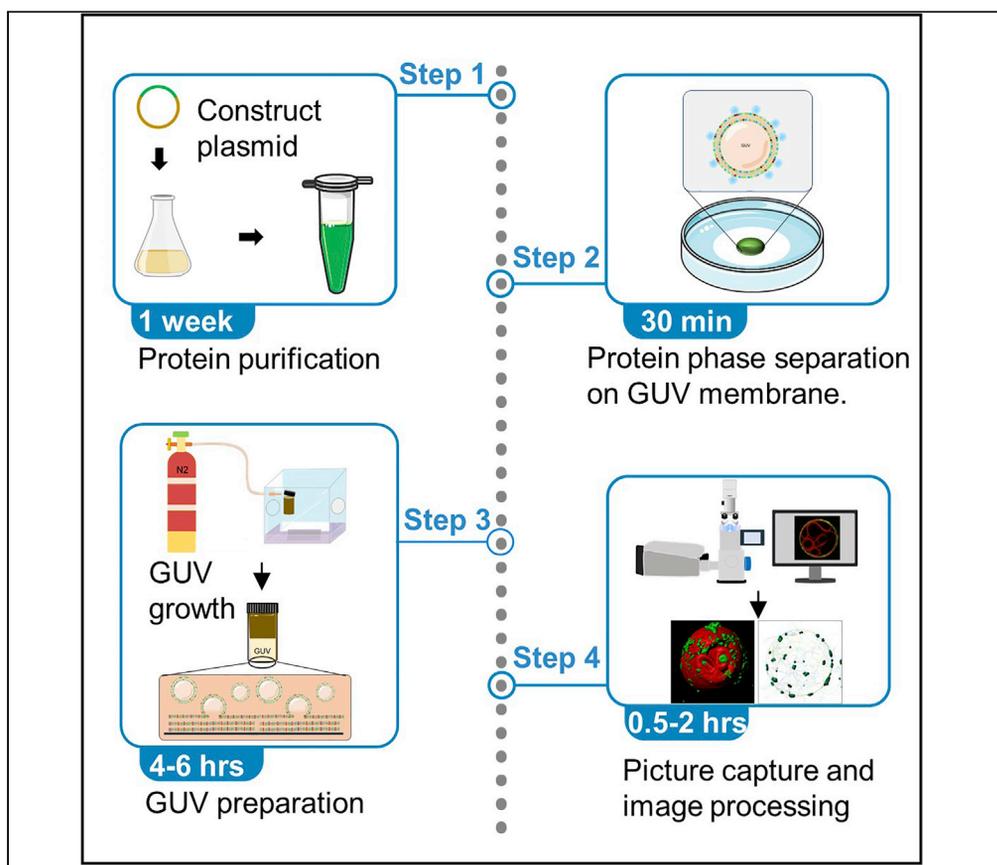


Protocol

A protocol for mimicking lipid-mediated phase separation on the membrane using giant unilamellar vesicles



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Highlights

Approach to prepare PI(4,5)P₂-containing GUVs and Ago1-GFP fusion protein

Procedure for using GUV to mimic Ago1 protein's phase separation on a membrane

Airyscan-based 3D time-lapse imaging of the phase-separated protein

Imaris-based processing and analysis of the 3D time-lapse images

Here, we present a general protocol for mimicking lipid-mediated phase separation on the membrane using giant unilamellar vesicles (GUVs). In this protocol, we use GUVs to mimic Ago1 protein's phase separation behavior on the membrane through binding with phosphoinositides (PIPs). We provide procedures to prepare fluorescent-labeled Ago1 protein and PI(4,5)P₂-containing GUVs, followed by steps to assess Ago1 protein's phase separation in 3D time-lapse images. This protocol can be applied to investigate a membrane-associated protein's behavior on the membrane.

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

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Protocol

A protocol for mimicking lipid-mediated phase separation on the membrane using giant unilamellar vesicles

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SUMMARY

Here, we present a general protocol for mimicking lipid-mediated phase separation on the membrane using giant unilamellar vesicles (GUVs). In this protocol, we use GUVs to mimic Ago1 protein's phase separation behavior on the membrane through binding with phosphoinositides (PIPs). We provide procedures to prepare fluorescent-labeled Ago1 protein and PI(4,5)P2-containing GUVs, followed by steps to assess Ago1 protein's phase separation in 3D time-lapse images. This protocol can be applied to investigate a membrane-associated protein's behavior on the membrane.

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

BEFORE YOU BEGIN

In addition to creating a physical boundary, cell membranes provide critical platforms for many biological processes. Due to the complexity of the membrane composition, it is a challenging task to study the membrane-associated proteins' behavior on the membrane. This protocol below describes detailed steps using GUV to mimic the Ago1 protein's phase separation behavior on the membrane through binding with phosphoinositides (PIPs). Before beginning, understanding the feature of your interest protein is very important. In our research, we found the Ago1 protein contains a lipid-binding domain which allows it to phase-separate on the endoplasmic reticulum (ER) membrane through binding with PI(4,5)P2 (Gao et al., 2022). Besides, you should be aware some tags used in protein purification (e.g., GFP and GST) may influence the protein's features, this must be considered before beginning the protocol. Before starting the experiment, all reagents should be prepared as described below.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>E. coli</i> DH5 α	CWBIO	CW0808H
<i>E. coli</i> BL21(DE3)	CWBIO	CW0809S
Chemicals, peptides, and recombinant proteins		
DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine)	Nippon Fine Chemical	Cat# 4235-95-4
DPPE(1,2-dipalmitoyl-sn-glycero-3-phosphocholine)	Nippon Fine Chemical	Cat# 63-89-8

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cholesterol	Nippon Fine Chemical	Cat# 57-88-5
PI(4,5)P2	Echelon Biosciences	Cat# P-4516
CM-Dil dye	Invitrogen	Cat# C7001
Chloroform	Keshi	Cat#67-66-3
Sucrose	Sigma-Aldrich	Cat# S0389
Tris Base	Sigma-Aldrich	Cat#V900483-5KG
Tris hydrochloride (1 M, pH 7.6)	Beyotime	Cat#ST776-500ml
HCl	Sinopharm Chemical Reagent	Cat# 10011018
NaOH	NJ-Reagent	Cat# 1310-73-2
NaCl	SCR	Cat#7647-14-5
Yeast Extract	Sigma-Aldrich	Y1625-1KG
Tryptone	Sigma-Aldrich	T7293-1KG
Isopropyl β-D-Thiogalactoside (IPTG)	Sigma-Aldrich	Cat# 10724815001
Phusion_high-fidelity DNA polymerase	New England Biolabs	Cat#M0530S
EcoR1	Thermo Fisher Scientific	Cat#FD0274
Xho1	Thermo Fisher Scientific	Cat#FD0694
Coomassie Brilliant Blue R-250.	Sangon Biotech	Cat#A100472
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	Cat#P7626
Imidazole	Sigma-Aldrich	Cat#792527-500G
Glycerol	Macklin	Cat#G810575-500ml
Kanamycin	Solarbio	Cat#YZ-1355006-200mg
Critical commercial assays		
FastPure Gel DNA Extraction Mini Kit	Vazyme	Cat#DC301-01
2 × Phanta Max Master Mix (Dye Plus)	Vazyme	Cat# P525-01
FastPure EndoFree Plasmid Maxi Kit	Vazyme	Cat# DC202-01
ClonExpress® Ultra One Step Cloning Kit vazyme	Vazyme	Cat#C115-01
Deposited data		
Raw and analyzed data	Gao et al. (2022)	https://doi.org/10.1016/j.molcel.2022.02.035
Oligonucleotides		
Ago1-F	Gao et al. (2022)	5'-CGCGGATCCGAATTCATGTATCCAGTTG-3'
Ago1-R	Gao et al. (2022)	5'-GCCCTTGCTCACCATGAACCAAACCTCGC-3'
GFP-F	Gao et al. (2022)	5'-GCGAGGTTTGTTTCATGGTGAAGGGC-3'
GFP-R	Gao et al. (2022)	5'-TGGTGGTGCTCGAGTTTACTTGTACAGCT-3'
Recombinant DNA		
pET-28a(+)	Yeasen	Cat#11905ES03
pEF-Flag-Ago1	Gao et al. (2022)	https://doi.org/10.1016/j.molcel.2022.02.035
pEF-Flag-GFP	Gao et al. (2022)	https://doi.org/10.1016/j.molcel.2022.02.035
Software and algorithms		
Zen blue 3.1 Software	ZEISS	https://www.zeiss.com/microscopy/int/products/microscope-software/zen/free-60-day-version-of-zen-blue-edition.html?vaURL=www.zeiss.com/trialzenblue
Imaris 9.0.1	Bitplane	https://imaris.oxinst.com/microscopy-imaging-software-free-trial
Snap Gene 5.2.3	SnapGene	https://www.snapgene.com/purchase/select-seats?planCode=sg_academic_yearly
Other		
Microscope cover glass (Φ18 mm)	Citoglas	Cat#12480
Microscope cover glass (20 × 20 mm)	Citoglas	Cat#12481
Glass bottom cell culture dish	NEST Scientific	Cat#801001
Eppendorf centrifuge	Eppendorf	Cat# 5425
Eppendorf centrifuge (low temperature)	Eppendorf	Cat# 5427R
Ni Sepharose High Performance(25 mL)	Cytiva (GE Life)	Cat#17526801
Vortex	Kylin-Bell	VORTEX-5
Brown screw bottle 5 mL	Wheaton	Cat#HB-0526-111

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Immersion oil	ZEISS	Cat# 444960-0000-000
Sonicator	Scientz	Scientz-650E
N ₂	TYHG	99.999%
Micro-Analytical Balance	METTLER TOLEDO	N/A
Milli-Q water	EMD Millipore	1060092500
Zeiss LSM 980 with Airyscan 2	ZEISS	N/A
AKTA pure	Cytiva (GE)	N/A
Fume hood	Dremlab Technologies	DL-150S
Drying oven	Yiheng (China)	DHG-9015A

Note: Immersion oil can cause skin irritation and is harmful to aquatic life, with long-lasting effects. Avoid its release into the environment and wear protective gloves. If it comes into contact with skin, wash with plenty of soap and water.

MATERIALS AND EQUIPMENT

Lysis buffer for protein purification

Reagent	Final concentration	Amount
Tris hydrochloride (1 M, pH 7.6)	50 mM	2.5 mL
NaCl (5 M)	500 mM	5 mL
Imidazole	20 mM	0.068 g
ddH ₂ O	N/A	Add up to 50 mL
Total		50 mL

Adjust the pH to 7.6 with NaOH or HCl at room temperature (22°C–28°C) and the buffer can be stored at 4°C for up to 1 month.

Binding buffer for protein purification

Reagent	Final concentration	Amount
Tris hydrochloride (1 M, pH 7.6)	50 mM	2.5 mL
NaCl (5 M)	500 mM	5 mL
Imidazole	30 mM	0.102 g
ddH ₂ O	N/A	Add up to 50 mL
Total		50 mL

Adjust the pH to 7.6 with NaOH or HCl at room temperature (22°C–28°C) and the buffer can be stored at 4°C for up to 1 month.

Washing buffer for protein purification

Reagent	Final concentration	Amount
Tris hydrochloride (1 M, pH 7.6)	50 mM	2.5 mL
NaCl (5 M)	500 mM	5 mL
Imidazole	50 mM	0.17 g
ddH ₂ O	N/A	Add up to 50 mL
Total		50 mL

Adjust the pH to 7.6 with NaOH or HCl at room temperature (22°C–28°C) and the buffer can be stored at 4°C for up to 1 month.

Elution buffer for protein purification

Reagent	Final concentration	Amount
Tris hydrochloride (1 M, pH 7.6)	50 mM	2.5 mL
NaCl (5 M)	500 mM	5 mL

(Continued on next page)

Continued

Reagent	Final concentration	Amount
Imidazole	300 mM	1.02 g
ddH ₂ O	N/A	Add up to 50 mL
Total		50 mL

Adjust the pH to 7.6 with NaOH or HCl at room temperature (22°C–28°C) and the buffer can be stored at 4°C for up to 1 month.

Note: The pH of the Tris is sensitive to the temperature, so it will be better to adjust the pH at room temperature.

Growth buffer (50 mL)

Reagent	Final concentration	Amount
Tris hydrochloride (1 M, pH 7.6)	100 mM	5 mL
NaCl (5 M)	50 mM	500 µL
Sucrose	50 mM	0.855 g
ddH ₂ O	N/A	Add up to 50 mL
Total		50 mL

Note: It is recommended to prepare fresh buffer because it can become contaminated with bacteria after long-term storage.

Dilution buffer (50 mL)

Reagent	Final concentration	Amount
Tris hydrochloride (1 M, pH 7.6)	100 mM	5 mL
NaCl (5 M)	50 mM	500 µL
ddH ₂ O	N/A	Add up to 50 mL
Total		50 mL

Adjust the pH to 7.6 with NaOH or HCl at room temperature (22°C–28°C) and the buffer can be stored at 4°C for up to 1 month.

Lipid solution

All phospholipids include DOPC, DPPC, PI(4,5)P2, and cholesterol. Stock solutions are prepared in chloroform.

Alternatives: The reagents can also be ordered from other companies such as Avanti Polar Lipids (Alabaster, AL).

Note: The stock solutions can be prepared fresh to use or prepared in a large quantity stock, the stock can be stored at –20°C.

△ **CRITICAL:** Chloroform can harm the eyes, skin, liver, kidneys, and nervous system. Chloroform can be toxic if inhaled or swallowed. This step should be carried out in a fume hood. Solutions should be disposed of following local regulations.

Data management

The image files are initially stored in the format of the microscope system software; this example uses ZEN3.1 blue for the Zeiss LSM980 with Airyscan 2 using the .czi file format. The images shown in the figures were compressed to JPEG format. We used Imaris 9.0.1 software to analyze the protein's

phase separation on the membrane for the 3D time-lapse analysis. The images shown in the figures used the .tif format, and the movie used the .mp4 format.

STEP-BY-STEP METHOD DETAILS

Figure 1 provides a schematic overview of the main steps in the procedure.

Purifying the protein of interest

⌚ Timing: ~1 week

This step describes how to purify the protein used for phase separation. Here, we describe the procedure for preparing the Ago1-GFP fusion protein using Ni-Sepharose to purify His-tagged protein.

Note: The details of the protein purification will depend on the identity of the desired protein.

Alternatives: MBP, Flag, or GST tag can also be used to purify the interested protein. Except for the bacteria purification, Expi293 GnTi cell or pichiaPink yeast expression system can also be used to purify the protein.

Expi293 GnTi expression system: <https://www.thermofisher.cn/order/catalog/product/A39250?SID=srch-srp-A39250>.

pichiaPink yeast expression system: <https://www.thermofisher.cn/cn/zh/home/life-science/protein-biology/protein-expression/yeast-protein-expression/pichiapink-yeast-expression-systems.html>.

1. Design the primers. The primer sequence is shown in Table 1.
 - a. Design the primers for the Ago1-GFP fusion gene using SnapGene software. Primer Ago1-F contains 15 bases of the pET28a plasmid for recombination. Primer Ago1-R contains at least 15 bases of GFP for recombination. Primer GFP-R contains 15 bases of the pET28a plasmid for recombination.
2. Construct the pET28a-Ago1-GFP plasmid.
 - a. Prepare two 50 μ L PCR reaction mixes in PCR tubes to amplify Ago1 and GFP, respectively. The PCR reaction mixes are shown in Table 2, and the reaction cycling conditions are given in Table 3.
 - b. Purify the PCR products using the Vazyme gel purification kit.

⏸ Pause point: The PCR products can be stored at 4°C overnight.
3. PCR product recombination with EcoR1/Xho1-digested plasmid.
 - a. Recombine the PCR products with EcoR1/Xho1-digested plasmid. The recombination reaction is described in Table 4.
 - b. Transform *Escherichia coli* (*E. coli*) DH5 α with the recombination mixture.
 - c. Pick a single colony and grow it in 1 mL of Luria-Bertani (LB) medium.
 - d. The LB was cultured at 37°C for 5–6 h, taking 1 μ L LB as a template and running PCR to identify positive clones.
 - e. Sequence to confirm the plasmid.
 - f. Extract the plasmid with FastPure EndoFree Plasmid Maxi Kit.
4. Express and purify Ago1-GFP protein.
 - a. Transform *E. coli* BL21 with the pET28a plasmid for the bacterial expression of the His-tagged fusion Ago1-GFP protein.
 - b. Pick a single colony and grow it in 5 mL of LB medium containing 100 μ g/mL kanamycin. Incubate at 37°C with shaking at 250 rpm.

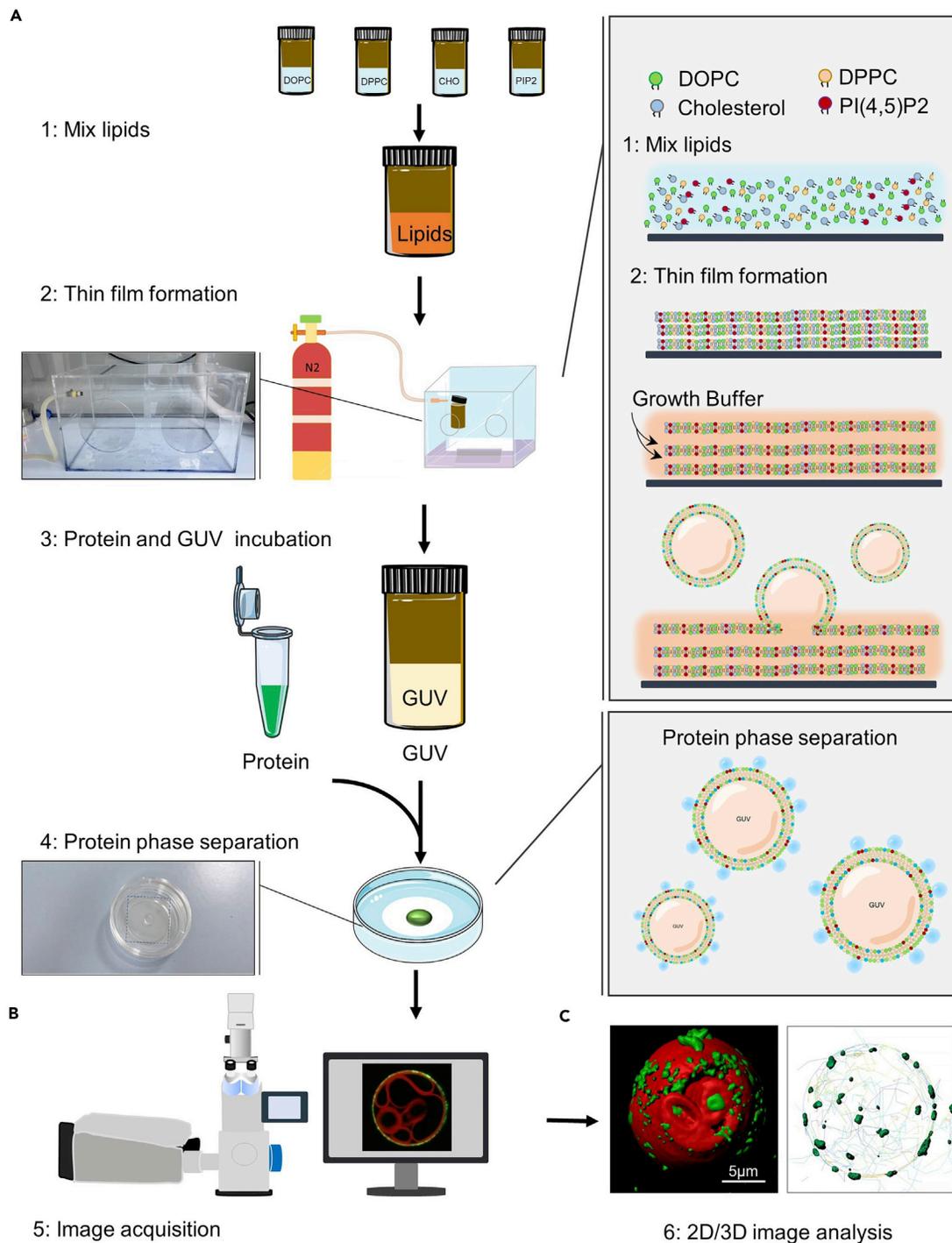


Figure 1. Outline of the overall procedure

(A) Prepare protein and GUV for mimicking protein's phase separation on the membrane. Under step 2 is a real photo of the apparatus for blowing N_2 .

(B) Image acquisition and processing.

(C) Image example, reprinted with permission from (Gao et al., 2022). Scale bars, 5 μm .

Table 1. Primers used to construct the Ago1-GFP plasmid

Primer	Sequence
Ago1-F	5'-CGCGGATCCGAATTCATGTATCCAGTTG-3'
Ago1-R	5'-GCCCTTGCTCACCATGAACCAAACCTCGC-3'
GFP-F	5'-GCGAGGTTTGGTTCATGGTGAGCAAGGGC-3'
GFP-R	5'-TGGTGGTGCTCGAGTTTACTTGTACAGCT-3'

Note: The antibiotic selection depends on the constructs that the users will employ.

- c. Dilute the overnight culture with 250 mL of fresh LB medium containing 100 µg/mL kanamycin and continue shaking at 37°C until $OD_{600nm} = 0.6$ (~3–5 h).
- d. Add 1 mM IPTG and shake for an additional 16 h at 16°C to induce Ago1-GFP protein expression. Collect the cells by centrifuging for 10 min at 15,000 × g at 4°C. Discard the supernatant.

Note: The bacterial cell pellet can be stored at –80°C for several weeks.

- e. Resuspend the bacterial cell pellet in 40 mL of lysis buffer. Sonicate the cells at 40% power using 5 s on/5 s off for 15–25 min.
- f. Centrifuge the lysate at 15,000 × g for 10 min at 4°C. Transfer the supernatant to a new 50 mL tube, and filter the supernatant through a 0.45 µm filter.
- g. Add 300 µL of Ni beads to a column and wash the beads with 10 mL of H₂O followed by 10 mL of binding buffer.
- h. Add the lysate to the column and incubate to allow binding between the His-tagged protein and the Ni beads.
- i. Wash the beads three times with 10 mL of washing buffer.

Note: Vary the imidazole concentration to achieve optimal washing.

- j. Elute the protein with elution buffer and separate the sample on SDS-PAGE. Stain with Coomassie blue G-250 to check the protein quality and quantity.
- k. Ultrafiltration or dialysis to remove the imidazole. The protein was diluted in the dilution buffer.

▣ **Pause point:** The protein can be used for the next step or can be stored at –80°C.

Note: The protein can be further purified with an AKTA purifier to improve the quality of the protein sample.

Alternatives: Dialysis or ultrafiltration to remove imidazole can also improve the quality of the protein.

Preparation of GUVs

⌚ **Timing:** 3–5 h

Table 2. PCR reaction master mixes

Reagent	Amount 50 µL
DNA template (pEF-Flag-Ago1 plasmid and pEF-Flag-GFP plasmid)	100 ng (1 µL)
DNA Polymerase mix (2 × Phanta Max Master Mix (Dye Plus))	25 µL
Forward primer	2.5 µL (10 µM)
Reverse primer	2.5 µL (10 µM)
ddH ₂ O	19 µL
Total	50 µL

Table 3. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	30 cycles
Annealing	56°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Hold	4°C	30 min	

A commonly used method to generate GUVs is the hydration method, which is based on the principle of membrane swelling (Reeves and Dowben, 1969). In this method, phospholipids and cholesterol are dissolved in an organic solvent such as chloroform that is then evaporated under a gentle flow of nitrogen until the solvent is removed. During the evaporation, a phospholipid film forms on the glass substrate. The film is then hydrated with an aqueous buffer, and GUVs begin to form owing to a combination of osmotic pressure, electrostatic interactions, and the hydrophobic effect (Stein et al., 2017; Tsumoto et al., 2009). The hydration method is easy and safe to prepare GUVs. This method not only allows the formation of charged lipids in GUVs but also avoids the disadvantages of the electro-formation method which might impact the oxidation of the lipids.

5. Dissolve the phospholipids or lipids in chloroform.
 - a. Weigh 2.4 mg of DOPC (MW 786.113, C₄₄H₈₄NO₈P) with Micro-Analysis Balance (METTLER TOLEDO) in 3 mL of chloroform to make a 0.8 mg/mL solution (Figure 1A).
 - b. Weigh 2.2 mg of DPPC (MW 734.04, C₄₀H₈₀NO₈P) with Micro-Analysis Balance in 3 mL of chloroform to make a 0.73 mg/mL solution (Figure 1A).
 - c. Weigh 1.2 mg of cholesterol (MW 386.65, C₂₅H₄₉O₁₉P₃) with Micro-Analysis Balance in 3 mL of chloroform to make a 0.4 mg/mL solution (Figure 1A).
 - d. Dissolve 0.1 mg of PI(4,5)P₂ (MW 746.57, C₂₅H₄₉O₁₉P₃) in 1.33 mL of chloroform to make a 0.075 mg/mL solution (Figure 1A). (The reagent contains 0.1 mg powder of PI(4,5)P₂ total).

Note: It is recommended to prepare a fresh solution because the chloroform is easily volatilized.

Low temperature can reduce the volatilization, the stock solution can be transferred to brown screw-top bottles and stored at –20°C for no more than 6 months.

Note: The users can also prepare a larger quantity of stock and store it at –20°C for no more than 6 months.

6. In a brown screw-top bottle, mix the lipids in the amounts shown in Table 5, the weight ratio of DOPC: DPPC: Cholesterol: PI(4,5)P₂ is 36:23:6:6. Vortex to fully mix (Figure 1A).

Note: During this procedure, it is recommended to keep the stock solutions on ice to minimize the volatilization of chloroform.

7. Remove the chloroform by blowing clean nitrogen (the purity is 99.999%) and further dry with nitrogen for at least 40 min.

Table 4. Recombination reaction system

Reaction component	
Ago1 fragment	2 μL
GFP fragment	2 μL
pET28a vector	1 μL
Recombination Mix (ClonExpress® Ultra One Step Cloning Kit vazyme)	5 μL

Table 5. Lipid mixture solution

Reagent	Amount
DOPC (0.8 mg/mL)	45 μ L
DPPC (0.73 mg/mL)	32 μ L
Cholesterol (0.4 mg/mL)	15 μ L
PI(4,5)P2 (0.075 mg/mL)	80 μ L

Note: The lipids dissolved in chloroform will be deposited onto the solid substrate. As the solvent evaporates, the amphiphilic structure of the lipids leads to the clustering of several stacks of bilayers (Figure 1A right panel).

△ CRITICAL: The chloroform must be completely removed, otherwise the growth buffer will not support good GUV growth.

8. Add 200 μ L of growth buffer and incubate for 1 h at 60°C in the drying oven (Figure 1A).

△ CRITICAL: As PI(4,5)P2 is a negatively charged lipid, the absence of salt in the growth buffer may prevent the incorporation of PI(4,5)P2 in the GUV (Beber et al., 2019). We recommend using the growth buffer containing at least 50 mM NaCl.

9. After incubation, transfer the GUV to a new 1.5 mL Eppendorf tube.

a. Centrifuge the GUV sample at 100 \times g for 10 min at room temperature to remove aggregated lipid species.

Note: The supernatant is used for the protein phase separation experiments (Figure 1A).

Note: Although the deposited lipids are uniformly mixed, each vesicle will vary slightly in composition.

△ CRITICAL: Chloroform can harm the eyes, skin, liver, kidneys, and nervous system. Chloroform can be toxic if inhaled or swallowed. This step should be carried out in a fume hood.

△ CRITICAL: The chloroform must be completely removed. Residual chloroform can affect GUV growth.

Ago1 forms phase separation on the PI(4,5)P2-containing GUV membranes

⌚ **Timing:** ~30 min

Incubate the purified protein with the GUVs to make the protein phase separation on the membrane.

10. Incubate the protein with GUVs (Figure 1A).

a. Remove the protein from -80°C storage and place it on ice to allow the sample to thaw slowly.

△ CRITICAL: GUVs (especially PI(4,5)P2-containing GUVs) must be freshly prepared because PI(4,5)P2 can migrate from the bilayer to the buffer (Beber et al., 2019).

Note: It is recommended to freshly prepare the protein. However, the purified protein can be stored at -80°C for a short period. In our hands, Ago1 protein in dilution buffer was stored at -80°C for 1 week and still works.

Table 6. Protein phase separation incubation system

Reagent	Amount
GUV	10 μ L
Protein	1 μ L
CM-Dil	1 μ L

Note: The concentration of the protein used may be different depending on the identity of the desired protein. For AGO1 protein, it works at a concentration between 0.3–3 mg/mL. High protein concentration is not essential for its phase separation on the membrane because there always be excess protein compared with PI(4,5)P2. We recommend using the protein approximately at 1 mg/mL concentration for the first time.

- b. Add the components separately as shown in [Table 6](#) in a 250 μ L PCR tube at room temperature.
- c. Transfer the mixture immediately to a glass cell culture dish and cover it with a square coverslip, seal the coverslip with nail polish to prevent liquid evaporation ([Figure 1A](#)).

Image acquisition: 3D and time-lapse imaging

⌚ Timing: 0.5–2 h

The image acquisition time depends on the microscope, the file size, the image software processing time, and the user's experience.

Here, we describe the confocal settings and software processing procedures. The images in this protocol apply ([Gao et al., 2022](#)) were acquired using a Zeiss LSM 980 with an Airyscan 2 confocal microscope. The microscope was controlled by ZEN 3.1 Blue software. We used Imaris 9.0.1 software for 3D reconstruction and movie export.

Alternatives: Other confocal microscopes such as Nikon or Leica and their related software is also OK.

11. Open the ZEN 3.1 software.
 - a. Open ZEN 3.1 (blue edition) by double-clicking the software icon on the desktop. Select "ZEN System" to initialize the hardware ([Figure 2A](#)).
12. Set up the microscope and locate the GUVs.
 - a. Click the **Locate** tab and find the GUVs through the eyepiece.

Note: First, use the 10 \times objective to find the GUVs, then change to the 40 \times oil objective. Put some immersion oil on the 40 \times oil objective before starting the imaging process.

- b. Go to the **Acquisition** mode and click the **Smart Setup** button. Select the channels needed (we mainly use 555 nm and 488 nm to observe the GUVs and proteins, respectively). Choose the **Airyscan**, **SNR/Sensitivity**, **smartest** (line) capture module ([Figures 2B and 2C](#)).

Note: The channel selection may be different depending on the fluorophore.

- c. Highlight a single track in the **Channels** window to set the parameters associated with the wavelength. Set the **Laser Power** to 0.4% initially. Increase the laser power if the detector gain cannot achieve the desired intensity. Values of 1%–3% are typically sufficient, but the

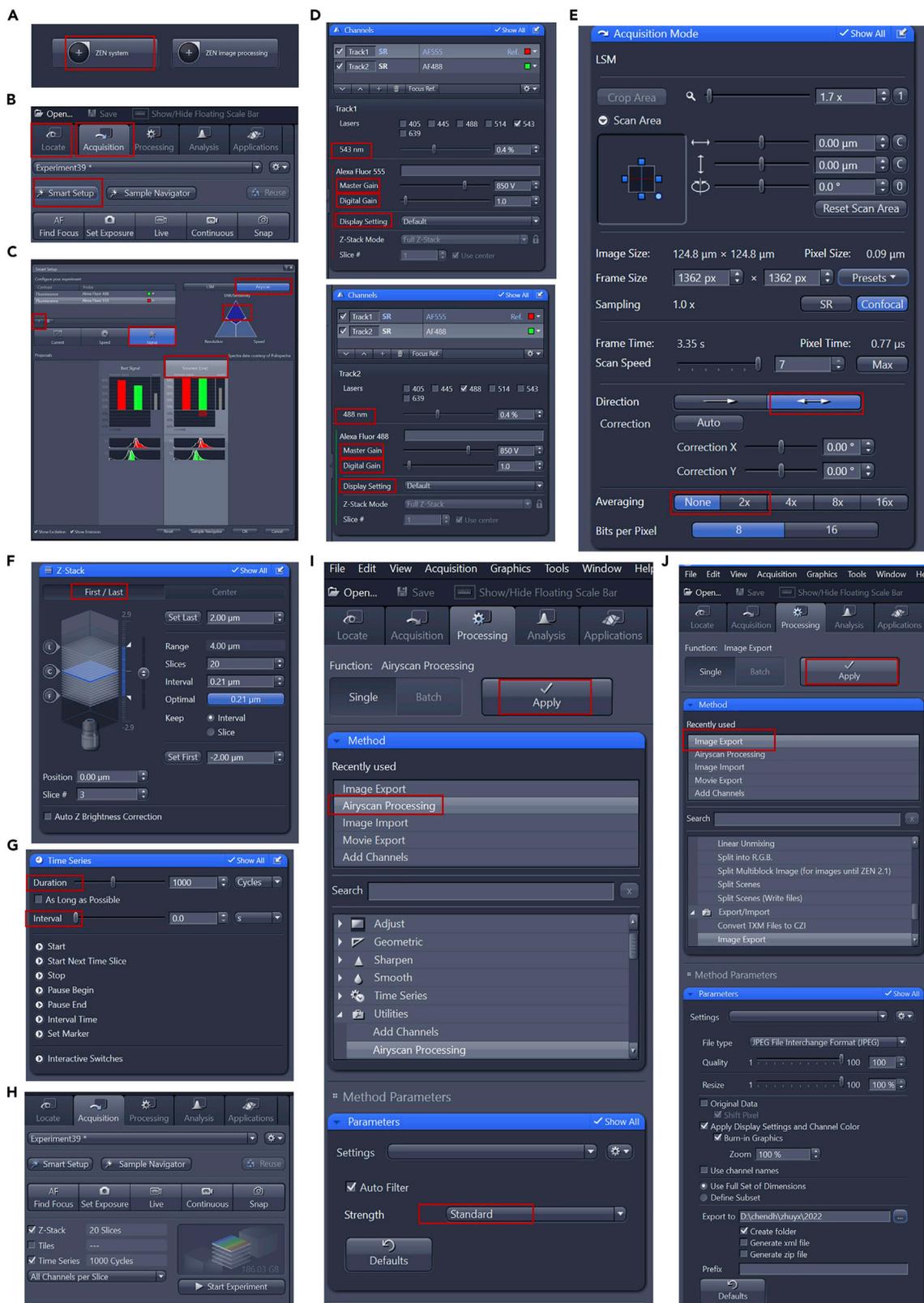


Figure 2. Setup of the Zeiss LSM 980 in ZEN3.1 Blue

- (A) Open ZEN blue software and select the ZEN system to initialize the hardware.
 (B and C) “Smart Setup” selection setup details.
 (D–H) Setup details of channels (D), Acquisition mode (E), Z-stack (F and H), and Time-series (G and H).
 (I) Setup details of Airyscan Method processing.
 (J) Details of exporting image.

Master Gain can be increased to obtain a stronger signal. The master gain should be set no higher than 850 V under the Airyscan module (Figure 2D).

Optional: Decrease the Digital Offset to reduce the background signal. Caution should be used here, as the true signal may be eliminated without altering the sensitivity. Increase the Digital Gain to amplify all signals and background, thereby stretching the histogram. Again, sensitivity is not directly altered with this function.

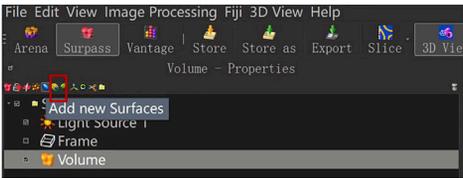
13. Adjust the scanning parameters.
 - a. Go to the **Acquisition Mode** window and select a suitable area and zoom setting to capture an area of interest. After a **Live** image has been stopped, use the **Crop** button to adjust the desired field of view (Figure 2E).
 - b. Select a **Frame Size** to represent the desired area per pixel. 1024 × 1024 is often used as a starting point (Figure 2E).
 - c. Adjust the **Scan speed** to 9 for a quick scan speed (Figure 2E).
 - d. Select **Bidirectional** scanning mode (Figure 2E).
 - e. Select a value for **Averaging** that can improve image quality. A small amount of averaging (2–4) is enough to improve the signal-to-noise ratio (Figure 2E).
 - f. Select the dynamic range with the **Bits per Pixel** setting. Eight-bit is often sufficient (Figure 2E).
 - g. Click the **Snap** button to acquire a single frame. For 3D or time-lapse imaging, please see the next step.
14. Z-stack and time-series set up.
 - a. Select the **Z-stack** and **Time-Series** checkbox in the main experiment tool area.
 - b. Go to the **Z-stack** window and select the preferred scanning mode; we use **First/Last** mode (Figure 2F).
 - c. Select the **Live** button, use the focus knob to locate one end of the specimen, then click the **Set First** button. Focus on another end of the specimen, then click the **Set Last** button (Figure 2F).
 - d. Set the **Interval** to define the step size of the Z-stack. For the best 3D reconstruction, use the value of the **Optimal** button (Figure 2F).
 - e. Go to the **Time-Series** window and set the **Duration** and **Interval** values (Figure 2G).
15. Find a **GUV** that contains phase-separated proteins. Start imaging by clicking the **Start Experiment** button (Figure 2H).
16. Files are processed by **Airyscan** and saved in **.czi** format (Figures 2J and 2K) which can be opened directly by **Imaris 9.0.1** (see [3D reconstruction](#) below).

3D reconstruction

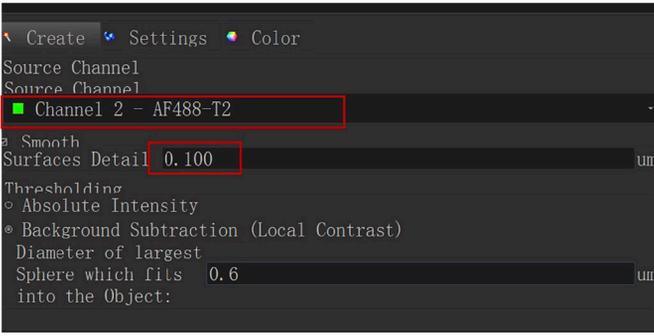
⌚ **Timing:** ~30 min

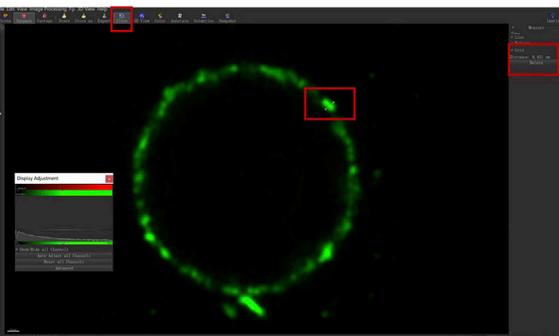
This procedure is for reconstructing 3D **GUVs** containing phase-separated protein and mainly uses the “surfaces” module of **Imaris**. This construction enables us to visualize the behavior of the **Ago1** protein on the **GUV** membrane.

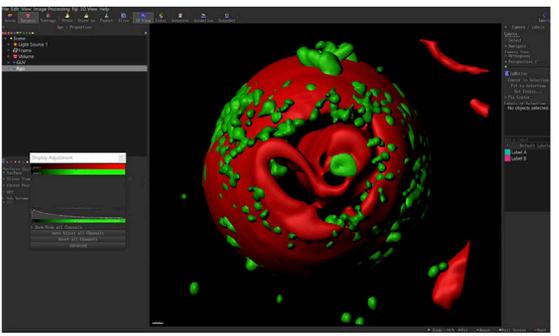
17. Open the **Imaris** software.
 - a. Open **Imaris 9.0.1** by double-clicking the software icon on the desktop.

A 

B 

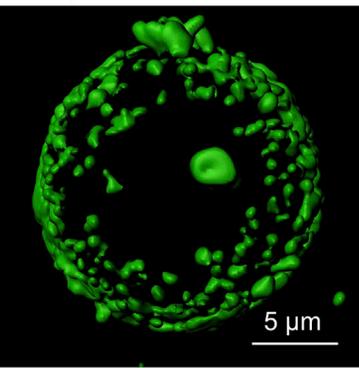
C 

D 

E 

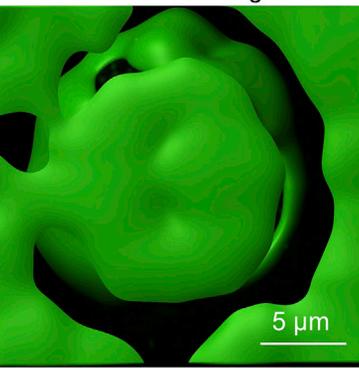
F

Surface details: 0.1 μm	Reason:	Reason:
Background Subtraction: 0.6 μm	Surface details: Too high value	Background Subtraction: Too High



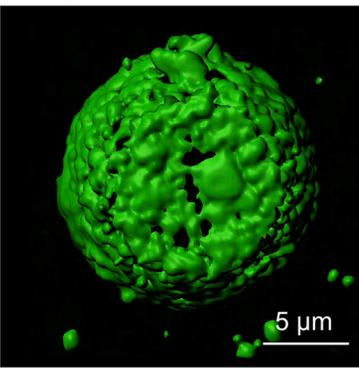
5 μm

sufficient reconstructions



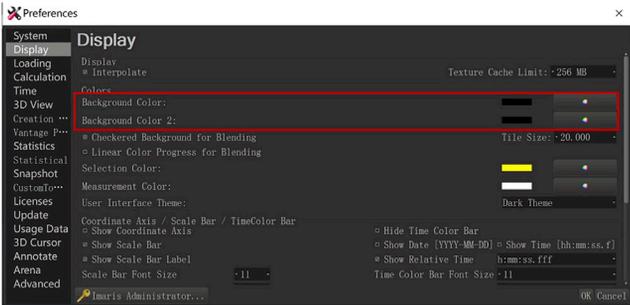
5 μm

insufficient reconstructions



5 μm

insufficient reconstructions

G 

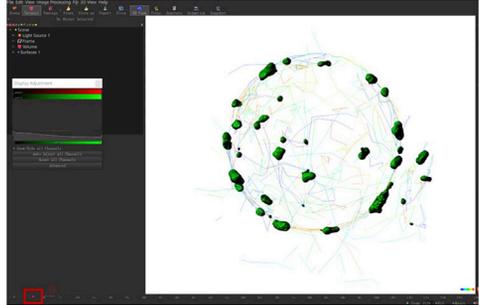
H 

Figure 3. 3D and time-lapse file reconstruction and processing using Imaris software

(A–D) Create new surfaces by clicking. Add new surface button (A) and go to the next step by clicking the blue button (B), choosing the channel and setting Surface Details and Background Subtraction (C and D).

(E) Example of created surfaces of GUV and proteins.

(F) Examples of sufficient and insufficient surface reconstructions. Scale bars, 5 μm .

(G and H) Setup details of the surfaces' background.

- b. Open the Z-stack file in Imaris by using the shortcut “Ctrl+O” or by dragging the file to the software to open it. Imaris supports the most common image formats.

Note: If the image is too large to be handled smoothly, crop it into multiple smaller images by clicking Edit and choosing Crop 3D.

18. Click the **3D View** button to view the file in 3D and click the play button to view the movie. Drag the file by clicking the left mouse button to access the 3D view of the GUV.
19. Create a new surface reconstruction.
 - a. Click on **Add new surface** in the top left corner (the blue button) of the Imaris menu (Figure 3A).
 - b. Click on the blue arrowhead in the bottom left to continue (Figure 3B).
 - c. Select the channel you want to reconstruct. Set the **Surfaces Detail** and **Background Subtraction**. For background subtraction, if you are the first to set this, click on **Slice mode** in the top menu; from slice view, you can look at the slices of your image. Measure distances by left-clicking on the image. Here, we have measured a general granule as 0.6 μm . Set the measured distance as the background subtraction and go to the next step (Figures 3C and 3D).

Note: The parameters are sensitive to the image quality. Choosing a proper setting for the reconstruction is an iterative process.

20. Click on either the blue or green arrow at the bottom to finish the surface reconstruction (Figures 3B and 3E). Ensure you are satisfied with the reconstruction by dragging and zooming in and out. Examples of sufficient and insufficient surface reconstructions are shown in Figure 3F.

Note: Select/unselect the fluorescence channels and the reconstructed surfaces to check whether there is sufficient overlap. If refinement is needed, start over from step 19. If you are satisfied with the result, export the image by clicking Store As and save the image.

3D time-lapse reconstruction and movie export

⌚ Timing: ~30 min

This procedure is for reconstructing a 3D time-lapse image, this construction enables us to visualize the dynamic change of the protein on the GUV membrane.

21. Open the 3D/time-lapse file in Imaris 9.0.1 as shown in step 17.
 - a. Click the **Animation** button to see the dynamic change of the phase-separated proteins. View the movie by pressing the left mouse button to see different slices changing over time (Methods video S1).
 - b. Create a new surface reconstruction as shown in step 19.

Note: Be careful when setting the Surface Detail. If the Surface Detail value is too low, it may result in a long time for reconstruction or even cause the software to crash. In addition, several tutorial videos can be useful for green hands: <https://imaris.oxinst.com/tutorials>.

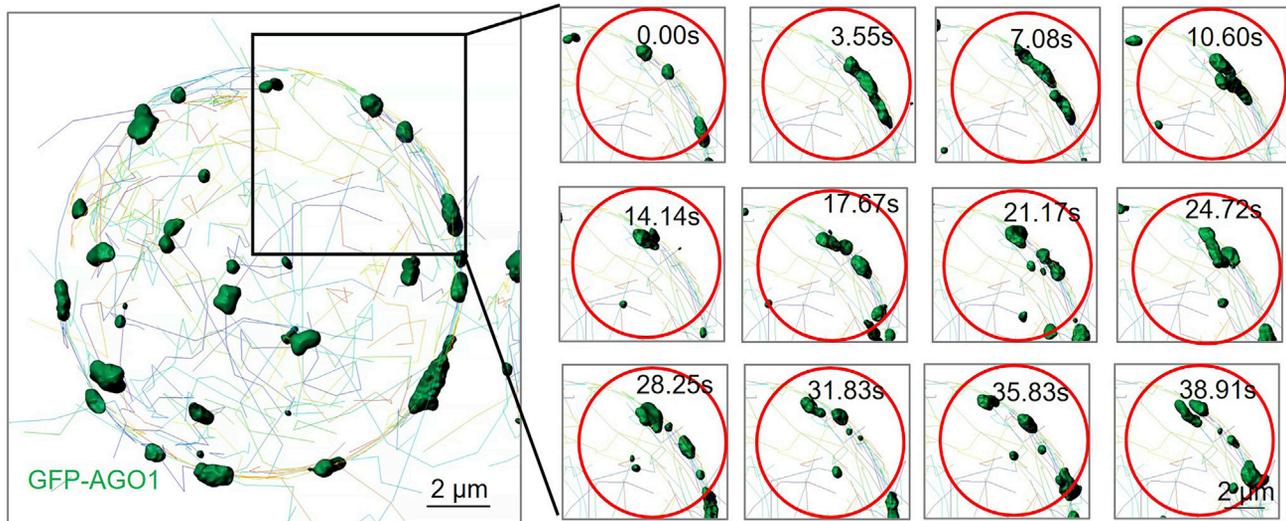


Figure 4. Ago1 protein can dynamically change on the membrane

The 3D time-lapse assay shows that the Ago1 protein can phase separate and can dynamically change on the *GUV* membrane. Scale bars, 2 μm .

- c. Click the **Snapshot** button to find the **Preferences** tab and click the **Display** button to change the background color. A white background can better display the moving granules (Figures 3G and 3H and Methods video S1).

Note: This step can only change the background of the created surface channels. The original file created by the confocal microscope cannot be changed.

- d. Click the red round button (Figure 3H) to export the movie. Rename the movie and save it in .mp4 format. The compression factor is 0; choose higher quality and a frame rate set to 10 frames per second. For the movie size, choose the original resolution ratio. Click the save button to export the movie (Methods video S1).

Quantification and data analysis

⌚ Timing: ~30 min

Imaris software contains the **Statistical analysis** module which can give us the ability to view segmented objects with any statistical value that has been calculated for that object (i.e., Size, Area, Intensity of single channel, Speed, and Volume of the objects). This procedure is describing how to annotate images with statistical values.

22. Click the **Surfaces** button, choose the **Statistics** button (in the object properties area on the bottom left of the screen), and click the **Specific Value**, choose the **Detailed** tab (Figure 5A).
23. Choose the value you want to check, here we choose the volume (Figure 5A).
24. The list gives us all the object's volume information, if the image is a time series, the statistical annotation is visible in the viewing area for all time points. Choose the objects you want to analyze by clicking on them in the statistics list, the volume of the object that consequently changes with time information can be seen in the viewing area. (Figures 5B–5D).
25. Click the tab **Export Statistics on Tab Display to File**, and choose the file you want to save (Figure 5B).

Figure 5. The statistical analysis of the phase-separated proteins

(A) Click on the statistics tab and select the volume analyze function.
(B–D) The selected object's volume consequently changes with time.
(E) How to export the Statistics data.

26. Imaris-generated data files are compatible with Excel, other statistical analysis programs such as Graphpad prism can also be used. A comprehensive step-by-step video tutorial on using the statistics function in Imaris can be found here: <https://imaris.oxinst.com/learning/view/article/statistics-in-imaris>.

EXPECTED OUTCOMES

This protocol provides detailed steps for mimicking lipid-mediated protein phase separation on the membrane using GUV. The protocol includes purifying the protein, preparing GUVs, performing protein phase separation on the GUV membranes, and three-dimensional reconstruction of phase-separated protein. Approximately ~3–5 mg of protein can be purified from 250 mL of bacterial culture. Typical GUVs are 5–20 μm in diameter and can be seen when labeled with CM-Dil. We rarely observe GUVs larger than 20 μm . Phase-separated Ago1 proteins can be seen (Figure 3D) on GUVs which were incorporated with PI(4,5)P2. The 3D reconstruction in Imaris is based on the fluorescence signals, the result shows that Ago1 protein can phase separate on the membrane (Figure 3E). The 3D time-lapse assay shows that the Ago1 protein can dynamically change on the membrane (Figures 3H and 4). This protocol enables us to investigate the Ago1 protein's behavior on the membrane in the presence of PI(4,5)P2.

LIMITATIONS

This method can be used to prepare GUVs containing charged PIPs (e.g., PI(4,5)P2-containing GUVs), but it has some limitations. First, the lipid composition of vesicles may vary owing to the inhomogeneity of the lipid film on the glass substrate. Second, it is difficult to control the precise size of the GUVs. Third, multilamellar vesicles or lipid debris may form except for the GUVs. Fourth, the in vitro assay may not reflect the real state of one protein in vivo as the complexity of the composition of the membrane and the cell niche. Fifth, the requirement for purified proteins is another potential limitation.

TROUBLESHOOTING

Problem 1

Recombinant protein aggregation. Sometimes the protein aggregates in the buffer but not on the membrane (related to purifying the protein of interest).

Potential solution

Protein may aggregate under some conditions, such as unsuitable pH, NaCl concentration, or too high protein concentration.

- Increase the NaCl (more than 150 mM) concentration or adjust the pH.
- Centrifuge the protein sample and discard the aggregated protein.
- Decreasing the concentration of the protein can also avoid aggregation.

Problem 2

Low or no GUV formation after adding the growth buffer (related to step 8).

Potential solution

There are many reasons for this:

- The chloroform was not completely removed. Residual chloroform can prevent the growth of the lipid layers.

- The salt concentration may be too high to form the protein's phase separation. The growth buffer must contain some ions to enable the charged PIPs to incorporate into the GUVs (Beber et al., 2019). Although GUVs grow well under low or no salt conditions, we recommend using 50 mM NaCl in the growth buffer.

Problem 3

The purified protein contains a high amount of co-eluted unwanted proteins (related to [purifying the protein of interest](#)).

Potential solution

Further purify the protein using an AKTA purifier (see protein purification step 4).

Problem 4

The GUVs were not spherical after 3D reconstruction (related to [Image acquisition](#)).

Potential solution

GUVs can move in the buffer, be careful to move the confocal dish slowly and wait until the GUVs become still (this may need 10 or more minutes).

Note: The coverslip should be sealed to prevent evaporation.

Problem 5

Cannot detect protein phase separation on the GUV membrane (related to [preparation of GUV](#)).

Potential solution

PIPs were not incorporated into the GUV. Check the ion concentration of the growth buffer. It is difficult for charged PIPs to incorporate into GUVs when the growth buffer does not contain sufficient ions (see protocol step 8).

Problem 6

Cannot create ideal surfaces (related to [3D reconstruction](#) and image processing).

Potential solution

Reset the **Surfaces Detail** and **Background Subtraction** (see protocol 19). The **surface details** and **Background Subtraction** depend on the image you take, so you must set them accordingly. For background subtraction, if you are the first to set this, click on **Slice mode** in the top menu; from slice view, you can look at the slices of your image. Measure distances by left-clicking on the image. Set the distance as the **Background Subtraction** and go on to the next step. Choosing a proper setting for the reconstruction is an iterative process (see [Methods video S1](#)).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dahua Chen (chendh@ioz.ac.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article by (Gao et al., 2022) includes all datasets generated or analyzed during this study. Original data have been deposited to Mendeley Data: <https://data.mendeley.com/datasets/ttkmsp5hsz>. This paper does not report original code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101709>.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.Z. and D.C.; methodology, Y.Z. and Y.G.; writing, Y.Z.; Q.S.; supervision, D.C. All authors critically read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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