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) Ca^{2+}-EGTA mixture:

1.46g CaCl2 99.4mM
3.8g EGTA 100mM
in 100 ml distilled water, store at RT.

(4) lipid solution

PE (L-a-phosphatidylethanolamine) 10mg/ml in Chloroform.
Store at –40°C.(Avanti Polar Lipids, INC #840025C)
PS(L- a-phosphatidylserine) 10mg/ml in Chloroform.
Store at –40°C (Avanti Polar Lipids, INC #840032C)
Before painting, mix 25µl PE with 25µl PS in a glass vial and dry under N_2, the dissolve in 20µl 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 250µm 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 900µ1 50 mM Cs^+ solution in *cis*, add 550 µ1 50mM Cs solution in trans.
5. Use glass rod painting lipid on aperture to get seal .
6. After seal, take 75 µ1 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 μg protein) in cis, mix it well.
8. When channel appear, remove chemical gradient by adding 50 μ1 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 μM,(68 μl, 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 buffe, final concentration is 1mM
D,Na_3VO_4 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 100μM.

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 lumenal 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, 4oC, 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, 4oC. Keep pellet, termed as the SR membrane.
9. Resuspend the pellet in freshly prepared resuspend solutoon.
10. Measure the concentration of protein, then aliquot the SR and frozen in liquid N_2. 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 –20oC freezer.

3. Reuspend solution

2.25g NaCl (0.9% NaCl)
25.67g sucrose (0.3M)
in 250 ml distilled water, store at 4oC.
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 heave 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 4oC.

5. The pellet will be resuspended in a resuspend solution containing 100μM 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_2HPO_4	141.96	3	425.9mg	851.8mg
KH2PO4	136.1	0.4	54.4mg	108.8mg
$MgCl_2.6H_2O$	203.3	0.5	101.6mg	203.3mg
$MgSO_4.7H_2O$	246.5	0.8	197.2mg	394.4mg

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

B. 1M $CaCl_2$

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+130μl Buffer B.

D.2.5% BSA

25mg/ml in Hanks'

E.10% pluronic F-127. Dissolve 100mg Pluronic in 1 ml DMSF at 40oC, keep at RT.(Puronic 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

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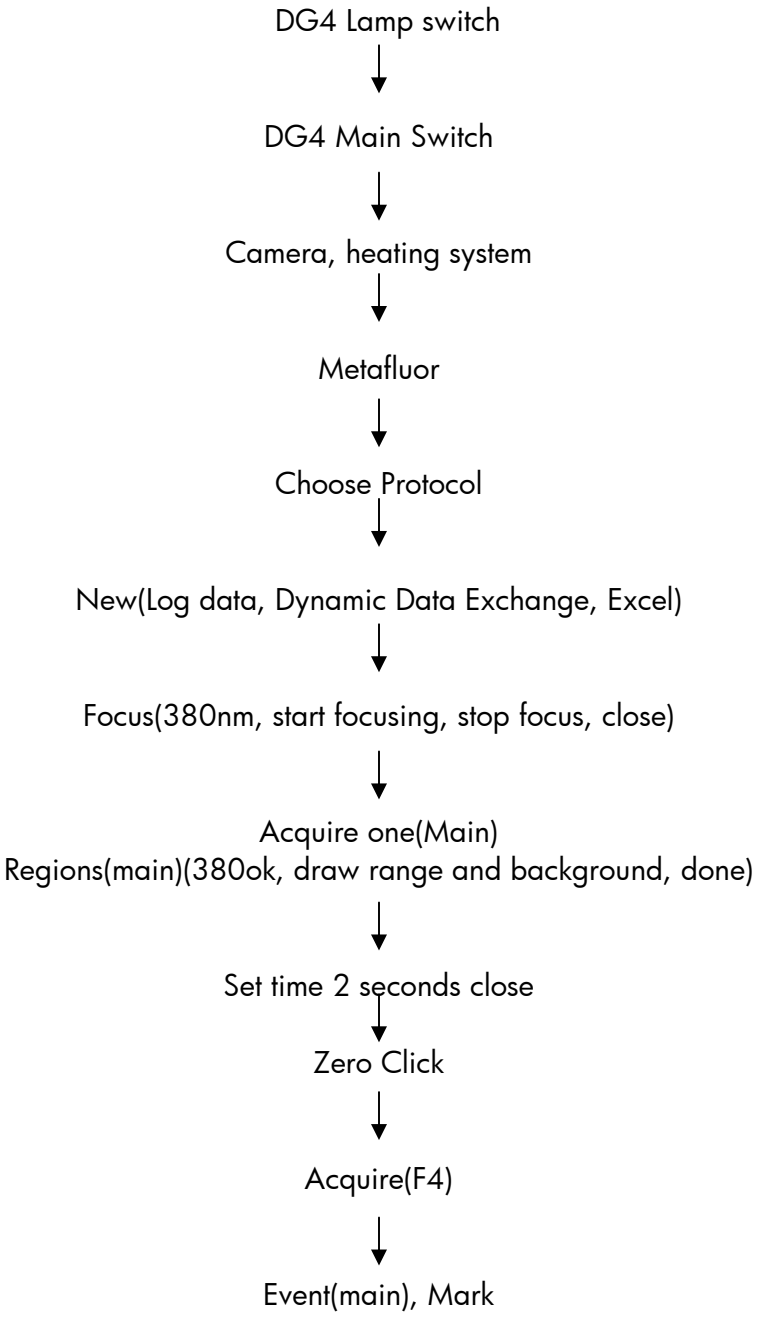
DG4 Lamp switch
DG4 Main Switch
Camera, heating system
Metafluor
Choose Protocol
New(Log data, Dynamic Data Exchange, Excel)
Focus(380nm, start focusing, stop focus, close)
Acquire one(Main)
Regions(main)(380ok, draw range and background, done)
Set time 2 seconds close
Zero Click
Acquire(F4)
Event(main), Mark