

# Calcium Phosphate Transfection of Neuronal Cells

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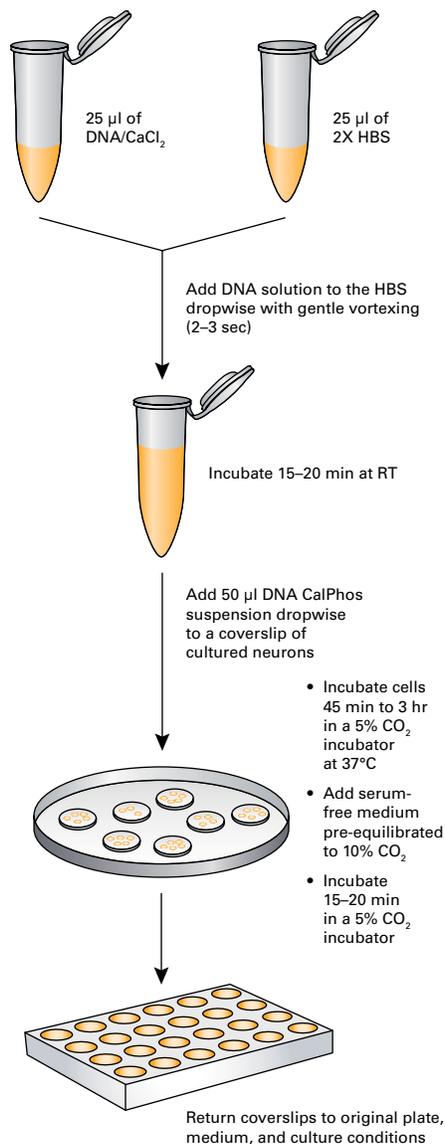
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We present a novel, highly efficient method for transfecting neuronal cells using Clontech's CalPhos™ Mammalian Transfection Kit. Our method produces transfection efficiencies that are typically tenfold higher than those of standard methods, while maintaining low cell toxicity. These improvements are largely accomplished by the modification of just two key steps. First, the DNA-Ca<sup>2+</sup> and phosphate solutions are gently mixed to obtain a very fine, homogeneous DNA-Ca<sup>2+</sup>-phosphate precipitate. Second, after incubation with the cells, the precipitate is dissolved in slightly acidic culture medium to reduce its toxicity. The high efficiency and low toxicity of this new protocol make it possible to readily transfect single autaptic neurons as well as mature neurons. (1)

Cultured neurons are among the most difficult cells to transfect with DNA; they are very sensitive to microenvironmental changes and tend to die soon after transfection. Calcium phosphate transfection is one of the most widely used methods for transfecting neurons because of its simplicity and low toxicity (2–7); transfection efficiencies, however, are typically very low (~1–5% on average) compared to those of other methods (2, 8, 9). The ideal transfection protocol would retain the ease of use and low toxicity of the calcium phosphate method while improving transfection efficiencies, thus broadening its application in functional genetic analyses.

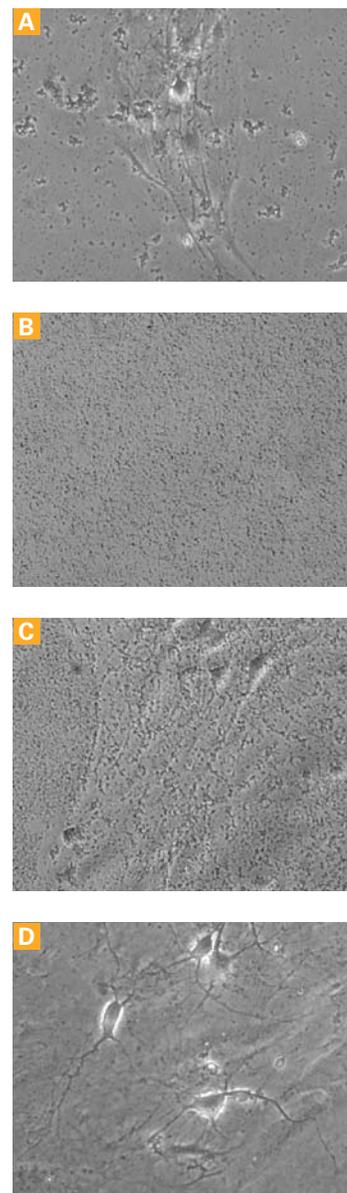
## Forming a Homogeneous DNA-Ca<sup>2+</sup>-Phosphate Precipitate

By identifying several critical factors that appear to have limited transfection efficiencies in previous protocols, we have developed a method that greatly improves transfection efficiencies yet retains minimal toxicity using Clontech's CalPhos™ Mammalian Transfection Kit (Cat. No. 631312) (Figure 1). For a complete step-by-step description of this protocol and a troubleshooting guide, please refer to Jiang, M. & Chen, G.,



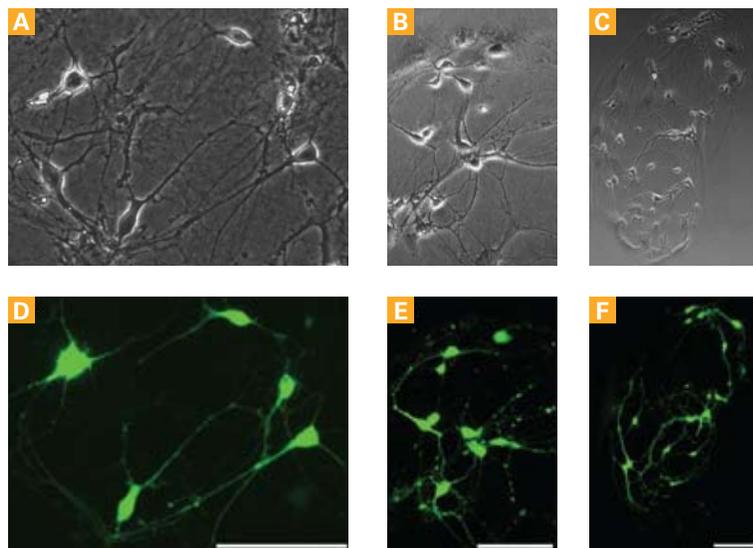
**Figure 1. Flowchart of our main Ca<sup>2+</sup>-phosphate transfection protocol.** For complete details, see Jiang, M. & Chen, G., 2006 (1).

2006 (1). In our protocol, one of the most important steps is the formation of a fine, homogeneous DNA-Ca<sup>2+</sup>-phosphate precipitate (Figure 2). It is believed that this precipitate enters cells through endocytosis, with some of the DNA molecules then making their way into the nucleus (8). We have found that the continuous vortexing used in other protocols often results in large and unevenly distributed precipitate particles, which we believe are



**Figure 2. Formation and subsequent dissolution of the DNA-Ca<sup>2+</sup>-phosphate precipitate.** **Panel A.** Continuous vortexing when mixing DNA with Ca<sup>2+</sup> and phosphate buffer results in large clusters of precipitate (examined after a 1 hr incubation). **Panels B & C.** Formation of an optimal DNA-Ca<sup>2+</sup>-phosphate precipitate through gentle vortexing during mixing (image taken after 1 hr incubation). **Panel D.** Dissolution of the precipitate with slightly acidic transfection medium pre-equilibrated in a 10% CO<sub>2</sub> incubator. Scale bar, 50 µm. (Reproduced from references 1 and 12).

## Calcium Phosphate Transfection of Neuronal Cells...continued



**Figure 3. High transfection efficiency is achieved in low-density hippocampal cultures with our improved protocol. Panels A–C.** Phase-contrast micrographs. **Panels D–F.** Fluorescent images of GFP-transfected cells in three independent transfections. Note that the majority of neurons in the local field (microislands) are transfected. Neurons were cultured for 10–15 days according to previously described methods (11, 12). (Reproduced from references 1 and 12.)

not endocytosed efficiently by the cells (Figure 2, Panel A). With gradual mixing of the DNA-CaCl<sub>2</sub> and 2X HBS solutions, and very mild vortexing, we are able to consistently form small precipitate particles that are more easily endocytosed (Figure 2, Panels B & C).

### Dissolving the DNA-Ca<sup>2+</sup>-Phosphate Precipitate

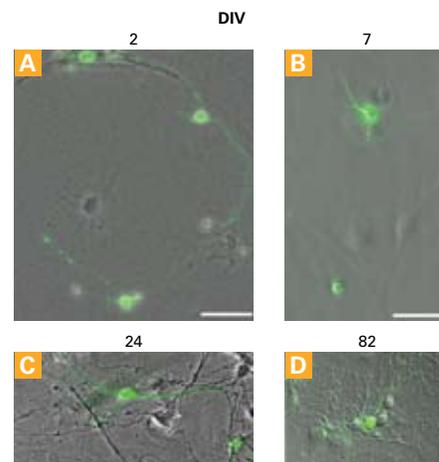
Another critical step in our protocol is the dissolution of the DNA-Ca<sup>2+</sup>-phosphate precipitate at the completion of the transfection incubation period. We found that the excess precipitate is not effectively removed by simply washing the cells with transfection medium, as is recommended in most protocols. We solve this problem by replacing the medium on the transfected cells with serum-free transfection medium that has been pre-equilibrated in a 10% CO<sub>2</sub> incubator, and then returning the plate to the 5% CO<sub>2</sub> incubator for a brief period of time (Figure 2, Panel D). Pre-equilibra-

tion in 10% CO<sub>2</sub> acidifies the medium slightly, which helps to dissolve the precipitate. This step significantly reduces neuronal toxicity, allowing the cells to be incubated with the precipitate much longer, and resulting in substantially increased transfection efficiencies.

Another important step in our procedure involves performing the transfection in a new culture plate (with the cells on cover slips) and then returning the cells to their original culture plate and medium once the transfection has been completed. With these improvements, we have successfully achieved transfection efficiencies of up to 60% in low-density primary neuronal cultures (Figure 3). Ours is the first method to achieve such high transformation efficiencies while maintaining low cell toxicity by dissolving the transfection precipitate. We have recently used this improved calcium phosphate transfection method to study endophilin function in synaptic vesicle endocytosis (10).

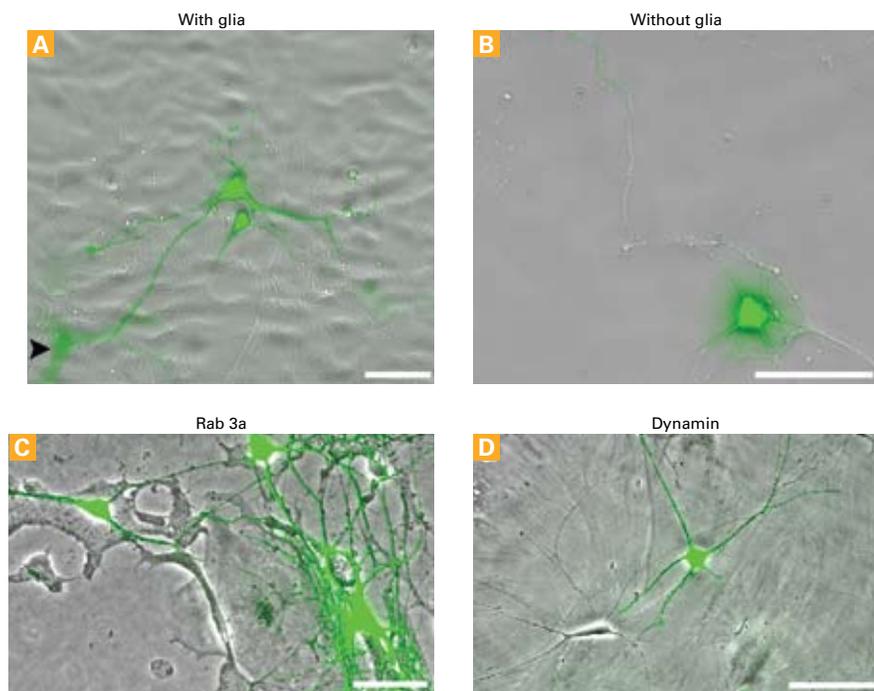
### High Transfection Efficiency in Low-Density Hippocampal Cultures

Different plasmids may require different incubation times for efficient transfection. In this procedure, plasmid incubation times can be optimized by varying the amount of time that the cells are exposed to the DNA-Ca<sup>2+</sup>-phosphate precipitate before it is dissolved. We have found incubation times of 45 min–3 hr to be optimal for highly efficient transfections and minimal cell toxicity. The high efficiency of our improved protocol is illustrated in Figure 3, in which the majority of neurons were successfully transfected with a GFP expression construct. One microisland contained a total of 22 neurons, among which 17 neurons were transfected, corresponding to a transfection efficiency of ~80% (Panels C & F). The entire coverslip contained 127 transfected neurons out of a total of 211, yielding a transfection rate of ~60.2%. This is far better than the average rates of 1–5% obtained in previous reports (2, 13, 14). Significantly, exogenous gene expression was stable and occurred very rapidly. GFP expression in



**Figure 4. Successful transfection of both mature and immature neurons. Panels A & B.** Transfection of young neurons with EGFP at 2 and 7 days *in vitro* (d.i.v.). **Panels C & D.** Transfection of mature neurons with EGFP at 24 and 82 d.i.v. Scale bar, 50 μm. (Reproduced from reference 1.)

## Calcium Phosphate Transfection of Neuronal Cells...continued



**Figure 5. Transfection efficiency in glial cells is very low.** Panel A. Only a few glial cells (arrowhead) are transfected in neuronal cultures with a monolayer of astrocytes. Panel B. In Banker-type cultures where neurons usually do not contact glial cells directly, transfection efficiency remains high (21.6%). Panels C & D. Many constructs can be successfully transfected using our protocol. Illustrated here are EGFP-Rab3a (Panel C) and EGFP-dynamin (Panel D). (Reproduced from reference 1.)

neurons was detected within 4 hr after transfection and persisted for more than one week.

### Transfection of Mature Neurons in Culture

Calcium phosphate transfection methods have often been used for transfecting young neurons (grown from 2–10 days in culture). Mature neurons have been more difficult to transfect because they tend to die shortly after transfection. Using our improved protocol, we found that neurons grown from 2 to 82 days in culture can be successfully transfected, enabling us to perform genetic analyses in cells that have well-established synaptic networks (i.e. cells that have been grown for more than 20 days in culture; Figure 4). The successful transfection of 82-day-old neurons is remarkable, however, because neurons rarely survive longer than 3 months

in culture. Transfection of these delicate cells is further evidence that our protocol is virtually free of toxic effects, even for mature neurons.

### High Transfection Efficiency in Neurons but not Glial Cells

Our neuronal cultures contain many glial cells because the neurons are plated on a monolayer of cortical astrocytes. Excessive astrocyte transfection is undesirable, as it could generate high background signals (e.g. for GFP) or even directly affect neuron function through neuron-glia interactions. Notably, with our method, few glia are transfected in these mixed cell cultures (Figure 5). This may be due to the presence of cytosine arabinoside, which is used to stop glial proliferation, and might also suppress transfection. Transfections were also effective for Banker-type cultures, where neuron-glia contact is minimal. In short, with our protocol, transfection

Product	Size	Cat. No.
CalPhos Mammalian Transfection Kit	each	631312

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efficiencies in low-density neuronal cultures seem to be consistent between those grown either with (25.2%) or without astrocytes (21.6%).

This protocol enables cultured neurons to be transfected with high efficiency and very low toxicity. The key features of the method are a carefully produced fine DNA-Ca<sup>2+</sup>-phosphate precipitate, and subsequent dissolution of the precipitate with a short exposure to slightly acidic medium. The method is successful for both mature and immature neurons, and its high efficiency allows even single autaptic neurons to be transfected for functional genetic analyses.

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