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Electroformation of Giant Unilamellar Vesicles and A Case Study of the Specific Host-Toxin Interaction of Lipid Membranes with Cassiicolin

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Detailed protocol

Electroformation of Giant Unilamellar Vesicles and A Case Study of the Specific Host-Toxin Interaction of Lipid Membranes with Cassiicolin

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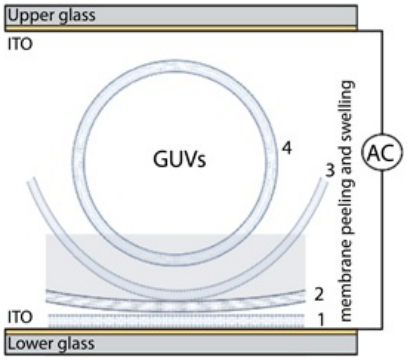
[Abstract] An alternating electric field is applied to induce swelling of thin lipid films and generation of giant unilamellar vesicles (GUVs) on an indium tin oxide (ITO)-coated glass surface. The process is, hereafter, referred to as the electroformation of GUVs. Several important parameters such as lipid manipulation, temperature, osmolarity and ionic strength of the solutions involved, and the electric field (current (DC, AC), amplitude, frequency) should be optimal for the successful electroformation of GUVs. In our case study, GUVs composed of lipid mixtures available in plant cells provide many benefits for studying the lipid-dependent pathogenicity of cassiicolin (Cas) toxins and thereby deciphering the host-selective toxin interaction of Cas toxins with the specific lipid membranes of plant cells. GUVs gently maintained in the solution furnish perfectly suspended and intact lipid membranes similar to cytoplasmic membranes enabling us to examine the selective binding of GFP-Cas1 and GFP-Cas2 to the specific lipid membranes. In this protocol, we briefly explain the principle of electroformation method and provide the experimental conditions and the manipulation for successfully making GUVs composed of plant lipids (DPPC, DPPC/DPPA, DPPC/MGDG, DPPC/DGDG, DPPC/stigmasterol, DPPC/sitosterol, DPPC/DGTS-d9, and DPPC/DGTS).



Keywords: Giant unilamellar vesicles, liposomes, cassiicolin, lipid-dependent pathogenicity, host specificity, electroformation, confocal microscopy, Vesicle Prep Pro

[Background] Cassiicolin (Cas), a toxin produced by the fungus *Corynespora cassiicola*, is responsible for corynespora leaf fall (CLF) disease in rubber trees. Cas toxin also causes leaf spot diseases in other host plants such as tomatoes, cucumbers, cotton, and soybeans (Dixon et al., 2009; Shrestha et al., 2017; Sumabat et al., 2018). To date, six Cas isoforms (Cas1 to Cas6) have been genetically identified by PCR-based detection from fungal isolates in various host plants and geographical origins (Déon et al., 2014, 2012). Nowadays, Cas toxin is widely accepted as the host-selective toxin in the specific plants. However, the molecular mechanisms underlying the host specificity of Cas isoforms have not been fully elucidated. Our recent study has successfully clarified the molecular mechanism underlying the lipid-dependent pathogenicity of Cas1 and Cas2 and cytotoxicity of these toxins on rubber leaves and thereby gaining insights into the molecular mechanism of the host specificity of Cas toxin (Ngo et al., 2021). Using STED confocal microscopy, we reveal that the binding of GFP-Cas1 and GFP-Cas2 on lipid membranes of GUVs are strongly dependent on the specific plant lipids. Membranes composed of negative phospholipids (DPPC/DPPA), glycerolipids (DPPC/MGDG, DPPC/DGDG), and sterols (stigmasterol, sitosterol) are more susceptible to the binding and disruption activities of Cas toxin than that composed of neutral phospholipids (DPPC) and betaine lipids (DPPC/DGTS-d9, DPPC/DGTS). For the first time, we successfully fabricated GUVs composed of different plant lipids and used them as a model of artificial cytoplasmic membranes to assay the binding activities of GFP-Cas1 and GFP-Cas2 using confocal microscopy, a powerful technique to study protein-lipid membrane interaction (Carvalho et al., 2008; Drücker et al., 2019). The results are summarized and clarified in further detail (Ngo et al., 2021). Importantly, the results produced by using GUVs system is complementary with that obtained by high-speed atomic force microscopy and low voltage Cryo-SEM in our case study, suggesting that GUVs mimicking cytoplasmic membranes of plant cells are very promising systems to study the lipid-dependent pathogenicity of not only mycotoxin but also other microbial toxins such as the pore-forming toxin (Drücker et al., 2019; Peraro and Van Der Goot, 2016).

Materials and Reagents

1. Biological materials

a. DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (Cat. 850355C-25mg, Avanti Polar Lipids, Alabama, USA, store at -20°C for months)

b. DPPA: 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (Cat. 830855P-25mg, Avanti Polar Lipids, Alabama, USA, store at -20°C for months)

c. Stigmasterol (Cat. 700062P-5mg, Avanti Polar Lipids, Alabama, USA, store at -20°C for months)

d. Sitosterol (Cat. 700095P-5mg, Avanti Polar Lipids, Alabama, USA, store at -20°C for months)

e. MGDG: Monogalactosyldiacylglycerol (Cat. 840523P-5mg, Avanti Polar Lipids, Alabama, USA, store at -20°C for months)

f. DGDG: Digalactosyldiacylglycerol (Cat. 840524P-5mg, Avanti Polar Lipids, Alabama, USA, store at -20°C for months)

g. DGTS-d9: 1,2-dipalmitoyl-*sn*-glycero-3-O-4'-[N,N,N-trimethyl(d9)]-homoserine (Cat. 857463P-1mg, Avanti Polar Lipids, Alabama, USA, store at -20°C for months)

h. DGTS: 1,2-dipalmitoyl-*sn*-glycero-3-O-4'-(N,N,N-trimethyl)-homoserine (Cat. 857464P-1mg, Avanti Polar Lipids, Alabama, USA, store at -20°C for months)

i. GFP-Cas1 and GFP-Cas2, Lab-made proteins, store at -80°C for months. The preparation of these proteins is reported (Ngo et al., 2021)

k. LB broth (Cat. L3022-1KG, Sigma, store at room temperature and dry condition for months)

l. BL21(DE3) (Cat. C2527H, NEB, store at -80°C for months)

2. Chloroform (Cat. 08402-55, nacalai tesque, Tokyo, Japan, store at room temperature)

3. HEPES (Cat. 02443-05, nacalai tesque, Tokyo, Japan, store at room temperature)

4. NaCl (Cat. 31320-05, nacalai tesque, Tokyo, Japan, store at room temperature)

5. EDTA (Cat. 15112-22, nacalai tesque, Tokyo, Japan, store at room temperature)

6. EGTA (Cat. 15214-92, nacalai tesque, Tokyo, Japan, store at room temperature)

7. D-Sorbitol (Cat. S1876-100G, Sigma, Tokyo, Japan, store at room temperature)

8. IPTG (Cat. I6758-5G, Sigma, store at -20°C for months)

9. Ampicillin sodium salt powder (Cat. A9518-5G, Sigma, store at 4°C for months)

10. KOH (Cat. 28616-45, nacalai tesque, Tokyo, Japan, store at room temperature)

11. $MgCl_2$ (Cat. 20935-05, nacalai tesque, Tokyo, Japan, store at room temperature)

12. Ethanol (99.5%) (Cat. 052-03343, Wako, Japan, store at room temperature)

13. 5 mol/l HCl (Cat. 081-05435, Wako, Japan, store at room temperature)

14. Milli-Q H_2O

Equipment

1. Vesicle Prep Pro (VPP) (Nanion, Germany)

2. STED confocal microscopy (Abberior Instruments, Germany)

3. Indium tin oxide (ITO)-coated glass slides (Nanion, Germany)

4. O-ring (16 mm) (Nanion, Germany)

5. Small glass bottlesマイティバイアル (Cat. 5-115-02 and 5-115-03, AXEL, Japan)

6. Sterile syringe filter (CA 0.22 µm) (Cat. 2-856-01, AS ONE Corporation, Japan)

7. Terumo syringe (20 mL) (JMDN code: 13929001, Terumo, Japan)

8. Micropipettes (2 – 20 µl) (Cat. 68401366, Monotaro, Japan)

9. Micropipettes (20 – 200 µl) (Cat. 68401375, Monotaro, Japan)

10. 200 µl Pipette tips natural (Cat. V-200BH, Monotaro, Japan)

11. 1000 µl Pipette tips natural (Cat. V-1000BH, Monotaro, Japan)

12. Disposable nitride gloves (Cat. EDGE® 82-133, Ansell)

13. Protective glasses (Cat. 54831745, Monotaro, Japan)

14. Kimwipes (Cat. NS3053900, CSC net, Japan)

15. Mettler toledo seveneasy S20 pH meter (Cat. Z654272, Sigma)

16. Ohaus® explorer® analytical balance (Cat. Z760420-1EA, MERCK)

17. Deep freezer (-25°C) (Model: GS-3120HC Nihon Freezer Co. Ltd., Japan)

18. Deep freezer (-80°C) (Model: CLN-35C, Nihon Freezer Co. Ltd., Japan)

19. Refrigerator (4°C – 8°C) (Model: SJ-56S-S, Sharp Co. Ltd., Japan)

20. Auto pure wd501 millipore (Cat. ZRQSVP03Y, Yamato, Japan)

21. FluoroDish cell culture dishes (FD35-100, WPI)

22. Aluminium foil (Sunfoil, Cat. 89842207, Monotaro, Japan)

22. Digital multimeter (Cat. DT-830B, Amazon)

23. Grease (Toray Molykote Silicone, Model FS-50, Toray, Japan)

24. Eppendorf tubes (Order Cat. 022431081, Eppendoft AG, Germany)

25. Ultrasonic cleaner (Cat. AUC-06L, AS ONE)

Software

1. Vesicle Control software compatible to windows operation system (Nanion, Germany).

2. Imspector software for STED confocal microscopy (Abberior Instruments, Germany)

3. Fiji-ImageJ (NIH, USA)

4. Illustrator (Adobe, USA)

Procedure

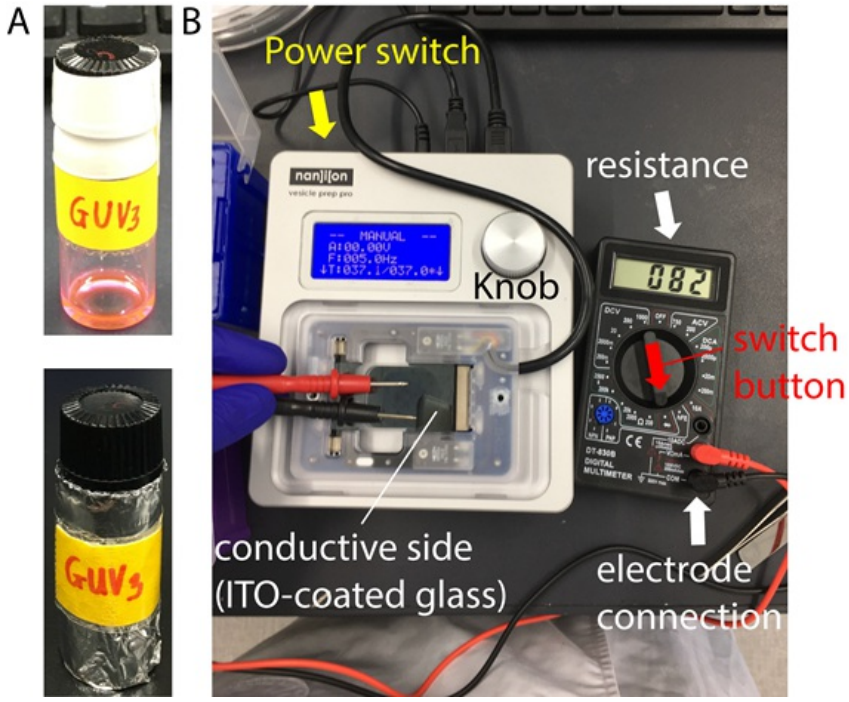


Figure 1. Preparation of lipid mixtures and general set up of the Vesicle Prep Pro (VPP) device for making thin lipid film on ITO-coated glass. (**A**) The glass bottle with a Teflon cap contains lipid mixtures before and after wrapping with an aluminum foil sheet. (**B**) Set up of the VPP device and the demonstration of how to check the conductive side of ITO-coated glass using a digital multimeter.

Principle: *We made the composite giant unilamellar vesicles (GUVs) composed of different plant lipids available in cytoplasmic membranes of plant cells for clarifying the binding of GFP-Cas1 and GFP-Cas2 to the specific lipid membranes using confocal microscopy. The electroformation methods for making GUVs have been well studied and established in several previous studies (Angelova and Dimitrov, 2007, 1986; Dimitrov and Angelova, 1988). Principally, an electric field (i.e., alternating current voltage (AC voltage) between 0 – 3 V amplitude and frequencies between 5 – 10 Hz) is applied and controlled by the VPP device between two electrodes contacting the conductive ITO sides of two glass surfaces (Eq. 1). The temperature of the system is determined based on the melting temperature (Tm) of lipids and set up at a value higher than the Tm of the major lipid component in the lipid mixture. Together, the high osmotic pressure in the electroformation buffer is also crucial to induce the peeling and swelling of the lipid bilayer under the applied electric field. High salt concentration hampers the peeling and swelling of lipid bilayers as interactions between the polar lipid headgroups are stabilized. The optimal electroformation buffer normally contains 0.2 – 1 M D-sorbitol or sucrose and 1 mM EGTA and 2 mM HEPES-NaOH (pH 7.4). Finally, the thickness (i.e., numbers of the lipid bilayers) of lipid thin films made on the ITO-coated glass should not be too thick or too thin. The optimal thickness should be approximately 50 – 100 bilayers and can be controlled by using 5 – 10 mM lipid mixtures and spreading out homogeneously on the ITO-coated glass. When using the VPP device (Nanion), the volume of chloroform lipid mixture and the size of the dry lipid film are predetermined depending on the size of the rubber O-ring and the applied volume of electroformation buffer.*

The voltage as a function of time V(t) is given by the equation:

$$V(t) = A * \sin(2\pi * f * t) \qquad \textbf{(Eq. 1)}$$

Where *A* is the maximum amplitude in V, *f* is the frequency in Hz, and *t* is time in second.

Hereafter, we will provide the detailed procedure and some tips in lipid manipulation to successfully make GUVs composed of various plant lipids.

A. Making composite lipid mixtures

***Note**: We prepared the composite lipid mixtures in pure chloroform as described in **Table 1**. We strongly recommend to use chloroform to dissolve lipid mixtures because this solvent is fastly volatile at room temperature. Despite the safety concerns of using chloroform, the handling of it in a small volume (~20 µl) is easy and can be done in the open Lab bench. We should wear protective glasses and gloves to manipulate chloroform. Some researchers suggest to use glass pipette to handle chloroform. However, we do not find any problem when using plastic pipette tip to manipulate precisely a small volume of chloroform. Importantly, chloroform is the best choice to dissolve lipids for the successful formation of thin lipid film on ITO-coated glass surface, a critical step in making GUVs.*

1. Dissolve composite lipids (Table 1) in chloroform to the desired concentration using a small glass bottle with a Teflon cap (1.5 mL or 4 mL) (Figure 1A). Wrap the bottle with an aluminum foil sheet to avoid direct contact with light.

2. Store the composite lipid solution in the deep freezer (-20°C – -25°C). When using, take the bottle out and warm it up at room temperature or in hand for approximately 10 min to prevent condensation of air moisture inside the lipid solution.

B. Making thin films on ITO glass

***Note**: Here, we describe the procedure to make GUVs using the "Vesicle Prep Pro (VPP)" device (Nanion). The device either works independently or controlled by the "Vesicle Control" software operated in Windows system.*

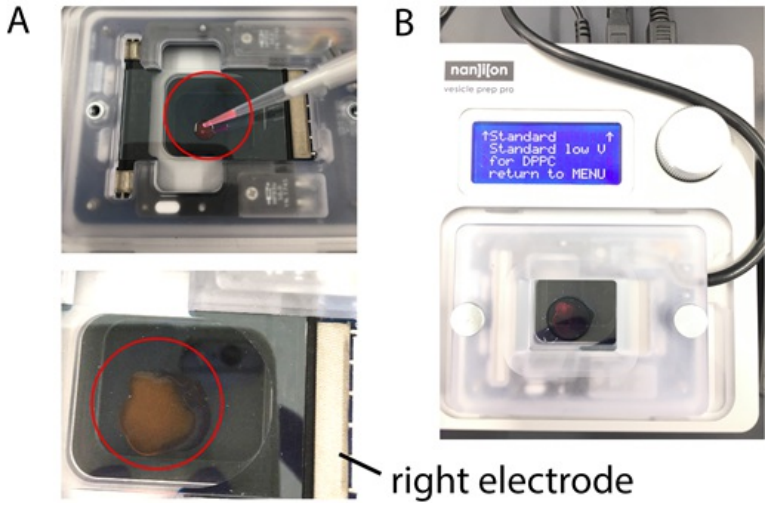


Figure 2. Making the thin lipid film on ITO-coated glass for electroformation of GUVs using the VPP device. (A) place and spread out the warm lipid mixtures dissolved in chloroform on the lower ITO-coated glass slide and let the solvent evaporate for approximately 10 min (B) place a rubber O-ring around the dry lipid film, add electroformation buffer inside the O-ring and cover it with the second ITO-coated glass slide with its conductive side facing down to furnish the elctroformation chamber between the two conductive sides of the glass surfaces. **Note**: the lower glass slide should not be hanged over the right electrode.

1. place an ITO-coated glass in the VPP chamber and confirm the conductive side of the glass surface using a digital multimeter (Figure 1B).

2. spread out 20 µl warm lipid mixture (~10 mM) on the conductive side of the ITO-coated glass surface with a pipette to yield the homogeneous lipid film slightly smaller than the O-ring used in the next step (Figure 2A).

3. let the chloroform evaporate completely for approximately 10 min at room temperature to obtain the dry and homogeneous lipid film on the ITO slide (Figure 2A).

4. slightly grease a rubber O-ring of the appropriate size on its lower side and place it around the lipid film on the first ITO-coated glass (Figure 2B).

C. Electroformation of GUVs

1. Apply the appropriate volume of electroformation buffer to the lipid film and close the chamber with the second ITO-coated glass slide. The conductive side of the upper ITO electrode has to face downwards, enabling the application of an AC voltage across the lipid film deposited on the lower ITO electrode (Figure 2B). Note: Avoid bubble formation in the chamber and use a high osmotic electroformation buffer for generating GUVs. We suggest an electroformation buffer containing 1 M D-sorbitol, 1 mM EGTA, and 2 mM HEPES-NaOH (pH 7.4) and apply a sonication for 1 min to disrupt any gas bubbles in solution using a bath sonicator type (Ultrasonic cleaner (AUC-06L, AS ONE). Do not use the low osmotic electroformation buffer containing less than 0.2 M D-sorbitol or sucrose because GUVs are poorly produced.

2. Close the VPP's chamber and start the predefined voltage protocol suited for the lipids of choice. The AC voltage, frequency and temperature can be defined via the Vesicle Control software or manually on the VPP device itself. We use the protocol for DPPC defined by the Vesicle Control software. Initial (0 V, 5 Hz, 50°C, 5 min); Start (3 V, 10 Hz, 50°C, 10 min); Main (3 V, 10 Hz, 50°C, 60 min); Fall (0 V, 10 Hz, 50°C, 5 min); End (0 V, 10 Hz, 20°C), referred to Figure 3. **Tips**: When closing the VPP's chamber, press and hold firmly and stably the chamber by one hand and sequentially fix the chamber with two screws by another hand to avoid shaking and forming gas bubbles inside.

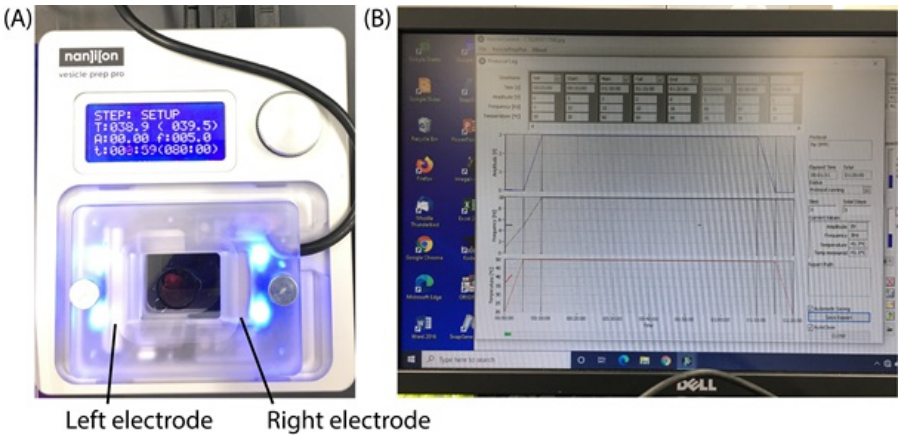


Figure 3. A typical setup for electroformation of GUVs. (A) The VPP device is set up and run with the predefined voltage protocol suitable for making GUVs composed of DPPC lipids. (B) The Vesicle Control software to control and record in real-time the predefined protocol (i.e., amplitude (*A*), frequency (*f*), and temperature). **Tips**: Make sure the conductive side of the lower glass slide that contacts only the left electrode and the conductive side of the upper glass slide contacts only the right electrode. If wrong, no voltage is applied across the thin lipid film, resulting in none or poor production of GUVs (Figure 3A).

3. Open the electroformation chamber by carefully removing the upper ITO-coated glass slide after the electroformation process is finished. Gently collect the GUV solution from the lower ITO-coated glass slide and transfer it to an Eppendorf tube. Store GUVs at 4°C until usage. GUVs can be kept well for one to two weeks under this condition.If sedimentation of GUVs happens, shake the tube gently before usage.

4. Clean the ITO slides and the O-ring with Milli-Q water and isopropanol after usage and verify the generation of GUVs under a microscope e.g. at 100x magnification.

D. Troubleshooting

1. The sorbitol or sucrose solutions used for electroformation are prone to contaminations when stored in a larger volume at room temperature. We recommend preparing these solutions, sterile filter them, and make small aliquots e.g. into 2 ml Eppendorf tubes, and keep them frozen until actual usage.

2. Contaminated lipid stock solutions are the main source for poor GUV quality after electroformation. Plastic containers or rubber sealings are not suited for the storage of the lipids dissolved in chloroform and will lead

to contamination of the lipid stock solutions. Thus exclusively use glass tubes or vials equipped with Teflon caps for storage of the lipid solutions.

3. GUVs tend to burst if the AC voltage is abruptly switched off. Please consider this effect and introduce voltage ramps in the beginning and – most importantly – at the end of each voltage protocol used for electroformation. The voltage can e.g. be ramped down continuously back to 0 V within 5 to 10 minutes for a gentle termination of the process. Do not apply the voltage amplitude (A) higher than 3 V because the higher voltage tends to create more gas bubbles during the electroformation of GUVs.

4. Store the generated GUVs in plastic containers like Eppendorf tubes at 4°C in the fridge until usage. GUVs solution cannot be frozen and thawed because these processes destroy the vesicles.

5. Make sure to carefully collect the generated GUVs solution from the lower ITO-coated glass e.g. with a 1 ml pipette as the vesicles might be damaged when exposed to large shear forces upon the usage of small pipette tips or syringes.

Representative Data

Several previous studies have been successfully made GUVs to study the protein-lipid interaction onto lipid membranes (Carvalho et al., 2008; Drücker et al., 2019). Recently, we have successfully fabricated the composite GUVs composed of the specific plant lipids to study the lipid-dependent pathogenicity of Cas1 and Cas2, as representatively shown in ***Table 1*** *and* ***Figures 4 and 5.*** *Data are adapted from our original work (Ngo et al., 2021).*

Table 1. Lipid compositions for making the composite giant unilamellar vesicles (GUVs). Data are adapted from Figure 4-table 1 (Ngo et al., 2021).

Plant Lipids	**DPPC (GUVs)**	**DPPC/ DPPA (GUVs)**	**DPPC/ MGDG (GUVs)**	**DPPC/ DGDG (GUVs)**	**DPPC/ Stigmasterol (GUVs)**	**DPPC/ Sitosterol (GUVs)**	**DPPC/ DGTS-d9 (GUVs)**	**DPPC/ DGTS (GUVs)**
DPPC	10 mM	8 mM	7.5 mM	7.5 mM	9 mM	9 mM	7.5 mM	7.5 mM
DPPA		2 mM						
MGDG			2.5 mM					
DGDG				2.5 mM				
Stimasterol					1 mM			
Sitosterol						1 mM		
DGTS-d9							2.5 mM	
DGTS								2.5 mM
18:1 Liss Rhod PE	0.1 mM	0.1 mM	0.1 mM	0.1 mM	0.1 mM	0.1 mM	0.1 mM	0.1 mM
16:0 Biotinyl PE	0.5 mM	0.5 mM	0.5 mM	0.5 mM	0.5 mM	0.5 mM	0.5 mM	0.5 mM

***Note**: Incorporation of the small amount of biotinyl-PE lipids into lipid membrane helps to absorb GUVs onto the glass-chemically modified surface using biotin-avidin conjugation. However, we can also simply absorb GUVs on the cleaned glass surface by a gravity force. We incorporate rhodamine-PE into lipid membrane to fluorescently image GUVs using STED confocal microscopy.*

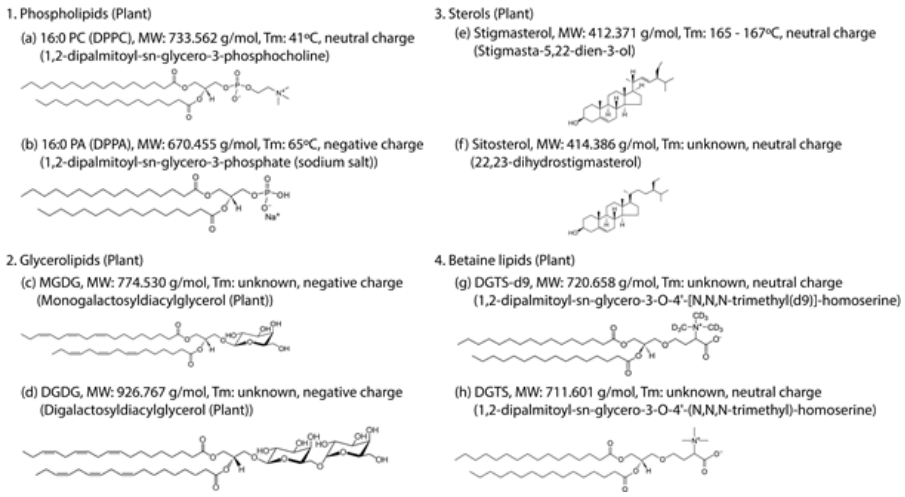


Figure 4. Structures and physical properties of different lipids available in cytoplasmic membranes of plant cells. We used these lipids to make the composite GUVs. Tm: melting temperature. Data are obtained in "safety data sheet" (Avanti Polar Lipids, Alabama, USA) and adapted from Figure 1-figure supplement 3 (Ngo et al., 2021).

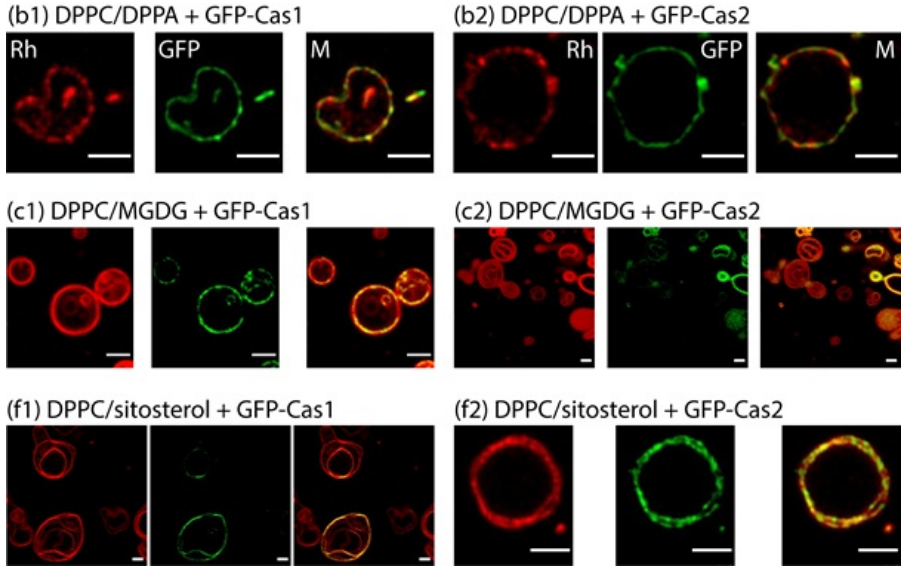


Figure 5. Representative confocal microscopic images of the binding of GFP-Cas1 and GFP-Cas2 to the composite GUVs composed of negatively charged phospholipid DPPC/DPPA (b1, b2), phospholipid/glycerolipid DPPC/MGDG (c1, c2), and phospholipid/sterol DPPC/sitosterol (f1, f2). Rh: rhodamine. GFP: GFP-Cas1 or GFP-Cas2. M: merged. Data are adapted from Figure 4 (Ngo et al., 2021).

Data analysis

Data processing, analyses, statistical tests, and the number of experimental replicases can be found in our original work (i.e., in the "Results" section: Figure 4 and Table 2, and in the "Methods" section: Confocal microscopy) (Ngo et al., 2021)

Notes

Electroformation methods of GUVs were originally studied and investigated by Angelova MI and Dimitrov DS (Angelova and Dimitrov, 1986). Nanion successfully exploits the principle and methods of this study to develop the Vesicle Prep Pro (VPP) device and protocol to make GUVs.

Recipes

1. Lab-made PcoldI GFP-Cas1 and PcoldI GFP-Cas2 plasmids

2. BL21 (DE3) competent cells

3. LB broth + Ampicillin

- Dissolve 20 g LB broth (L3022-1KG, Sigma) in 1L water.

- Autoclave at 121°C for 15 min.

- Cool down LB broth to room temperature and add ampicillin to a final concentration of 100 µg/ml.

Note*: The detailed preparation of GFP-Cas1 and GFP-Cas2 can be found in "Materials" and "Protein prepation" sections (Ngo et al., 2021).*

4. Electroformation buffer

- 1 M D-sorbitol

- 1 mM EGTA

- 2 mM HEPES-NaOH (pH 7.4)

Note*: We should sterilize buffer by using sterile syringe filter (CA 0.22 µm) and make small aliquots (e.g., 0.5 – 1 ml) and store them at -20°C. When using, we can warm up one small aliquot at 37°C for approximately 30 min.*

5. Imaging buffer

- 5 mM HEPES-NaOH (pH 7.4)

- 5 mM NaCl

- 0.05 mM EDTA

- 1 M D-sorbitol

Note*: We should sterilize buffer by using sterile syringe filter (CA 0.22 µm). We can use this buffer to image the GUVs on the cleaned glass surface without chemical modification. In most cases, GUVs weakly absorb onto the glass surface by gravity force. When imaging the negatively charged DPPC/DPPA GUVs we should add salts (e.g., 300 mM NaCl and 10 mM $MgCl_2$) into the imaging buffer for better absorption of these GUVs onto the glass surface.*

Acknowledgments

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Competing interests

The authors declare no competing interests.

Ethics

This protocol does not include any human subject and does not require the specific ethics committee that approved the experiments.

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