

Real-time analysis of nanoscale dynamics in membrane protein insertion via single-molecule imaging

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Abstract Membrane proteins often need to be inserted into or attached to the cell membrane to perform their functions. Understanding their transmembrane topology and conformational dynamics during insertion is crucial for elucidating their roles. However, it remains challenging to monitor nanoscale changes in the insertion depth of individual proteins in membranes. Here, we introduce two single-molecule imaging methods, SIFA and LipoFRET, designed for *in vitro* observation of the nanoscale architecture of membrane proteins within membranes. These methods have demonstrated their efficacy in studying biomolecules interacting with bio-membranes with sub-nanometer precision.

Keywords Membrane protein, Insert, Real time, Single molecule, Nanoscale

INTRODUCTION

Membrane proteins play essential roles in various cellular processes, including ligand-receptor recognition and activation, intercellular communication, and ion transport (Almen *et al.* 2009; Bretscher and Raff 1975; Hegde and Keenan 2022). These proteins often undergo subtle conformational changes to perform their functions. Therefore, it is crucial to dissect the nanoscale architecture and dynamics of membrane proteins when they integrate into the membrane. Over the years, many structural techniques, for instance, X-ray crystallography (Andersson *et al.* 2019; Lieberman *et al.* 2013) and cryo-EM (Cheng 2018; Yao *et al.* 2020), have offered valuable insights into the structures of membrane-interacting macromolecules. However, capturing the real-time conformational changes of membrane proteins remains to be a significant challenge.

Herein, we introduce two single-molecule methods for directly observing the nanoscale dynamics of

membrane proteins interacting with bio-membranes, surface-induced fluorescence attenuation (SIFA) (Li *et al.* 2016; Ma *et al.* 2019b) and FRET with quenchers in liposomes (LipoFRET) (Ma *et al.* 2019a). SIFA is based on the fluorescence energy transfer between a fluorophore and monolayer graphene oxide (GO) (Hong *et al.* 2012), so it is a sensitive point-to-plane distance indicator (Figs. 1A and 1B). For two single-point coupled dipoles, Like FRET (Förster Resonance Energy Transfer), the emission rate follows a d^{-6} dependence. Replacing one dipole with a line of dipoles results in a d^{-5} scaling upon integration. Similarly, for a two-dimensional array of dipoles on a graphene oxide sheet, a d^{-4} scaling is obtained (Gaudreau *et al.* 2013). Therefore, the distance d between fluorophores and monolayer GO, can be calculated according to the d^{-4} scaling formula:

$$d = d_0 \left(\frac{F/F_0}{1 - F/F_0} \right)^{\frac{1}{4}},$$

where F and F_0 are the fluorescence of fluorophores in

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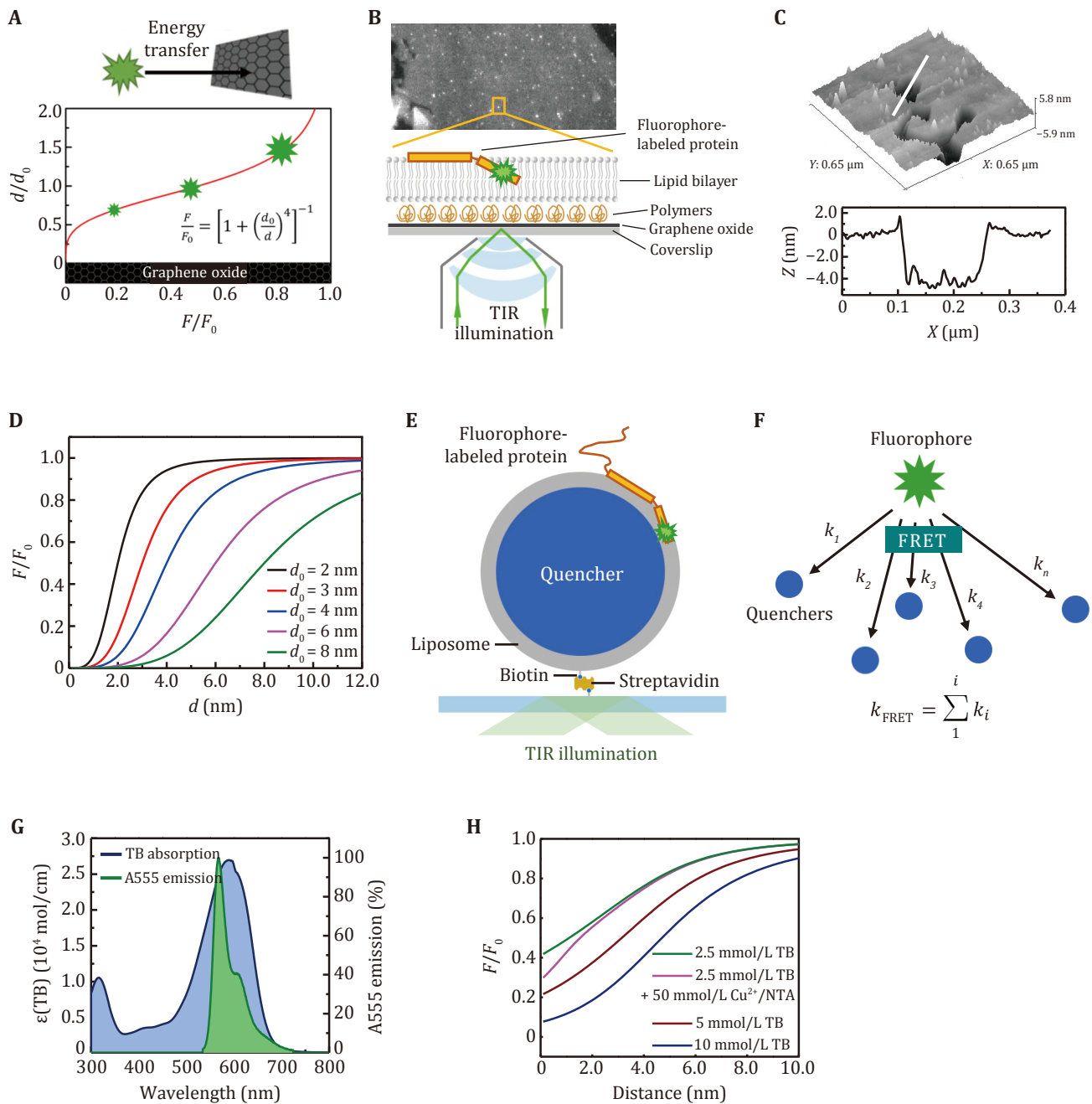


Fig. 1 The principle of SIFA and LipoFRET. **A** The fluorescence of a fluorophore changes rapidly with its distance to a graphene oxide layer. **B** The experimental setup of SIFA on TIRF microscopy. **C** The morphology (upper panel) and the profile of the thickness (lower panel) of a lipid bilayer composed of DOPC/DOPA. **D** The dependence of the relative intensity on distance in SIFA with different critical distances d_0 (Li *et al.* 2016). **E** The scheme of LipoFRET. **F** FRET from one donor to multiple acceptors. **G** The absorption spectra of TB (blue) and the emission spectra of the donor Alexa Fluor 555-MAL (green). **H** The dependence of the relative intensity on the distance to the inner surface of the liposome membrane in LipoFRET (Ma *et al.* 2021)

the presence and absence of GO, respectively, and d_0 is the characteristic distance at which energy transfer efficiency reaches 0.5. Therefore, SIFA is a powerful tool for measuring the orientation and depth of insertion of

membrane proteins in supported lipid bilayers (SLBs) produced by direct vesicle fusion on top of a GO layer modified with PEG (Figs. 1C and 1D). LipoFRET was developed based on liposomes (Fig. 1E), a bio-mimic

system with surface curvature and unrestricted membrane fluidity (Ma *et al.* 2019a). It is based on the principle of the FRET from one donor to multiple acceptors (quenchers) encapsulated in unilamellar liposomes (Figs. 1F and 1G). Unlike SIFA, LipoFRET does not have a clear calculation formula for distance versus intensity. This is because the distance between quenchers is comparable to d_0 in LipoFRET experiments, the quencher solution cannot be treated as a continuous medium. Instead, Monte Carlo simulations can be utilized to calculate the relative intensity-distance relationships. Fluorophores attached to membrane proteins at different penetration depths (or distances) in the liposome lipid bilayer show different intensities based on their energy transfer efficiency (Fig. 1H). SIFA and LipoFRET can be applied, progressively, to investigate membrane proteins in solid-supported lipid bilayer systems and liposome systems. The techniques utilize the energy transfer between fluorophores and quenchers, and are able to track the axial movement of a single fluorophore-labeled protein in membrane.

MATERIALS

Biological materials

- *E. coli* BL21 strain

Reagents

- 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG; Avanti Polar Lipids)
- 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC; Avanti Polar Lipids)
- 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE; Avanti Polar Lipids)
- 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4',5'-biphosphate) (PI(4,5)P2; Avanti Polar Lipids)
- 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS; Avanti Polar Lipids)
- Cardiolipin (CL; Avanti Polar Lipids)
- 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC; Avanti Polar Lipids)
- 1,2-dioleoyl-sn-glycero-3-phosphate (sodium salt) (DOPA, Avanti Polar Lipids)
- 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (Biotinyl Cap PE; Avanti Polar Lipids)
- Trypan Blue (Merck) or Blue dextran, MW 10000 (Merck)
- Chloroform (Fisher Chemical)
- Methanol (Fisher Chemical)

- Acetic acid (Merck)
- 3-(Triethoxysilyl)propylamine (Merck)
- mPEG-SVA, MW 5000 (Laysan Bio)
- Biotin-PEG-SVA, MW 5000 (Laysan Bio)
- Alexa Fluor 555 C₂ maleimide (Thermo Fisher)
- PD-10 Desalting Column (Cytiva)
- Superdex™ 75 Increase 10/300 GL (Cytiva)
- Isopropyl β -D-1-thiogalactopyranoside (IPTG; Sigma Aldrich)

Software and Algorithms

- MATLAB R2021b (MathWorks)
- Origin 2021 (OriginLab)
- ImageJ 1.52v (National Institutes of Health, USA)

Equipment

- Ultrasonic cleaner
- Total internal reflection fluorescent microscope (TIRFM; Nikon Ti2)
- EMCCD IX897 (Andor)
- Oil immersion objective (100x, N.A. 1.49; Nikon)
- QBIS LS 532 nm (CW Solid State Lasers; Coherent)
- Filter setup (49907 ET; Chroma)
- Langmuir-Blodgett (KSV NIMA)
- Extruder kits (Avanti Polar Lipids)

PROCEDURE

Procedure of SIFA imaging: detecting dynamics of membrane protein on the supported lipid bilayers

Protein expression and purification [TIMING ~3 d]

(A) Clone the cDNA that encodes the N-terminal fragments MLKL₁₋₁₅₄ fused with a GST tag into the pGEX-4T-2 vector.

[CAUTION!] The Alexa Fluor maleimides dyes specifically couple to the thiol groups on Cysteine residue of target proteins. Therefore, the site of the target protein for fluorescent labeling must have a single Cysteine residue. To ensure specific labeling, all other Cysteine residues should be mutated. In this manuscript, S55C, S92C and S125C of MLKL₁₋₁₅₄ are the sites that were specifically labelled.

(B) Express the recombinant MLKL in *E. coli* BL21 cells. Grow the cells in Super Broth with 100 μ g/mL ampicillin and shake until the OD₆₀₀ reaches 0.8 at 37 °C.

- (C) Protein expression was induced with 0.5 mmol/L IPTG at 18 °C, shaken for a further 16 h.
- (D) Harvest cells by centrifugation and lyse them by sonication in a purification buffer containing 10% glycerol.
[RECIPE] Purification buffer: 25 mmol/L HEPES, 150 mmol/L NaCl, 0.5 mmol/L TCEP, PH 7.4.
- (E) Purify the proteins using glutathione-sepharose beads and then cleave the GST tag through thrombin.
- (F) Purify the proteins using size exclusion chromatography with a Superdex-75 10/300GL size exclusion column.

Protein labelling [TIMING ~15 h]

- (A) Mix 10 µmol/L MLKL₁₋₁₅₄ with 100 µmol/L Alexa Fluor 555-MAL (Alexa Fluor 555 C2 maleimide) dyes at a molar ratio of 1:10 for site-specific fluorescence labeling. The mixture was reacted under 4 °C for 12 h in the dark.
[CAUTION!] Sonicate the dissolved dye in an ultrasonic bath for 15 min to completely dissociate dye oligomers before adding it to the target protein, which can avoid cross-linking of dyes (Zosel *et al.* 2022).
- (B) Remove the extra unreacted Alexa Fluor 555-MAL dyes through PD-10 desalting column and the labelled proteins were flash frozen in liquid nitrogen and stored at -80 °C until required.

Preparation of LUVs solutions [TIMING ~10 h]

- (A) Mix the phospholipids POPC, POPE, DOPS, PI(4,5)P2, CL, and POPG in chloroform at a molar ratio of 35/20/20/10/10/5.
[CAUTION!] Chloroform is highly volatile and toxic. Chloroform can dissolve plastics and phospholipids should be mixed in a sealed glass bottle.
[TIP] Note that the composition of the lipids depends on the protein for study.
- (B) Dry the chloroform with nitrogen and place the lipids mixture in vacuum pumps overnight to remove residual chloroform.
- (C) Suspend the dried lipid films in imaging buffer to achieve a final lipid mixture concentration of 1–2 mg/mL. Freeze the liquid in liquid nitrogen, then thaw them in a 37 °C water bath. Undergo ten freezing thawing cycles.
[RECIPE] Imaging buffer: 25 mmol/L HEPES, 150 mmol/L NaCl, PH 7.4.
- (D) The lipids liquid was forced to go through a polycarbonate filter with 100 nm pore size using a mini-extruder kit for 21 times in order to form large unilamellar vesicles (LUVs) solutions.

Preparation of GO-PEG-supported lipid bilayers

[TIMING ~20 h]

- (A) Produce ultra-large graphene oxide (GO) flakes by using the modified Hummer's method and collected by centrifugation (Zhao *et al.* 2010).
- (B) Clean the coverslips by ultrasonication in acetone and methanol for 30 min each, then treat them with piranha solution at 95 °C for 2 h. After each cleaning step, rinse the coverslips with de-ionized water and dry them out with nitrogen gas.
[RECIPE] Piranha solution: H₂SO₄ (98%) and H₂O₂ (30%) at 7:3 volume ratio.
[CAUTION!] Piranha solution is highly corrosive, must slowly add H₂O₂ to concentrated H₂SO₄ while stirring slowly with a glass rod to prevent excessive temperatures and solution splash.
- (C) Use the Langmuir-Blodgett to deposit GO monolayer on the surface of the cleaned coverslips and then heat the coverslips under vacuum at 85 °C for 2 h to remove residual solution.
- (D) Use double-sided tape to glue the GO-covered coverslip and clean slide into a flow chamber and also glue undeposited GO coverslips and clean slide into chambers for measuring the intrinsic intensity of the fluorophore F_0 .
- (E) Produce a hydrophilic monolayer on the GO-covered coverslips using the cross-linked compound of 1-Aminopyrene and hydroxyl-PEG-NHS ester (AP-PEG). Incubate the coverslips with AP-PEG for 30 min, then wash away the unreacted AP-PEG with an imaging buffer.
- (F) Inject the LUVs solution slowly into the GO-PEG chamber and incubate at 37 °C for 12 h to form GO-PEG-supported lipid bilayers. Wash away extra vesicles with an imaging buffer. Repeat these steps for the undeposited GO chamber.
[CAUTION!] The chamber should be used within two days after the bilayer preparation.

Single molecule SIFA imaging and analysis

[TIMING ~12 h]

- (A) Mount the GO-PEG chamber onto the stage of the TIRFM.
- (B) Seek Large and non-overlapping monolayer GO in the field of view.
[TIP] GO has obvious autofluorescence and the intensity of autofluorescence can be used to determine whether it is a monolayer GO.
- (C) Inject ~1 nmol/L labeled MLKL into the imaging GO-PEG chamber and record the fluorescence signal with an EMCCD camera at a frame interval of 30 ms.

Output the data as 16-bit TIFF movies for further analysis.

[TIP] Alexa Fluor 555-MAL fluorophores were excited with a 532-nm laser, as were alternative fluorophores like Cy3, ATTO 555, and TAMRA. The filter setup 49907 ET is also suitable for these fluorophores. The frame interval usually varies from 30 to 100 ms as required.

- (D) Repeat the same imaging steps with an undeposited GO chamber for the measurement of the intrinsic intensity of the fluorophore.
- (E) Extract the trajectories of fluorophores using a plugin called Particle Tracker in ImageJ and obtain the fluorescence intensity from tiff film based on the trajectory coordinates using a customized MATLAB script. Intrinsic intensity F_0 was determined through control measurement and relative intensity ratio F/F_0 indicates axial movement of target protein upon lipid bilayers (Fig. 2A).

[TIP] To use the Particle Tracker plugin, open your movie and select Particle Tracker from Plugins -> Particle Detector & Tracker. Key parameters include Radius (particle size in pixels, 3 in this manuscript), Cutoff (score threshold, default 0), Percentile (intensity cutoff for candidate particles, usually 0.2–2), Displacement (max pixel movement, default 2) and Link Range (frames for matching, default 10). Adjust and preview as needed.

Procedure of LipoFRET imaging: detecting dynamics of membrane protein on the liposome

Preparation of biotin-carrying liposomes [TIMING ~12 h]

- (A) Mix DOPC, DOPA and Biotinyl Cap PE at 7:3:0.002 molar ratio and dried into lipid film as protocol described.
- (B) Resuspend the lipid film with imaging buffer containing 5 mmol/L quencher (Trypan Blue or Blue dextran). Also, resuspend the lipid film with imaging buffer without a quencher for the control experiment.
- (C) Have the lipid mixture solution undergo ten freezing thawing cycles.
- (D) Extrude the lipid mixture as described in protocol. For the liposomes containing quenchers, remove the quencher outside the liposomes with PD-10 desalting columns.

[TIP] In previous studies, liposome size was shown not to affect the intensity-distance curve of LipoFRET (Ma *et al.* 2019a). Additionally, the extrusion procedure is capable of producing uniform liposomes, which enables us to adjust the size of the

prepared liposomes. This adjustment ensures that the curvature of the prepared liposome is suitable for the target membrane proteins to perform their functions.

Coverslip modification [TIMING ~7.5 h]

- (A) Clean coverslips as described in protocol till the completion of the piranha solution procedure.
- (B) Put the coverslips in the vacuum dryer and let them cool down to room temperature.
- (C) Mix the APTES at the volume ratio of 1% in a mixture of methanol and acetic acid (95:5, volume ratio). Incubate coverslips with liquid mixture for 10 min.
- (D) Discard the liquid, rinse the coverslips with deionized water, and dry them out with nitrogen gas.
- (E) Dissolve mPEG-SVA and Biotin-PEG-SVA in the PEG buffer. For 1 mg PEG powder, it would need 10 μ L PEG buffer to dissolve it. Mix the mPEG-SVA and Biotin-PEG-SVA at the volume ratio of 99:1.
[RECIPE] PEG buffer: 0.6 mol/L K_2SO_4 and 0.1 mol/L $NaHCO_3$.
- (F) Add 50–100 μ L PEG solution onto one piece of coverslip, then put another piece to stack onto it.
- (G) Incubate the coverslips in a humid and dark environment for 2.5 h. Then separate the coverslips, rinse them and dry them out. The coverslips may be stored in a vacuum at $-20^\circ C$.

Single-molecule imaging and analysis with LipoFRET [TIMING ~12 h]

- (A) Mount the PEG-coverslip-assembled flow chamber onto the stage of TIRFM.
- (B) Add streptavidin at 0.01 mg/mL into the chamber and incubate for 10 min. Wash the chamber with the buffer to remove excess streptavidin.
- (C) Dilute the quencher-encapsulated liposomes to ~ 0.01 mg/mL, and inject them into the chamber. Incubate for 5 min. Wash the chamber again with the buffer to remove unbound liposomes.
[TIP] Because of the wide adsorption spectra and autofluorescence of the quencher, one may observe the liposomes in an emission channel that fits Cy5 dye to determine if the sufficient density of the liposomes is reached.
- (D) Add the target protein to the channel to interact with the liposomes, and record images or films. In this example, α -synuclein labeled by Alexa Fluor 555-MAL was also obtained by a similar purification and labelling procedure as described in the protocol. The labeled α -synuclein (α -synuclein K10C-Alexa

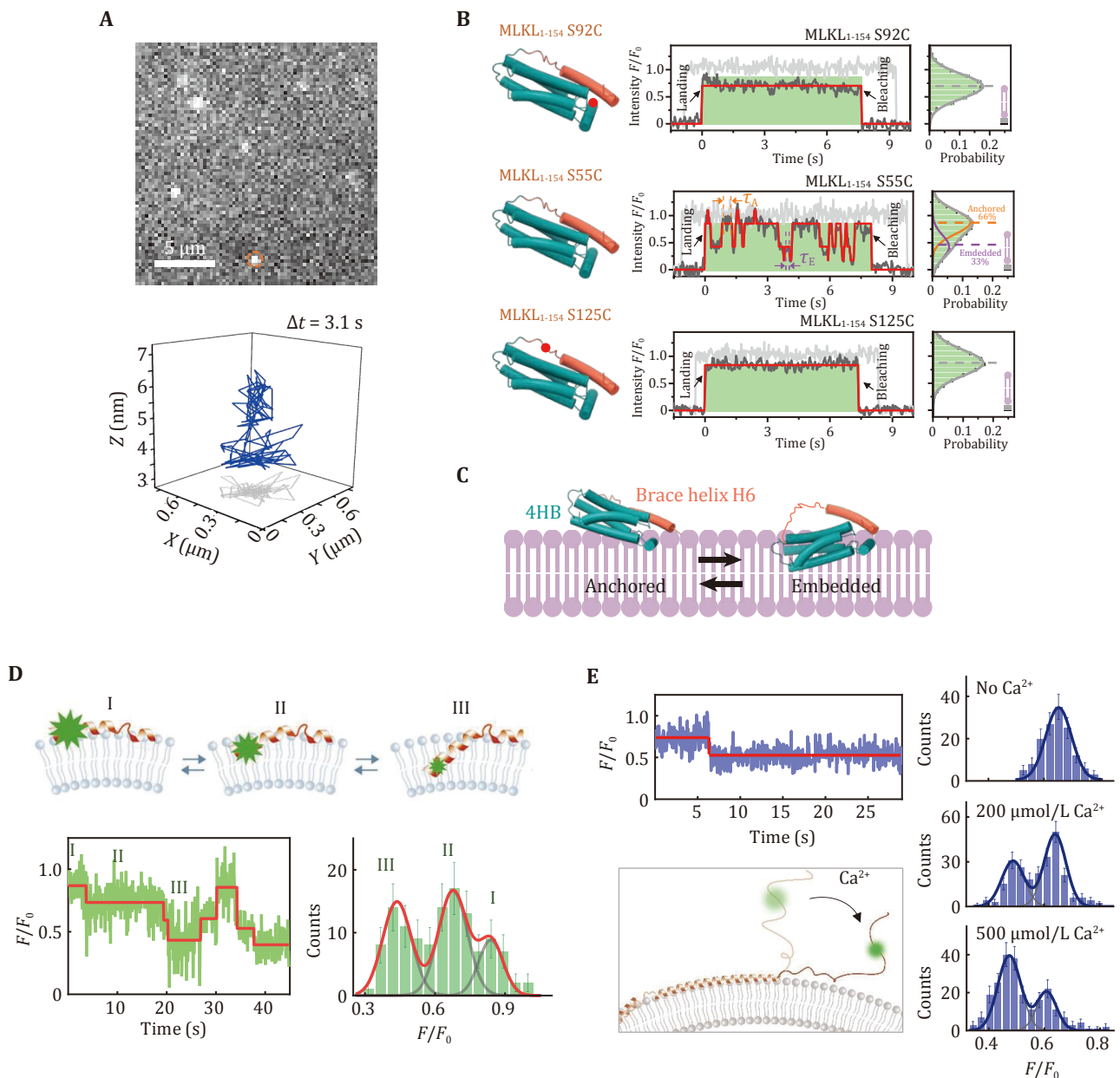


Fig. 2 Observing the interaction of membrane protein with membranes with SIFA and LipoFRET. **A** A 3D trace of a singly labelled MLKL protein. The in-plane positions (the x and y coordinates) were read from the image, and the z coordinates were derived from the intensity $F(x,y)$ of the fluorophore. **B** Typical fluorescence trajectory (middle) and statistics of intensity (right) of S92C, S55C and S125C labelled MLKL₁₋₁₅₄ (left), respectively. **C** Cartoon representation of the two states. **D** The spontaneous membrane insertion dynamics of the N-terminal of α -synuclein (upper panel) reflected by intensity trace (lower left) and the histogram (lower right). **E** The position changes of the C-terminal of α -synuclein upon calcium adding (left) and the position distribution characterized by the intensity distribution (right) (Ma *et al.* 2019a; Yang *et al.* 2023)

555, α -synuclein T72C-Alexa 555, or α -synuclein S129C-Alexa 555) was added at ~ 1 nmol/L.

- (E) Repeat the above imaging steps with the liposomes that do not contain a quencher for the measurement of the intrinsic intensity of the fluorophore.
- (F) Extract the intensity of the fluorophores with the

software ImageJ and MATLAB. Gaussian fitting can be applied to derive the intrinsic intensity of the fluorophore-labeled proteins (F_0) without a quencher. Then the fluorescence intensity (F) of the proteins on quencher-containing liposomes is also analyzed. The normalized intensity F/F_0 and its

changes are compared to the intensity-distance curves to derive the position changes of the labeled site during the conformational motion of the protein.

ANTICIPATED RESULTS

In this protocol, we describe the procedure of SIFA and LipoFRET in detail. As shown in Fig. 2, SIFA enables us to track the three-dimensional movement of MLKL on supported lipid bilayers with an experimental accuracy of ~0.6 nm for axial movement and ~25 nm for lateral movement (Fig. 2A). Applying SIFA to different interest site of MLKL (Figs. 2B and 2C), two states “Anchored” and “Embedded” were captured, even though only nanoscale change in architecture between two states, and nanoscale dynamics of MLKL undergo conformational change were detected. Whereas LipoFRET distinguishes the penetration depths of the proteins on the membrane of liposomes. Application of LipoFRET on α -synuclein could detect the spontaneous intensity alteration of α -synuclein K10C-Alexa 555, which represents the shift among three penetration depths (Fig. 2D). The intensity of α -synuclein S129C-Alexa 555 can also be seen higher than that of α -synuclein T72C-Alexa 555. In addition, the C-terminal of the protein and the membrane surface could be observed to move to a closer position relative to the membrane surface upon the Ca^{2+} addition (at 0.1 to 1 $\mu\text{mol/L}$ concentration) when applying the method to α -synuclein S129C-Alexa 555 (Fig. 2E).

PERSPECTIVES

Single-molecule fluorescence imaging has long been applied in membrane protein studies, including techniques like FRET and FIONA (Fluorescence Imaging with One Nanometer Accuracy). FRET has been widely used to probe the folding of membrane proteins, intramolecular movements of domains, and inter-molecular movements of interacting proteins (Mercier *et al.* 2020; Shi *et al.* 2024). FRET is suitable for a detection range of approximately 3 to 8 nm (Ha 2001). FIONA excels in single-molecule localization, providing axial accuracy of 0.5 nm and lateral accuracy of 1–2 nm, offering unparalleled precision in localizing individual molecules and tracking their movements over time (Park *et al.* 2007; Selvin *et al.* 2007; Wang *et al.* 2014).

SIFA and LipoFRET are capable of detecting the insertion of membrane proteins into biological membranes with sub-nanometer experimental accuracy. SIFA is suitable for tracking the three-dimensional

movement of target proteins (Jiang *et al.* 2022; Ma *et al.* 2018, 2019b; Yang *et al.* 2023), while LipoFRET is ideal for detecting the location of the curvature-sensitive membrane proteins inserted in bio-membranes (Ma *et al.* 2019a, 2020). Each technique has unique strengths, making them suitable for different experimental needs.

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Compliance with Ethical Standards

Conflict of interest Chenguang Yang, Dongfei Ma, Shuxin Hu, Ming Li and Ying Lu declare that they have no conflict of interest.

Human and animal rights and informed consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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