PROTOCOL FOR FLUORESCENCE IMAGING SYSTEM

Microscope and Software Setup:

General Switch (1), (2)
↓
DG4 Lamp switch (3)
↓
DG4 Main Switch (4)
↓
LAMBDA 10-2 (5)
↓
Camera (6)
↓
Computer (7)
↓
heating system –general switch(8)→Warner switch (9)
↓
Metafluor
↓
Protocol (main left) → Fura.FSF (for $[Ca^{2+}]_i$ only)
DAF-Fura.FSF (for NO and $[Ca^{2+}]_i$)
↓
New (main left) → log Data → Dynamic Data Exchange (DDE) → Excel 97→back to Metafluor
↓
Focus → 380 → start focusing → stop focus → close
↓
Acq one (main)
↓
Regions (main) → 380 (OK) → draw range (chose cells) and background (for cell) → Done
↓
Reference (main) → 340/380/480 region No (for cells) → Subtract Background → Close
340/380/480 constant (~~for~~ vessel)
↓
Set Time 2 secods close
↓
Zero Click
↓
Acquire (F4)

↓

Event (main) → Mark (F5)

Dataswitch: A imaging system, B printer

IMPORTANT: After you turn off the DG4, DO NOT turn on again in 30 min

Buffer for $[Ca^{2+}]_i$ and NO

A. Ca^{2+}-free Hanks':

	FW	mM	1000 ml	2000 ml
NaCl	58.44	137	8.0g	16.0 g
Glucose	180.2	10	1.8 g	3.6 g
HEPES	238.3	20	4.77g	9.54 g
KCl	74.44	5.4	402.5 mg	805.0 mg
$NaHCO_3$	84.01	4.2	352.8 mg	705.6 mg
Na_2HPO_4	141.96	3	425.9 mg	851.8 mg
KH_2PO_4	136.1	0.4	54.4 mg	108.8 mg
$MgCl_2.6H_2O$	203.3	0.5	101.6 mg	203.3 mg
$MgSO_4.7H_2O$	246.5	0.8	197.2 mg	394.4 mg

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

B. 1 M $CaCl_2$

$CaCl_2.H_2O$ Fw: 147 14.7 g / 100 ml = 1 M

Adjust pH to 7.4 Keep at room temperature

C. Hanks' Buffer

100 ml Buffer A + 130 ul Buffer B

D. 2.5 % BSA

25 mg / ml in Hanks'

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

F. 1 M EGTA (Fw: 380.4)

(19.02g EGTA /50 ml H_2O, adjust pH to > 8.0 with 1 M NaOH, When all EGTA dissolved into solution, then adjust pH to 7.4, keep at RT

Coverslip and Dye preparation

Coverslip and cell preparation:
cut coverslip into 0.6 × 0.6 cm^2 (in order to insert in the chamber), put the coverslips into a 35 mm dish, add 75% alcohol to sterilize the coverslip. Wash alcohol out with sterilized water. Then transfer coverslip by sterilized forceps to other 35 mm dishes (4-6 coverslips in each dish). Seed the cells into the dishes. When the cells are about 40-50% confluent, it is time to do experiment.

Dye preparation:
1. Fura 2-AM from Molecular Probes (F-1221, 20 × 50 μg)
Add 25 μl DMSO and 25 μl 10% pluronic in each tube (50 μg), the concentration is 1 mM
Aliquot to 1.5 ml-eppendorf tubes (10 μl/each), keep at –20°C

2. DAF-2 DA (5 mM in DAMSO) from Sigma (D-225)
Aliquot to 1.5 ml-eppendorf tubes (2 μl/each), keep at at 4 °C

Loading

Tansfer coverslips from cultured dish to another 35 mm dish, wash three time with Full Hanks'.

Procedure for Ca^{2+} loading only

1. Take 10 μl of fura 2-AM (1 mM) from frezer (-20^{0}C), add 1000 μl Hanks' buffer and 40 μl 2.5% BSA. (final concentration is fura 2-AM 10 μM, BSA 0.1%, * pluronic 0.05%). Sonicate for 2min.
2. Add mix above to dish to load cells (or vessel) for 1 h at RT at dark room
3. Wash dish three times with Hanks' (or Ca2+-free Hanks' buffer)

Procedure for simultaneous loading both NO and $[Ca^{2+}]_i$

1. Take 10 μl of fura 2-AM (1 mM) from frezer (-20^{0}C), add 500 μl Hanks' buffer and 40 μl 2.5% BSA. (for Ca^{2+})
2. Take 2 μl of DAF-2 DA (5 mM) from 4^{0}C refrigerate and add 500 μl Hanks' (for NO)
3. Mix two dyes together and sonicate for 2 min
4. Add mixture to loading cells (or vessel) for 1 h at RT at dark room
5. Wash dish three times with Hanks' (or Ca^{2+}-free Hanks' buffer)

(The final concentration is fura 2-AM 10 μM, DAF 10 μM, BSA 0.1%, pluronic 0.05%)

*. Pluronic is used to help the dye dissolves more even, and to avoid dye compartmentalization)

Process for Measurement

Insert coverslip (or vessel) into chamber.

↓

Chose the right objective lens (Fluor 40 for cell, and Fluor 20 for vessel)

↓

Turn on visible light

↓

Focus and chose good area

↓

Turn off visible light

↓

Protocol for Fluorescence Imaging system

↓

Recording for 5 min to get the baseline

↓

Add agonist (direct add to both solution or by infusion) and recording for some time

↓

Add ionomycin 5 μM and $CaCl_2$ 2.5 mM to get Maximal $[Ca^{2+}]_i$

↓

Add ionomycin 5 μM + EGTA 5 mM in Ca^{2+}-free Hanks' to get Minimal $[Ca^{2+}]_i$

Data Acquisition and Analysis

1. Excel data:

A. Because the file is very easy to be lost, so when you open Excel (as mentioned in *Protocol for Fluorescence Imaging system*), you should save this file and open and save another Excel file before your recording. The first one for your data recording, the second one for your picture saving (by pasting).

B. When you are recording, only use SAVE or PASTE button with excel files, DO NOT use other functions such as OPEN, SAVE AS, NEW and et al, otherwise, you will lose all your data.

C.Once you find that the data is lost, DO NOT use SAVE, on the other hand, SAVE AS a new name. You still can find your old data (saved last time) in your old file. After finish, you can link two files together.

2. For NO picture, please DO NOT use AUTOSCALE. Select a suitable scale before recording.

3. Select ratio Scale Bar range: Configure → Image Display Control→ ratio 1 (IMD Display is much better than Pseudocolor Display)→ impute the number you wanted.

4. Use Print Scrn SysRq key to copy whole screen, and paste to your excel

5. Select Scar Bar: File → references →Scale Bar

6. Change graphs scale: Graphs → Configure Graphs

Pay attention!

1. Turn on switches in right order, especially, turn on DG4 Lamp switch before DG4 Main Switch. Computer is last one.
2. Do not forget to turn all switches after you finish experiment:

Computer, heating system
↓
Camera, LAMBDA 10-2
↓
DG4 Main Switch
↓
DG4 Lamp switch
↓
General Switch

3. The life of Xenon lamp is limited; so just turn on system 15 min before you start.
4. Do not over-focus, otherwise you will break the chamber or objective lens.
5. Infuse solution carefully, do not overfill
6. Do not put tray in wrong position (middle is right position)