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Overexpression of Proteins in Neurons Using Replication-Deficient Virus

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Summary

Overexpression of proteins is a powerful way to determine their function. Until recently, the low efficiency of neuronal transfection has made it difficult to use overexpression and structure–function studies to investigate the role of neuronal proteins in their native environment. The development of neurotrophic viral systems has overcome the obstacle of low efficiency and allows for unprecedented opportunities to use biochemical and electrophysiological techniques to assess the effects of overexpressing wild-type or mutant proteins in neurons. Here, a general protocol for the production of replication-deficient Semliki Forest virus constructs directing the overexpression of proteins of interest in cultured mammalian neurons is described.

Key Words: Cultured neurons; infection; overexpression; replication-deficient virus; Semliki Forest virus; structure/function; virions.

1. Introduction

Structure–function studies provide valuable information about the mechanisms underlying protein function. Most structure–function studies to date have used heterologous expression systems that place constraints on the proteins that can be studied and the questions that can be addressed. This has been a particular problem for the study of neuronal protein function given the highly specialized nature of the neuron itself. One of the greatest concerns with the use of heterologous expression systems for overexpression and structure–function studies of neuronal proteins is the possibility that the protein will not be properly processed or trafficked outside its native environment. In addition, proteins that may ordinarily associate with the protein of interest (POI) may not be expressed in the heterologous system. To get around these problems, we

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overexpress wild-type and mutant versions of neuronal proteins with functions we are interested in studying in cultured mammalian central nervous system neurons using replication-deficient virus.

Here, we describe the generation of replication-deficient Semliki Forest virus virions. The first step is the polymerase chain reaction (PCR)-based subcloning of the nucleotide sequence encoding the POI into a mammalian expression vector, pIRES2-EGFP (enhanced green fluorescent protein). The pIRES2-EGFP vector directs the production of the POI separately from a reporter protein (EGFP) through an intervening internal ribosomal entry site (IRES) sequence. The first subcloning is followed by a second subcloning of the POI-IRES-EGFP cassette into the replication-deficient Semliki Forest virus vector (pSFV). Virions are produced after electroporation of RNA derived from the POI-IRES-EGFPpSFV1 construct, along with helper RNA, into baby hamster kidney (BHK) cells. Virions are activated and used to infect cultured neurons. Finally, immunocytochemistry is performed to verify expression of the POI.

2. Materials

2.1. PCR Amplification of POI Complementary DNA

1. Molecular biology grade (MBG) water (Eppendorf, Westburg, NY).
2. Thermostable proofreading DNA polymerase: Vent polymerase and buffer (New England Biolabs, Ipswich, MA).
3. Dimethyl sulfoxide (DMSO).
4. dNTPs: mix of 10 m*M* each, from 100 m*M* deoxynucleotide 5'-triphosphate (dNTP) set, PCR grade (Invitrogen, Carlsbad, CA) in MBG water.
5. Project-specific primers (Integrated DNA Technologies, Coralville, IA).
6. Template DNA encoding POI.
7. Mineral oil.
8. 1X Tris-borate ethylenediaminetetraacetic acid (EDTA) (TBE) buffer: 89 m*M* Tris-borate, 2 m*M* EDTA (from 10X TBE; Fisher, Pittsburgh, PA).
9. 10 mg/mL ethidium bromide stock (Sigma, St. Louis, MO; use caution as this is toxic).
10. 1% agarose (Invitrogen) gel in 1X TBE with 4 μL ethidium bromide stock/100 mL.
11. 10X dye loading buffer: 15% Ficoll-400, 0.25% bromophenol blue, 0.25% xylene cyanol FF in MBG water.
12. 5X PCR loading buffer: 1:1 mixture of 10X dye loading buffer and 10X TBE.
13. 1 Kb DNA ladder (Invitrogen): run 0.5 μg/lane using 5X PCR loading buffer.
14. QIAquick PCR Purification Kit (Qiagen, Germantown, MD).

2.2. Subcloning of PCR-Generated POI Complementary DNA Into the pIRES2-EGFP

1. pIRES2-EGFP vector (Clontech, Mountainview, CA).
2. Project-specific restriction enzymes (REs; New England Biolabs, Roche, Indianapolis, IN).

3. 10X bovine serum albumin (BSA): make from 100X BSA (10 mg/mL; New England Biolabs) in MBG water.
4. Calf intestine alkaline phosphatase (CIAP; MBI Fermentas, Burlington, Ontario, Canada).
5. 1X Tris-acetate EDTA (TAE) buffer: 40 m*M* Tris-acetate, 1 m*M* EDTA (from 10X TAE; Fisher).
6. 1% low-melting-point agarose (LMPA; Invitrogen) gel in 1X TAE with 4 μL ethidium bromide stock/100 mL.
7. Phenol/chloroform/isoamyl alcohol 25:24:1 (Sigma Fluka, St. Louis, MO; use caution as this mix is caustic).
8. Chloroform/isoamyl alcohol 49:1 (Sigma Fluka).
9. Pellet Paint coprecipitant (Novagen, Madison, WI).
10. Millipore UltraFree FilterSpin columns (Fisher).
11. Rapid DNA Ligation Kit (Roche).
12. One Shot competent *Escherichia coli* (Invitrogen).
13. Luria-Bertani (LB) medium.
14. Bacto Agar for plates (BD Biosciences, Clontech).
15. LB with kanamycin (KAN; 50 μg/mL).
16. KAN plates: LB agar plates with kanamycin (50 μg/mL).
17. QIAprep Spin Miniprep Kit (Qiagen).

2.3. Subcloning of Complementary DNA for POI-IRES-EGFP Into pSFV

1. REs and other materials as in **Subheading 2.2.**
2. pSFV and pSFVHelper2 vectors (Invitrogen; *see* **Note 1**).
3. Guanosine 5'-triphosphate (GTP)/cytidine 5'-triphosphate (CTP) mix: Mix of 1 m*M* each from PCR grade 100 m*M* dNTP set (Invitrogen) in MBG water.
4. Large (Klenow) fragment DNA polymerase I (Klenow; New England Biolabs).
5. LB with ampicillin (AMP; 150 μg/mL).
6. AMP plates: LB agar plates with ampicillin (150 μg/mL).

2.4. Linearization of Template DNA

1. Restriction enzyme: *Spe*I preferred (New England Biolabs, Roche).
2. Phenol/chloroform/isoamyl alcohol 25:24:1 (Sigma Fluka).
3. Chloroform/isoamyl alcohol 49:1 (Sigma Fluka).
4. Pellet Paint coprecipitant (Novagen).

2.5. RNA In Vitro Transcription

1. SP6 mMessage mMachine Kit (Ambion, Austin, TX).
2. 1X MOPS buffer: from 10X MOPS buffer (Fisher) in MBG water.
3. Denaturing formaldehyde gel: Place 0.5 g agarose in 35 mL MBG H_2O, heat in microwave, and cool to approx 60°C, and add 10 mL formaldehyde (*do not microwave formaldehyde*) and 5 mL 10X MOPS.
4. RNA loading buffer: 72 μL formamide, 26 μL formaldehyde, 16 μL 10X MOPS buffer, 8 μL glycerol, 18 μL MBG H_2O, and 2 μL ethidium bromide stock (10 mg/mL).
5. 10X dye loading buffer (*see* **Subheading 2.1.**, **item 10**).

2.6. Virion Production and Activation

1. BHK cells (ATCC, Manassas, VA).
2. Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen).
3. BHK medium: 95 mL DMEM, 5 mL fetal calf serum (Gibco), 0.25 mL pen/strep antibiotic (Gibco/Invigrogen).
4. Trypsin/EDTA (0.25% trypsin/1 m*M* EDTA; Invitrogen).
5. 1X Ribonuclease (RNase)-free phosphate-buffered saline (PBS): from 10X PBS (Fisher) in MBG water.
6. Chymotrypsin (Worthington Biochem, Lakewood, NJ): 2 mg/mL in PBS with 0.9 m*M* $CaCl_2$ and 0.5 m*M* $MgCl_2$.
7. Aprotinin (Roche): 6 mg/mL in PBS with 0.9 m*M* $CaCl_2$ and 0.5 m*M* $MgCl_2$.

2.7. Infection of Neuronal Culture

1. Cultured neurons.
2. Activated virions.

2.8. Immunocytochemical Confirmation of Protein Expression

1. Fix solution: 4% paraformaldehyde plus 4% sucrose in PBS.
2. Permeabilization buffer: 2% Triton X-100 in PBS.
3. Blocking buffer: 5% BSA (Sigma) in PBS.
4. 1° antibody solution: approx 1 μL antibody/mL in blocking buffer.
5. 2° antibody solution: approx 2 μL antibody/mL in blocking buffer.

3. Methods

Be aware that most institutions require permission to work with replication-deficient viruses. For replication-deficient semlike forest virus, the National Institutes of Health recommends biosafety level 3 practices in a biosafety level 2 environment, including use of a type II/class A biosafety cabinet.

3.1. PCR Amplification of POI Complementary DNA

It is almost always necessary to use PCR to introduce the three RE sites required for the two sequential subcloning steps employed for generation of the viral construct (*see* **Fig. 1**).

1. Primer design: select three RE sites to add to either side of the POI. Complementary DNA (cDNA) sequence in the PCR product: two at the 5' end, upstream of a Kozak consensus sequence and the Start codon, and one at the 3' end, immediately following the Stop codon. RE sites 1 and 2 are used to subclone the PCR product, and RE site 3 is used for the subsequent subcloning of the POI-IRES-EGFP cassette into the pSFV viral vector (*see* **Subheadings 3.2.** and **3.3.**). A Kozak sequence (GCCACC) is introduced just before the Start codon to ensure robust expression of the POI. The 5' primer encodes RE site 1, RE site 3, the Kozak sequence, the Start codon, and 15–20 additional bases perfectly matched to the POI cDNA sequence. The 3' primer

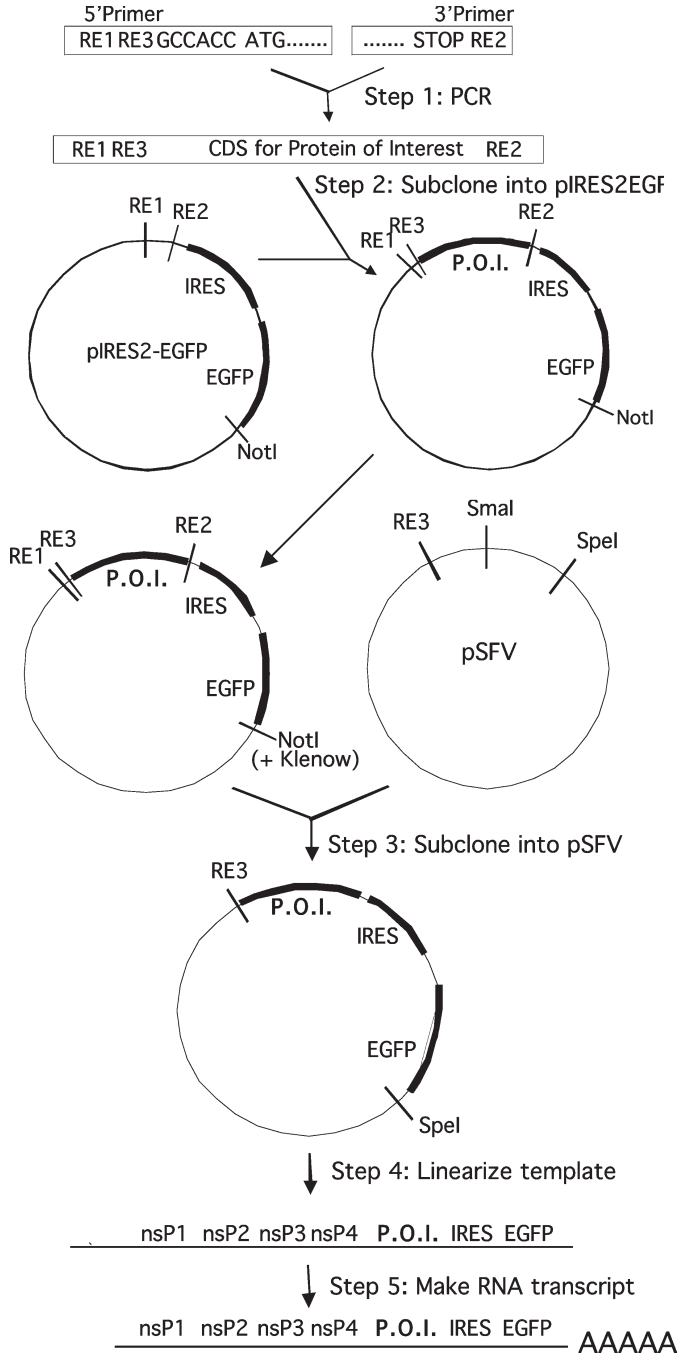

Fig. 1. Flowchart of **Subheadings 3.1–3.5.**

encodes 15–20 bases perfectly matched to the POI cDNA sequence, the Stop codon, and RE site 2. RE sites 1–3 must not be present in the cDNA encoding the POI. Be careful to note whether the new sequence created by the primers introduces any additional restriction sites. A GC lock at the 3' end of each primer is recommended.

In choosing RE site 3, note that the multiple cloning site (MCS) for pSFV contains just three restrictions sites: *Bam*HI, *Sma*I (a blunt cutter), and *Xma*I (an isoschizomer of *Sma*I that leaves an overhang). We now use a homemade pSFV variant with an improved MCS; we also use a homemade pIRES2-EGFP variant in which the *Bam*HI at the end of the MCS site has been removed, allowing us to use *Bam*HI as RE3.

Keep the initial melting temperature of your primers 65°C or above. Melting temperature is determined by the number and composition of nucleotides in the primers. There are many ways to estimate the melting temperature, but a quick and easy way is to multiply the number of A or T bases by 2, multiply the number of G or C bases by 4, then add the two numbers (this method will overestimate the melting temperature for long sequences, but it usually does not matter). To calculate the initial melting temperature, use only the perfectly matched bases between the primers and the POI template DNA sequence (i.e., do *not* include the bases encoding the RE sites or the Kozak sequence unless already present). After the first few cycles of PCR, sufficient PCR product builds up to serve as template itself; the primers have a much higher melting temperature with this PCR-derived template because of the additional bases of perfectly matched sequence that are not included in the initial melting temperature calculation.

2. PCR: to a PCR tube, add 34.5 μL MBG H_2O, 5 μL 10X Vent polymerase buffer, 2.5 μL DMSO, 1 μL dNTPs (10 m*M* each), 1 μL template DNA encoding POI, and 1 μL Vent polymerase for a total reaction volume of 45 μL. Overlay with 50 μL mineral oil. Place tube in PCR machine and start program (*see* below). After the PCR machine reaches 80°C, start the reaction by adding 2.5 μL of each primer (20 μ*M* stock in MBG water), for a final reaction volume of 50 μL. This "hot start" method increases the specificity and yield of the PCR product, as does the addition of DMSO.

   The following is an example PCR program for a 2.3-kb POI cDNA:

   1 cycle:
   - 95°C for 1.5 min (melting step)
   - 60°C for 2 min (annealing step)
   - 72°C for 2 min 20 s (extension step)

   2 cycles:
   - 95°C for 30 s
   - 60°C for 2 min
   - 72°C for 2 min 20 s

   22 cycles:
   - 95°C for 30 s
   - 65°C for 1 min
   - 72°C for 2 min 20 s

   Hold at 4°C

The annealing step temperature is raised (and the duration reduced) after the first three cycles because sufficient PCR product, perfectly matched to the entire length of the 5' and 3' primers, has been generated to serve as a template for subsequent rounds. The duration of the extension cycle is calculated assuming a polymerization rate for Vent polymerase of 1000 bases/min.

3. Diagnostic gel: verify the quality and yield of the PCR product (4 μL PCR product + 1 μL 5X PCR loading buffer) on a 1% agarose TBE gel in TBE running buffer using 1-kb DNA ladder. 5X PCR loading buffer ensures that low-molarity PCR samples are retained in the gel wells.
4. Purification of the PCR product: purify the PCR product with Qiagen's QIAquick PCR Purification Kit following the manufacturer's instructions. Purification is necessary to remove the proofreading Vent polymerase, which will remove overhangs created during the restriction digest described in the **Subheading 3.2.**

3.2. Subcloning of PCR-Generated POI cDNA Into the pIRES2-EGFP

1. Preparation of the PCR insert and vector: both the PCR product and the pIRES2-EGFP vector are cut with REs 1 and 2. Cut 14 μL purified PCR product for the insert (less if yield is very high). Cut 2 μg pIRES2-EGFP vector DNA brought up to a total of 14 μL volume with MBG water. After digestion with the REs, the vector is treated with phosphatase to reduce background (vector religating without insert). To a final volume of 20 μL, add 14 μL PCR DNA (or 2 μg pIRES2-EGFP in 14 μL MBG H_2O), 2 μL 10X restriction buffer, 2 μL BSA (if required), 1 μL RE 1, and 1 μL RE 2. Incubate restriction digest at 37°C for 2 h. Include BSA if either enzyme calls for it. After 2 h, add 0.5 μL CIAP to vector only (do *not* add CIAP to PCR DNA digest) and incubate for an additional 0.5 h at 37°C.
2. LMPA gel purification of PCR insert: add 2 μL 10X dye loading buffer to the restriction digest and run the cut PCR product out on a 1% LMPA gel in TAE running buffer. Cut DNA band out of the gel (using an adjustable ultraviolet illuminator, if possible, to minimize exposure of the DNA to ultraviolet light), mince the band, and place in a Millipore FilterSpin column; spin at maximum speed for 15 min.
3. Organic extraction of vector: add 80 μL MBG H_2O to digested pIRES2-EGFP sample to bring the volume up to 100 μL. Add an equal volume (100 μL) phenol/chloroform/isoamyl alcohol (caution: caustic). Vortex for 1 min. Spin for 3 min at maximum speed in a microcentrifuge. Transfer 100 μL of the aqueous (upper) phase to a fresh microcentrifuge tube. Add 100 μL of chloroform/isoamyl alcohol, vortex 1 min, spin 3 min, and transfer 90 μL of the aqueous phase to a fresh microcentrifuge tube.
4. Precipitate vector: follow manufacturer's instructions for Novagen's Pellet Paint and resuspend pellet in 40 μL MBG H_2O. Pellet Paint allows for a quick and efficient precipitation.
5. Ligation of PCR insert into pIRES2-EGFP vector: ligate the gel-purified cut PCR DNA into the prepared pIRES2-EGFP vector following manufacturer's instructions for the Roche Rapid DNA Ligation Kit. Briefly, add to make a final reaction volume

of 21 µL, 1 µL digested and purified pIRES2-EGFP, 7 µL digested and purified insert DNA for POI, 2 µL solution 2, 10 µL solution 1, and 1 µL solution 3 (enzyme). Incubate at room temperature for 15 min. For a vector-only control (to test the background occurrence of vector closing without an insert by performing the ligation reaction without any insert DNA added), put all of the above in a separate tube but replace PCR insert DNA with 7 µL MBG H_2O.

6. Transformation of competent *E. coli*: follow manufacturer's instructions for transformation of One Shot competent cells. Plate all of transformation mix on KAN plates and place in 37°C incubator overnight.
7. DNA miniprep: start four to six miniprep cultures with LB with KAN and put tubes in 37°C shaking incubator overnight (14–18 h). Purify plasmid DNA using QIAprep Spin Miniprep Kit according to manufacturer's instructions.
8. Diagnostic restriction digest and gel electrophoresis: cut the prepped DNA with REs 1 and 2 for 1 h at 37°C in a 10 µL reaction containing 4 µL DNA, 3 µL H_2O, 1 µL 10X restriction buffer, 1 µL BSA (as needed), and 0.5 µL REs 1 and 2. At end of digest, add 1 µL 10X dye loading buffer to sample and run cut product out on 1% agarose TBE gel (*see* **Subheading 3.1.**, **step 3**).
9. Sequence plasmid: sequence one (or more) of the plasmids having the correct restriction digest band pattern to verify that no base changes have been introduced by PCR (*see* **Note 2**).

3.3. Subcloning cDNA for POI-IRES-EGFP Into pSFV

The cDNA encoding the POI and EGFP, and the intervening IRES sequence, is excised from pIRES2-EGFP, purified, and inserted into the pSFV vector.

1. Restriction digest and Klenow treatment of POI-IRES-EGFP insert: in a reaction with a final volume of 27 µL, mix POIpIE (2 µg) in 20 µL MBG H_2O, 3 µL 10X restriction buffer, 3 µL 10X BSA, and 1 µL *Not*I (*see* **Note 3**). Cut for 1 h at 37°C. Add 1 µL 1 m*M* GTP/CTP mix (*Not*I site is all Gs and Cs) and 1 µL Klenow enzyme. Incubate for 30 min at room temperature to fill in *Not*I-generated overhang and create a *Sma*I-compatible blunt end (*see* **step 2**). After 30 min, inactivate Klenow by incubating at 75°C for 10 min. After cooling to 37°C, add 1 µL RE 3 and cut for 1 h at 37°C (*see* **Note 4**).
2. Restriction digest and phosphatase treatment of pSFV vector: to a final volume of 20 µL, add 14 µL pSFV (1 µg) in MBG H_2O, 2 µL 10X restriction buffer, 2 µL 10X BSA (if required), 1 µL RE 3, and 1 µL *Sma*I. Incubate for 2 h at 37°C, then add 0.5 µL CIAP and incubate for an additional 0.5 h at 37°C.
3. LMPA purification of insert (*see* **Subheading 3.2.**, **step 2**): use 3 µL 10X dye loading buffer.
4. Organic extraction and Pellet Paint precipitation of pSFV vector (*see* **Subheadings 3.2.**, **step 3** and **3.2.**, **step 4**).
5. Ligation of insert into vector (*see* **Subheading 3.2.5.**, **step 5**): use 7 µL POI-IRES-EGFP insert.

6. Transformation of competent *E. coli*: follow manufacturer's instructions for transformation of One Shot competent cells. Plate all 300 μL of transformation mix on AMP plate and place in 37°C incubator overnight.
7. DNA miniprep: Start four to six miniprep cultures with LB with AMP and put tubes in 37°C shaking incubator overnight (14–18 h). Purify plasmid DNA using QIAprep Spin Miniprep Kit according to manufacturer's instructions.
8. Diagnostic restriction digest and gel electrophoresis: cut with RE 3 and *Spe*I (*see* **Subheading 3.4.**, **step 1**) to identify correct POI-IRES-EGFPpSFV constructs.

3.4. Linearization of Template DNA

Linearized template DNA is used for in vitro transcription of RNA that is electroporated into BHK cells to make the virions. Prepare template DNA of your construct and pSFVHelper2 by cutting plasmid DNAs with *Spe*I (*see* **Note 5**).

1. Linearize DNA in a final volume of 40 μL by adding 13 μg DNA in 30 μL MBG H_2O, 4 μL10X restriction buffer, 4 μL 10X BSA, 2 μL *Spe*I. After 2 h, add 60 μL MBG H_2O to bring total volume to 100 μL.
2. Organic extraction: *see* **Subheading 3.2.**, **step 3**.
3. Precipitate linearized DNA with Pellet Paint: *see* **Subheading 3.2.**, **step 4** Resuspend in 15 μL MBG H_2O.

3.5. RNA In Vitro Transcription

Use gloves and RNase-free tubes during this and subsequent steps to prevent RNA degradation by the RNases that are ubiquitously present on skin.

1. In vitro RNA transcription from template DNA: follow manufacturer's instructions for SP6 mMessage mMachine. To a final reaction volume of 20 μL, add 10 μL 2X NTP/CAP solution, 2 μL 10X reaction buffer, 4 μL linear template DNA, 2 μL GTP (required for long transcripts), and 2 μL SP6 enzyme mix. Incubate for 2 h at 37°C, store at −20°C (or colder).
2. Denaturing gel electrophoresis assessment of RNA quality and quantity: remove 1 μL RNA reaction mix and add to 3 μL RNA dye loading buffer; run out on a denaturing formaldehyde gel to test for quality and quantity. Load one lane with 1 μL 10X loading buffer to monitor progress through the gel. Good RNA will run as a tight, bright band (sometimes as a doublet).

3.6. Virion Production and Activation

POI-IRES-EGFPpSFV RNA and SFVHelper2 RNA are electroporated into BHK cells to produce *inactive* replication-deficient virions encoding the POI and EGFP. A safety feature of the SFV system is that chymotrypsin treatment is required to activate the virions before they are able to infect neurons. Check with your local safety administrators for the requirements your institution may have for working with the replication-deficient Semliki Forest virus.

1. Grow and harvest BHK cells: grow BHK cells in BHK medium at 37°C in 175-cm^2 tissue culture flasks until 80–100% confluent. One 175-cm^2 flask will yield approx 0.5–2 × 10^7 cells at 80–100% confluence (~1 × 10^7 cells are required for each batch of virions). Remove BHK medium and briefly rinse flask bottom with approx 2–5 mL DMEM (or other serum-free solution). Add 5–7 mL trypsin/EDTA to the flask and incubate 5 min to allow cells to lift from the flask bottom. Firmly tap the flask on the side to completely free the cells. Add 5 mL DMEM, rinsing the surfaces of the flask, and transfer the contents of the flask to a 15-mL conical vial. Spin 4 min in a clinical centrifuge to pellet cells.

   Carefully pour off the supernatant from the previous spin. Add 5 mL DMEM and triturate until cells are fully resuspended (taking care to avoid air bubbles). Add another 5 mL DMEM for a total volume of 10 mL. Spin 4 min in the clinical centrifuge to pellet cells. Carefully pour off the supernatant. Resuspend all cells in 10 mL RNase-free PBS (combining cells from multiple flasks). Place a drop of the cell suspension on a hemocytometer and count cells. Spin remaining suspension for 4 min in a clinical centrifuge to pellet cells, pour off supernatant, and resuspend cells in an appropriate volume of RNase-free PBS to give a concentration of approx 1 × 10^7 cells/mL.
2. Electroporation of RNA into BHK cells: add 0.8 mL BHK cell suspension to a cuvet and then add 9 μL POI-IRES-EGFP RNA and 9 μL SFVHelper2 RNA. Place cuvet on ice.

   This procedure assumes use of a Bio-Rad Gene Pulser II and Capacitance Extender Plus. Electroporate immediately after adding the RNA with Gene Pulser set at 0.4 kV and 900 μF. The time constant should read 12–15 ms. Place the cuvet on ice for 5 min. To a 60-mm tissue culture dish, add 5 mL BHK medium and the contents of the cuvet, avoiding the mucilaginous debris produced during electroporation that floats at the surface (*see* **Note 6**).
3. Production of virions: incubate transfected BHK cells for 48 h at 31°C and harvest the medium (which contains the released virions). Growing the cells at 31°C after electroporation increases the titer of the virion stocks. Freeze virion stocks overnight at −20°C, then store at −80°C.
4. Activation and storage of virions: to 0.5 mL virion stock, add 50 μL chymotrypsin and incubate for 40 min at room temperature. After 40 min, add 55 μL aprotinin and incubate for 5 min at room temperature. Store activated virion stock at −20°C for up to a month (*see* **Note 7**).

3.7. Infection of Neuronal Culture

Add 5–50 μL activated virion stock per milliliter neuronal culture medium and incubate for 4–48 h. Infection efficiency for virion stocks varies widely from virion prep to virion prep, and the appropriate volume for each batch must be determined empirically. In addition, different neuronal culture preparations can be more or less amenable to infection; this also can only be determined empirically. It takes about 10 h for the EGFP to become clearly detectable

in infected neurons, and cell health often declines 24–48 h after infection. Adding more virion stock will increase the number of infected neurons (and start to infect astrocytes), but cell health is often compromised; this may be acceptable for biochemical assays for which maximal infection efficiency is required, and harvesting of neurons can take place earlier than 10 h (significant amounts of protein are made even in the first few hours after addition of the virion stock, although they may not have a chance to be trafficked to their proper destination).

3.8. Immunocytochemical Confirmation of Protein Expression

Immunocytochemistry is used to confirm expression of the POI. Once protein expression has been confirmed for a particular POI-IRES-EGFPpSFV construct, GFP fluorescence is sufficient to indicate the presence of POI.

1. Fix cells: replace culture medium with appropriate volume of fix solution and incubate at room temperature for 20 min. Replace solution with permeabilization buffer and incubate at 4°C for 10 min. Replace solution with blocking buffer and incubate at 4°C for 1 h.
2. Label cells with primary antibody: replace blocking buffer with an appropriate volume (just enough to cover is usually sufficient) of 1° antibody solution for 1–12 h at 4°C. Remove 1° antibody solution and wash three times with blocking buffer for 5 min at room temperature, rocking slowly.
3. Labeling cells with secondary antibody: replace blocking buffer with 2° antibody solution and rock slowly in the dark (or cover with foil) for 1 h at room temperature. Wash three times with PBS, rocking slowly in the dark for 10 min at room temperature.
4. Mount cover slips on slides and visualize using appropriate detection technique (e.g., fluorescent microscopy).

4. Notes

1. Invitrogen has discontinued the SFV Gene Expression System.
2. So-called silent mutations, in which a base change does not alter the amino acid sequence, are acceptable as long as the mutation does not introduce any new unwanted restriction sites.
3. If there is a *Not*I site in the coding sequence of your POI, then modify the *Not*I-plus-Klenow strategy using the next unique downstream restriction site in pIRES2-EGFP; note that the *Xba*I site just beyond the *Not*I site is methylated and will not cut unless DNA is grown in a dam$^-$ host.
4. Depending on the size of the sequence encoding your POI, it may be difficult to distinguish your "insert" band from the (unwanted) "left-over pIE vector" band when you run the cut plasmid out on an LMPA gel (**Subheading 3.3.**, **step 3**) if the POI-pIRES2-EGFP is cut with only two enzymes. For these cases, when you add RE 3 to the restriction digest mix, include an additional enzyme that cuts the vector at an appropriate location to allow ample separation of bands.

5. *Spe*I is the manufacturer's recommendation for the linearizing enzyme. If you have an *Spe*I site within the coding sequence for your POI, then you must choose an alternate unique cutter downstream of *Spe*I. Although the SFV Gene Expression System manual suggests using *Sap*I, we have found that this enzyme cuts at locations other than its predicted restriction sites; other enzymes to consider are *Pvu*I, *Xmn*I, and *Sph*I. We have successfully generated virions using template linearized with *Sph*I, the site farthest from the *Spe*I site.
6. For a quick and crude assessment of transfection efficiency, add a sterile 12-mm cover slip to the tissue culture dishes; this cover slip can be removed 24–48 h after electroporation and inspected under fluorescence to check for production of GFP. In our best preps, at least 70% of the BHKs are green.
7. Virion stocks lose their infection efficiency with time, even when stored at –80°C without thawing. Some virion stocks go bad after a few months; others remain usable for up to a year. Repeated freezing and thawing reduces infection efficiency, so small working aliquots are recommended once approximate infection efficiencies have been empirically determined.