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Protocol

Introduction of Green Fluorescent Protein (GFP) into Hippocampal Neurons through Viral Infection

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INTRODUCTION

Expression of green fluorescent protein (GFP), its more fluorescent mutant forms (e.g., EGFP [enhanced GFP]), or their fusion protein derivatives, affords a number of informative possibilities in cellular neuroscience. EGFP is a soluble protein and appears to be homogeneously distributed within the cytosol of neurons when expressed. Thus, it reveals the structure of the neuron, including the cell body, and axonal and dendritic arbors. It is also sufficiently bright to reveal detailed structures such as axonal boutons and dendritic spines. When expressed as a fusion protein, EGFP can provide information about the distribution characteristics of the proteins within neurons. Furthermore, during single-cell electrophysiological studies, such expression can direct the investigator to record from a cell carrying a foreign gene. In this protocol, we describe the use of the Sindbis pseudovirus expression system to deliver GFP to neurons. Sindbis is a member of the alphaviruses, which are plus-stranded RNA viruses. This protocol uses the DH(26S) strain, which preferentially infects neurons over glia (50:1). Two infection methods are given: one for dissociated hippocampal cultured neurons and one for organotypic hippocampal slices.

RELATED INFORMATION

A number of methods have been used to express heterologous proteins in neurons. These include viral transfection (de Hoop et al. 1994; Pettit et al. 1995; Moriyoshi et al. 1996; Goins et al. 1997), lipofection (Holt et al. 1990), calcium phosphate (Watson and Latchman 1996), biolistic (Lo et al. 1994), and electroporation approaches. A full list of available protocols for neural slice culture and neuronal cell culture methods can be found at http://cshprotocols.cshlp.org/cgi/collection/neural_cell_culture. In particular, see **Preparation of Rodent Hippocampal Slice Cultures** (Fuller and Dailey 2007). A description of useful EGFP mutants can be found in Cubitt et al. (1995) and Heim et al. (1995).

Sindbis is related to the Semliki Forest virus that has previously been used for heterologous expression in neurons (de Hoop et al. 1994). The Sindbis virus infects a wide range of species including mammals, birds, reptiles, amphibians, and insects (Schlesinger 1993), and different strains can be used to selectively infect different cell types (Corsini et al. 1996). To generate infective Sindbis virus particles that express a gene of interest, we have essentially followed the methods described by Bredenbeek et al. (1993). The methods have been used to express and analyze the expression of several proteins in various preparations (Shi et al. 1999; Hayashi et al. 2000; Zhu et al. 2000; Esteban et al. 2003).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Baby hamster kidney (BHK)-21 cells, plated in 10-cm (see Step 3) and 35-mm (see Step 8) dishes

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<**!**>Fast Green, 0.1% (Optional; see Step 16)

Helper virus plasmid DH(26S)

The helper virus plasmid contains the gene for the structural proteins necessary for the production of virus particles (Schlesinger 1993). It is advantageous to use a highly expressing helper RNA containing mammalian tRNA sequence in its 5′ region (Bredenbeek et al. 1993). An alternative helper, termed DHBB (tRNA/TE12), contains the 5′ tRNA sequence as well as neurotropic glycoprotein genes and can be used for efficient production of pSinRep(nsp2S^{726}) virus for infection of neurons (J Kim and P Osten, unpubl.).

<**R**>MEM for neuron culture (for infecting dissociated neurons; see Steps 12 and 13)

Neural tissue sources

E19 rat hippocampi (source of dissociated neurons; see Steps 12 and 13)

P1 rat pups (source of astrocytes for plating dissociated neurons; see Steps 12 and 13)

PND6 (postnatal day 6) to PND7 rat pups (source of hippocampal slices; see Steps 14-17)

A full list of available protocols for neural slice culture and neuronal cell culture methods can be found at http://cshprotocols.cshlp.org/cgi/collection/neural_cell_culture.

Recombinant Sindbis plasmid vector pSinRep5 (Bredenbeek et al. 1993), containing the gene of interest, EGFP cDNA (derived from pEGFP-N1; Clontech)

The EGFP gene is cloned between the subgenomic promoter and a poly(T) sequence. An alternative Sindbis expression vector, pSinRep(nsp2S^{726}), can be used for prolonged expression with decreased cytotoxicity in dissociated cultured neurons (see Steps 12 and 13). This vector is based on a mutant Sindbis virus containing a single-amino residue change of P to S at position 726 (Dryga et al. 1997). The P726S mutation results in decreased viral replication and thus a lower-titer viral preparation.

The low level of pathogenicity of Sindbis virus in humans has allowed it to be classified as a Biosafety Level-2 (BL-2) agent by the National Institutes of Health (NIH) Recombinant DNA Advisory Committee. Nevertheless, before any constructs are synthesized, experiments should be cleared through an institutional biosafety committee. All personnel working with the Sindbis Expression System (Invitrogen) should be properly trained to work with BL-2 organisms. BL-2 precautions include the use of laminar flow hoods, laboratory coats, appropriate gloves and eye protection, and decontamination of infectious wastes. Sindbis virus can be inactivated by organic solvents, bleach, or autoclaving. In addition, the components of the Sindbis Expression System have been designed to guard against any potential health threats.

Restriction enzymes appropriate to the recombinant Sindbis vector and the helper virus plasmid (see Step 1)

RNA polymerase (e.g., Ambion SP6 transcription kit) and other reagents for in vitro transcription of DNA

Equipment

Capillary, glass, ~20 µm tip (for infecting hippocampal slices; see Steps 14-17)

Coverslip, 25-mm glass (Belco) coated with poly-L-lysine (for infecting dissociated neurons; see Steps 12 and 13)

Electroporator (see Step 3)

Equipment for in vitro transcription of DNA

Incubator preset to 37°C

Incubator preset to 35°C, 5% CO_2

Microinjection equipment (for infecting hippocampal slices; see Steps 14-17)

Millicell cell culture insert plates (Millipore) (for infecting hippocampal slices; see Steps 14-17)

Ultracentrifuge tubes

Ultracentrifuge with swinging-bucket rotor (e.g., Beckman SW41)

METHOD

Preparation of Sindbis Virus Expressing EGFP

In Vitro Transcription

1. Linearize the recombinant Sindbis vector and the helper virus plasmid with appropriate restriction enzymes. Use 1 µg of each DNA.

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2. Transcribe the linearized DNAs using a standard in vitro transcription protocol with an appropriate RNA polymerase.

   The RNA thus produced is capped and polyadenylated. The Sindbis construct produces a recombinant transcript encoding the gene of interest and the native components essential for viral (and hence transgene) replication. This transcript does not encode the viral structural proteins necessary for the production of virus particles (Schlesinger 1993). These are encoded by the helper virus transcript (Bredenbeek et al. 1993).

Transfection

3. Use ~10 µg of each transcript to cotransfect BHK-21 cells (1×10^7 cells per sample, plated in a 10-cm dish) by electroporation.

4. Incubate the transfected cells at 37°C. After 24 h, estimate the efficiency of transfection by examining the cells for cytopathic effects (elongation, detachment from substrate, etc.).

   By this time, most cells should be showing cytopathic effects.

5. Return the cells to the incubator for a further 12-24 h.

   For constructs driving EGFP expression, the efficiency of the electroporation step can easily be checked by examining the cells under an inverted microscope equipped for EGFP fluorescence imaging. Alternatively, if antibodies are available, immunocytochemistry can be performed on the cells after the viral supernatant has been removed. Using this method, >90% of cells should show the exogenous protein. This level of efficiency is necessary for the production of high-titer virus suspension. After 36-48 h, the culture medium containing the viruses is collected (designated unpurified infective supernatant), and this can be used to infect other cells, such as neurons. At this time, almost all cells should exhibit cytopathic effects.

6. Collect the cell culture medium, containing the recombinant virus particles, and centrifuge in an ultracentrifuge using a swinging-bucket rotor at 160,000*g* for 90 min at 4°C.

7. Store the resulting viral suspension at -80°C.

   Essentially no helper virus RNA is packaged into the newly produced viral particles, because the helper RNA either completely lacks a packaging signal, as in the case of the DH-BB(tRNA/TE12) helper, or contains the signal, but packages very inefficiently due to other deletions in the Sindbis genome (Bredenbeek et al. 1993). This prevents any further replication of the virus and leads to the production of heterologous protein only when host cells are infected with the virus.

Titration of Sindbis Pseudovirus Suspension

8. Infect a known number of BHK-21 cells (10^5 cells/35-mm dish) with an unknown number of virus particles in a known volume of culture supernatant (e.g., 0.5-50 mL).

   Because the Sindbis pseudovirus particles do not undergo a second round of infection, and thus do not form plaques, the titer of the virus solution cannot be determined with a conventional plaque assay.

9. Incubate for 24 h at 37°C and then determine the number of infected cells as a portion of the total number of cells.

   The proportion of infected cells versus the total number of cells in several microscope fields can be determined either by fluorescence (for EGFP or its fusion protein), by X-gal staining (for LacZ), or by immunostaining (for any protein for which an antibody is available).

10. Calculate the virus titer as follows:

    Virus titer = (Proportion of infected cells per visual field $\times 10^5$)/(Total number of cells per visual field × Volume of virus solution)

    A typical yield from this protocol is 10^6 to 10^7 infective particles per milliliter, but in some cases, it can be as high as 10^8 infective particles per milliliter. Although the source of this variability is not entirely understood, it is due in part to the quality of RNA, viability of BHK-21 cells used for electroporation, and the length and species of cDNA used.

    The amount of virus-containing solution required to generate an appropriate infection (i.e., injection in slices, as described below) is often determined empirically, without precise titration.

11. (Optional) If necessary, concentrate the viral suspension.

    Concentration may be necessary for some experiments in which a particularly efficient infection is required (e.g., infection of slices). Using this step, the titer of virus can be increased by 50- to 100-fold.

    i. Remove cell debris by centrifuging at 400*g* for 10 min and transfer the supernatant to an ultracentrifuge tube.

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ii. Centrifuge the supernatant in an ultracentrifuge using a swinging-bucket rotor at 160,000*g* for 90 min at 48°C.

iii. Aspirate the supernatant from the top, leaving ~200 mL.

iv. Resuspend the pellet (which is usually invisible), aliquot into small amounts (~5 mL), and store at -80°C.

Infection of Neurons with Recombinant Sindbis Virus

Steps 12 and 13 describe infection of dissociated hippocampal cultured neurons. Steps 14-17 describe infection of organotypic hippocampal slices.

Infecting Dissociated Hippocampal Cultured Neurons

In this procedure, dissociated hippocampal neurons are infected with Sindbis virus. A confluent monolayer of cortical astrocytes, derived from P1 rat pups (100,000 cells), is formed on a 25- × 25-mm coverslip coated with poly-L-lysine. Neurons are dissociated from E19 rat hippocampi by trypsin digestion, followed by trituration and plated onto the astrocyte layer at 68,000 cells/coverslip (Banker and Goslin 1991; Maletic-Savatic and Malinow 1998).

12. Culture cells as follows:

i. Plate astrocytes and neurons on a 25-mm-square glass coverslip.

If neurons are plated without an astroglial feeder layer (i.e., directly on poly-L-lysine), Sindbis infection efficiency will be greatly reduced. Because Sindbis is a membrane-enveloped virus, it probably sticks to poly-L-lysine. Use an alternative substrate for plating neurons directly on cell culture plates (e.g., collagen).

ii. Maintain cultures in MEM for neuron culture (Banker and Goslin 1991) for 7-14 d at 35°C in an atmosphere of 5% CO_2.

13. Infect the cultured neurons by adding an aliquot (usually 5-50 mL) of the unpurified infective supernatant suspension (as prepared in Steps 1-5 above).

Detectable expression is seen within 6 h of infection. After 3 d, there are few obvious adverse effects on cell morphology. After 5 d, however, some cells show clear toxic effects. One study reports infection of cultured dorsal root ganglion cells with Sindbis virus, and expression of lacZ could be detected for more than 1 mo (Corsini et al. 1996).

Infecting Organotypic Hippocampal Slices

Organotypic hippocampal slices are prepared as described by Stoppini et al. (1991). This protocol was optimized by taking animals of various ages and varying incubation and virus application times.

14. Prepare organotypic hippocampal slices from PND6 to PND7 rat pups on Millicell cell culture insert plates, as described by Stoppini et al. (1991), in a medium described by Musleh et al. (1997).

A detailed method for slice preparation, also based on Stoppini et al. (1991), can be found in ***Preparation of Rodent Hippocampal Slice Cultures*** *(Fuller and Dailey 2007).*

15. Maintain the tissue slices at 35°C in an atmosphere of 5% CO_2. Change medium two to three times a week.

16. After 1-10 d, inject Sindbis-EGFP virus particles (prepared in Steps 1-11) using a fine (tip ~20 µm) glass capillary by applying repeated pressure pulses (5 msec, 2 psi).

Inject virus into slices as described by Pettit et al. (1995).

Visualization of virus solution with 0.1% Fast Green helps handling.

A minimum of 4-5 d after preparation is necessary for the slices to become adherent to the substrate filters.

17. Perform imaging and/or electrophysiological analysis 12 h to 5 d after infection.

DISCUSSION

Imaging

Expression of EGFP in the manner described here produces very bright neurons, comparable to neurons loaded intracellularly with a high concentration of fluorescein. Neurons expressing EGFP can be imaged under epifluorescence and two-photon laser-scanning microscopy. For epifluorescence, both xenon 75-W and mercury 100-W light sources can be used.

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For epifluorescence, filter sets from Chroma Technology, e.g., 31001 (D480/30x Exciter [EX]; 505DCLP Dichroic [BS]; D535/40m Emitter [EM]), or Omega, e.g., XF23 (Exciter 485DF22; Dichroic 505DRLP; Emitter 535DF35), are suitable. These filter sets produce very little (<1%) overlap between EGFP and Texas Red signals. There can be significant overlap between the EGFP signal (which appears green to the eye) and background autofluorescence (which appears more yellow and is generally restricted to astrocytes).

Advantages and Limitations of the Sindbis Virus System

Several limitations to the use of Sindbis virus should be kept in mind. First, there is a limit to the size of the construct inserted in the viral genome. This is ~6 kb. Second, cell toxicity is associated with infection. Different tissues have different limits. Dissociated cultured neurons infected with pSinRep5 virus do not survive more than ~2 d following infection, whereas cells infected with the attenuated pSinRep(nsp2S^{726}) virus are viable for ~3-5 d. Neurons in cultured organotypic slices survive ~4-5 d following infection, even with the pSinRep5 virus. We have found that neurons infected with GFP for <3 d have no difference in membrane or synaptic properties compared with noninfected neurons. Neurons in animals 12-21 d old infected in vivo (see Takahashi et al. 2003) do not survive more than ~72 h after infection. Neurons in older animals are more poorly infected, and infection causes more toxicity earlier. In slice cultures, we have not been able to express two different proteins in individual neurons by coinfection. There are reports, however, that dissociated neurons in culture can be coinfected with distinct Sindbis viruses (e.g., see Perez et al. 2001). These limitations can be overcome by using biolistic delivery, electroporation, or other viral vectors (e.g., herpes simplex virus [HSV]) (for details, see Carlezon et al. 2000).

The delivery of recombinant proteins to neurons can be difficult, variable, and time-consuming. However, the Sindbis expression system provides a relatively easy and reproducible means of achieving such delivery. Generation of the infective particles can be rapid (within 1 wk of having an appropriate subcloned construct). The strain used here is neurotropic, which is a particular advantage because it does not infect glial cells, and imaging provides better resolution. Infected cells remain viable for several days (at least) after infection and a large fraction of neurons can be infected simultaneously. Infection can be anatomically targeted to a small group of neurons, and gene expression is relatively rapid (detectable within hours). In conclusion, the Sindbis expression system has several features that make it preferable over other vector systems for some applications.

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