KRANIAS LABORATORY:

Calcium Uptake Procedure (Mouse Protocol)

1. The night before the experiment, make up the following:
   a. Make wash solution (20 mM Tris HCl, 2 mM EGTA, pH 7.0). Use 50x stock to make up 1.1 L to use (1078 ml dH_2O + 22 ml 50x stock).
   b. Set up scintillation vials (5x per buffer [1 std, 1 blank, 3 for time course]) and buffer tubes (26 total [Label 1-13 twice]).
   c. Place the instruments for homogenization in the freezer.
   d. Determine decay factor of ^{45}Ca.
2. On day of experiment, make up the homogenization buffer using 5ml column:

Homogenization Buffer:

[Final]	Stock	10 ml	5 ml
50 mM	0.5 M phosphate buffer, pH 7.0	1 ml	0.5 ml
10 mM	0.1 M NaF	1 ml	0.5 ml
1 mM	0.5 M EDTA	0.02 ml	0.01 ml
0.3 M	Sucrose	1.03 g	0.52 g
0.3 mM	0.02 M PMSF	0.15 ml	0.075 ml
0.5 mM	0.1 M DDT	0.025 ml	0.0125 ml

3. Make up the reaction mixture (RM) using the 30x column.

Reaction Mixture:

[Final]	Stock	1.5 ml	15x	30x
40 mM	80 mM Imidazol, pH 7.0	0.75 ml	11.25 ml	22.5 ml
95 mM	1 M KCl	0.1425 ml	2.138 ml	4.275 ml
5 mM	0.1 M NaN_3	0.075 ml	1.125 ml	2.25 ml
5 mM	0.1 M $MgCl_2$	0.075 ml	1.125 ml	2.25 ml
0.5 mM	0.5 M EGTA	0.0015 ml	0.023 ml	0.045 ml
5 mM	0.1 M K^+ oxalate	0.075 ml	1.125 ml	2.25 ml
		1119 ul total vol. (2 x 559.5 ul)		

4. Set up the 13 buffer tubes with duplicate tubes (26 tubes). Add 1119 ul RM to each one.
5. Make up the ruthenium red solution daily. You want 400 ul of 0.114 mM (1 uM Final). Stock is 0.875 mM. Therefore, add 52 ul of 0.875 mM with 348 ul of water.
6. Add the following to each tube (you can add the 10.88 mM calcium and water in the main lab):

Buffer #	pCa	10.88 mM $CaCl_2$	dH_2O	^{45}Ca (40 uCi/ml)	Total (ul)
1	8.0	2.4	228.5	1.9	232.8
2	7.5	7.1	220	5.7	232.8
3	7.2	12.8	209.8	10.2	232.8
4	7.0	18.3	199.9	14.6	232.8
5	6.8	25.2	187.4	20.2	232.8
6	6.6	33.1	173.2	26.5	232.8

7	6.4	41.2	158.6	33.0	232.8
8	6.3	45.1	151.6	36.1	232.8
9	6.2	48.9	144.8	39.1	232.8
10	6	55.6	132.7	44.5	232.8
11	5.8	61.4	122.3	49.1	232.8
12	5.5	68.7	109.1	55.0	232.8
13	5	82.6	84.1	66.1	232.8

7. Make up a 40 uCi/ml stock solution.
8. Add the ^{45}Ca to the buffer tubes (IN THE RADIOACTIVE LAB!!).
9. Do the homogenizations of the mouse heart in the cold room:
    a. For every heart, use 1 ml homogenization buffer (put in 2 ml tube).
    b. Homogenize 4x5 passes with 10 seconds resting interval in between.
    c. Run protein assay to determine concentration (do when tubes are in the scintillation counter).
    d. For experiment, need 75 ul * 26 tubes = 1950 ul
        So, use 450 ul homogenate + 2550 ul homogenization buffer (put in 15 ml conical tube)
10. Place 0.45 uM Millipore filters (Fisher HAWP02500) on filtration rack for the first buffer tube.
11. Wash each filter 2x with 2.5 ml wash solution.
12. Add 13.2 ul of 0.114 mM Ruthenium Red (1 uM Final) to first buffer tube.
13. Add 75 ul of homogenates to first buffer tube.
14. Incubate for 30 secs @ 37^0C, then remove an aliquot of 290 ul for N.S. binding (also called blank). Put aliquot through a 0.45 uM Millipore filter.
    a. Wash each 2x with 2.5 ml wash solution.
15. Add 60 ul 100 mM ATP, [Final] = 5 mM.
16. Take out 300 ul at 30, 60, and 90 secs.
    a. Wash each 2x with 2.5 ml wash solution
17. Place filters in the scintillation vials.
18. Repeat steps #10-#16 for the remaining buffer tubes.
19. When finished, add 60 ul from the reaction mixture to the standard vial for each buffer level.
20. Add 10 ml ScintiVerse BD (Fisher SX18-4) to each scintillation vial.
21. Count using program #3 on the Beckman LS 3801 model.
22. Calculate :

    Ca Uptake (nmol/mg) = [(sample cpm – NS cpm)/(Std cpm * 5)] * [Calcium conc. (nmol)/protein conc. (mg)]

    Then take linear regression over 0.5, 1, and 1 min for rate. If regression isn't linear (approx. 0.90 or better), throw out that pCa.

23. Survey room according to map.