Protocol for calcium phosphate transfection of primary neurons

1. Make DMEM/KY solution: Add 50 mL of 10x KY solution from Appendix 1 to 450 mL of DMEM without pen-strep and without glutamine.
2. Make Neuronal Growth Media (see Primary Striatal Dissection protocol) but without serum. Instead of including bovine calf serum, replace the serum component with an equivalent amount of BME.
3. Make two types of 2x HBS (Hepes buffered saline) solutions. One will be used in the shock solution (see below) and the other one will be specifically used for forming the calcium crystals. This second HBS solution must have exactly the right pH, which can only be known empirically. Both solutions are made with the same basic components. Add all the ingredients below and bring up to just under 500 mL with water, pH with 10N and 5N NaOH, and then bring the solution up to a final volume of 500 mL.

2x Hepes Buffered Saline Component	Amount
NaCl	8 g
KCl	0.355 g
Na2HPO4	0.19 g
D-Glucose	1.35 g
HEPES (free acid)	5 g

4. pH one solution of HEPES to 7.4. The other solution should be split into 50 mL aliquots and pH'd so that each aliquot is 0.02-0.05 pH units away from each another and range the spectrum from pH 6.8-7.25. To implement this protocol, you will have to do pre-testing to see which of these specially pH'd aliquots forms the best calcium crystals and gives the most reliable transfections. I will call this

aliquot the pH-optimized aliquot of 2x HBS. If the calcium precipitate that forms (see below) is not optimal, consider trying a different pH 2x HBS solution as the "pH-optimized HBS." Other things that affect precipitate formation include the quality/type/amount of DNA, the pH of the media you are adding the DNA/calcium solution to (higher pH = faster precipitate formation), and the temperature of the media you are adding the DNA/calcium solution to (higher temperature = faster precipitate formation). You may need to adjust each of these variables in any given transfection to optimize precipitation so that it forms a fine, sandy layer over the neurons, but isn't so thick that it induces toxicity.

5. Make the shock solution: Combine all the ingredients below.

Shock solution component	Amount
2x HBS (the solution where the pH is unimportant)	50 mL
Sterile H2O	38 mL
Cell-Culture Grade DMSO	2 mL
DMEM/KY Solution	10 mL

6. Place the shock solution in the 37°C water bath.
7. For each 24-well plate that needs to be transfected:

Place 50 mL of DMEM/KY into a 15 cm plate (so that there is maximal exposure of the DMEM/KY solution to the air).

Place 50 mL of DMEM into a 15 cm plate.

Place 25 mL of Neuronal Growth Media without serum into a 10 cm dish.

Place the DMEM/KY dish and the DMEM dish into an incubator at 37°C and 10% CO_2.

Place the Neuronal Growth Media without serum dish into a regular 37°C, 5% CO_2 incubator.

8. Be sure you have available an additional incubator at 37°C but with room air composition (i.e. negligible CO_2).
9. Place your remaining DMEM/KY solution in a 37°C water bath (be sure to check that the water bath is at the right temperature!) with the lid loosely on (so that air can circulate into the bottle, but no bugs can get in).
10. Take your 24-well plate and replace the Neuronal Growth Media with 0.5 mL of DMEM/KY. Remove the DMEM/KY and replace with 1 mL DMEM/KY.
11. Place the plates back in the incubator and place the bottle of DMEM/KY back in the 37°C water bath with the slid slightly loosened.

Preparing DNA/Calcium solution:

1. Calculate compositions for each DNA/calcium solution. Calculate how many wells need to be transfected with a given DNA construct and then use the following formula (don't mix these components yet!):

Component	Amount Needed per Well of 24-Well Plate
DNA	Varies, but usually between 0.5 ug and 3 ug per construct; no more than 5 ug of total DNA per well of a 24-well plate.
Sterile H_2O	= 12.375 uL – volume of DNA (DNA + H_2O + $CaCl_2$ = 13.75 uL)

2.5 M CaCl2	1.375 uL
HBS (pH-optimized solution)	13.75 uL
Total precipitate	27.5 uL

2. In a 5 mL polystyrene pop-top tube, add DNA, then add water, then add calcium chloride; gently tap the tube to mix the solution until the calcium is thoroughly mixed in.
3. Drip the HBS into DNA/H2O/calcium solution (slowly; drop-wise) while grating the polystyrene tube on a grated surface (to help agitate the solution).
4. After all the HBS has been dripped into the tube, gently tap the tube to make sure the solution is mixed. Then push down any drops that are stuck to the side of the tube with a pipette tip.
5. Place the DNA/calcium solution in the dark and leave for 10-15 minutes.
6. Take the 24-well plate out of the incubator and wash each well with the DMEM/KY from the water bath, using 0.5 mL per well. Wash one more time and be sure that the final amount of DMEM/KY in the well is 0.5 mL. It is CRITICAL to have this amount in the well! Be sure to let the plate stand for at least 5 minutes after adding the last round of DMEM/KY – you want to let the wells alkanalize to a degree.
7. Take out the DNA/calcium precipitate from the dark and gently tap the tube to mix.
8. Add 25 uL of the solution to each well. Be sure to add the precipitate to the CENTER of the well. Also be sure NOT TO touch the DMEM/KY solution in the well with the pipette tip. The pH of the well solution is different than the pH of the DNA/calcium solution and you therefore don't want to contaminate the DNA/calcium solution with your pipette tip as you alternate between the DNA/calcium tube and the wells on the plate.

9. After every 4-6 wells, swirl the plate to make sure the precipitate is evenly distributed across the wells.
10. Work quickly as it is important to try and have all wells "see" precipitate at about the same time. This helps prevent some wells from forming too much precipitate while other wells haven't formed enough precipitate.
11. When all the precipitate has been added to the wells, swirl the plate one more time and then place the plate in a 37°C incubator WITH ATMOSPHERIC CO2 (not a 5% CO2 incubator!). This step is also critical. Calcium crystals form at alkaline pH, so you want to minimize the exposure of the DMEM/KY media to the high CO2 content of a typical incubator, which will excessively acidify the solution.
12. Wait 15 minutes and then check on the status of the crystals. The goal is to have a fine, sand-like pattern to the crystals. It will take trial and error to discover how much crystallization is enough. If you have too few crystals, your transfection efficiency will be really low. If you have too many crystals, your toxicity will be high. There is a very fine sweet-spot that takes trial and error to achieve. In general, the time the neurons see calcium crystals is less important to overall toxicity than how heavy the calcium crystal precipitate is when you go to wash it off.
13. If the precipitate isn't yet heavy enough, return the plate to the 37°C atmospheric incubator and keep rechecking every 10-15 minutes. You don't want the precipitation to go too long, so be sure to check frequently enough.

Taking off the calcium precipitate:

1. Just before the precipitate is heavy enough, take the DMEM/KY solution from the 37°C/10% CO2 incubator and place the solution into sterile bottle such that the surface area exposed to air is

minimized. The goal here is to keep this solution at an acidic pH for as long as possible while it is out of the incubator.

2. When the precipitate is heavy enough, remove the DMEM/KY and replace with 0.5 mL of shock solution, which should have been preincubating in the 37°C waterbath. Keep the shock solution on each well for 3-4 minutes (starting from the time it first gets introduced onto the well).
3. Remove the shock solution and wash with 1 mL of the DMEM/KY solution from the 10% CO_2 incubator. Remove the DMEM/KY and wash again with the DMEM/KY solution from the 10% CO_2 incubator.
4. Place the plate back in the 37°C/5% CO_2 incubator and leave for 30 minutes.
5. Check the wells after 30 minutes under the microscope to see if the calcium crystals have fully dissolved. If they haven't, return the plate to the incubator for another 10-30 minutes, depending on how much calcium crystal precipitate is left. Warning: if you see calcium crystals after the first 30 minute incubation, it is likely you'll see higher toxicity for your transfection.
6. Just before you are ready to wash the DMEM/KY solution off, take the DMEM solution that is in the 37°C/10% CO_2 incubator and place it in a bottle that minimizes surface area exposed to air.
7. Remove the DMEM/KY and replace with 1 mL DMEM from the 10% CO_2 incubator. Wash the DMEM off and again replace with 1 mL DMEM from the 10% CO_2 incubator.
8. Check under the microscope; there should be zero calcium crystals left. If there are, you may see higher toxicity. Consider (although not necessary) washing several more times with DMEM that has been equilibrated at 10% CO_2.
9. Replace the DMEM with 1 mL Neuronal Growth Media without serum that had been pre-incubating in the 37°C/5% CO_2 incubator.