

BILAYER CHANNEL RECONSTITUTION

(1) 1% Agar Salt Bridge

1.0g Agar

3.75g KCl

in 100ml distilled water, store at 4°C.

(2) Cs solution: (Cesium Methanesulfonate)

1) 50 mM Cs⁺ solution

0.209 MOPS, 10mM

1.14 g CH₃SO₃Cs, 50mM

in 100 ml distilled water, adjust to pH 7.2 by CsOH, store at RT.

2) 3M Cs⁺ solution

6.84 g CH₃SO₃Cs, 3M in 10 ml distilled water, store at RT.

(3) Ca²⁺-EGTA mixture:

1.46g CaCl₂ 99.4mM

3.8g EGTA 100mM

in 100 ml distilled water, store at RT.

(4) lipid solution

PE (L- α -phosphatidylethanolamine) 10mg/ml in Chloroform.

Store at -40°C.(Avanti Polar Lipids, INC #840025C)

PS(L- α -phosphatidylserine) 10mg/ml in Chloroform.

Store at -40°C (Avanti Polar Lipids, INC #840032C)

Before painting, mix 25 μ l PE with 25 μ l PS in a glass vial and dry under N₂, then dissolve in 20 μ l n-decane. Lipid final concentration is 25 mg/ml. Lipid concentration of 25-30mg/ml is OK.

(5)Electrode:

Polished the silver wire by sand paper, and then soaked in the Chlorox Bleach for 2-3 min. Clean it and then put Ag/AgCl in salt bridge tube. Fix it with parafilm.

Procedures for lipid bilayer experiments

1. Warm up the Axopatch Intergrating Patch Clamp, and digidata 1322A system for 20min.

2. Fix 250 μ m aperture delrin cuvette in trans chamber.

3. connect one electrode from *cis* to headstage input, connect another electrode from *trans* to headstage ground.

4. Add 900 μ l 50 mM Cs⁺ solution in *cis*, add 550 μ l 50mM Cs solution in *trans*.

5. Use glass rod painting lipid on aperture to get seal .

6. After seal, take 75 μ l 50mM Cs solution from *cis*, then add 75 3 M Cs⁺ solution in *cis*. The final concentration of Cs⁺ in *cis* is 300mM.

7. Add SR(50-100 μ g protein) in cis, mix it well.
8. When channel appear, remove chemical gradient by adding 50 μ l 3M Cs solution in trans solution. The final concentration of Cs⁺ in trans is 300mM. Channel should be disappeared.
9. Add voltage from -40mv to get amplitude value of channels. Record the channels around -40mv as control, and then add doses of drugs to cis (or sometimes to trans) and record again.

Preparation of SR from VSM

Solution

1. PBS solution:

Dry powder in foil pouches from Sigma, each pouch dissolved in 1 liter deionized water. Store at 4°C.

2. Homogenate buffer, Sucrose buffer(pH=7.4)

20 mM HEPES

1 mM EDTA

255 mM Sucrose

Adjust pH by adding NaOH to pH 7.4, then filter the solution, and store in 20°C freezer.

Before using, add proteinase inhibitor(PI) as follows, always use 80ml for two cow hearts vessel homogenizing.

A, Leupeptin, final concentration 2 μ M, (68 μ l, 1mg/ml, in 80 ml homogenate buffer)

B, 2 or 3 pellets of cocktail proteinase inhibitor to 80 ml homogenate buffer

C, PMSF dissolve in EtOH first, and then add to buffer, final concentration is 1mM

D, Na₃VO₄ final concentration is 1 mM.

3. Resuspend solution

2.25g NaCl (0.9%NaCl)

25.67g sucrose(0.3M) in 250ml distilled water, store at 4°C.

Before using, add 4l 100 mM PMSF in 4ml resuspend solution, the final concentration of PMSF is 100 μ M.

Procedure

1. separate cow heart coronary artery(actually take all the artery for enough amount) in 15 to 20 minutes for per heart. Put vessel in PBS solution on ice.
2. Clean vessel in PBS solution by removing fat and other tissue out of the vessel. Then cut the vessel in luminal way and scratch several times to remove the endothelia cells away, proceeding on ice. Put in PBS solution for 20min around.
3. Transfer vessel to homogenate buffer at high speed for 2 min. further treated by hand in Dounce homogenizer.
5. Sonicate homogenate for 20 seconds x 3times.

6. centrifuge the homogenate at 4000g, i.e. 7140 rpm of JA20 rotor, 20 minutes, 4°C, discard pellet (Check the conversion from RCF to RPM by Beckman centrifuge website on line).

7. Centrifuge the supernatant at 8000g, i.e. 10100 rpm of JA20.1 rotor, 35min, 4°C. Keep pellet, termed as the SR membrane.

9. Resuspend the pellet in freshly prepared resuspend solution.

10. Measure the concentration of protein, then aliquot the SR and frozen in liquid N₂. Store at -80°C until use.

Purification of SR from VSM

Solution

1. **Sucrose solutions** (Percent by weight) plus 10 mM HEPES, pH 7.0, the concentration are 45%, 40%, 35%, 30% and 27% respectively.

2. Sucrose buffer (pH=7.4) for dilution.

20mM HEPES

1mM EDTA

255mM Sucrose

Adjust pH by adding NaOH to pH 7.4, then filter the solution, and store in -20°C freezer.

3. Resuspend solution

2.25g NaCl (0.9% NaCl)

25.67g sucrose (0.3M)

in 250 ml distilled water, store at 4°C.

Before using, add 4µl 100mM PMSF in 4ml resuspend solution, the final concentration of PMSF is 100µM.

Procedure:

1. the pellet, termed the SR membrane, will be resuspended in a small volume (1-2ml) of resuspend solution.
2. formed discontinuous sucrose gradient in a SW 32 centrifuge tube (Beckman). From bottom to the top, sucrose solutions were layered sequentially, 2 ml of 45%, 3ml of 40%, 4ml of 35%, 3ml of 30%, and 2ml of 27%.
3. 15mg of unfractionated SR was layered on the top of the gradient and then spun at 64,000g, (SW32, 22,800 rpm) for 14hrs.
4. Fractions from 30-33% sucrose and 37-40% sucrose contained the purified light and heavy SR fractions, respectively. Separated the heavy and light SR fractions, diluted by 10 volume of 0.25M sucrose buffer, then centrifuged at 40,000g for 90 min at 4°C.

5. The pellet will be resuspended in a resuspend solution containing 100 μ M phenylmethylsulfonyl fluoride, aliquoted, frozen in liquid N₂, and stored at -80°C until use.

Fluorescence image Measurements

Solution

A.

	FM	mM	1000ml	2000ml
NaCl	58.44	137	8.0g	16.0g
Glucose	180.2	10	1.8g	3.6g
HEPES	238.3	20	4.77g	9.54g
KCl	74.44	5.4	402.5mg	805.0mg
NaHCO ₃	84.01	4.2	352.8mg	705.6mg
Na ₂ HPO ₄	141.96	3	425.9mg	851.8mg
KH ₂ PO ₄	136.1	0.4	54.4mg	108.8mg
MgCl ₂ .6H ₂ O	203.3	0.5	101.6mg	203.3mg
MgSO ₄ .7H ₂ O	246.5	0.8	197.2mg	394.4mg

Adjust pH to 7.4 with NaOH and HCl, keep at 4°C.

B. 1M CaCl₂

CaCl₂.H₂O FW:147 14.7g/100ml=1M

Adjust pH to 7.4 keep at room temperature

C. Hank's Buffer

100 ml Buffer A+130 μ l Buffer B.

D.2.5% BSA

25mg/ml in Hanks'

E.10% pluronic F-127. Dissolve 100mg Pluronic in 1 ml DMSF at 40°C, keep at RT.(Pluronic help the dissolve better, improve loading, and reduce dye compartmentalization)

F.1M EGTA(FW:380.4)

19.02g EGTA/50ml H₂O, adjust pH to >8.0 with 1 M NaOH, when all EGTA dissolved into solution, then adjust pH to 7.4, keep at RT.

Protocol for Fluorescence image system

General Switch



