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Plasma Membrane Preparation from *Lilium davidii* and *Oryza sativa* Mature and Germinated Pollen

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[Abstract] Pollen germination is an excellent process to study cell polarity establishment. During this process, the tip-growing pollen tube will start elongating. The plasma membrane as the selectively permeable barrier that separates the inner and outer cell environment plays crucial roles in this process. This protocol described an efficient aqueous polymer two-phase system followed by alkaline solution washing to prepare *Lilium davidii* or *Oryza sativa* plasma membrane with high purity.
Keywords: Mature pollen grains, Germinated pollen grains, Plasma membrane, Aqueous polymer two-phase system, Alkaline solution

[Background] Pollen plasma membrane contains various proteins that are vital for pollen tube growth and fertilization, such as receptor-like kinases (Wang *et al.*, 2016) and ion channels (Hamilton *et al.*, 2015). Isolating pure plasma membrane (PM) is the premise for the comprehensive PM proteome analysis. There are mainly four methods for PM preparation: differential centrifugation, density gradient centrifugation, preparative free-flow electrophoresis and the aqueous polymer two-phase system. Normally, differential centrifugation is often combined with density gradient centrifugation together to separate the subcellular components according to their size, shape and density. This technique is rapid, but due to the organelle density's overlap, the resultant PM yield and purity are low (Schindler and Nothwang, 2006). Both free-flow electrophoresis and the aqueous polymer two-phase system separate membrane vesicles according to their surface properties. These two methods can enrich PM pure enough for proteomic analysis (Alexandersson *et al.*, 2007). However, the instrument for the free-flow electrophoresis is complicated to operate (Sandelius *et al.*, 1986). On the contrast, the aqueous polymer two-phase system can be performed easily and rapidly with centrifugation, making this method more convenient for PM preparation. PM enriched by the aqueous polymer two-phase system present in the form of vesicles which contain some cytoplasm contaminations (Alexandersson *et al.*, 2008). Treatment with alkaline solution (100 mM Na_2CO_3, pH 11.5) can open these vesicles into sheets to release the contaminations (Fujiki *et al.*, 1982).

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Materials and Reagents

1. 1,000 µl pipette tips (Corning, Axygen®, catalog number: T-1000-B)
2. 20 x 10 cm envelope
3. 50 ml tube (Corning, catalog number: 430829)
4. 60 x 15 mm Petri dish (Corning, catalog number: 430196)
5. 150 x 25 mm Petri dish (Corning, catalog number: 430599)
6. Gauze
7. 10 ml tube (Biosharp, catalog number: BS-100-M)
8. 100 µm cell strainer (Corning, Falcon®, catalog number: 352360)
9. 2 ml microtube (SARSTEDT, catalog number: 72.694.005)
10. 10.4 ml ultracentrifuge tube (Beckman Coulter, catalog number: 355603)
11. 26.3 ml ultracentrifuge tube (Beckman Coulter, catalog number: 355654)
12. Lily mature pollen grains harvest according to Han *et al*. (2010)
13. Rice mature pollen grains harvest according to Dai *et al*. (2007)
14. Boric acid (H_3BO_3) (Sigma-Aldrich, catalog number: B9645)
15. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9541)
16. Calcium chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: C2661)
    Note: This product has been discontinued.
17. Sucrose (Sigma-Aldrich, catalog number: S7903)
18. Calcium nitrate tetrahydrate, $Ca(NO_3)_2{\cdot}4H_2O$ (Sigma-Aldrich, catalog number: C1396)
19. Thiamine hydrochloride (VB_1) (Sigma-Aldrich, catalog number: T4625)
20. Poly (ethylene glycol), average M_n 4,000 (PEG 4000) (Sigma-Aldrich, catalog number: 81240)
21. 3-(N-Morpholino)propanesulfonic acid (MOPS) (Sigma-Aldrich, catalog number: M1254)
22. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E6758)
23. DL-Dithiothreitol (DTT) (Sigma-Aldrich, catalog number: D0632)
24. Phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: P7626)
25. cOmplete, EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics, catalog number: 04693132001)
26. L-ascorbic acid (VC) (Sigma-Aldrich, catalog number: A7506)
27. Poly (vinylpolypyrrolidone) (PVPP) (Sigma-Aldrich, catalog number: 77627)
28. Potassium phosphate tribasic (K_3PO_4) (Sigma-Aldrich, catalog number: P5629)
29. Polyethylene glycol, average mol wt 3,350 (PEG 3350) (Sigma-Aldrich, catalog number: P4338)
30. Dextran T-500 (Pharmacia, catalog number: 17-0320-01)
31. Sodium carbonate (Na_2CO_3) (Sigma-Aldrich, catalog number: S7795)
32. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A1933)
33. Lily pollen germination medium (see Recipes)
34. Rice pollen germination medium (see Recipes)
35. Homogenate buffer (see Recipes)

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36. Plasma membrane isolation buffer (see Recipes)
37. Aqueous polymer two-phase system (see Recipes)
38. Dilution buffer (see Recipes)
39. Washing buffer (see Recipes)

Equipment

1. Pipette (Gilson, model: P1000N)
2. Vortex
3. Balance
4. Centrifuge (Beckman Coulter, model: J2-HS)
5. Homogenizer (MP Biomedicals, model: FastPrep®-24)
6. Ultra-centrifuge (Beckman Coulter, model: Optimal™ L-80XP)
7. Microscope with 5x and 10x objective (Carl Zeiss, model: Axio Imager 1)

Software

1. ZEN lite software (2012, blue edition)

Procedure

A. Mature pollen grain collection
   1. Use 20 x 10 cm envelope to collect the mature pollen grains (MPGs) at noon (11:00 AM-1:00 PM) when the anthers are dehiscing.
   2. Transfer the MPGs to 50 ml tubes.
   3. Use the MPGs immediately or add allochroic silica gels to them and then store at -80 °C.

B. *In vitro* pollen germination

   The initiation procedures of *in vitro* germination are different for lily and rice pollen. For lily pollen, there should be a pretreatment to wash out the thick lipid coat, while the thin-walled rice pollen does not need this wash.

   1. *Lilium davidii* pollen germination

      Note: This procedure was modified from Ren et al. (1998) and Prado et al. (2004).

      a. Transfer the -80 °C stored MPGs to -20 °C in the dark for 2 weeks before the *in vitro* germination.
      b. Put 0.5 g MPGs into a dark, humid environment at 4 °C for 10 h.

      Note: The dark, humid environment is custom made as follows: Put MPGs in a Petri dish (60 x 15 mm) (Figure 1A), covered with gauze (Figure 1B), and then placed them in a larger Petri dish

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(150 x 25 mm) with 50 ml ddH_2O (Figure 1C). This arrangement only permitted gauze contact with the ddH_2O, do not let the MPGs contact the ddH_2O directly.

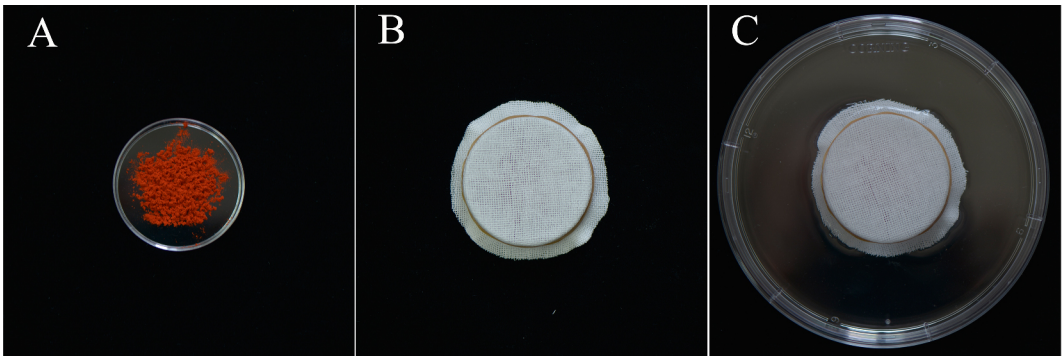

Figure 1. Set up of the humid environment. A. Put 0.5 g lily MPGs in a 60 x 15 mm Petri dish; B. Cover the Petri dish with four layers of gauzes; C. Put the set of B into a 150 x 25 mm Petri dish with 50 ml ddH_2O.

c. Wash the MPGs twice with 5 ml germination medium in a 10 ml tube and discard the flow-through after centrifuging at room temperature at 4,500 *x g* for 5 min.
d. Put the washed MPGs (Figure 2A) into 100 ml germination medium in a Petri dish (60 x 15 mm), and then incubate them in the dark at 28 °C for 2 h with gentle shaking at 75 rpm/min.

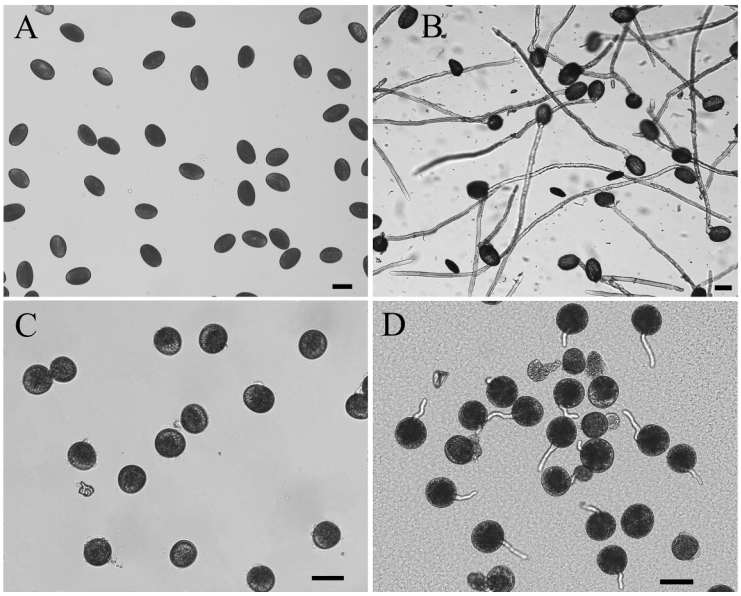

Figure 2. Morphology of *Lilium davidii* and *Oryza sativa* MPGs and GPGs. A. *Lilium davidii* MPGs; B. *Lilium davidii* MPGs *in vitro* germinated for 2 h; C. *Oryza sativa* MPGs; D. *Oryza sativa* MPGs *in vitro* germinated for 15 min. Bars = 50 μm.

e. Use microscope with 5x objective to observe the germination rate, make sure the germination rate is more than 90% and the pollen tube is approximately 500 μm long (Figure 2B).

f. Collect the germinated pollen grains (GPGs) by using 100 μm cell strainers, discard the flow-through that contains the ungerminated MPGs.

g. Use the GPGs immediately or store them (transfer the mass retained by the strainer to 50 ml tubes, do not add any solution) at -80 °C.

2. *Oryza sativa* pollen germination

Note: This procedure was modified from Dai et al. (2007).

a. Put 0.5 g freshly collected MPGs (Figure 2C) immediately into 100 ml germination medium in a Petri dish and culture them at room temperature with gentle shaking for about 15 min.

Note: Rice MPGs must be freshly collected, if not, they will lose the activity for germination.

b. Use microscope with 10x objective for observation to get synchronously germinated pollen tubes (Figure 2D).

Note: If pollen germination is not synchronized, or the germination rate is less than 80%, the material should not be used for GPGs PM proteomic analysis.

c. Collect the GPGs through centrifugation at 4 °C, 1,000 *x g*, 5 min.

d. Use the GPGs immediately or store at -80 °C.

C. Microsomal vesicles isolation

1. Use 6 ml homogenate buffer to suspend 0.5 g pollens (MPGs or GPGs) in a 50 ml beaker, and then transfer the suspension to three 2 ml microtubes with equal volume.

Note: Lily MPGs should be washed as step B1c before starting step C1.

2. Homogenize the pollens in 2 ml microtubes by using the FastPrep®-24 at the speed of 6.5 m/sec for 120 sec.

Note: For rice pollens, using the speed of 6.5 m/sec for 30 sec is enough.

3. Centrifuge the suspension at 4 °C, 1,500 *x g* for 5 min to remove cell debris, and then transfer the supernatants to new tubes.

4. Centrifuge the resulting supernatants at 4 °C, 12,000 *x g* for 20 min to remove mitochondria, then transfer the supernatants to new tubes.

5. Centrifuge the resulting supernatants at 4 °C, 31,000 *x g* for 15 min to remove other organelle contaminants, then transfer the supernatants to ultracentrifuge tubes.

6. Ultracentrifuge the resulting supernatants at 4 °C, 100,000 *x g* for 1 h, the resulting pellets are the total microsomal vesicles (MSVs).

D. Plasma membrane enrichment

Aqueous polymer two-phase system is an effective tool to enrich plasma membrane.

The following protocol was designed according to the principle of Schindler and Nothwang (2006).

1. Suspend the MSVs in 1.2 ml plasma membrane (PM) isolation buffer.

2. Put 1.0 g suspended MSVs into the 8.0 g aqueous polymer two-phase system, mix by vortex and then centrifuge at 4 °C, 4,200 *x g* for 30 min.

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Note: The aqueous polymer two-phase system were prepared by using the electronic balance, because the concentrations of the components PEG 3350 and Dextran T-500 were mass ratios (see Recipes).

3. Transfer 3.0 ml of the top layer into another 8.0 g aqueous polymer two-phase system, mix and then centrifuge at 4 °C, 4,200 *x g* for 30 min.
4. Repeat step D3 once.
5. Transfer 3.0 ml of the top layer to an ultracentrifuge tube, dilute it 3-5 folds with dilution buffer, centrifuge at 4 °C, 200,000 *x g* for 1 h. The resulting pellets are PM vesicles.
6. Suspend the resulting pellets with 50 µl dilution buffer for immediate use or store this suspension at -80 °C.

E. Plasma membrane purification

Alkaline solution can change the PM vesicles into PM sheets to remove the imbedded cytoplasm. The following protocol was mainly according to Fujiki *et al*. (1982).

1. Dilute the PM vesicles with the washing buffer to protein concentration at 0.02~1.00 µg/µl.
2. Keep in an ice-bath for 30 min with occasionally vortexing at every 10 min.
3. Centrifuge at 4 °C, 50,000 *x g* for 1 h. Discharge the supernatants and the resulting pellets are the purified PM.
4. Wash the purified PM pellets gently with cold ddH_2O, don't suspend the pellets, and then discard the ddH_2O.

   Note: Usually, starting with 0.5 g pollen can get about 20 µg purified PM proteins. Their purity can be tested through Western blot by using antibodies for PM-specific P-type H^+-ATPase and some organelle specific proteins, such as nuclear protein histone H1.
5. Use the purified PM immediately or store the pellets at -80 °C.

Data analysis

Digital images of lily and rice germinated pollens were obtained using an upright light microscope (Axio Imager 1, Carl Zeiss, Germany) and ZEN lite software (2012, blue edition).

Notes

Don't over homogenize the pollens. Too much cell debris will reduce the capacity of aqueous polymer two-phase system for plasma membrane purification. The homogenization will be fine when 80% pollens (8 out of 10 pollens) are broken observed under microscope.

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Recipes

Note: Use MilliQ water to prepare the following solutions, do not need to autoclave or sterilize by filtration.

1. Lily pollen germination medium
   1.6 mM H_3BO_3
   1.0 mM KCl
   500 μM $CaCl_2$
   15% (w/v) sucrose
2. Rice pollen germination medium
   40 mg/L H_3BO_3
   3 mM $Ca(NO_3)_2{\cdot}4H_2O$
   3 mg/L VB_1
   10% (w/v) PEG4000
   250 mM sucrose
3. Homogenate buffer
   250 mM sucrose
   Note: For lily pollen, use 15% (w/v) sucrose.
   50 mM MOPS, pH 7.8
   1 mM EDTA
   1 mM DTT
   1 mM PMSF
   1x protease inhibitor cocktail
   Note: For lily pollen, add 5 mM ascorbic acid, 0.6% (w/v) PVPP, 1% (m/v) PMSF.
4. Plasma membrane isolation buffer
   250 mM sucrose
   5 mM potassium phosphate, pH 7.8
   1 mM DTT
   1 mM PMSF
5. Aqueous polymer two-phase system
   6.5% (w/w) PEG3350
   6.5% (w/w) Dextran T-500
   250 mM sucrose
   5 mM KCl
   5 mM potassium phosphate, pH 7.8
   1 mM DTT
   Note: For lily, use 6.3% (w/w) PEG3350 and 6.3% (w/w) Dextran T-500.
6. Dilution buffer
   250 mM sucrose

50 mM MOPS/KOH, pH 7.8
1 mM DTT
1 mM PMSF

7. Washing buffer
   100 mM sodium carbonate, pH 11.5

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