Introduction of green fluorescent protein into hippocampal neurons through viral infection

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Introduction

A number of methods have been used to express heterologous proteins in neurons. These include viral (deHoop 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), and biolistic approaches (Lo et al, 1994). Expression of green fluorescent protein (GFP), its more fluorescent mutant forms (e.g., EGFP, Cubbit et al., 1995; Heim et al, 1995), or as a fusion protein, afford a number of informative possibilities in cellular neuroscience. EGFP is a soluble protein and appears to be homogeneously distributed within the cytosol of hippocampal neurons when expressed. Thus it reveals the neuronal structure including cell body, and axonal and dendritic arbors. It is also sufficiently bright to unveil detailed structure like axonal boutons and dendritic spines (unpublished observations). When expressed as a fusion protein, EGFP can inform us about the distribution characteristics of the proteins within neurons. Furthermore, during single-cell penetration electrophysiological studies, such expression can direct the investigator to record from a cell carrying a foreign gene. In this chapter we describe the use of the Sindbis pseudo-virus expression system to deliver green fluorescent protein to neurons.

Sindbis Virus

Sindbis is a member of the alphaviruses which are plus-stranded RNA viruses. Sindbis is related to the Semliki Forrest virus that has previously been used for heterologous expression in neurons (deHoop et al, 1994). The Sindbis virus infects a wide range of species including mammals, birds, reptiles, amphibians, and insects (Schlesinger, et al., 1993). Different strains of Sindbis virus can be used to infect selectively different cell types (Corsini, et al., 1996). We have concentrated on one strain that preferentially infects neurons over glia. To generate infective Sindbis virus particles that express a gene of interest, we have essentially followed the methods described by Bredenbeek et al., 1993 and also described step by step in the Web site for Invitrogen (http://www.invitrogen.com/cat_sindbis.html).

Making Sindbis virus expressing EGFP

Here we describe briefly the basic methods of making a Sindbis virus that expresses EGFP. The gene of interest, EGFP cDNA (derived from pEGFP-N; Clonetech), is cloned into the Sindbis plasmid vector, pSinRep5 (Bredenbeek et al, 1993). This construct is linearized and from it RNA is transcribed *in vitro*. The RNA thus produced is capped, polyadenylated and contains sequences for the inserted gene and also the Sindbis viral

genome components that are essential for the replication of viral genome and the overproduction of mRNAs of the foreign gene. The transcribed RNA will not contain the gene for the structural proteins that are necessary for the production of virus particles (Schlesinger, et al., 1993). These are provided by another capped and polyadenylated RNA which is similarly produced by the *in vitro* transcription of a linearized helper defective virus plasmid DH(26S) (Bredenbeek et al, 1993). Both RNA species are then simultaneously transfected into BHK-21 cells by electroporation. In BHK-21 cells the transfected RNA directs the production of new genomic viral RNAs (containing the gene of interest) which are packaged into virus particles and bud off into the BHK-21 cell culture medium. The culture medium containing the viruses is collected (designated unpurified infective supernatant) and this can be used to infect other cells. This newly produced recombinant virus consists of envelope and coat proteins (which were encoded by the helper RNA) and the genomic RNA (containing the inserted gene). No helper virus RNA is packaged in the genome of the newly formed viruses because the helper RNA lacks a packaging signal. This prevents any further replication of the virus and only leads to the production of heterologous protein when host cells are infected with the virus.

Titration of Sindbis virus

The Sindbis pseudovirus particles do not undergo a second round of infection and thus do not form plaques. Therefore, the titer of the virus solution cannot be determined with a conventional plaque assay. Instead, the number of infected cells are counted and is employed as an indication of the number of infective Sindbis virus particles. A known number of BHK-21 cells (10^5 cells / 35 mm dish) are infected with an unknown amount of virus particles. After one day, the proportion of infected cells versus the total number of cells in several microscopic fields is determined either by fluorescence (for EGFP or its fusion protein), X-gal staining (for LacZ), or by immunostaining (for any protein for which an antibody is available). Virus titer is calculated as,

virus titer = (Number of infected cell per visual field X 10^5)/ (Number of total cell per visual field X volume of virus solution)

We typically get 10^6-10^7 infective particles / ml but in some cases, it can be as high as 10^8 infective particles / ml. While we do not know the source of this variability, some is due to quality of RNA, viability of BHK-21 cells used for electroporation, and the length and species of cDNA used. We note that in many cases, we decide empirically the amount of virus-containing solution required to generate appropriate infection, without precise titration.

Concentration of virus solution

For some experiments in which confluent infection is desired, one may need to concentrate the virus solution. To maximize the titer of virus, we combine a discontinuous sucrose density gradient followed by ultrafiltration. Unpurified infective supernatant is overlayed on a 20% sucrose layer, which itself is on a 55% sucrose

cushion. After centrifuging at 160,000 x g for 90 min, the interface between two sucrose layers is recovered. This solution can be used for injection, if the high sucrose concentration is not a cause for concern. To maximize the infectivity, the sucrose solution can be further ultrafiltrated using Centricon 100 (Millipore) centrifuged at1000 x g for 3 h (done intermittently, see Centricon protocol). These procedures can increase the titer 10-50-fold.

Infection of dissociated hippocampal cultured neurons

Dissociated hippocampal neurons are generated from 19-day rat embryos (Banker and Goslin, 1990) and plated onto a confluent monolayer of astrocytes. Astrocytes and neurons are plated at 50,000 and 30,000 cells, respectively, per 25 mm square glass coverslip (Belco). Cultures are maintained in serum-free medium (Banker and Goslin, 1990). We infect neurons by adding an aliquot (usually 5-50 uL) of the unpurified infective supernatant solution (described above). We see detectable expression within 6 hours of infection. We have monitored cells up to 5 days after infection and see little obvious adverse effect on morphology in the first 3 days. After this period some cells show clear toxic effects. One study reports infection of cultured dorsal root ganglion cells with Sindbis virus expressing lac-Z lasting over one month (Corsini et al., 1996). We use a strain of Sindbis, DH(26S), that confers relative neurotropism. We find that with dissociated cultured hippocampal neurons, the infection of neurons to glia is ~50:1.

Infection of organotypic cultured hippocampal slices

Organotypic hippocampal slices are prepared as described by Stoppini et al 1991. We have made slices from animals of various ages, incubated for various times, and applied virus after various times, but have generally settled on the following protocol:

1) organotypic slices are prepared from P7 to P14 animals; P7 slices yield better infection rate and slices survive better; P14 slices have better synaptic transmission in that lower stimulus intensities are required to elicit transmission.
2) slices are injected with Sindbis-EGFP one to 10 days after slices are prepared; at least one day is necessary to allow slices to become adherent to the substrate filters. Injection of virus into slices is conducted as described in Pettit et al (1995). One can also infect slices by immersing a thin strip of filter membrane in viral solution and then applying directly on the region of the slice where infection is desired. The membrane strip is removed when EGFP observation is conducted.
3) slices are imaged or electrophysiology is conducted from 12 hrs to 5 days after infection.

Imaging

Expression of EGFP in this manner produces very bright neurons. By comparison, the expression is as bright as a neuron loaded intracellularly with high concentration fluorescein (unpublished results). We have imaged neurons expressing EGFP under epifluorescence and two-photon scanning laser microscopy (in collaboration with Karel

Svoboda). For epifluorescence, we have used both xenon 75W and mercury 100W light sources.

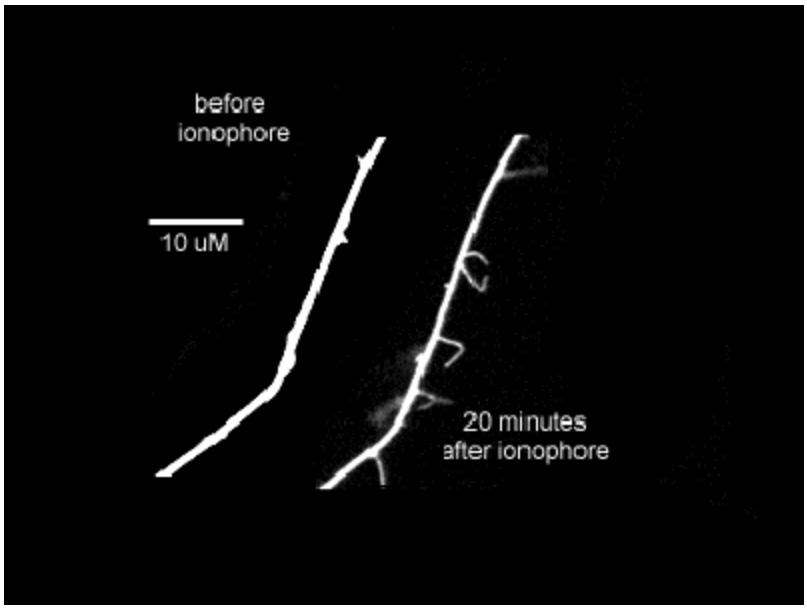
Filters

For epifluorescence, we have used filters from Chroma Technology or Omega. From Omega we have used their set XF23 (Ex 485DF22; Di 505DRLP02; Em 535DF35). From Chromatech we have used their set 31001 (Ex D480/30; Di 505DCLP; Em D535/40).With these filter sets we find very little (<1%) overlap between EGFP and Texas Red signals. There can be significant overlap between EGFP signal (which looks green to the eye) and background autofluorescence (which looks more yellow, and is generally restricted to astrocytes).

Conclusion

Delivery of recombinant proteins to neurons can be difficult, variable and time consuming. We have found that 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 week of having an appropriate subcloned construct). The strain we use is neurotropic. Infection appears not to be toxic (at least for several days after infection). Infection of a large fraction of neurons is possible; and infection can be anatomically targeted to a small group of neurons. Expression is relatively rapid (within hours). In conclusion, this method has several features that may make it preferable over other vector systems for some applications.

Figure: Fluorescent micrograph of a small region of a dissociated hippocampal neuron infected with Sindbis-EGFP. Images were obtained with AT200 Photometrics cooled CCD camera, Zeiss Axioscop, FITC optics, before (left) and 20 minutes after (right) 30 second exposure to calcium ionophore A23187 (1uM).



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