Preparing Samples for NPY assay:

To prepare ahead of time: water bath turned on to 37˚C, 50 ml tube labeled with HBSS and 50 ml tubes labeled for each stimulant, placed in water bath.

Six well plates of cells of interest, top row labeled 1-3, lower row labeled 4-6. #1-3 will be basal release, #4-6 will be stimulated release.

With a 6-well plate, you will need 6 polystyrene tubes and 2 sets of 6 eppendorf (eppi) tubes = 12 tubes/plate. Label one set of 6 eppi tubes with round tag placed on top:

Identifying stimulus or cell type, #1-6, Date

Label the second set of 6 eppi tubes (with black sharpie on lid directly):

Name of stimulant or cell type, #1-6

In each eppi tube rack, place the two separate sets of eppi's labeled for the stimulant, in the same order of #1-6 (double check before pipetting samples).

Do not label the polystyrene tubes yet, as the trifluoroacetic acid (TFA) that needs to be added to them will remove the marker. Instead, label the paper horizontal tags with:

Name of stimulant or cell type, #1-6, date

In the lab book, write down that the NPY samples were pulled, the type of stimulant used, with the date done.

Place the polystyrene tubes in a rack in the fume hood, and calculate the amount of TFA that you will need:

~0.32 mls per tube. So, 36 tubes x 0.32 mls = 11.52 + 4 mls for error = ~15mls poured into a glass beaker that is clean.

Use a 10 ml pipette with the red bulb to suction up ~10mls into the pipette and add ~0.3 mls of TFA to each of the tubes, working quickly and trying to spill as little as possible. You will need to do this twice to get all of the tubes. If it is too little TFA, NPY will be degraded, and too high will interfere with the measurement.

Wait to add the paper labels to the tubes until after the solutions have been added to a set of tubes and they have been capped (below).

Gently cover the rack of tubes with parafilm to keep the TFA from evaporating. In the hood, place the bag of blue tops, a 1 ml pipettor and a blue rack of 1 ml tips, and a trash container to collect the tips, as well as a Styrofoam tube rack to hold the tubes with samples. You will also need the labels.

On the work bench, place a clean glass beaker with about 50 mls of 18 Mohm water. Get out one cell scraper per plate.

Get a suction set up, with a clean Louis Pasteur pipette for removing volumes of solutions.

Get a timer, one location set to 5 min and another to 15 min. Get ice bucket with ice.

To pull the samples from cells:

Get a plate (or two) of cells from the incubator.

Remove all media from one plate of cells with suction, and add 1 ml of HBSS to each well (gently-so as not to disturb the cells, add along the side of plate) with one pipette tip without changing tips in between cells. Label top of plate with HBSS.

Incubate 37 degree water bath (gently rocking or not), for 5 min, set timer.

Remove the HBSS from each well of cells with suction. Add 1 ml of HBSS to the top three wells labeled #1-3 (basal release), and add 1 ml of stimulating solution to the lower three wells labeled #4-6. Label as HBSS and stimulant (eg: ATP, nicotine, cytisine)

Replace in the water bath at 37˚, for 15 min, set timer.

With a 1 ml pipette tip, remove all media from cells and place each well solution into the appropriately labeled eppi tube that has a label written on the lid directly. Spin the tubes at 0.1 ref or 1 rpm in eppendorf table top centrifuge for 3 min (orient the hinge at the top of the centrifuge rotor so that the pellet (if there are any residual cells) is always at the bottom side of the hinge).

While spinning, add 1 ml of 18 Mohm water to each well of the plate, label

across the plate as WATER, and set aside. The cells can be scraped with the cell scraper now or after the spin.

After the spin, place the eppi's back into the eppi rack and take them to the hood with the waiting tubes containing the TFA. With the 1 ml pipettor set to 950µl, remove 950µl of basal or stimulated solution from the possible pellet of cell debris and place in order to 6 waiting polystyrene tubes with TFA. Know the order you added the solutions from the wells #1-6.

Set the eppi's aside. Top each polystyrene with a blue cap, keeping the same order. Use a different tip for each of the wells, switching between and not touching the tip to the TFA (the pipettor shouldn't even go into the tube, just the tip to eject).

Add the appropriate label to each tube.

Place the completed 6 tubes into the Styrofoam rack and place into the -20 freezer. As each set of tubes are finished, add the set to the waiting Styrofoam tube rack. The last set in 36 will stay in the plastic rack and the sets from the Styrofoam in the -20 freezer will be transferred back to the plastic rack and the entire set transferred to the -80 freezer with eppi tubes for protein. This must be done as soon as the last set of tubes are finished, and not left at -20 overnight.

Take the eppi's back to the bench, scrape the wells of cells if not already done, and transfer all of the scraped floating cells to the waiting eppi with 1 ml pipettor to the already-pelleted cell debris, being sure to keep the well #1 correlated to eppi #1.

Place tubes on ice and fill another eppi tube with water and label it B. Use sonicator with settings already set (3 up/downs) and washing with the B tube in between each of the samples. Wipe dry with kimwipes. Be sure to turn off when done.

Spin the tubes for 10 min on max speed using the centrifuge in the cold box. When done, transfer the supernatant (~0.9-1 ml) to the waiting sterile eppi's labeled with stimulant, date and well number. Place these tubes into the -20 freezer in a box that is labeled as the type of stimulant used, and the date on the top using tape. When finished with all samples, transfer these eppi's (for protein determination) and the plastic rack of polystyrene tubes (with the NPY) to the -80 freezer.