# A Reliable Protocol for transfection of mature primary hippocampal neurons using a neuron-glia co-culture system

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Received December 26, 2006 / Accepted February 8, 2007

DNA transfection is a powerful tool for studying gene functions. The $Ca^{2+}$-phosphate precipitation remains one of the most popular and cost-effective transfection techniques. Mature neurons are more resistant to transfection than young ones and most other cell types, and easy to die if microenvironment changes. Here, we report a transfection protocol for mature neurons. The critical modifications are inclusion of glial cells in culture and careful control of $Ca^{2+}$-phosphate precipitation under microscope. Cerebral glial cells were grown until ~70-80% confluence in DMEM/10% horse serum, which was thereafter replaced with serum-free Neurobasal/Ara-C, and E19 hippocampal neurons were plated onto the glial layer. Formation of fine DNA/$Ca^{2+}$-phosphate precipitates was induced using Clontech CalPhos™ Mammalian Transfection Kit, and the size (0.5-1 ㎛ in diameter) and density (about 10 particles/100 ㎛$^2$) were carefully controlled by the time of incubation in the medium. This modified protocol can be reliably applied for transfection of mature neurons that are maintained longer than two weeks *in vitro*, resulting in 10-15 healthy transfected neurons per a well of 24-well plates. The efficacy of the protocol was verified by punctate expression of pEGFP-CaMKIIα, a synaptic protein, and diffuse expression of pDsRed2. Our protocol provides a reliable method for transfection of mature neurons *in vitro*.

**Key words** – $Ca^{2+}$-phosphate precipitation, neuron-glia coculture, pEGFP-CaMKIIα, transfection

## Introduction

Transfection is one important approach to analyze and manipulate function of target genes [13]. Basically, two different transfection principles exist: viral and non-viral transfection [17]. Virus-mediated transfection such as adenoviral vectors [1] or herpes simplex vectors [15] provide high gene transfer efficiency. However, construction of these vectors is time consuming, and strong laboratory security measures have to be met. Some non-viral transfection methods have been developed such as the $Ca^{2+}$-phosphate transfection method, the DEAE-Dextran approach, ultrasound, lipofection and electroporation [6,9,12]. Among these, $Ca^{2+}$-phosphate precipitation have been the most popular and cost-effective techniques. However, low efficiency and cytotoxic effect limit the $Ca^{2+}$-phosphate method [18].

Neurons have historically proven refractory to easy genetic manipulation. They are more resistant to transfection than most other cell types. Also, neurons must be grown more than two weeks *in vitro* before being matured morphologically. The problem is that transfection efficiency goes further down with mature neurons. To circumvent this problem, transfection was carried out with early DIV neurons (9-12 DIV) and further grown to mature. However, this trick is not always satisfactory because transfected neurons do not survive long.

Glial cells are non-neuronal cells that provide support and nutrition, maintain homeostasis, form myelin, and participate in signal transmission in the nervous system. Some glia function primarily as physical support for neurons. Others regulate the internal environment of the brain, especially the fluid surrounding neurons and their synapses, and provide nutrition to nerve cells. We reasoned that glial cells in neuron-glia co-cultures would protect neurons from cytotoxicity and keep them healthy after transfection procedure. Indeed, we find this modification permits transfection of neurons that are maintained longer than two weeks *in vitro*.

## Materials and Methods

### Plasmids

pEGFP-CaMKIIα, α-isoform of Type II calcium/calm-

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odulin-dependent protein kinase, was a kind gift from Dr. Tobias Meyer (Stanford University School of Medicine, USA). The pEGFP and pDsRed2 were from Clontech.

### Glial culture

Primary glial cultures were obtained from cerebral hemispheres of embryonic day 20 (E20) - postnatal day 2 (P2) rats. After animal decapitation the skull was opened, meninges were removed and brain hemispheres were dissected into small pieces, treated with 0.25% trypsin-EDTA (Invitrogen) for 10 min at 37°C. After trituration with a fire-polished Pasteur pipette, tissue debris was let to settle down. The supernatant containing dissociated cells were collected with a Pasteur pipette, counted, and seeded on coverslips coated with poly-DL-lysine (1 mg/ml; Sigma) at a density of 57,000 cells/$cm^2$ in 24-well culture plates with MEM (Invitrogen) containing 0.5% D-(+)-glucose (Sigma), 1.0 mM sodium pyruvate (Invitrogen), 2 mM/ml glutamax I (Invitrogen), 10% horse serum (Invitrogen), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin. Serum was decomplemented by heating at 56°C for 30 min. Plates were incubated overnight at 37°C in a 5% $CO_2$-humidified atmospheric air. Next day, entire media (0.5 ml) containing unattached cell debris were replaced with new fresh media, and continued to incubate. The first change of culture medium (1/3) was made after six days and, thereafter, every three days. When they reached 70-80% of the confluence, which usually takes about 2 weeks, the entire media were replaced with Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), 0.5 mM glutamine, and cytosine-β-arabinofuranoside (Ara-C, final conc. 4 mM/ml). Sometimes we used once-split glia, which we do not find any apparent differences from unsplit ones.

### Neuronal culture

Embryonic day 18 (E19) rat hippocampal cells were dissociated by triturating trypsin-treated tissues, and were plated on poly-DL-lysine-coated coverslips (1,000~1,500 cells/$mm^2$) in the Neurobasal medium supplemented with B27, 0.25 mM glutamax I, and 12.5 μM glutamate (Invitrogen, Carlsbad, CA), as previously described [3,5]. One third of the culture media was replaced with the same fresh one but glutamate every 3 days.

### Glia-Neuron co-culture

Dissociated embryonic (E19) hippocampal neurons were seeded onto the glial monolayer that reached 70-80% of the confluence and maintained (less than one week at this confluence) in Neurobasal medium supplemented with B27 (Invitrogen), 0.5 mM glutamine, and Ara-C (4 uM/ml). One third of the culture media was replaced with the same fresh one but glutamate every 3 days.

### Transfection Procedure

Transfection was carried out as described by Jiang *et al.* [8], who substantially modified the $Ca^{2+}$-phosphate transfection method from the Clontech CalPhos™ transfection protocol. Briefly, coverslips containing cell cultures were transferred from their original well to a new 24-well plate with 0.5 ml prewarmed transfection medium, which is similar to the culture medium except that it is serum free. Both the original and the new plate were returned to the incubator. The DNA/$Ca^{2+}$-phosphate precipitate using Clontech CalPhos™ Mammalian Transfection Kit (BD Bioscience, Palo Alto, CA, USA), according to the instruction. Typically, we use make 25 $\mu\ell$ of solution A (DNA 1.0 $\mu$g, 2 M $CaCl_2$ 2.2 $\mu\ell$, $H_2O$ to make final volume 25 $\mu\ell$) and 25 $\mu\ell$ of solution B (HEPES-buffered saline, pH 6.98, 17.5 $\mu\ell$, $H_2O$ 7.5 $\mu\ell$) for transfection of one well of 24-well plates. The solution A was transferred to solution B by quickly pipetting small aliquots (about 1/8 volume of solution A at a time) several times, vortexed briefly at lowest speed, and incubated at 19-20°C for 20 min without any further vortexing to form fine particles of precipitate. The DNA/$Ca^{2+}$-phosphate suspension solution was added dropwise to each coverslip (50 $\mu\ell$/coverslip), and the plates were incubated for 40 min to 1.5 h in 5% $CO_2$ culture incubator at 37°C. After incubation, the precipitate was dissolved by incubating the precipitates with transfection medium pre-equilibrated in a 10% $CO_2$ incubator for 10-20 min in 5% $CO_2$ culture incubator rather. The transfected coverslips were transferred back to their original wells containing the original culture medium, and continued to incubate in 5% $CO_2$ culture incubator. Live digital images were acquired using a fluorescence microscope (Leica DM IRE, Wetzlar, Germany).

## Results and Discussion

DNA transfection is a powerful tool for studying gene functions. Among various methods to introduce exogenous genes into the target cells, $Ca^{2+}$-phosphate precipitation re-

mains one of the most popular and cost-effective techniques when transfecting neurons. Neurons are very sensitive cells and easy to die if microenvironment changes. Since the $Ca^{2+}$-phosphate precipitation method inevitably accompanies such microenvironmental changes, neurons tend to die after transfection procedure. Glia are neurosupportive in nature. Therefore, we took advantage of glia in designing a new protocol that would allow neuronal survival after transfection.

We used cerebrum for glial source. Astroglial cells, the main population of neuroglia, transform the bipolar raial glial cells during prenatal and early postnatal development. The shift from the radial glial fiber system to a diffuse glial network is achieved largely in the E17-P2 interval in the mouse [14]. Therefore, we used E20-P2 rat brains for glial sources. It took about 2 weeks for cerebral glial cells to grow until ~70-80% confluence. We often use once-split glial cultures, in which case we seed them so that they reach the required confluence in a couple of days. However, repeated split/transfers of glial cells seems not effective in neuroprotection. Once glial cells grow upto semiconfluence, the media were replaced with Neurobasal, a serum-free medium, in which Ara-C was added to prevent further proliferation (Fig. 1A). Hippocampal neurons, dissociated from E19 rat brains, were plated onto the glial layer. As shown in Fig. 1B, neurons developed well with robust processes, as judged morphologically. In this system neurons routinely survived longer than 3 weeks.

The critical factors in $Ca^{2+}$-phosphate precipitation method are the formation of homogeneous snow-like precipitate with particle size about 1-3 ㎛ and the subsequent removal of the precipitate [8]. However, the size and density of particles varied significantly even if we followed the reported protocol as closely as we can. When best conditions are met, about 10 particles/100 $㎛^2$ are formed after 30 min incubation in culture media (Fig. 2A). In this instances we incubate the plates for 40-60 min. However, in many instances, the particle formation was far from satisfactory (less than 1 particle/100 $㎛^2$) and the size was smaller than usual ones (Fig. 2B). Longer incubation time did not help forming particles in these instances, and transfection did not work. When the particle density was in the range of 3-6 particles/100 $㎛^2$ (Fig. 2C), longer incubation in culture medium did help form more particles (Fig. 2D). In these instances we incubate the plates up to 1.5 h before dissolution of the particles in 10% $CO_2$-preequilibrated media.

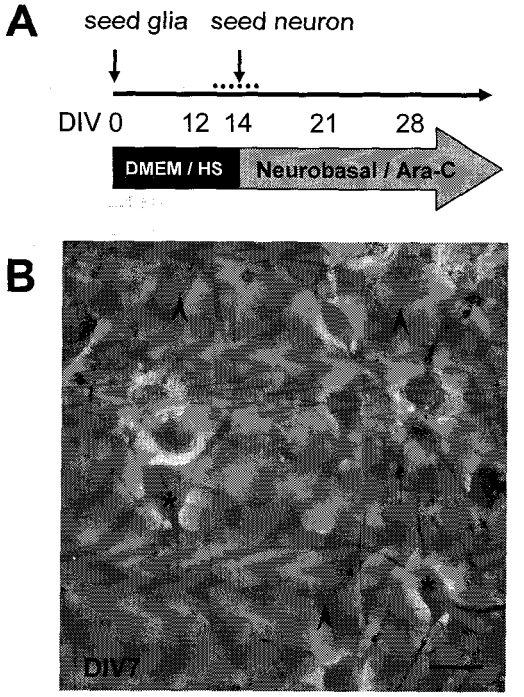


Fig. 1. Neuron-glia cocultures. A, Schematic represention of culture schedule. Glial cells from cerebri of E19-P2 were seeded on poly-D/L-lysine-coated coverslips in DMEM containing 10% horse serum. Once the culture reaches at 70-80% confluence (about two weeks, dotted line), media were replaced with Neurobasal containing Ara-C. Hippocampal neurons were seeded onto glial monolayer. B, A photomicrograph showing the coculture on DIV7. Neurons and glia are marked by asterisk and arrowheads, respectively. Bar, 20 ㎛.

When optimal particle density was achieved, as short as 5 min incubation in 10% $CO_2$-preequilibrated media was long enough to dissolve the particles (Fig. 2E).

Examples of transfected neurons were shown in Fig. 3. Cultures were transfected on DIV14 with pEGFP-CaMKIIα and pDsRed2 plasmids. Images were obtained after 24 h expression. The α-isoform of Type II calcium/calmodulin-dependent protein kinase is a typical postsynaptic membrane protein enriched in the postsynaptic density (PSD) [2,11]. As expected, EGFP-CaMKIIα fusion protein formed punctae along dendritic trees (Fig. 3A), which is a characteristic expression pattern of CaMKIIα [19]. This expression pattern of CaMKIIα exhibits only when a neuron matures, which is achieved in approximately 2 weeks *in vitro*. Neurons grown less than 2 weeks *in vitro* would not exhibit such patterns. Another example where DsRed2 was expressed was shown in Fig. 3B. DsRed2 is not a synaptic protein but diffusely distributed throughout the cell.

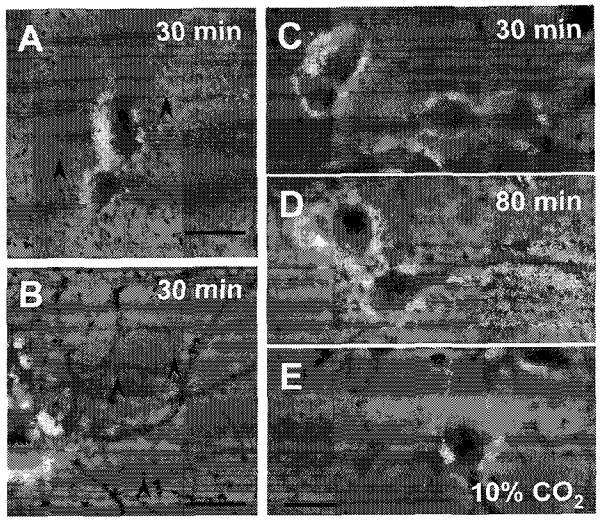


Fig. 2. Photomicrographs showing formation and removal of DNA/Ca2+-phosphate precipitate. A, Optimal formation of the precipitate was formed in 30 min. B, Little fromation of precipitates. Particles are marked by arrowheads in A and B. C. Formation of precipitates at intermediate density in 30 min. D. Longer incubation of C. E. Removal of precipitates by dissolving with slightly acidic transfection medium preequilibrated in a 10% CO2 incubator. Scale bar, 20 ㎛.

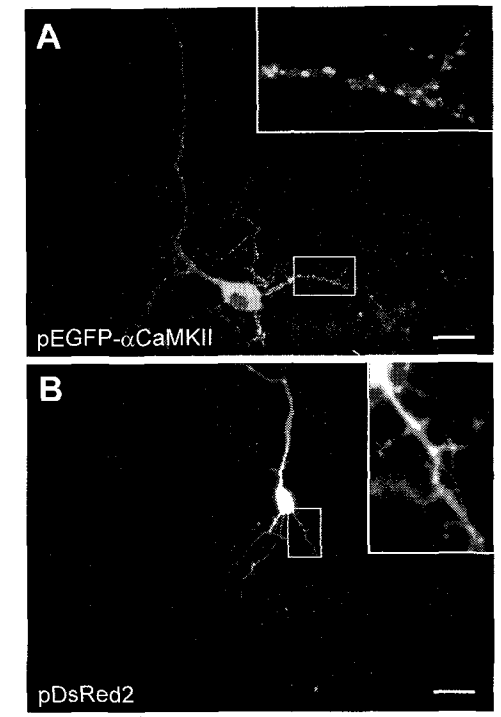


Fig. 3. Photomicrographs showing transfected neurons. Cultures were transfected with pEGFP-CaMKIIa (A) and pDsRed2 (B) on DIV14 and imaged after 24 hrs. Box areas were enlarged and shown in insets. Note punctate expression of the synaptic protein CaMKIIa fused with EGFP (A), and diffuse expression of non-synaptic protein RFP (B). Scale bar, 20 ㎛.

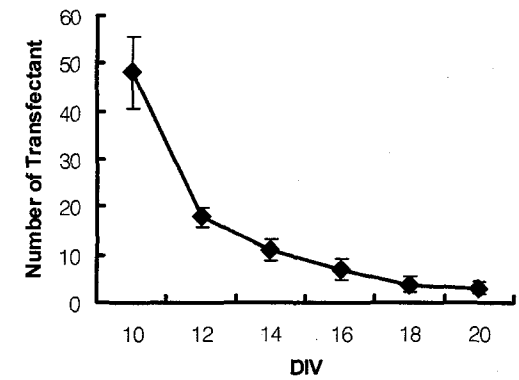


Fig. 4. Efficiency of transfection depending on DIV. Cultures were transfected on indicated DIV. Transfected neurons were identified in a fluorescence microscope.

The transfection efficiency drops down rapidly after about DIV10. The efficiency of transfection using cultures earlier than DIV8 can be as high as 60% [8]. However, when cultures longer than DIV10 is used, most cells die due to cytoxicity of the procedure. We tested the efficacy of the current protocol in application to older cultures. When neurons were plated at a density of 57,000 cells/$cm^2$ in 24-well culture plates onto monolayer of glial cells, there appeared several transfected neurons in cultures maintained up to DIV18 (Fig. 4). This was impossible with previous available protocols in our hands.

DNA transfection is a powerful tool for studying gene functions. Although $Ca^{2+}$-phosphate precipitation have been the most popular and cost-effective techniques, the cytotoxic effect of the procedure limited its application to sensitive cells such as neurons. The transfection rate of the $Ca^{2+}$-phosphate method is usually low, about 1-5% in average [4]. There have been many attempts in various labs to improve the $Ca^{2+}$-phosphate transfection efficiency in neurons [10,16]. However, the efficiency reported is far less than other methods such as viral infection, which often reaches more than 40% of transduction efficiency [7] Although Jiang *et al.* [8] reported much high efficiency (up to 60%) using hippocampal neurons cultured in microislands, its applicability to usual culture plates such as 24-well ones is not known. Therefore, our protocol that takes advantage of glial cells provides a novel method that can be reliably used for transfecting mature neurons *in vitro*. In conclusion, taking advantage of glial cells and controlling the precipitate formation, we have developed a reliable protocol that enables transfection of mature neurons.

## Acknowledgement

This work was supported by the research program of Dongguk University.

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## 초록 : 신경세포-신경교세포 공동배양을 이용한 성숙한 해마신경세포의 효율적인 형질전환 방법

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형질전환은 유전자의 기능을 이해하는데 매우 중요한 기법이다. $Ca^{2+}$-인산 침전법은 시간과 비용이 저렴하여 가장 흔히 사용된다. 그러나 성숙 신경세포는 어린 신경세포나 다른 세포종에 비하여 형질전환이 어렵고 쉽게 죽는다. 본 연구에서는 Clontech사의 CalPhos™ Mammalian Transfection 방법을 수정하여 성숙한 신경세포를 효율적으로 형질전환할 수 있는 방법을 고안하였다. 대뇌 신경교세포를 DMEM/10% 말혈청에서 70-80% confluence까지 키우고 배지를 혈청이 첨가되지 않은 Neurobasal/Ara-C로 바꾸어 주어 더 이상 신경교세포가 분열하지 않게 한 다음, 여기에 E19 해마신경세포를 접종하여 배양하였다. DNA/$Ca^{2+}$-인산 침전물은 Clontech사의 CalPhos™ Mammalian Transfection Kit을 이용하여 크기(0.5-1 ㎛ in diameter) 및 농도(약 10 particles/100 $㎛^2$)를 배지에서 배양시간을 변화시켜 적당히 조절하였다. 이렇게 하면 *in vitro*에서 2주 이상 배양한 신경세포도 24-well plate 한 well 당 10-15개의 형질전환된 건강한 신경세포를 얻을 수 있었다. 이 방법의 효용성을 검증하기 위하여 연접단백질인 EGFP-CaMKIIα 융합단백질과 RFP 단백질 유전자(각각 pEGFP-CaMKIIα 및 pDsRed2)를 형질전환한 결과 전자는 점박이 모양, 후자는 세포전체에 퍼진 양상의 표현을 관찰할 수 있었다. 따라서 본 연구는 성숙한 신경세포를 효율적으로 형질전환할 수 있는 방법을 제공한다.