Fura-2 Imaging Protocols-Amy Harkins lab (based on Aaron Fox protocols).
9/19/06

Mix fura-2 AM (Molecular Probes, #F1221, 50 ug lyophilized) by adding 50 ul of DMSO and vortexing. Store at -20 ^{o}C wrapped in foil for light protection.

Mix Hank's Buffered Salt Solution (HBSS-10X)-with final 1x concentration:

NaCl (FW 58.44) 80 g – 137 mM
KCl (FW 74.55) 4 g – 5.4 mM
$MgCl_2$-$6H_20$ (FW 203.3) 1g – 0.5 mM
$MgSO_4$-$7H_20$ (FW 246.5) 1g – 0.4 mM
KH_2PO_4 (FW 136.1) 0.6 g – 0.44 mM
Na_2HPO_4-$7H_20$ (FW 268.1) 0.9 g – 0.34 mM

For 1000 ml, use 850 mls dH_20 (18 Mohm), add in order, stirring. Bring to 1000ml final volume. Store at 4 ^{o}C.

Mix HBSS-1x solution:

Mix 100 mls of the 10X HBSS solution with 800 mls of dH_20. Add 0.14 g of anhydrous $CaCl_2$ (FW 111, 1.3 mM), 1g of d-glucose (FW 180.2, 5.5 mM) and 0.35 g $NaHCO_3$ (FW 84.01, 4.2 mM). Bring to 1000 ml volume with dH_20, pH to 7.4 and store for ~1 week at 4 ^{o}C.

Mix 50 mM KCl:

NaCl (FW 58.44) 5.0843 g (87 mM)
KCl (FW 74.56) 3.728 g (50 mM)
$MgCl_2$ (FW 203.3) 0.2033 g (1 mM)
$CaCl_2$ (1 M Stock from certified volumetric stock) 5 ml for 5 mM
HEPES (FW 238.3) 2.833 g (~12 mM)
Glucose (180.16) 1.8016 g (10 mM)

1 liter volume, pH 7.35, ~290-310 mOsm. Store 4 ^{o}C.

Replating: One to two days prior to experiments, replate cells to collagen coated coverslips (round, glass, sterilized with ethanol, dried) placed in 35 mm tissue culture dishes. These dishes are placed in 150 mm Petri dishes to be used as a microincubator and carrier between incubator and experiments. When replating, place cells in center of round glass coverslips for imaging to be optimal. Empirically determine the density of cells and how to replate to have cells stick to glass through the washes and the perfusion of solutions.

On day of experiment:
Take the HBSS and 50 mM K solutions out of the refrigerator, turn on equipment, perform calibration curve, check that cells are well adhered to glass.
Take 2, 50 ml Falcon tubes, label as HBSS and HBSS+BSA. Pour HBSS from the HBSS container into the 50 ml tube labeled as HBSS.

Mix HBSS+BSA: Measure out ~45 mg of BSA (from Sigma, #A6003-5g BSA-essentially fatty acid free) and add to the empty tube labeled HBSS + BSA. Transfer ~45 mls of HBSS and mix gently so as not to make a lot of bubbles, but to mix the BSA. This should be a 1mg/ml mixture of BSA and HBSS.

Mix Fura-2 + HBSS+BSA: Thaw the fura-2 AM 50 ul DMSO mix in the dark. Pipette 6-8 ul of this mixture into each of two 15 ml Falcon tubes. Add 2 mls of HBSS+BSA into each of the tubes. Get two dishes of coverslips from the incubator, then vortex the 15 ml tubes together on high for 1 min. Set in rack with the 2, 50 ml tubes (loosen/remove lids to all Falcon tubes).

Load fura: With 1 ml sterile pipette tips, remove all media from one 35 mm dish of cells and eject into a waste container. With the same tip, bring up 1 ml of HBSS and gently add to the cells, being careful to place along the side and gently to not cause disruption of plated cells. Pull up the same 1 ml of HBSS and eject into the waste container, bringing up the next 1 ml of HBSS with the same tip and placing gently on the cells. Remove again, and discard tip. With a new sterile pipette tip, pipette up 1 ml of HBSS+BSA and wash gently, removing to waste. With the same pipette tip, repeat 2 more times to wash the cells 3x with HBSS + BSA. With the same tip, immediately add 1 ml of the 2 mls of fura +HBSS+BSA that was vortexed for 1 min. Add the second 1 ml of fura to the cells and label the lid of the coverslip dish with the time. Typically, we load for 45 min, but this might need to be varied as might the 6-8 ul of fura-2 in 2 mls of HBSS+BSA. Repeat with second coverslip/dish. Replace both dishes in CO_2/37 ^oC incubator for the 45 min loading time.

Wash: remove both dishes and gently with a new sterile tip, pull 1 ml of the fura-HBSS+BSA mix to waste, and then the second. With a new tip, wash the cells 3-4x with 1 ml of HBSS (not HBSS+BSA) each time gently, placing the wash in the waste. After the 4^{th} wash, leave on 1 ml of HBSS and add a second ml of HBSS. Label the time and replace in the incubator for 30 min-45 min.

I usually get the first coverslip of cells about 25 min into the wash and get it set up on the imaging rig in the chamber. I perform the imaging experiment in 10-15 min max and then get the second dish from the incubator. I do all experiments in the dark.

I constantly perfuse the cells with HBSS on the recording chamber for resting Ca measurements and to stimulate, I use 50 mM K solution. To stimulate, I have used in the past, 20 mM, 40 mM and 60 mM KCl solutions as well, each time replacing NaCl with equivalent amounts of KCl.

To obtain a positive control for imaging, I use 20 uM working concentration ionomycin (Calbiochem #407950, 5 mg free acid mixed to 20 mM in DMSO) by placing the appropriate volume into the volume of the chamber while perfusing.

To continue through the day, I begin the next 2 coverslips of cells loading about the same time I wash the first sets, and just keep track of loading/washing and knowing how long it takes to switch coverslips and do the imaging experiments.