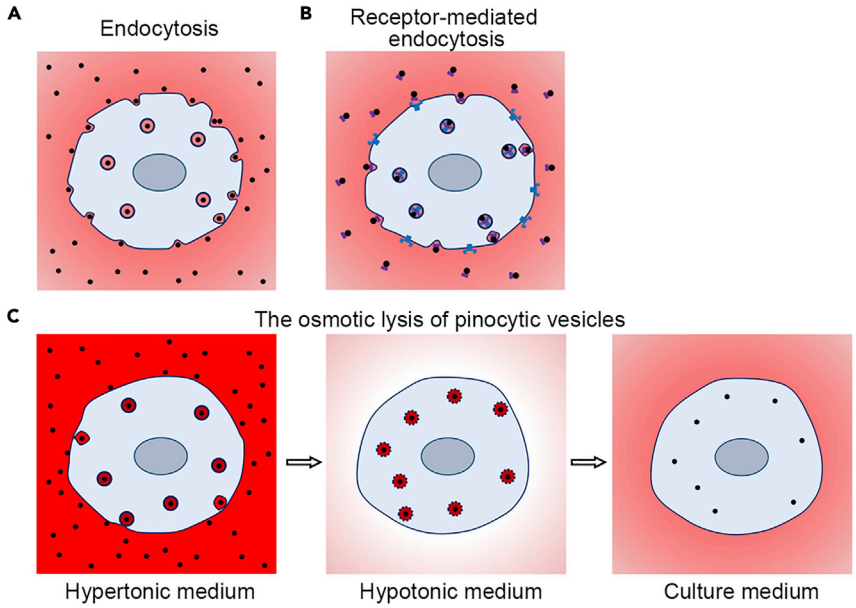
⚠ CRITICAL: Confluency that is too high might result in cell overlays, which can affect the imaging quality.

Loading fluorescent particles into cells

Timing: 1–4 h

Three methods are used to introduce fluorescent particles into cells as probes to study different forms of transport in cells.

4. Labeling of intracellular vesicles via the endocytosis of fluorescent particles (Figure 2A) (troubleshooting 1, 2, and 3).



D

Methods / Properties	Endocytosis	Receptor-mediated endocytosis	The osmotic lysis of pinocytic vesicles
Concentration of particles	~5 nM	~1 nM	~10 nM
Time	1~4 h	~1 h	~20 min
Destination	Vesicle	Vesicle	Cytosol

Figure 2. Schematic of delivering particles into living cells

(A) By coculture with cells, fluorescent particles enter cells in the form of endocytic vesicles.

(B) Fluorescent particles are internalized by receptor-mediated endocytosis.

(C) The fluorescent particles are delivered into the cytoplasm via lysis of pinocytic vesicles. Cultured cells are exposed to hypertonic medium with high concentration of fluorescent particles, which is delivered into cells via pinocytic vesicles (left panel). Then when the cells are placed in hypotonic medium, the pinocytic vesicles burst and release the particles in the cytosol (middle panel). At last, the cells are incubated in culture medium to recover at least 10 min (right panel).

(D) Comparison of three methods of internalizing fluorescent particles.

Note: Living cells are constantly exchanging substances with the culture medium, so when the fluorescent particles are mixed with the culture medium, some particles may be endocytosed in the form of vesicles (Rejman et al., 2004).

a. Prewarm the stock solution containing 100-nm fluorescent beads with carboxylate coating (Molecular Probes, F8803) at 37°C for approximately 10 min.
b. Centrifuge the stock solution in a microfuge at 5,000 × *g* for 3 min.

⚠ CRITICAL: Centrifugation is performed to avoid aspirating the sediment from the solution.

c. Then aspirate 0.5 μL of the supernatant reagent using a pipette.
d. The reagent is mixed with 2 mL of complete DMEM at a final concentration of 9×10^6 microspheres/mL in a 15 mL centrifuge tube (Figure 2D).

⚠ CRITICAL: To mix the reagent and medium well, we cannot add the reagent to the Petri dish directly.

e. Pipette up and down at least 10 times to mix the liquid with the medium.
f. Replace the medium in the Petri dish with the mixed medium.
g. Culture the cells in an incubator for approximately 4 h.
h. Discard the medium and rinse the cell 3 times with 4 mL prewarmed PBS to remove excess fluorescent beads (troubleshooting 3).

Note: Make sure to rinse the cells to remove beads attached to the cell membrane.

i. Incubate at least 10 min before imaging.

5. Labeling of intracellular vesicles via the endocytosis of epidermal growth factor receptor (EGFRs), as shown in Figure 2B (Li et al., 2018; Lidke et al., 2004) (troubleshooting 1, 2, and 3).

Note: In response to the ligand EGF binding, the transmembrane protein EGFR is activated and undergoes endocytosis with the EGF. By labeling the EGFR with biotinylated EGF and streptavidin-coated fluorescent probes consecutively, the intracellular vesicle containing the endocytic complex is labeled.

a. Culture the cells in serum-free DMEM with 10 nM biotin-EGF on ice for 15 min (Figure 2D).
b. After being washed with cold PBS three times, incubate the cells with 0.5–1 nM streptavidin-coated quantum dots (QDs, Invitrogen, Q10123MP) for 5 min.

Note: QDs are commonly used fluorescent probes in single-particle tracking, because of their high brightness and photostability (Pinaud et al., 2010).

c. Then, wash the cells three times with cold PBS again to remove unlabeled streptavidin-QDs (troubleshooting 3).
d. Transfer the cells to 37°C to initiate the internalization of EGF-QDs for at least 1 h before imaging.

6. Delivery of fluorescent particles into the cytoplasm via lysis of pinocytic vesicles, as shown in Figure 2C (Jiang et al., 2020; Li et al., 2015; Okada and Rechsteiner, 1982) (troubleshooting 1, 3, and 4).

Note: To maintain the osmotic pressure balance, the cells will undergo pinocytosis under the culture of hypertonic solution so that the hypertonic solution mixed with fluorescent particles will be engulfed by the cells in the form of vesicles, and then the hypotonic solution will cause the vesicles to rupture in the cytoplasm, releasing fluorescent particles into the cytoplasm.

a. Make the hypertonic loading medium and hypotonic medium following the protocol of pinocytic cell-loading reagent (I-14402).
b. Repeat steps 4a and 4b to centrifuge the stock solution of fluorescent particles, before aspirate the supernatant for the next step. Both the fluorescent beads (F8803, ~100 nm in diameter) and QDs (Q10123MP, ~25 nm in diameter) could be applied.
c. Add approximately 0.2 μL of fluorescent particles to a 0.5 mL centrifuge tube containing 100 μL of hypertonic loading medium (Figure 2D).

⚠ CRITICAL: Because fluorescent particles are only introduced into the cells at the 10-mm bottom well of the Petri dish, 100 μL of solution is enough to fill the bottom well.

d. Discard the medium in the dish. Then, quickly, but gently, pipette the hypertonic medium containing the particles to the dish.
e. Culture for 10 min in the incubator.

⚠ CRITICAL: The concentration of particles in the hypertonic solution determines the efficiency of particle entry into cells.

Note: Concentrations that are too high or incubation times that are too long can cause excessive particles to adhere to the glass bottom and cell surfaces, which can interfere with imaging.

f. Discard the hypertonic solution in the Petri dish. Then, quickly add 2 mL of hypotonic solution (serum-free medium and deionized water in a 6:4 ratio) and incubate for 2 min.

⚠ CRITICAL: Longer exposure to the hypotonic lysis medium may result in blebbing of the cell membranes and loss of cell viability.

Note: If the cells are sensitive to osmotic pressure, the incubation time can be reduced to approximately 1 min.

g. Aspirate the hypotonic solution with a pipette, add 2 mL of complete medium, and incubate in the incubator for 10 min.
h. Rinse the cells at least 3 times with PBS, add 2 mL PBS each time and shake the dish gently for approximately 10 s to remove the particles adhered to the cell membranes (troubleshooting 3).
i. The cells are then left in complete DMEM in an incubator to recover for 10 min before imaging.

Prewarm the incubation chamber on microscope

Timing: 0.5 h

This step allows the incubation chamber's temperature and carbon dioxide concentration to stabilize before the experiment begins.

7. Prepare the following equipment and materials.
   a. An on-stage incubation system (TOKAI HIT).
   b. 5% CO_2 tank.
   c. Distilled water 20 mL.

   Note: Live cell imaging is performed at 37°C with 5% CO_2, which is important for maintaining the physiological environment of cells.

8. Add 20 mL distilled water to the bath of the incubator to keep the humidity.
9. Open the valve of the gas cylinder and the pressure relief valve in sequence to maintain the CO_2 supply of the incubator. Adjust the valve to set the pressure to be approximately 0.1 MPa.

   Note: Keep the pressure reducing valve closed when opening the valve of the gas cylinder to avoid the disconnection of the pipe from the controller due to excessive pressure.

10. Turn on the power of the incubation system. Place an empty Petri dish in the chamber. Set the temperature of the top heater, stage heater, bath heater and lens heater to 37°C for half an hour before live cell imaging.

Note: The temperature setting for different heaters is instrument dependent and not necessarily generalizable.

Note: Placing a Petri dish in the chamber can prevent water vapor from spreading to the lens.

Note: Turn on the incubation system to maintain a stable culture environment before the experiment.

Opening the focus locking system

Timing: 0.5 h

This step achieves the focus-locking and prevent the vertical drift.

11. Turn on the 940 nm laser light source, piezoelectric stage and focus-locking software.
12. The working distance of the piezo stage is 100 μm, so we set the initial position of the stage at 50 μm so that the working range of the platform is ±50 μm.

Note: We need to adjust the focal plane by approximately 50 μm and focus on the target plane again.

13. Start the imaging of the infrared spot, which is reflected by the interface between the specimen and coverglass. Set the laser intensity, gain, and exposure time for best imaging.

Note: The principle of focusing is to detect the vertical drift of the lens according to the position shift of the infrared spot and then adjust the piezoelectric stage to compensate for the drift of the lens.

14. Set the parameters on the focus lock panel.
    a. Threshold: For 8-bit images, set a threshold between 0–255. Pixels with intensities above the threshold are used to locate the centroid of the imaged points. Setting this parameter ensures that there are enough pixels to fit the center of the spot. We usually set this parameter to 100.
    b. Step: Set the step size of the piezo stage. Setting this parameter ensures that the piezo stage is fast enough to compensate for lens drift. We usually set this parameter to 20 nm.
    c. Sensitivity: Changes in the distance exceeded the sensitivity cause the piezo stage to compensate for the drift. Setting this parameter determines the sensitivity to lens drift. We usually set this parameter to 1 pixel.
15. Click lock to start focus.

Note: Except for the calibration experiment, we need to lock the focus after setting the focal plane.

3D single-particle tracking in living cells

Timing: 2 h

We generally apply HILO imaging to excite fluorescent probes inside cells while achieving a high signal-to-noise ratio (Figure 1). This step includes the imaging of immobilized fluorescent particles on glass, calibration and 3D single-particle tracking in living cells.

16. Place the dish in the observation chamber on the microscope (troubleshooting 5, 6, and 7).

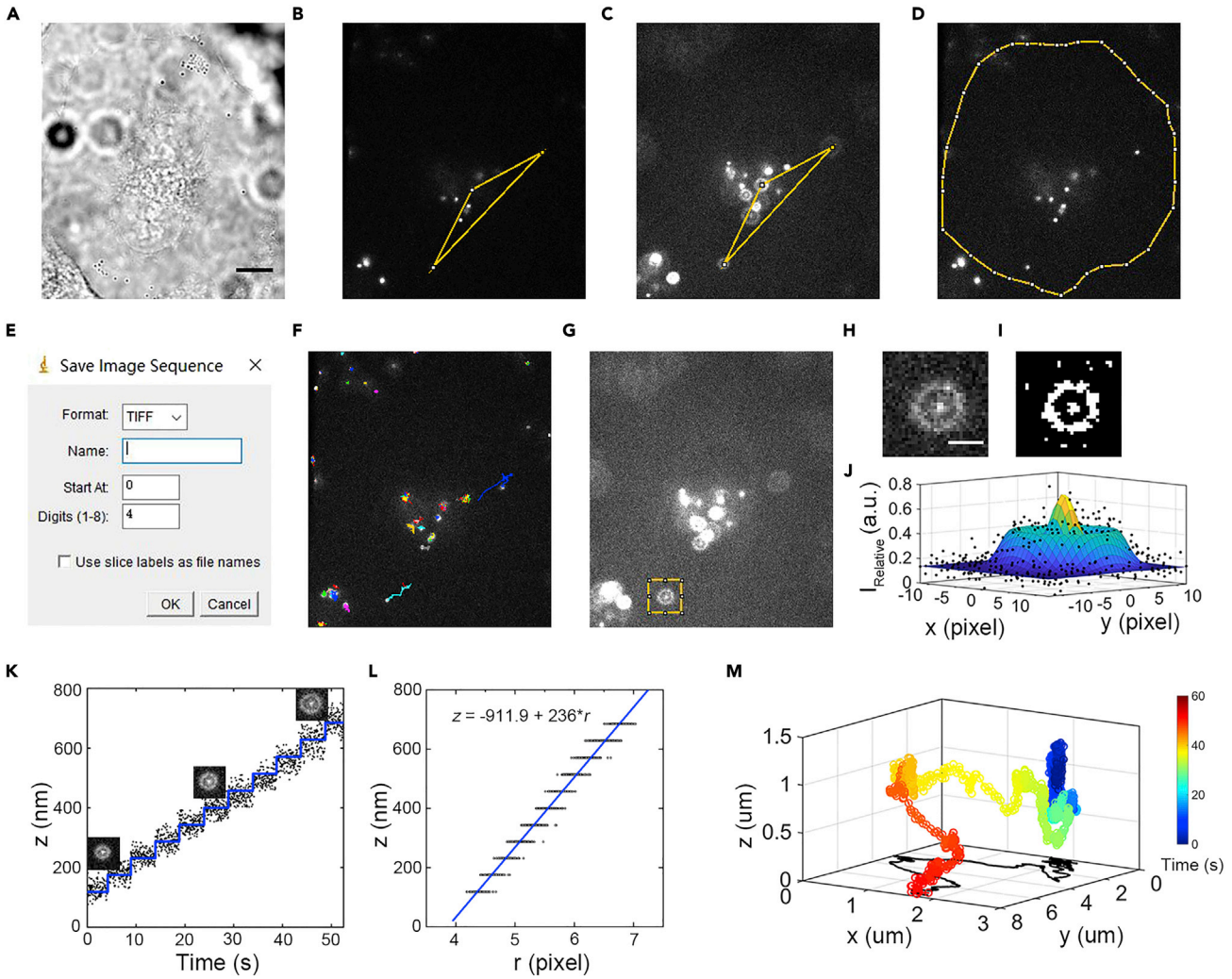


Figure 3. Imaging and 3D trajectory reconstruction

(A) Bright-field image of an A549 cell. Scale bar, 10 μm.

(B and C) The transformation matrix between the focused and defocused planes.

(D) Focused image of particles in a cell. The cell boundary is marked in yellow.

(E) The dialog screen of the the File–Save As–Image sequence tool in imageJ.

(F) Trajectories extracted by the plugin particle tracker in ImageJ.

(G) Defocused image of particles in a cell.

(H) A typical diffraction ring indicated by the yellow box in (G). Scale bar, 3 μm.

(I) Binarized image of the diffraction ring in (G). The radius of the ring measured by the binarized image is 6.5 pixels, which can be used as the initial value of the Gaussian fitting.

(J) 2D Gaussian Fitting of Image Intensity Based on Spatial Location. The measured radius of the diffraction ring is 6.62 pixels.

(K) Sample data of the stage positions in the z direction (blue line) and the radii of the diffraction rings (black points).

(L) Calibration data between the z coordinate and the measured radius (r) of diffraction rings.

(M) Plot of a typical trajectory with the time indicated by color.

17. Turn on the brightfield light source and look down the eyepieces to focus the sample and position on a region of interest (Figure 3A–3D).
18. Turn off the brightfield light source. Use a laser that is compatible with the fluorescent particles and start at a moderate power.

Note: We usually choose the weakest possible power of the laser to image the fluorescent particles, which reduces the photobleaching of fluorescent particles and avoids damage to cells.

19. Use the appropriate optical path, open the shutter, and detect the fluorescent signal with the EMCCD. Focus on the fluorescent particles on the bottom and adjust the illumination angle for HILO imaging.

Note: The HILO mode is ideal for single-particle tracking because it can significantly improve the imaging signal-to-noise ratio.

⚠ CRITICAL: HILO mode may cause uneven light field.

20. Cool down the EMCCD to −75°C. Set the camera exposure time at 30 ms. Set the gain of the EMCCD between 0–300. Select the frame transfer of the EMCCD (troubleshooting 8).

Note: The linear signal amplification range of CCD is 0–300, so we should set the gain in this range. For the fluorescence bead experiment, the gain can be set to approximately 50. For the QD experiment, because the brightness of the quantum dot is relatively low, setting the gain value above 250 can obtain a good imaging effect. For different probes, the key to setting the gain is that the CCD can detect clear diffraction rings.

21. Set the repeat to be 2,000 times (e.g., total acquisition duration is 60 s under the 30 ms interval). Choose the stream mode to realize the fastest frame rates.

Note: We have tried intervals of 30 ms and 100 ms, which are able to track the movements of the labeled vesicles and fluorescence particles. It should be noted that a shorter time interval means a shorter exposure time, which will reduce the signal-to-noise ratio of the imaging.

22. 3D tracking in living cells. Select a cell containing fluorescent particles to take 2,000 frames of video. After taking the video, switch to brightfield illumination and take a single snapshot of the cell. It can be used to further determine the position of the nucleus and the cell outlines, which are important for analyzing the spatial distribution of intracellular transport (troubleshooting 9).

⚠ CRITICAL: The number of fluorescent particles in different cells varies greatly, so we usually select cells with no more than 30 particles to avoid overlapping diffraction rings.

Note: Do not touch the optical table during the experiment, which may cause vibrations of the imaging platform.

23. To determine the localization precision of the imaging platform, we image the immobilized particles under the same conditions and protocol as that for living cells. If the system is not stable, the coordinates of the fixed point will exhibit movements instead of fluctuation around the center.

Note: During the experiment, focus locking is very useful to avoid measurement bias in the z direction.

24. To determine the calibration between the z position and radius of the diffraction ring of the fluorescent particles, we take 2,000 frames of video of the fixed particles and simultaneously lift the lens in 50 nm steps every 5 s (troubleshooting 10).
25. For each sample, we usually finish imaging in 2–4 h to avoid impairing cell function. Finally, save all the images in TIFF format for the following processing.

3D trajectory reconstruction

Timing: 1 day

Reconstruct 3D trajectories from video using ImageJ and MATLAB. Extract the lateral trajectory from the focal plane through the ImageJ plugin particle tracker and then determine the axial coordinates by fitting the diffraction ring with the script written in MATLAB.

26. Select a region consisting cells, duplicate the video and save the file.

Note: The raw video contains images on both the focused and defocused planes. For the convenience of processing, we duplicate and save the videos of the region of interest on the two planes.

a. Open images using ImageJ, an open-source image analysis software program.
b. Adjust the video brightness through the Image–Adjust–Brightness/Contrast tool to make the image clear.
c. Use the Rectangle Tool to draw a region of interest containing cells on the focus plane. Duplicate the image stack of the selection with the Image-duplicate tool.
d. Move the rectangular region 256 pixels horizontally to the defocused plane with the Edit–Selection–Specify tool and then use the duplicate tool to duplicate the defocused image stack.
e. Save the duplicated stacks of the focused plane and defocused plane areas to a folder.
f. Create a new subfolder named "def". Use the File–Save As–Image sequence tool to save the defocused image stack in the folder "def" in the form of an image sequence.

Note: Set the stack's name to empty on the dialog screen (Figure 3E).

27. Obtain the transformation matrix between the two planes.
    a. On the stack of the focused plane, use the polygon selection tool to select at least three bright spots to construct a polygon, then use the magnifying glass tool to zoom in on the image, and adjust the position of the polygon vertices to be approximately in the center of the bright spots (Figures 3B and 3C).

Note: Fitting the diffraction ring in subsequent steps would recalculate the center position of the diffraction ring. Therefore, the vertex position of the polygon does not need to be 100% in the center of the bright spot.

    b. Copy the polygon to the defocused plane stack with the Edit–Selection–Restore Selection tool. Similarly, adjust the position of polygon vertices with the magnifying glass tool.

Note: Display the same frame image on the focused and defocused stacks.

    c. Save the polygon coordinates on the two stacks using the File–Save As–XY Coordinates tool.

28. Extract motion trajectories from the focused stack using ImageJ's plugin Particle Tracker (Sbalzarini and Koumoutsakos, 2005) and draw the region occupied by the intracellular trajectories (Figure 3F).
    a. Select the focused stack, start the plugin Particle Tracker, which displays a dialog screen. The dialog has two parts: "Particle Detection" and "Particle Linking".
    b. In the Particle Detection part, we need to set Radius, Cutoff and Percentile.
        i. The radius is set to determine the radius of the particle, and we usually set it to 3, to ensure that the circle with the given radius could cover the entire light spot.

ii. Cutoff is set to remove the possibly few aggregations as well as the blurred particles, and we usually set it to 0.
iii. Percentile (percent %) is the intensity threshold. The local region that has an intensity above the threshold is recognized as a particle. We usually set this parameter to 0–5 depending on the total intensity of the stack and the density of the particles.

Note: When we set the percentile, we should use the Preview Detected button and the slider to view the detected particles in other frames.

c. In the Particle Linking part, we need to set the displacement and the link range.
   i. Displacement is set to 2 or 3.
   ii. The link range is set to 2 or 3.

Note: These parameters need to be optimized by checking the extracted trajectories in the following procedures. Here, a link range over 1 could bridge QD blinking events.

d. After completing the particle tracking, the result window will be displayed.
e. Click on the *Visualize All Trajectories* button to open a new window that visualizes all the trajectories and the original movie frames in an overlay.
f. After opening the visualization window, click the filter button at the bottom. We set the minimum length (in frames) to be 50 for trajectories to be displayed (the default is to display all), and trajectories below that length will be excluded.

Note: Check the trajectories optically for inappropriate linking and optimize the linking parameters repeatedly. These parameters could be adjusted on the menu bar of the result window.

g. In the trajectory window, select a region that covers all the trajectories by clicking and dragging the mouse.
h. Click on the Trajectories in Area Info button, which displays the information of all trajectories in the selected area on the verbose information panel. In this panel, trajectory information can be exported to any text editor.
i. The trajectory information includes the frame number, the xy coordinates and the intensity parameters.

Note: A trajectory will have at least 2 rows. Each row represents a frame in this trajectory and holds the information about the particle related to this trajectory in that frame. The first column indicates the frame number, starting at 0 for each trajectory. The second and third columns contain the x- and y-coordinates of the particle, respectively. The x-axis points top-down, and the y-axis is oriented left-right in the image plane. The 4th and 5th columns contain the intensity moments of orders 0 and 2, respectively (m0, m2). The 6th column contains the non particle discrimination score. When more than one trajectory is displayed, a blank line separates each individual trajectory.

j. Select the All Trajectories visual window. Capture the image and use polygon selections to draw a polygon region that covers all the trajectories needed.

Note: With the region, immobilized trajectories and trajectories that do not belong to this cell can be excluded.

29. Cut and save the ROI image.
    a. Import the trajectory information into MATLAB and store it in a structure and the coordinates of the region into MATLAB and store them in arrays.

b. Exclude trajectories whose mean coordinates are not within the polygon region (Figure 3D).
c. Import the coordinates of the two polygons into MATLAB and store them in arrays.
d. Through simple matrix operations, calculate the transformation matrix between the two planes.
e. Using the transformation matrix, transform the *xy* coordinates of the trajectory on the focus plane to the defocus plane (Figure 3G).
f. Create a subfolder named "Image" to store diffraction ring images.
g. For each trajectory, read the image in file "Dec" frame by frame, and the intensity of the image is stored in an intensity matrix I0.
h. Define a square region of interest (ROI) with a side length of 31 pixels (Figure 3H).
i. For the kth point on the trajectory, obtain the value of the intensity matrix I0 of the size of the ROI centered at (x_k, y_k).
j. Convert the ROI matrix to a grayscale image and save the image named "ith track kth frame" in the folder "Image".

Note: The results of each trajectory can be previewed through the ROI images in the folder. Some images with overlapping or poor imaging results can be deleted manually.

30. Calculate the radius of the diffraction ring from MATLAB (troubleshooting 11).
    a. For each trajectory, read the ROI image frame by frame and store in a matrix *I*.
    b. Through a real-space bandpass filter, obtain a matrix I_1 with an improved signal-to-noise ratio.
    c. Set the appropriate threshold to binarize the matrix and save it as a new matrix I_2.
    d. Use the circleHough function to obtain the radius r_0 of the binary image, as shown in Figure 3I (Wang and Boyer, 2012).
    e. Perform fitting to the matrix I_1 with the following equation, which is a Gaussian peak surrounded by a ring of radius *r*:

$$I = c_0 + c_1 * \exp\left[- c_2 * \left((x - x_0)^2 + (y - y_0)^2 \right) \right] + c_3$$

$$* \exp\left[- c_4 * \left(\left((x - x_0)^2 + (y - y_0)^2 \right)^{\frac{1}{2}} - r \right)^2 \right]$$

where *I* is the intensity matrix of the ROI, x_0 and y_0 are the coordinates of the ring center, and *r* is the ring radius (Jiang et al., 2020; Toprak et al., 2007).

Note: Cftool can directly generate the fitting code.

⚠ CRITICAL: For the fitting of such a complex function, it is easy to get stuck. The try catch end structure can be used to skip the error operation and continue the subsequent fitting.

    f. The loop is used to set different fitting initial values for fitting, and the result with the largest r-square is selected (Figure 3J).
    g. Save the frame number, *xy* coordinates and radius of each track in an array.
    h. For each trajectory, plot the radius as a function of frame number and use the brush tool to remove discontinuous points in the array.

Note: Fitting images with overlapping or poor signal-to-noise ratios may produce points with discontinuous radii.

Note: Usually, there will be more than 10 trajectories in a cell, which will generate more than 10,000 frames of images to be processed. When using a computer with an i5 3.2 GHz processor and 16 Gb of RAM, and the script will take more than 5 h.

31. Determine the calibration between the ring radius and z position.
    a. In the defocus plane, the multipoint tool is used to directly select the center position of the diffraction ring.
    b. Use the File–Save As–Image Sequence tool to save the image stack as an image sequence to a folder named "Def".
    c. Import the position of the diffraction ring to MATLAB.
    d. Repeat steps 29f-29j to save the ring images and repeat step 30 to calculate the ring radius.
    e. Plot the radius as a function of frame number.
    f. With the function getline, obtain the averaged value of each step (Figure 3K).
    g. With cftool, linearly fit the value with the z position of the piezo stage recorded in the experiment. The slope is the calibration between the ring radius and the z position (Figure 3L).
    h. The axial coordinates (*z*) are obtained by multiplying the ring radius by the slope of the calibration.
32. Determination of localization precision.
    a. For the fixed points on the glass, repeat steps 26-30 to obtain the *xy* coordinates and radius.
    b. For a fixed point, MSD = $2*\sigma^2$ in each direction, where σ is the localization precision (Savin and Doyle, 2005).
    c. Randomly select 10 fixed points and calculate the average MSD curve in the *xyz* three directions to determine the localization precision.
33. The z-coordinate of the trajectory can be calculated from the ring radius obtained in step 30 and the calibration obtained in step 32. In this way, a complete 3D trajectory with *xyz* coordinates over time can be reconstructed (Figure 3M).

Dynamic analysis of 3D trajectories

Timing: 4 h

Dynamics analysis of 3D trajectories is performed with a custom script written in MATLAB.

34. Select the trajectories with track lengths longer than 50 frames.

Note: Selecting trajectories longer than 50 frames can ensure that there are enough points to compute the 1 s (33 frames) MSD curve.

35. The MSD curve is calculated by the equation $MSD(\tau) = |r(t + \tau) - r(t)|^2$, where *r* is the 3D coordinates and τ is the lag time (Lerner et al., 2020). Calculate the mean squared displacement between all point locations in each direction (*x, y, z)* with time interval τ=n*0.03 s (n=1, 2, 3, … 33) along the entire trajectory.

36. The diffusion coefficient and the anomalous exponents are calculated by fitting the MSD to lag time.
    a. The MSD of each trajectory is plotted as a function of lag time in log-log. Observe whether the MSD curve is a straight line or a curve. If it is a straight line, then the motion patterns at different time scales are consistent, and the dynamic parameters of the entire curve can be directly fitted. If the MSD curve changes at different time scales, it means that the motion of particles follows different motion modes at different time scales; then, the parameters should be fitted separately at different time scales (Figure 4A).
    b. To clearly determine if MSD changes linearly with time, plot MSD/t as a function of t in a logarithmic coordinate system (Figure 4B).

Note: With the plot of MSD/t, the negative slope of MSD/t indicates the subdiffusive motion. The positive slope indicates superdiffusive motion. The increased slope suggests that the motion gradually alters to normal diffusive motion.

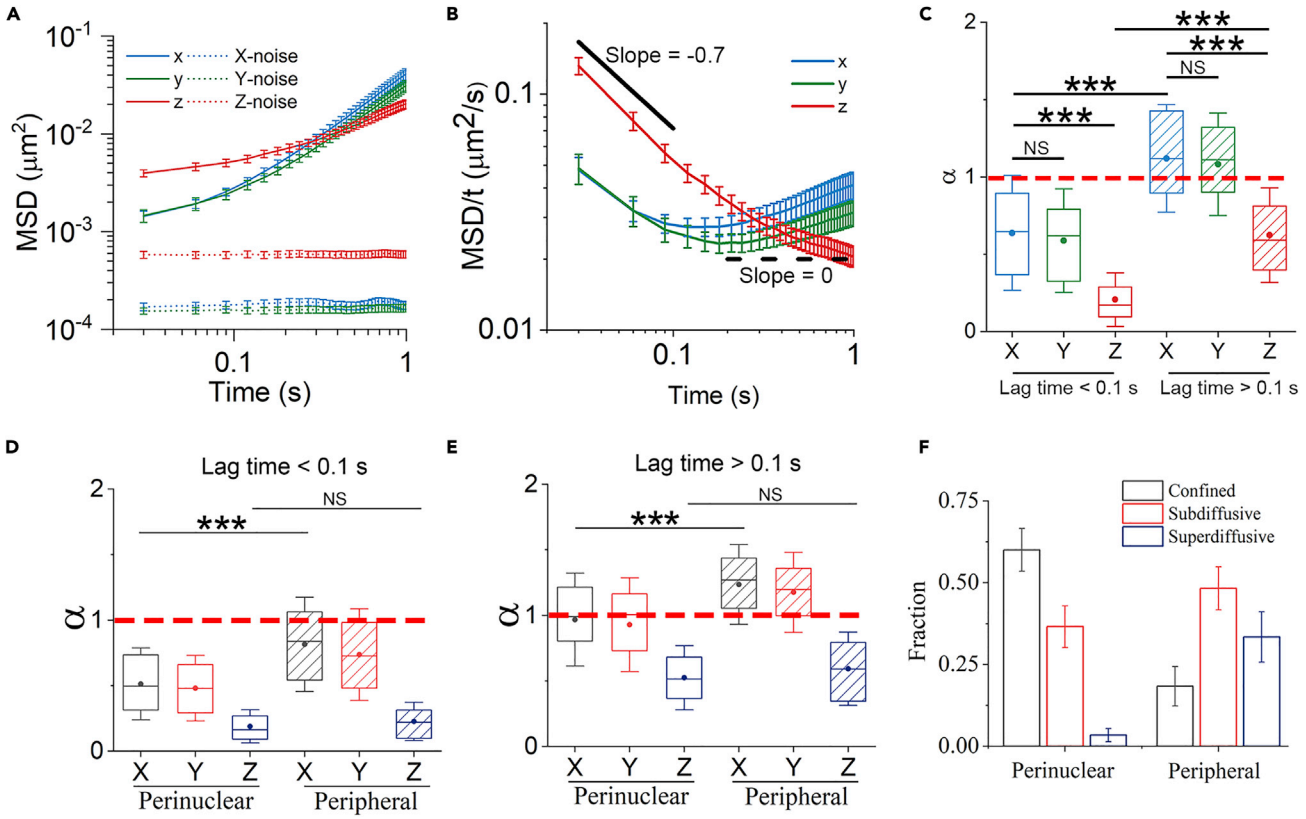


Figure 4. Dynamic analysis of the 3D trajectories

(A) Comparison of the particle MSD in each direction as a function of lag time in A549 cells (solid lines, 107 trajectories from 6 cells) and on the cover glass (noise floor, dashed lines, 15 trajectories).

(B) Plot of MSD/t versus time. The solid and dashed lines in black represent slopes of -0.7 and 0, respectively. Error bars indicate the SEM.

(C) Comparison of the exponent α in each direction at short and long timescales. For boxplots, the line in the box indicates the median value, the dot indicates the mean value, box edges correspond to 25% and 75% of the dataset, and error bars indicate the SD. ***p < 0.001; NS, not significant.

(D and E) Comparison of the exponent α for each direction between the perinuclear and peripheral regions. Exponent α determined at short timescales (B) and long timescales (C).

(F) Fraction of confined (a < 0.5), subdiffusive (0.5<a < 1), and superdiffusive (1<a < 2) motions in the two different regions. Figure (D–F) is reproduced from Jiang et al. (2022).

c. Each 1D MSD can be fitted with MSD = $2*D_\alpha*t^\alpha$. α is the anomalous exponent: $\alpha \approx 1$ is free Brownian motion (e.g., pure random walk or free diffusion), $\alpha < 1$ subdiffusion (e.g., diffusion within a confined area), $\alpha > 1$ superdiffusion (e.g., diffusion overlaid with directed motion), and $\alpha \approx 2$ directed motion, D_α [$\mu m^2/s^\alpha$] is the anomalous exponent-dependent diffusion coefficient, which describes the ability of the particle to diffuse (Figures 4C and 4F).

Note: For superdiffusive and subdiffusive motions, the units of diffusion coefficient D_α [$\mu m^2/s^\alpha$] are α-dependent and cannot be used directly to evaluate dynamics. Here, we suggest using α to compare the dynamics in different conditions. For trajectories on longer time scales, since alpha is close to 1, the apparent diffusion coefficient D_{app} can be calculated with uniform units [$\mu m^2/s$]. For 1D MSD, calculate D_{app} by fitting MSD with MSD=2* D_{app} *t, and set α=1.

d. Finally, dynamic parameters α in different directions at different time scales are presented as box plots.

e. The position of the nucleus and the outline of the cell can be identified according to the captured brightfield image of the cell. The perinuclear region and the peripheral region

can be defined according to the distance of the particle to the nucleus, and then the difference in transport in the two regions can be compared for further analysis (Figures 4D–4F).

EXPECTED OUTCOMES

Here, we provide a detailed, step-by-step protocol that enables us to characterize the 3D spatiotemporal transport dynamics of macromolecules in living cells. With this method, we discovered the quasi-2D diffusion of QDs in adherent cells. We also found spatiotemporal differences in vesicle trafficking in the lateral and axial directions and observed the diffusion behavior of fluorescent particles within vesicles after microtubule disruption. Being able to label fluorescent particles to specific biomolecules (such as mRNAs and proteasomes) in cells and directly observe their three-dimensional trajectories will be crucial for understanding the functions of the biomolecules.

LIMITATIONS

Since the temporal resolution of the EMCCD we used is approximately 100 Hz, we cannot track very fast-moving probes. In space, the imaging range of the axial direction is only approximately 2 μm. In addition, the overlapping of the diffraction rings when the two molecules are close makes it impossible to extract the 3D trajectory of this part, so it is impossible to observe the interaction between the two particles. Furthermore, since there is only one EMCCD, 3D tracking of particles and imaging of subcellular structures cannot be achieved simultaneously.

TROUBLESHOOTING

Problem 1

Fluorescent particles aggregate into clusters.

Potential solution

Centrifuge before use and aspirate the supernatant of the particle solution.

Problem 2

An insufficient number of beads is endocytosed into cells.

Potential solution

Increase the concentration of beads in the coculture medium.

Problem 3

Fluorescent particles may attach to cell membranes.

Potential solution

Before imaging, wash the cells thoroughly with PBS 3 times.

Problem 4

An insufficient number of quantum dots is introduced into cells.

Potential solution

Appropriately increase the concentration of quantum dots. Carry out 2–3 cycles of the osmotic lysis of pinocytic vesicles for the introduction.

Problem 5

Air bubbles are generated after dripping oil on the lens.

Potential solution

Pop the air bubbles using a small pipette tip. Wipe off the oil droplets and re-drop.

Problem 6
No laser is emitted from the lens.

Potential solution
Adjust the incident angle of laser. Fine-tune the mirror position for the laser to gradually increase the incident angle until TIRF occurs, and then carefully decrease the angle to achieve HILO for optimal imaging.

Problem 7
Lens drift during the imaging.

Potential solution
Before experiments, waiting for over 30 min after tuning turning on the microcopy microcope and incubation chamber. Use the focus-lock system during the imaging.

Problem 8
Insufficient or overexposed fluorescence in the image.

Potential solution
Increase or decrease the gain of the EMCCD. Adjust the laser intensity or change the attenuators in the light path.

Problem 9
The position of the brightfield image and the fluorescence video is misaligned.

Potential solution
Before and after taking video, without changing the imaging device, directly switch the light source to the image.

Problem 10
The calibration relationship of different experiments is different.

Potential solution
Calibration experiments are performed for each dish.

Problem 11
The diffraction ring images of fluorescent particles overlap each other.

Potential solution
Appropriately reduce the number of fluorescent particles in cells to avoid the occurrence of overlapping. For the videos having few frames with overlapping diffraction rings, delete the frames where the diffraction rings overlap, and divide the trajectory into segments.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Hui Li (huili@bnu.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability

The datasets and code generated in this study are available on GitHub: https://github.com/chaojiang1901/3D-particle-tracking-MATLAB. The datasets and code are also available at Zenodo: https://doi.org/10.5281/zenodo.7091266.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.L.; methodology, C.J. and H.L.; experiments, C.J.; analysis, C.J., S.X.D., P.Y.W., and H.L.; resources, S.X.D., P.Y.W., and H.L.; writing and editing, C.J., S.X.D., P.Y.W., and H.L.; supervision, P.Y.W. and H.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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