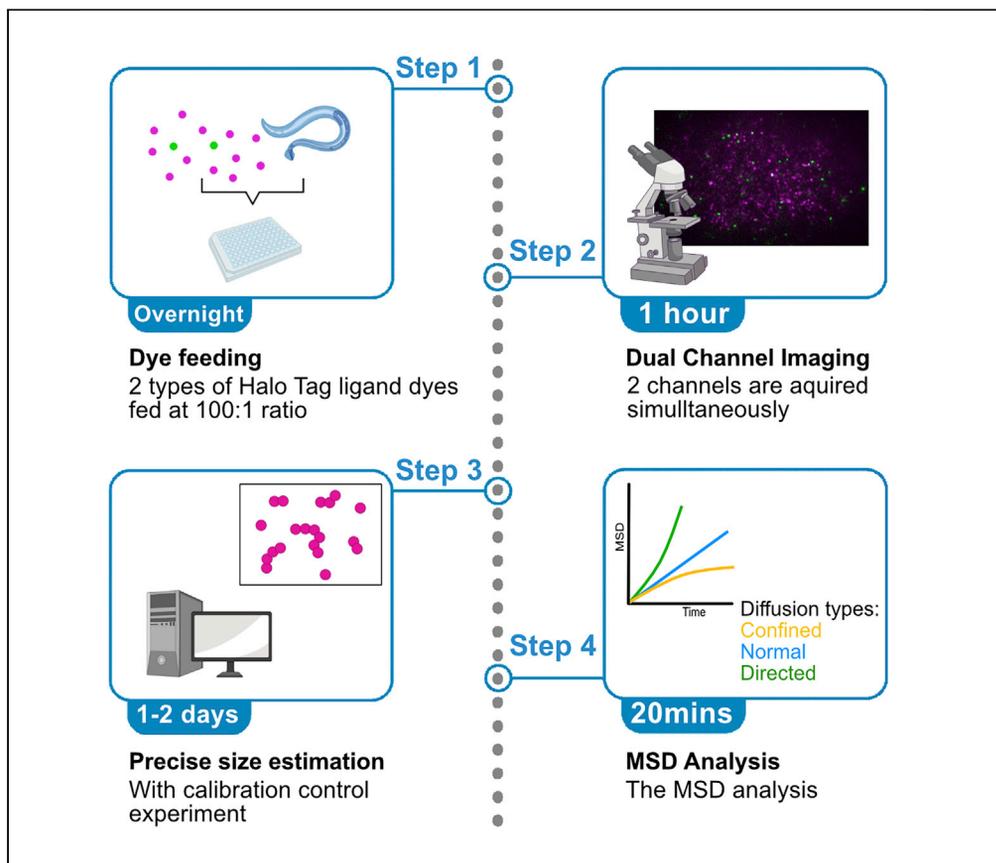


Protocol

Non-invasive chimeric HaloTag labeling to study clustering and diffusion of membrane proteins



As live imaging plays an increasingly critical role in cell biology research, the desire to label and track individual protein molecules *in vivo* has been growing. To address this, in this protocol we describe steps for sparse labeling using two different HaloTag ligand dyes in *C. elegans*. This labeling approach is simple, is non-invasive, and preserves the view of the bulk protein population. We further describe how to carry out single-particle tracking experiments and extract information about particle diffusion behavior.

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

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Highlights

Protocol for chimeric labeling with HaloTag system for non-invasive sparse labeling

Step-by-step instructions for particle tracking and MSD analysis

Calibration control experiments available for determining the precise cluster size

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Protocol

Non-invasive chimeric HaloTag labeling to study clustering and diffusion of membrane proteins

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<https://doi.org/10.1016/j.xpro.2022.101857>

SUMMARY

As live imaging plays an increasingly critical role in cell biology research, the desire to label and track individual protein molecules *in vivo* has been growing. To address this, in this protocol we describe steps for sparse labeling using two different HaloTag ligand dyes in *C. elegans*. This labeling approach is simple, is non-invasive, and preserves the view of the bulk protein population. We further describe how to carry out single-particle tracking experiments and extract information about particle diffusion behavior.

For complete details on the use and execution of this protocol, please refer to Chang and Dickinson (2022).¹

BEFORE YOU BEGIN

This protocol describes how to use two different HaloTag ligand dyes to achieve sparse labeling, and how to perform particle tracking and MSD analysis to analyze particle diffusion and clustering. We developed these approaches using *C. elegans* zygotes, but the approaches we describe here should be adaptable to other systems with minor modifications.

Institutional permissions

Institutional permissions are not required for working with *C. elegans*.

HaloTag basics

HaloTag is a genetically encoded tag based on the bacterial enzyme haloalkane dehalogenase, which can be fused to proteins of interest and can be functional in both live and fixed cells.² HaloTag irreversibly attaches to its synthetic ligand by forming a covalent bond (Figure 1A). A wide variety of HaloTag ligands are available, including fluorescent dyes spanning the visible spectrum. Since each HaloTag molecule can bind a single ligand dye molecule, labeling cells with a mixture of two different fluorescent dyes produces a HaloTag-fused protein population labeled with two different colors simultaneously.¹ We primarily use Janelia Fluor dyes due to their high quantum yield and high efficiency, although in principle any combination of dyes could be used in our approach.^{3–5}

Example application: PAR-3 segregation in single-cell polarity

Before the first cell division of the *C. elegans* zygote, PAR polarity proteins are transported to either anterior or posterior domains to establish the anterior-posterior axis. During this process, the scaffold protein PAR-3 plays a crucial role. PAR-3 forms a heterogeneous population of oligomers of many different sizes, and these clusters are physically transported to the anterior of the cell during polarization^{1,6} (Figure 1B). To study the physical basis for PAR-3 transport, we needed an approach to track individual PAR-3 clusters on the cell membrane. We developed a chimeric labeling protocol



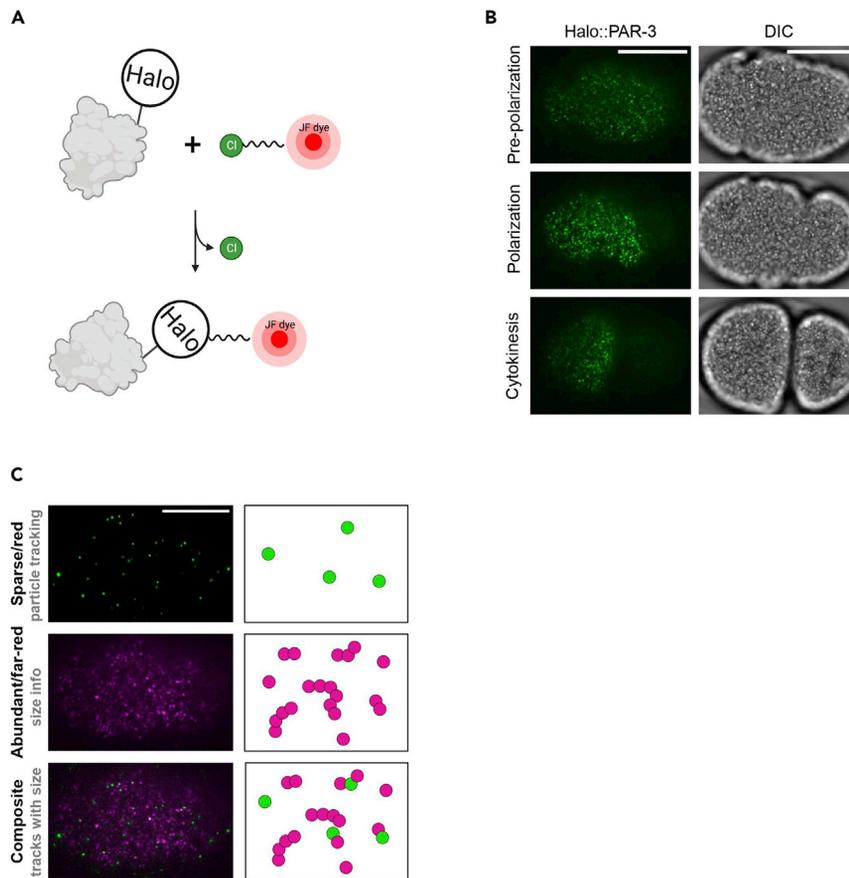


Figure 1. An overview of the study background and dual-labeling design, related to ‘before you begin’

(A) A cartoon illustration of how Halo works: the HaloTag binds covalently to its ligand dye.

(B) Live images of PAR-3 segregation during *C. elegans* zygote polarization. Left panels: cortical Halo::PAR-3 labeled with JF646. Right panels: DIC channel images during polarization process. Scale bar: 10 μ m. Figure modified from Chang and Dickinson.¹

(C) TIRF images and cartoon illustrations of the dual-labeling experiment. Magenta represents far-red/abundant channel and green represents red/sparse channel. Scale bar: 10 μ m. Figure modified from Chang and Dickinson.¹

to overcome the dense and heterogeneous nature of the PAR-3 population, allowing us to 1) sparsely label and track PAR-3 clusters in a non-invasive manner, and 2) visualize the bulk protein population and estimate the number of PAR-3 molecules in each cluster¹ (Figure 1C).

Design and construction of endogenously Halo tagged *C. elegans* strains

⌚ Timing: 4–6 weeks

Cells expressing HaloTag fusion proteins are required for this approach. *C. elegans* strains carrying HaloTag insertions into native genes can be made using CRISPR protocols and reagents previously published by our laboratory.^{7–10} We use NEBuilder HiFi DNA Assembly to generate a plasmid construct that contains the HaloTag and a selectable marker, flanked by 500–1,500 bp of unmodified genomic homology arms. This repair template construct, a Cas9–sgRNA expressing vector, and fluorescent extrachromosomal array markers are co-injected into the syncytial gonads of young adult *C. elegans* hermaphrodites. Cas9 cleavage of the target locus triggers DNA repair by homologous recombination, resulting in incorporation of the repair template, and knock-in animals are isolated from the F2 progeny of injected animals using hygromycin selection and a phenotypic marker. After each knock-in strain is isolated, the selectable marker is removed by Cre-Lox excision.

Preparation and storage of HaloTag ligand dye aliquots

⌚ Timing: 2 h

- Order dyes from the Lavis lab, Tocris, Promega or from another source. When a new vial of dye is received:
 - Dissolve in acetonitrile to 1 mM.
 - Make 2 μ L aliquots in PCR tubes.

Note: It is not necessary to wrap tubes in foil or otherwise protect the solution from light during this step, but work quickly to avoid excessive exposure to ambient light.

- Evaporate acetonitrile using a speedvac.

Note: We run the speedvac for 30 min with vacuum on and 45°C baking. If the PCR tubes are too small to fit into the speed vac, place 1.5 mL microcentrifuge tubes in the rotor, and place an 0.2 mL microcentrifuge tube inside each 1.5 mL tube to make smaller holders.

- Store dried aliquots in a small foil-wrapped jar with desiccant at -20°C . Dyes are stable for 6–12 months when stored in this way.

Preparation of VALAP

⌚ Timing: 10 min active, 4–6 h total

- Mix equal amounts by weight of Petrolatum (a.k.a. Vaseline), Lanolin and Paraffin wax in a large beaker.
- Melt and stir together over low heat (80°C – 100°C) on a stirring hot plate. Be patient, it may take several hours for everything to melt depending on the amount. Do not overheat.
- Let cool, cover with foil and store at 20°C – 25°C . Stable indefinitely (several years).
- Before use, scoop a small amount into a microcentrifuge tube and melt in a heat block at 70°C .

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>E. coli</i> OP50 standard food	Caenorhabditis Genetics Center	OP50
<i>E. coli</i> HB101 Liquid Culture food	Caenorhabditis Genetics Center	HB101
Chemicals, peptides, and recombinant proteins		
Janelia Fluor® 585	Laboratory of Luke D. Lavis	Grimm et al., ³ Grimm et al., ⁴ and Grimm et al. ⁵ 2016
Janelia Fluor® 646	Laboratory of Luke D. Lavis	Grimm et al., ³ Grimm et al., ⁴ and Grimm et al. ⁶
Polylysine	Sigma	SLCC5591
DMSO (critical reagent)	Sigma	D2650-5X5ML
Acetonitrile	Sigma	SHBJ9782
Petrolatum	Acros Organics	417090010
Lanolin	Alfa Aesar	A16902
Paraffin wax	Acros Organics	416770020
Experimental models: Organisms/strains		
<i>C. elegans</i> strain with endogenously tagged Halo::PAR-3 par-3(cp323[HaloTag-GLO [^] PAR-3]) III, L4 stage hermaphrodites	Dickinson et al. ⁶	LP621

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
ImageJ	N/A	https://imagej.net/
MATLAB	Mathworks Inc.	https://www.mathworks.com/products/matlab.html
Utrack	Jaqaman et al. ¹¹	Version 2.2.0; https://github.com/DanuserLab/u-track
DualLabeling_Analysis	This paper	https://doi.org/10.5281/zenodo.7178745
MSDanalyzer	Tarantino et al. ¹²	https://tinevez.github.io/msdanalyzer/
SLM toolbox	D'Errico ¹³	https://www.mathworks.com/matlabcentral/fileexchange/24443-slm-shape-language-modeling
Other		
TetraSpeck microspheres	Invitrogen	1929718
Spacer Grade 23–26 μm Soda Lime Solid Glass Microspheres	CoSpheric	S-SLGMS-2.5 23-26um
Speedvac	Eppendorf	Vacufuge plus
Plasma cleaner	PlasmaEtch, Inc.	PE-25
UV ozone cleaner	Jelight, Inc.	Model 18

MATERIALS AND EQUIPMENT

Dual-view TIRF microscope

We performed single-particle tracking via near-TIRF microscopy.¹ Tracking of two different fluorescent dyes simultaneously requires a microscope with a dual-view emission path design. A dual-view image splitter is easy to construct using off-the-shelf components that are relatively inexpensive. A parts list and instructions for construction and alignment are provided in the Supplemental Material (Methods S1). Our microscope uses a micromirror illumination path design that is optimal for imaging multiple wavelengths simultaneously,^{14,15} although this is not strictly necessary; any TIRF illumination system that can deliver 561 nm excitation is sufficient. The total system magnification should be chosen such that the image pixel size is 60–70 nm; the slight over-sampling in this configuration improves particle tracking performance (our unpublished observations). This degree of pixel representation can be achieved by using 100× magnification with an sCMOS camera that has 6.5 μm pixels, or by using 150× magnification with a camera that has 11 μm pixels.

S Medium		
Reagents	Final concentration	Final amount
S Basal solution (5.85 g/L NaCl, 1 g/L K ₂ HPO ₄ , 6 g/L KH ₂ PO ₄)	N.A.	10 mL
5 mg/ mL cholesterol	5 μg/mL	10 μL
1 M potassium citrate pH 6.0	10 mM	100 μL
trace metals solution (1.86 g/L EDTA, 0.69 g/L FeSO ₄ · 7H ₂ O, 0.2 g/L MnCl ₂ · 4H ₂ O, 0.29 g/L ZnSO ₄ · 7H ₂ O, 0.025 g/L CuSO ₄ · 5H ₂ O)	N.A.	100 μL
1 M CaCl ₂	3 mM	30 μL
1 M MgCl ₂	3 mM	30 μL
Total	N.A.	10.3 mL

Note: We recommend to first make 1 L of S basal solution and 1 L of trace metal solution as stock. Then make a 10.27 mL S medium stock as shown in the table, and aliquot into 1.5 mL microcentrifuge tubes. The S basal solution can be kept at 4°C until micro-organism start to grow in it. The Trace metals solution should be wrapped with foil and kept in dark at 20°C–25°C, which will be stable for years. The S medium stock made can be kept at 4°C for 3 months.

Egg Buffer		
Reagents	Final concentration	Final amount
1 M HEPES	25 mM	375 μ L
5 M NaCl	118 mM	453 μ L
1 M KCl	48 mM	720 μ L
1 M CaCl ₂	2 mM	30 μ L
1 M MgCl ₂	2 mM	30 μ L
Water	N.A.	13.5 mL
Total	N.A.	15 mL

Note: We recommend to make a 15 mL stock and aliquot into 1.5 mL microcentrifuge tubes. The stock can be kept on a shelf at 20°C–25°C for 6 months.

VALAP		
Reagent	Final concentration	Final amount
Petrolatum (also known as Vaseline)	33%	333 g
Lanolin	33%	333 g
Paraffin Wax	33%	333 g
Total	N.A.	1 kg

Note: We recommend making 1 kg of VALAP stock, which can be stored on a shelf at 20°C–25°C for several years.

STEP-BY-STEP METHOD DETAILS

Preparing *C. elegans* for HaloTag imaging

⌚ Timing: 30 min active, overnight incubation (1–7 days the first time) (for step 1)

The HaloTag labeling can be achieved by feeding in this protocol. This step shows how to feed the two different HaloTag ligand dye to *C. elegans* in liquid culture to achieve the labeling of target protein population.

1. Maintain the desired *C. elegans* strain on the standard NGM plates with OP50 bacteria feeding. For additional details regarding *C. elegans* culturing and handling, please refer to the Wormbook “Maintenance of *C. elegans*”.
2. Prepare HB101 liquid culture for feeding.

Note: HB101 is easier for worms to eat and improves worm health in liquid culture, but OP50 can be used instead if desired.

- a. Streak out bacteria from the glycerol stock in the –80°C freezer onto an LB plate (with no antibiotics) and grow 12–18 h at 37°C.
 - b. Pick a single colony and inoculate in 5 mL of LB liquid medium, shake at 37°C for 12–18 h.
 - c. Store the culture at 4°C for up to 3 months (longer times have not been tested).
3. Prepare S medium and egg buffer by mixing the ingredients listed in the Key Resource Table. These solutions are stable at 20°C–25°C for several months.
 4. Feeding dyes to worms in mini-scale liquid culture (Figure 2).
 - a. Prepare the feeding culture mix.
 - i. Prepare two 1.5 mL tubes, label as JF585 and JF646.
 - ii. Add 1 mL* of HB101 liquid culture in each tube, spin down the bacteria by centrifuge at 1500 x g for 3 min, remove the supernatant.

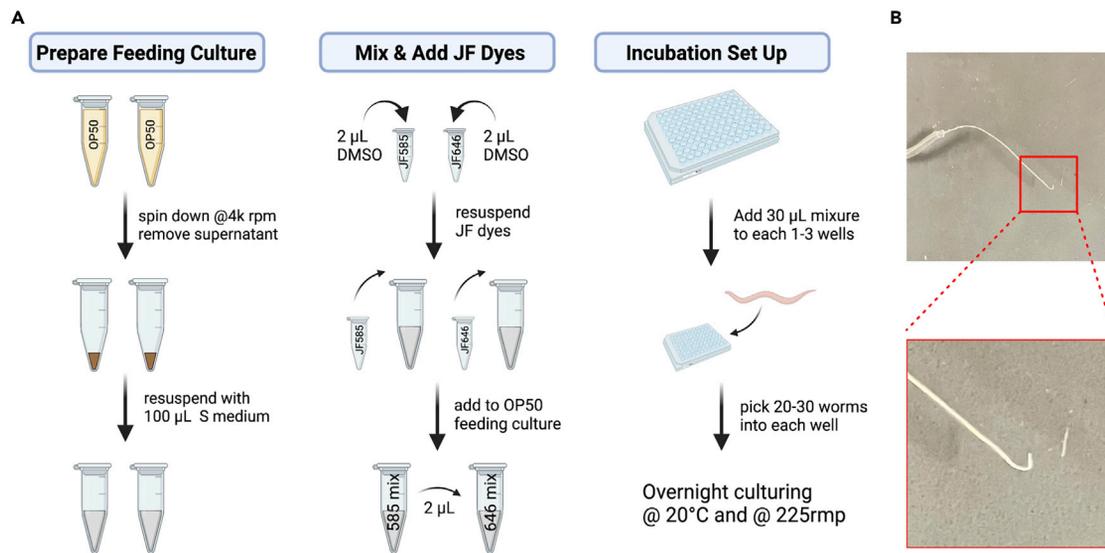


Figure 2. Standard operating procedure for labeling the HaloTag in *C. elegans*, related to 'preparing *C. elegans* for HaloTag imaging'
(A) Cartoon illustration of the procedure for JF dye feeding in *C. elegans*. Created with [BioRender.com](https://www.biorender.com).
(B) A hook shaped worm pick. The tip of the worm pick is zoomed out in the lower panel.

Note: The volume of bacteria to use depends on the density of the culture. After adding the worms in step 4c, you should be able to see them swimming under a stereomicroscope. If the culture is too turbid, reduce the amount of bacteria used.

- iii. Add 100 μL S media to each tube, resuspend by pipetting up and down.
- b. Add JF dyes to the culture mix.
 - i. Dissolve one aliquot each of JF585 and JF646 in 2 μL DMSO to reconstitute a 1 mM stock.

Note: A working aliquot of DMSO can be kept at 20°C–25°C for 2 weeks; keep additional aliquots at –20°C. Discard the room-temperature DMSO aliquot and replace with fresh aliquot from the freezer or from a newly opened DMSO ampule every 2 weeks.

△ CRITICAL: Fresh DMSO is required for *C. elegans* survival. If experiencing a worm survival issue, see [troubleshooting](#) section for more information.

- ii. Add 1.5 μL of dissolved JF585 and JF646 to each of their respective tubes of bacterial suspension to reach a final concentration of 15 μM .
- c. Culture set up and incubation.
 - i. Add 2 μL of JF585-medium-bacteria mix to 100 μL JF646-medium-bacteria mix to obtain a 1:50 ratio* of JF585:JF646 in the final culture.
 - ii. Add 30 μL of the dye mixture to one well of a round-bottom 96-well plate.
 - iii. Pick 20–30 L4 worms into the culture.
 - iv. Add a total of ~200 μL water to adjacent wells or seal with parafilm to prevent drying out.
 - v. Shake at 225 rpm in a 20°C incubator for 16–18 h.

Note: Fluorescent dyes differ in their cell permeability and affinity for HaloTag, and this ratio has been optimized for the combination of JF585 (sparse) and JF646 (abundant). If different dye combinations are to be used, perform a titration experiment to determine the ideal ratio.

Microscope settings

⌚ Timing: 10 min

The protein population labeled chimerically by HaloTag ligand dyes can be imaged using a dual-view imaging system. This step describes how to acquire images, using open-source Micro-Manager software as an example. These instructions should be adaptable to other microscope control software.

In order to achieve the high frame rate required for diffusion analysis while also imaging a second fluorescence channel, the two channels need to be acquired simultaneously (not in an alternating fashion). We achieved this by constructing a dual-view image splitter (see the [‘materials and equipment’](#) section, above and the [supplemental information](#)), but a dual camera setup is also possible. To avoid unnecessary delays, turn on the microscope and configure your acquisition before preparing your sample.

5. Align the 2 channels with TetraSpeck fluorescent beads. This calibration step should be done at the beginning of each day, before starting the experiment.
 - a. Before use, dilute TetraSpeck fluorescent glass beads 1:50 in water.
 - b. Plasma treat a glass coverslip for 3–5 min on max power.

Note: Alternatively, if you do not have access to a plasma cleaner, prepare the coverslip by soaking in 1 M KOH for 20–30 min and then rinsing with plenty of water. This step renders the glass surface hydrophilic so that the TetraSpeck beads will stick to it.

- c. Vortex the TetraSpeck beads vigorously.
- d. Transfer 15–20 μL beads to the prepared coverslip.
- e. Place the coverslip on a cold hot plate, turn on, and leave until the liquid evaporates (20–30 s).
- f. Add 10 μL of egg buffer, PBS or water.
- g. Using a dry pipette tip, add a small amount of glass spacer beads by dipping the pipette tip into the vial of beads and then quickly touching the tip to the liquid droplet.
- h. Cover with a microscope slide and seal with VALAP. The prepared bead slide can be kept on the benchtop at 20°C–25°C for 4–6 weeks.
- i. On the dual-view microscope, view live images of the beads with the red and far-red channels overlaid.

Note: 50 ms exposure with 5 mW 561 nm laser power is usually sufficient to visualize both red and far-red fluorescence from the beads.

- j. Adjust the image splitter so that the 2 channels are perfectly aligned.
 - k. Acquire a reference image of the beads to document the alignment and to use for image registration corrections later, if needed.
6. Set the laser to desired powers.

Note: The laser power will be instrument-dependent and needs to be determined empirically. To determine the appropriate laser power, excite the desired sample with a 561 nm laser, and gradually increase the power until single molecules can be visualized clearly by eye in the live images. If phototoxicity is observed, lower the laser power as needed for embryo survival. The final settings should yield a particle brightness (‘amplitude’) of 12–14 a.u. after particle tracking is performed (see “Particle Tracking”, below). Note that we do not use 638 nm excitation to visualize the far-red dye in these experiments; both red (sparse) and far-red (abundant) dyes are efficiently excited by 561 nm light, and their emission signals are separated by the image splitting optics.

7. Set exposure time to 50 ms.
8. Set imaging frequency to 20 frames / second.
9. Set acquisition length according to the cell stage of interest.

Dissecting, mounting and imaging

⌚ Timing: 40 min (for step 10)

This step provides detailed step-by-step instructions on how to harvest, mount, and image *C. elegans* embryos.

10. Prepare coverslip for imaging.
 - a. Add 11 μ L droplet of polylysine on a microscope slide.
 - b. Place a 22 mm \times 22 mm coverslip on top of the droplet.
 - c. Bake on hot plate at $>100^{\circ}\text{C}$ for ~ 30 s until all liquid evaporates.
11. Dissect worms to obtain embryos.
 - a. Take 1 worm out of the 96-well plate using a hook shaped worm pick (Figure 2B) and check the stage. Worms should ideally be young adults with a single row of 8–10 eggs.
 - b. If the culture is at the desired stage, use a glass pipette to transfer the whole 30 μ L liquid culture onto an OP50 NGM plate, let the liquid dry out and let the worms crawl on plate for a minimum of 30 min before imaging. This step eliminates excess unbound dye and allows clean visualization of the HaloTag.
 - c. Add 30 μ L of egg buffer into a watch glass slide, and transfer 2–3 labeled worms from the NGM plate. Cut each worm in half with a pair of 26-gauge needles to release the embryos.
12. Mounting the embryos.
 - a. Take the coverslip from step 10 and make sure the polylysine-coated side is facing up.
 - b. Add a 10 μ L droplet of egg buffer onto the coverslip.
 - c. Using a dry pipette tip, add a small amount of 23–26 mm glass spacer beads by dipping the pipette tip into the vial of beads and then quickly touching the tip to the liquid droplet.
 - d. Transfer an embryo of the desired stage onto the coverslip using a glass micro needle.
 - e. Gently place a microscope slide on the coverslip.
 - f. Seal with VALAP.
13. Imaging.
 - a. If not already done, set up the microscope as described in the previous section ('microscope settings').
 - b. Locate the embryo under the microscope.
 - i. Under a stereomicroscope, locate embryos at desired stage and draw a cross mark on the slide to mark the position of each embryo.
 - ii. Place the slide on the microscope stage.
 - iii. Move the objective lens to the upper left quadrant of the cross and view live images using brightfield illumination.
 - iv. Turn off any autoscaling function (which adjusts the image display based on the brightness) in the imaging software.
 - v. Move the stage towards the right slowly until the image darkens; this corresponds to the shadow of the cross mark made in step 13.b.i.
 - vi. Move the imaging field down (along y axis) while scanning left & right (along x axis) until the embryo is located.
 - c. Focus.
 - i. While still viewing live brightfield images, focus at the lower cortex of the embryo.
 - ii. Turn off the brightfield channel, switch to the far red (abundant dye) channel and take snapshots while adjusting the focus until fluorescent foci are in focus.

Note: By taking snapshots rather than imaging continuously, photo-bleaching and phototoxicity will be limited.

- d. Adjust the penetration depth until cortical proteins are clearly visualized and the background signal (visible as rapidly-diffusing, out-of-focus signal from the cytoplasm) is as low as possible.

Note: Imaging is performed using near-TIRF rather than true TIRF, due to the thickness of the eggshell. The penetration depth will need to be adjusted for each individual embryo, as the eggshell thickness can vary.

- e. Using the red+far red dualview imaging channel, acquire timelapse images as configured in "Microscope settings," above.

Data analysis: Particle tracking with Utrack

⌚ Timing: 20 min to several hours (for step 14)

This section provides detailed instructions on how to perform particle tracking in the software Utrack (Figure 3A). Accurate particle tracking analysis is required for particle diffusion analysis. Therefore, this section also includes how to examine the tracking results to make sure the tracking was done correctly.

14. Software download and installation.
 - a. Download and install ImageJ.
 - b. Download and install MATLAB.
 - c. Download Utrack from <https://github.com/DanuserLab/u-track>.
 - d. Add Utrack to the MATLAB file folder, where all other scripts are stored.
 - e. Launch MATLAB, locate 'u-track-master' in the file navigator, right click -> 'Add to Path' -> 'Selected Folders and Subfolders'.
 - f. Download the 'DualLabeling_Analysis' package from https://github.com/dickinson-lab/DualLabeling_Analysis. Add to MATLAB file folder and 'add to path'.
15. Prepare the movie for particle tracking.
 - a. Use ImageJ to split the channels in the movie into two different movies.

Note: If images were acquired side-by-side, this simply requires duplicating the image and cropping each channel. If the two channels were overlaid as a hyperstack, use the 'Color -> Split Channels' command.

- b. Use the 'Save -> Image Sequence' command to export each channel to an image sequence in its own separate folder. This will result in each folder having a series of single-plane TIFF files from one channel.
16. Track sparsely labeled particles with Utrack.
 - a. In MATLAB, use command

```
movieSelectorGUI
```

to launch Utrack. A GUI window will appear (Figure S1A).

- b. Movie Selection.
 - i. Click 'New', a new window 'Movie edition' will appear (Figure S1B).
 - ii. In the 'Channels' section, click 'Add Channel', select the folder containing the image sequence of sparse/JF585 channel.

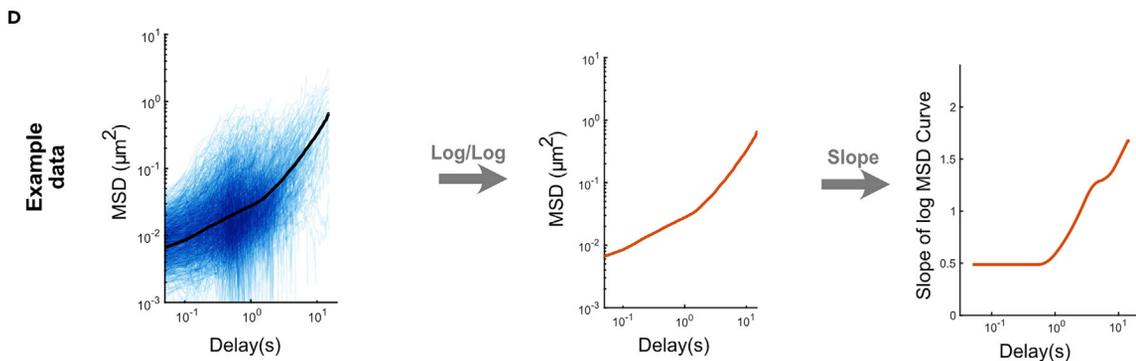
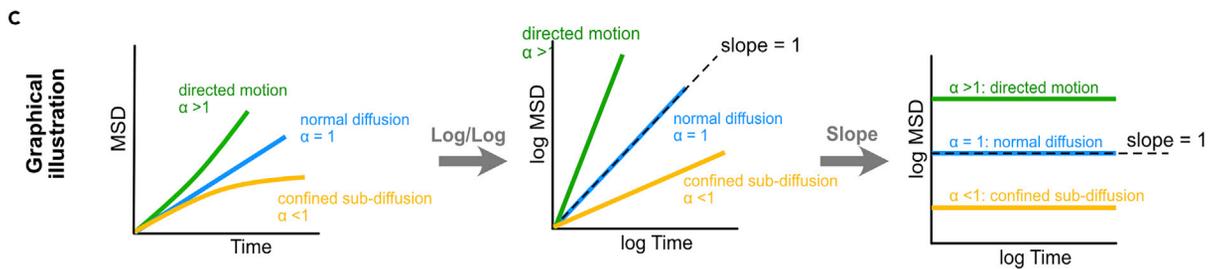
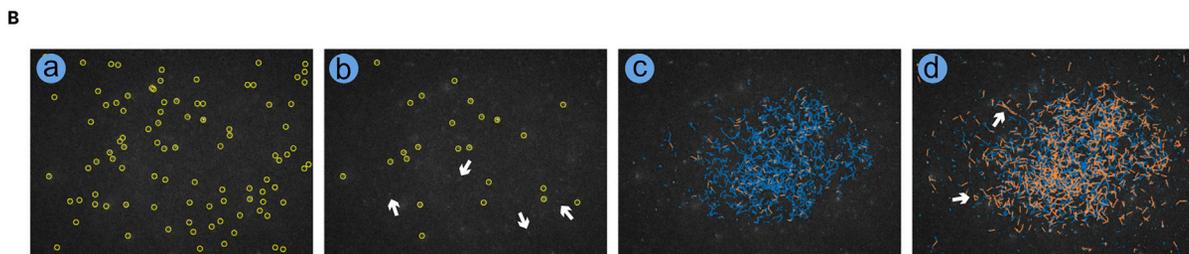
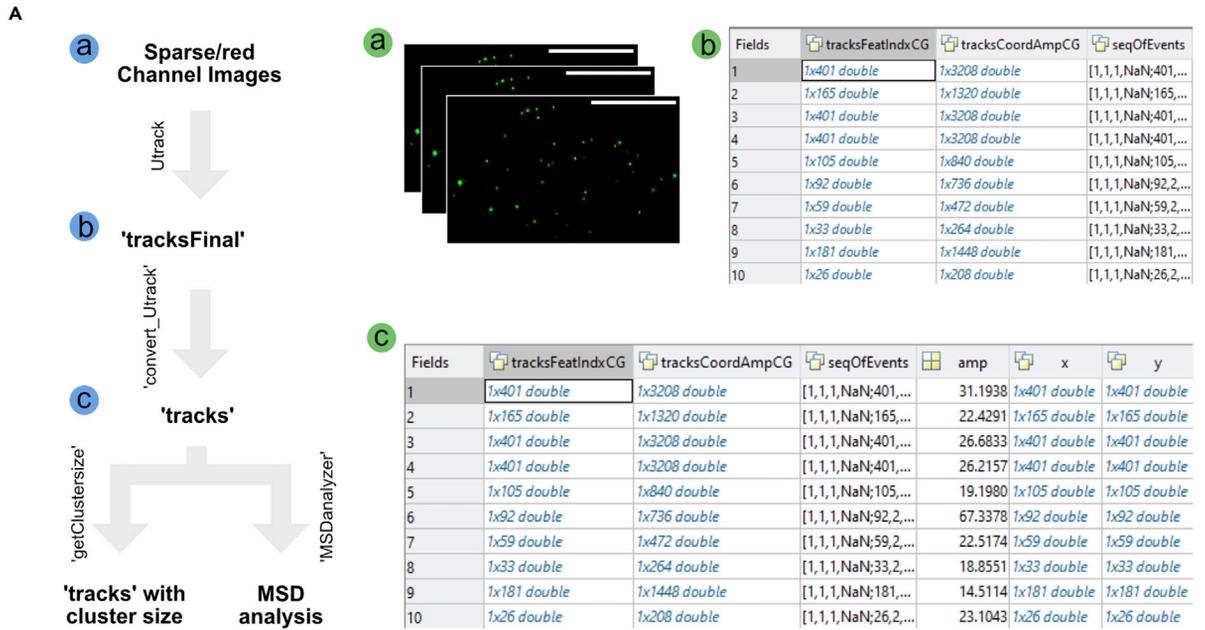


Figure 3. The workflow and interpretation of the MSD analysis, related to 'Data Analysis'

(A) An illustration of the data analysis workflow. The appearance of the example data/results are shown to the right. Scale bar: 10 μm .
 (B) Correct and erroneous results from Utrack particle tracking. (a) Correct detection step result. (b) Erroneous detection step result: dimmer foci are not detected (white arrows). (c) Correct linking step result, orange lines represent the gaps in the tracks that have been connected by the software. (d) Erroneous linking step result: long straight lines and/or sharp turns (white arrows), and extensive gap filling (orange lines).
 (C) Graphical illustration of the interpretation of MSD plots and anomalous parameters. Figure modified from Chang and Dickinson.¹
 (D) An example illustration of the MSD analysis. Engineered Halo::PAR-3 clusters were labeled with JF585, data were pooled from 3 embryos. Left: the MSD curves. Blue lines are the MSD curve of each cluster tracked. Black line represents the averaged MSD curve. Middle: the averaged MSD curve. Right: the first derivative, or the slope, of the averaged MSD plot. Figure modified from Chang and Dickinson.¹

- iii. Click 'Advanced Channel Settings', enter the requested information.
- iv. Enter the requested information in the 'Movie Information' section.
- v. Select your 'Output Path' where data will be saved.
- c. Particle tracking.
 - i. In the 'Movie selection' window, click 'Continue'. A new window will appear. Select '[2D] Single particles' and click 'OK' (Figure S1C).
 - ii. A new window 'Control Panel - U - Track' will appear (Figure S1D).
 - iii. Check 'Step 1: Detection' and click on 'Settings'.
 A new window will appear, select 'point source detection' and click 'Settings...' (Figure S1E).
 A new window will appear (Figure S1F), in the 'Parameters for' section, set 'Alpha' to 0.03*. Click 'Apply'.

Note: Alpha is the key parameter that sets the sensitivity threshold for particle detection (for a more detailed description, see.¹¹ In our experience, an Alpha of 0.05 resulted in detection of background noise, therefore we reduced Alpha to 0.03 for cleaner detection. The most appropriate alpha should be determined for different noise levels, fluorescence intensity and/or particle density, by analyzing a few samples and visually inspecting the particle detection.

- All other settings are left as default.
- iv. Check 'Step 2: Tracking' and click on 'Settings'.
 A new window will appear (Figure S1G).
 In the 'Parameters' section, set 'Maximum Gap to Close' to 3, and 'Minimum Length of Track Segments from First Step' to 2. Click on 'Apply'.

△ CRITICAL: The maximum GAP should be adjusted according to particle density. A denser population would require a lower 'max GAP to close' to avoid false linking between different particles. See 'checking tracking results' for more details.

- v. Click 'Run'.
17. Checking tracking results.
 - a. Click 'Results' for step 1.
 - i. 4 windows will appear.
 - ii. In the 'Viewer' window (Figure S1H), check and uncheck 'point sources' a few times while looking at the 'Movie' window to make sure the particles are detected correctly (Figure 3Ba).

△ CRITICAL: If background noise is falsely detected as particles, decrease the value of Alpha in the particle detection settings. If some real particles are missed, increase the value of Alpha (Figure 3Bb).

- b. Click 'Results' for step 2.
 - i. 4 windows will appear.

- ii. In the 'Viewer' window (Figure S1H), check 'Static tracks' and examine the track appearance in the 'Movie' window.

△ **CRITICAL:** An appropriate 'Maximum Gap to Close' will result in relatively smooth tracks (Figure 3Bc), with the length of each track segment being roughly the same. Outstanding long straight lines or sharp angles are indications of false linking between different particles (Figure 3Bd), the 'Maximum Gap to Close' should be reduced if this is observed.

- c. Extracting track information from Utrack:
 - i. In your output data folder, locate the results file:
Output path -> TrackingPackage -> tracks -> Channel_1_tracking_result.mat.
 - ii. Drag this file into MATLAB workspace. A cell array named 'tracksFinal' will be imported (Figures 3Aa and 3Ab).
 - iii. Use command.

```
tracks = convert_Utrack(tracksFinal);
```

to organize 'tracksFinal' into a more readable format (Figures 3Ab and 3Ac).

Data analysis: Particle diffusion analysis

The MSD plots provide information about particle diffusion behavior via the anomalous parameter α . An $\alpha < 1$ suggests confined or sub-diffusive motion, where the movement of particles are restricted; an $\alpha = 1$ indicates normal Brownian diffusion; and an $\alpha > 1$ represents a directed motion, where particles move in a directed manner due to an external force (Figure 3C). These steps shows how to determine the diffusion coefficient and anomalous parameter from the MSD analysis.

Perform MSD analysis and anomalous parameter visualization:

18. Software download and installation.
 - a. Download 'MSDAnalyzer'¹² from <https://tinevez.github.io/msdanalyzer/>, add to MATLAB file folder and 'add to path'.
 - b. Download and install 'SLM toolbox' from <https://www.mathworks.com/matlabcentral/fileexchange/24443-slm-shape-language-modeling>.
19. Launch MATLAB, if not done so already, re-organize Utrack result by using the command.

```
tracks = convert_Utrack(tracksFinal)
```

20. Perform MSD analysis.
 - a. Our dual-labeling analysis package includes a script to generate normal and log-log MSD plots. After preparing the data and generating particle tracks as described above, use the command.

```
msd = runMSD(tracks);
```

to generate linear MSD plots, and transform plots to log-log scale using:

```
set(gca, 'YScale', 'log');  
set(gca, 'XScale', 'log');
```

Note: Before running this command, we usually remove short tracks (<0.5 s) to reduce the background noise and the signal from cytoplasmic PAR-3. This can be accomplished by using the script 'PickLongTracks' included in the 'DualLabeling_Analysis' package:

```
Tracks = PickLongTracks(10, tracks);
```

Where the first argument indicates the minimum number of frames (10) that a track should have in order to be included in the analysis.

b. Inspect the plots to identify the type of motion a particle is experiencing (Figure 3C).

Note: A straight line on the linear-scale plot indicates that the particle is undergoing normal diffusion, while concave-up or concave-down curves indicate directed or confined motion, respectively (Figure 3C, left). Straight lines on the log-log plot indicate time-invariant motion; that is, particles move in the same way on short and on long timescales, with a slope indicating the type of motion (Figure 3C, center). MSD curves that are not straight lines on a log-log plot indicate that a particle is undergoing different kinds of motion on different timescales. For example, engineered hexameric PAR-3 exhibits confined diffusion on short timescales but directed motion on longer timescales (Figure 3D).

21. Estimate the diffusion coefficient.

a. Determine the diffusion coefficient of a membrane-bound species by fitting the linear-scale MSD curves to the 2D diffusion equation:

$$\text{MSD} = 4Dt^\alpha.$$

where D is the diffusion coefficient, t is the time step and α is the anomalous parameter. Fitting can be done using the curve fitting toolbox within MATLAB or a third-party tools like ezyfit (<http://www.fast.u-psud.fr/ezyfit/>).

△ CRITICAL: For cases where the log-log MSD plot shows different kinds of motion on different timescales, the data will be poorly fit by the diffusion equation, since this equation assumes that the diffusion of a particle is time-invariant. In such a situation, estimate the diffusion coefficient by fitting only the first few time steps of the MSD curve when the motion should be close to normal diffusion (see Figure S3A of Chang and Dickinson, 2022 for an example).

22. Estimate the anomalous parameter.

For cases where the diffusion of a particle is time-invariant, the anomalous parameter α will be obtained from the fit in step 21a and is equivalent to the slope of the log-log MSD curve. For situations where particles undergo different types of motion on different timescales, the anomalous parameter at different time lags can be visualized by estimating the derivative of the log-log MSD plot.

a. Check that the 'msd' variable, from step 20a above, is still defined in the MATLAB workspace. If not, run step 20a again.
b. Extract the log mean MSD curve using the command.

```
logMeanMSD = log10(msd.getMeanMSD);
```

c. Visualize different values of the anomalous parameter (indicating different kinds of motion) on different timescales.

i. Estimate the slope of the log-log MSD curve by taking the first derivative of a spline fit to the data. This can be done in MATLAB using the SLM toolbox. Start by running the following command:

```
s1m = slmengine(logMeanMSD(:, 1), logMeanMSD(:, 2), ...  
'plot', 'on');
```

Note: This will plot the data and a fit.

- ii. Plot the first derivative (which gives the value of the anomalous parameter) by choosing “First Derivative” from the “Plot” menu of the figure window.
- iii. To extract the first derivative to a vector for further analysis, plotting or manipulation, use:

```
alpha = slmeval(logMeanMSD(:, 1), slm, 1);
```

where the final argument ('1') indicates to evaluate the first derivative.

- d. Tips for getting a good SLM fit that accurately represents the data:

- i. Depending on the data, you may want to limit the fit to only shorter timescales where the data are not too noisy. This can be done by constraining the range of values passed to `slmengine`. For example, to plot and fit only the first 100 time delays, use:

```
slm = slmengine(logMeanMSD(1:100, 1), logMeanMSD(1:100, 2), ... 'plot', 'on');
```

- ii. The SLM toolbox has many additional options that can be used to constrain properties of the fit. These are specified by passing additional arguments as key/value pairs to the `slmengine` command. An option that we use frequently is `'increasing'`, `'on'`, which constrains the fit to be an increasing function. This constraint is physically realistic, since diffusing molecules always have an increasing MSD with increasing time step. Other options are described in the SLM toolbox documentation.

Data analysis: Calculating cluster intensity for particles labeled in the abundant channel

PAR-3 is an oligomeric scaffold protein, in which the segregation behavior varies for different cluster sizes.¹ Therefore, observing the diffusion pattern with cluster size information was critical for our study. Many other membrane proteins also oligomerize,¹⁶ and so measuring the intensity of clusters that contain a tracked tracer molecule is of general value. The following are used to extract the cluster intensity from the abundant channel.

- 23. If you have not done so already, prepare the **abundant/far-red** channel as an image sequence using ImageJ as explained above.

Note: You also need to have completed tracking for the sparse/red channel prior to this step.

- 24. Enter the command.

```
tracks = getClustersize_10frames(tracks);
```

Note: This script eliminates any tracks that are less than 10 frames long and uses the first 10 frames to calculate the cluster intensity. We usually remove short tracks (<0.5 s) to reduce the background noise and the signal from cytoplasmic PAR-3. If analysis of short tracks or estimating from the 1st frame only is needed, use:

```
tracks = getClustersize(tracks);
```

instead.

- 25. A window will appear. Select the folder containing the **abundant/far-red** channel image sequence.
- 26. The result will be shown as an additional column in 'tracks', named [size_far-red].

Data analysis: Estimating the number of molecules per cluster with a calibration experiment

The intensity of single fluorophores varies systematically from embryo to embryo due to variations in mounting, focus and eggshell thickness. However, the intensities of the red and far-red channels vary

in tandem, and their *ratio* of intensities is constant. Therefore, we performed calibration experiments in which both JF585 and JF646 were diluted to single-molecule levels. With this dataset, we are able to use the red dye, which present as single molecules in both calibration and tracking experiments, as an internal standard to correct the embryo-to-embryo variations in far-red dye intensity. This section allowed accurate estimation of the number of far-red molecules per cluster based on the far-red particle intensity.

27. Worm feeding and movie acquisition:
 - a. Prepare 3 microcentrifuge tubes as described above.
 - i. Tube 1: JF585-bacteria-S medium mix.
 - ii. Tube 2: JF646-bacteria-S medium mix.
 - iii. Tube 3: bacteria resuspended in S medium.
 - b. Transfer 2 μ L of tube 1 mix and 1 μ L of tube 2 mix into tube 3*.

Note: If other dyes or other model organisms are used, these dilutions should be re-determined by titration.

- c. Feed worm as described in the ‘[preparing C. elegans for HaloTag imaging](#)’ section above.
 - d. Acquire movies as described above in the ‘[dissecting, mounting and imaging](#)’ section. Repeat for 3 embryos.
28. Extract intensity information from the calibration experiment for both channels.
 - a. Save the first frame of each channel as a single-plane TIFF file.
 - b. Feed the first frame of each into Utrack as described above, run the ‘detection’ step only, and reorganize the results using the “convert_Utrack” script.
 - c. The cluster intensity information can be found in the [amp] column in converted result ‘tracks’.
 - d. Copy all [amp] intensity datasets for all repeats in both channels into an Excel spreadsheet.
29. Calculate the cluster size of far-red clusters.
 - a. With the double-dilution calibration experiment, determine the ratio of average single far-red dye intensity to the average single red dye intensity. Because the intensities of single particles in TIRF images are log-normally distributed,¹⁷ we perform this calibration in log space.
 - i. Convert each intensity dataset to log scale. The fluorophore intensity should appear as a normal distribution. Each step of this calibration can be visualized by plotting individual values in Graphpad Prism or another graphing software ([Figures S2A and S2B](#)).
 - ii. In log space, calculate the mean (log) intensity for each channel and each embryo ([Figure S2B](#)).
 - iii. In log space, normalize each red channel mean intensity to 1 by performing the following calculation for each data point:
Normalized red intensity = red intensity/red channel average (from the same movie).
 - iv. In log space, normalize the corresponding far-red channel average from the same embryo accordingly by using:
normalized farred intensity = farred intensity/red channel average (from the same movie) ([Figure S2C](#)).
 - v. In log space, pool all replicate datasets together, and calculate the ratio of the average single far-red dye intensity to the average single red dye intensity ([Figure S2D](#)).
 - b. Calibrate the sparse/abundant dual-labeling data (steps i–ii should already been done in the previous steps).
 - i. If not done so already, extract the intensity information of red dyes in each sparse/abundant dual-labeling experiment. This can be done by converting the Utrack tracking result using ‘convert_Utrack’ script, and [amp] can be found in the converted cell array ([Figure S2E](#)).
 - ii. If not done so already, for each corresponding farred foci, determine the farred channel intensity with the ‘getClustersize’ script we developed ([Figure S2E](#)).
 - iii. Convert each intensity dataset to log scale, and calculate the mean (log) intensity for each dataset ([Figure S2F](#)).

- iv. In log space, normalize all red channel averages to 1 by using:
Normalized red intensity = red intensity/red channel average (from the same movie) (Figure S2G).
- v. In log space, correct the corresponding far-red cluster intensity by using:
Calibrated farred cluster intensity = $1 * \log \text{ratio}(\text{mean single far-red: mean single red}) * \text{far-red intensity}$.
- vi. Convert calibrated far-red cluster intensities back to normal scale by using:
Calibrated absolute farred cluster intensity = $e ^ {(\text{calibrated log farred cluster intensity})}$.
- c. Calculate the cluster size by using:
farred cluster size = calibrated absolute farred cluster intensity / e .

EXPECTED OUTCOMES

Firstly, this protocol introduces a non-invasive sparse labeling tool. In previous studies, sparse labeling of protein has been achieved by either microinjection¹⁸ or low level transgenic expression followed by partial photo-bleaching.¹⁹ Dual labeling allows one to achieve the same result noninvasively with an easier procedure. In addition, the labeling in both channels enables easier tracking without losing the visualization of the bulk protein population. Secondly, we also provide guidance for MSD analysis, which is used to calculate the anomalous parameter from and thus determine the mode of particle diffusion. Lastly, estimating cluster size accurately from fluorescent intensity has been challenging. In this study, we developed a control calibration experiment to account for variations across multiple experiment repeats, so that an accurate estimation of cluster size can be expected.

QUANTIFICATION AND STATISTICAL ANALYSIS

All software and data analysis procedures are explained in detail in the text above.

LIMITATIONS

The dual-labeling technique described in this protocol is developed for and optimized in *C. elegans*. Although dual-labeling was not tested in other systems, this technique can be potentially adapted to any organism in which tagging by HaloTag has been established. There are a few likely modifications needed: first, the concentration of dyes in the culture mix should be re-determined due to the differences in cell permeability between different model systems; second, the culture conditions should be adapted for the organism, including but not limited to: temperature, culture medium, oxygen level; third, the incubation time should be adjusted accordingly.

We exclusively used TIRF imaging in our study, because we were exploring PAR-3 cluster movement on the cell cortex. For tracking in internal organelles such as the nucleus, epifluorescence imaging can be used,²⁰ although tracking may be improved by using a light sheet microscope. We have not tested the dual-labeling experiment with microscopy systems other than TIRF, but in principle the dual-labeling method can be compatible with any imaging system with minor modifications.

TROUBLESHOOTING

Problem 1

Worms died during overnight liquid culture incubation, related to “[preparing *C. elegans* for HaloTag imaging](#)” (step 4).

Potential solution

It is essential to use fresh, high-quality DMSO, sold in ampules. Old or low-purity DMSO contains contaminants produced by oxidation, which are toxic to worms.

Problem 2

Sparse labels are too sparse or too dense, related to “[dissecting, mounting and imaging](#)” (step 13).

Potential solution

In our hands, the dye labeling efficiency gradually decreases over time. If the sparse labels are more scarce than expected, adjust the ratio of the two dyes. A simple titration experiment can quickly establish the correct ratio for a given pair of dyes.

Problem 3

Unable to see any labeled particles in the sparse channel, related to “[dissecting, mounting and imaging](#)” (step 13).

Potential solution

- Adjust the TIRF penetration depth: Eggshell thickness can vary from day to day and embryo to embryo, which affects the ability to see single molecules at the cell cortex. If a suitable penetration depth cannot be found for a given sample, try a new embryo.
- Gradually increase the laser power until sparse labels become visible.

Problem 4

Too much autofluorescent dirt on the coverslip, related to “[dissecting, mounting and imaging](#)” (step 13).

Potential solution

- Clean the glass using a UV/Ozone cleaner or by oxygen plasma treatment.
- If you do not have access to a UV/Ozone or plasma cleaner, incubate with 1 M KOH for 30 min, then rinse with distilled water.

Problem 5

Particles are not detected in Utrack, related to “[Data Analysis: Particle Tracking with Utrack](#)” (steps 14–17).

Potential solution

- Make sure ‘point source’ mode is used for particle detection.
- Gradually increase the alpha value during the detection step.
- Acquire data using a higher laser power to ensure sufficient signal-to-noise.

RESOURCE AVAILABILITY

Lead contact

Requests for resources and further information should be directed to, and will be fulfilled by, the lead contact, Daniel J. Dickinson (daniel.dickinson@austin.utexas.edu).

Materials availability

C. elegans strains generated in this study are available from the [lead contact](#) upon request.

Data and code availability

Original microscopy data are available from the [lead contact](#) upon reasonable request.

All original code generated in this study is available via Github (see the [key resources table](#) for links).

Any additional information required to reanalyze the data reported in this work paper is available from the [lead contact](#) upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101857>.

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AUTHOR CONTRIBUTIONS

Y.C. and D.J.D. designed the experiments. D.J.D. designed and built the TIRF microscope. Y.C. performed the experiments. Y.C. and D.J.D. analyzed the data. D.J.D. supervised the project and secured funding. Y.C. and D.J.D. co-wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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