Journal of NeuroVirology, 13: 353–363, 2007

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280701393204

informa
healthcare

Semliki Forest virus vectors with mutations in the nonstructural protein 2 gene permit extended superinfection of neuronal and non-neuronal cells

Markus U Ehrengruber and Alan L Goldin

Department of Microbiology and Molecular Genetics, University of California, Irvine, California, USA

Semliki Forest virus (SFV) vectors are widely used in neurobiological studies because they efficiently infect neurons. As with any viral vector, they possess a limited cloning capacity, so infection with different SFV vectors may be required to introduce multiple transgenes into individual cells. However, this approach is limited by superinfection exclusion. The authors examined marker expression in baby hamster kidney cells, mouse cortical neurons, and rat hippocampal neurons using different fluorophore-encoding vectors that are based on the wild-type SFV4 strain and on the less cytopathic SFV4(PD) mutant, which carries two point mutations in nonstructural protein 2. For every fluorophore tested, SFV4(PD) gave higher (up to 22-fold) expression compared to SFV4. In infections using two and three different vectors, SFV4 caused relatively few multifluorescent baby hamster kidney cells when applied at 0-s, 15-min, or 2-h intervals. In contrast, SFV4(PD) permitted significantly enhanced marker coexpression, resulting in 46% doubly and 21% triply fluorescent baby hamster kidney cells, and 67% to 78% doubly fluorescent cortical and hippocampal neurons. At 15-min or 2-h addition intervals, SFV4(PD) still permitted 23% to 36% doubly fluorescent baby hamster kidney cells. The increased efficiency of SFV4(PD) in coexpressing separate markers from different viral particles suggests that mutations in nonstructural protein 2 affect alphaviral superinfection exclusion. The results demonstrate that SFV4(PD) is well-suited to coexpress multiple proteins in neuronal and non-neuronal cells. This capability is particularly valuable to express the various components of heteromeric protein complexes, especially when the individual cDNAs cannot be combined into single SFV particles. *Journal of NeuroVirology* (2007) **13,** 353–363.

Keywords: alphavirus; baby hamster kidney 21 (BHK) cell; homologous interference; monomeric red fluorescent protein; mouse cortical neuron; superinfection exclusion

Introduction

Semliki Forest virus (SFV) belongs to the alphavirus genus of the Togaviridae family and contains an 11.4-kb positive-strand RNA genome, which encodes both nonstructural proteins 1 to 4 (nsP1–4) required for viral replication and structural proteins that participate in viral assembly. During infection of the

Address correspondence to Dr. Markus U. Ehrengruber at his current address: Kantonsschule Hohe Promenade, Postfach, CH-8090 Zürich, Switzerland. E-mail: markus.ehrengruber@hopro.ch

This paper is dedicated to Drs. James H. and Ellen G. Strauss in vivid remembrance of their common ornithological excursions during Markus U Ehrengruber's time at Caltech—with a look onto alphavirsues from the avian side.

The authors thank Eric Sung for designing the sequencing primers, Joyce Iping for technical help with the preparation of SFV vectors and cortical cell cultures, Sheri L. McKinney for supplying dispersed hippocampal neurons, and Drs. K. George Chandy and George A. Gutman for access to the multiwell plate reader. The authors are grateful to Dr. Sondra Schlesinger for critically reviewing the manuscript. This work was supported by grants from the NIH (NS48336), the McKnight Endowment Fund for Neuroscience, and the National Multiple Sclerosis Society. M.U.E. acknowledges Starbucks Co. for providing a comfortable environment in which to write this paper.

Received 31 January 2007; revised 22 March 2007; accepted 29 March 2007.

host cell, the nsP1–4 polyprotein is translated and the nsP4 polypeptide is cleaved from the nsP1–3 polyprotein, after which this protein complex participates in the synthesis of full-length minus-strand viral RNA. In many vertebrate cells, the viral polyprotein is cleaved into its single protein components and the viral proteinase nsP2 is released by 3 h after infection. The minus-strand RNA molecules then serve as templates for the synthesis of both full-length genomic RNA and a 4.1-kb subgenomic plus-strand RNA from an endogenous subgenomic RNA promoter. After translation of the structural proteins from the subgenomic RNA and encapsidation of the genomic plus-strand RNA molecules, infectious viral particles begin to be released at 4 to 6 h post infection (Strauss and Strauss, 1994).

Based on wild-type SFV, Liljeström and Garoff (1991) designed a relatively biosafe vector system in which the 4.1-kb structural genes of the viral particles were replaced by a transgene. This self-replicating vector is referred to as a replicon. A similar expression system was developed for the closely related Sindbis virus (Bredenbeek *et al*, 1993). Both vectors are characterized by a rapid onset and high-level of transgene expression as well as a broad host cell range (Schlesinger, 2000; Lundstrom, 2007). Importantly, when applied to central nervous system (CNS) tissue preparations containing neurons and glial cells, both conventional SFV and Sindbis virus vectors exhibit a marked preference for neuronal infection (Ehrengruber *et al*, 1999, 2001; Maletic-Savatic *et al*, 1999), so they have become the vectors of choice for many neurobiological applications (Ehrengruber, 2002a, 2002b). However, a difficulty with the use of these vectors has been their cytotoxicity both *in vitro* (Strauss and Strauss, 1994) and *in vivo* (Graham *et al*, 2006). This problem was at least partially solved by introducing point mutations in the non-structural genes, notably for nsP2, to generate less and noncytotoxic vectors (Agapov *et al*, 1998; Frolov *et al*, 1999; Perri *et al*, 2000; Fazakerley *et al*, 2002; Lundstrom *et al*, 2003).

A limitation in the use of both SFV and Sindbis virus vectors is that it is difficult to express multiple transgenes, which is essential to express functional heteromeric protein complexes. Two approaches have been used to solve this problem. The first solution is to introduce separate cDNA molecules using single alphaviral vectors with two subgenomic RNA promoters or a cap-independent internal ribosome entry site (Ehrengruber, 2002a). Although effective, this approach cannot be used for large proteins because of the limited cloning capacity of the vectors. A second solution is to introduce distinct transgenes by coinfection with diverse viral particles, each expressing a different transgenic molecule. However, this approach is not always successful because of superinfection exclusion, also called homologous interference, in which cells infected with an alphavirus quickly become refractory to infection by other alphaviruses (Strauss and Strauss, 1994). At a moderate concentration of wild-type SFV or Sindbis virus (10 to 25 viral particles per cell), superinfection exclusion is established within 15 min (Johnston *et al*, 1974; Singh *et al*, 1997). One explanation for rapid superinfection exclusion is that 15 min is a sufficient amount of time for enough nsP2 protein to be produced by the first virus so that it cleaves the newly synthesized nsP1–4 polyprotein from the second virus before it is able to synthesize the minus-strand RNA (Strauss and Strauss, 1994).

In this study, we examined SFV superinfection exclusion by employing a vector array of four different fluorescent marker genes as reporters for viral infection and protein expression. The four marker genes were monomeric red fluorescent protein 1 (mRFP1), enhanced yellow fluorescent protein (EYFP), enhanced green fluorescent protein (EGFP), and enhanced cyan fluorescent protein (ECFP). We compared the marker expression patterns obtained with wild-type SFV4 vectors and the less cytopathic SFV4(PD) vector, which carries two point mutations in nsP2 (Lundstrom *et al*, 2003).

Results

To directly visualize the coexpression of multiple transgenes in individual cells and thus indirectly examine cellular infection with several viral particles, we constructed conventional and less cytopathic SFV vectors encoding the fluorescent markers mRFP1, EYFP, EGFP, or ECFP. The conventional, wild-type SFV vector plasmid that we used (pSFV2gen) is based on pSFV2, which was originally developed for the SFV vector system (Liljeström and Garoff, 1991). As the full sequence for pSFV2gen has not been published, we sequenced and compared it to the available SFV sequences (see Materials and Methods). Our data confirm that pSFV2gen was derived from the wild-type SFV4 strain, so we have used the term SFV4 for all wild-type vectors obtained with pSFV2gen. The SFV4(PD) vectors are less cytopathic because of two amino acid substitutions in nsP2 (S259P and R650D) (Lundstrom *et al*, 2003), and these mutations were confirmed by sequencing (GenBank EF535150).

Kinetics of transgene expression

To characterize the transgene expression patterns obtained in host cells upon infection with SFV4 and SFV4(PD) virus-like particles, we first measured the time course and expression levels in infected baby hamster kidney 21 (BHK) cells by quantifying fluorescence using a multiwell plate reader. Figure 1 shows that the onset of transgene expression for both SFV4 and SFV4(PD) vectors started within 10 h post infection, which is characteristic for SFV vectors (Ehrengruber, 2002a). For SFV4, maximal expression was reached at 36 h post infection, whereas SFV4(PD)-infected BHK cells showed

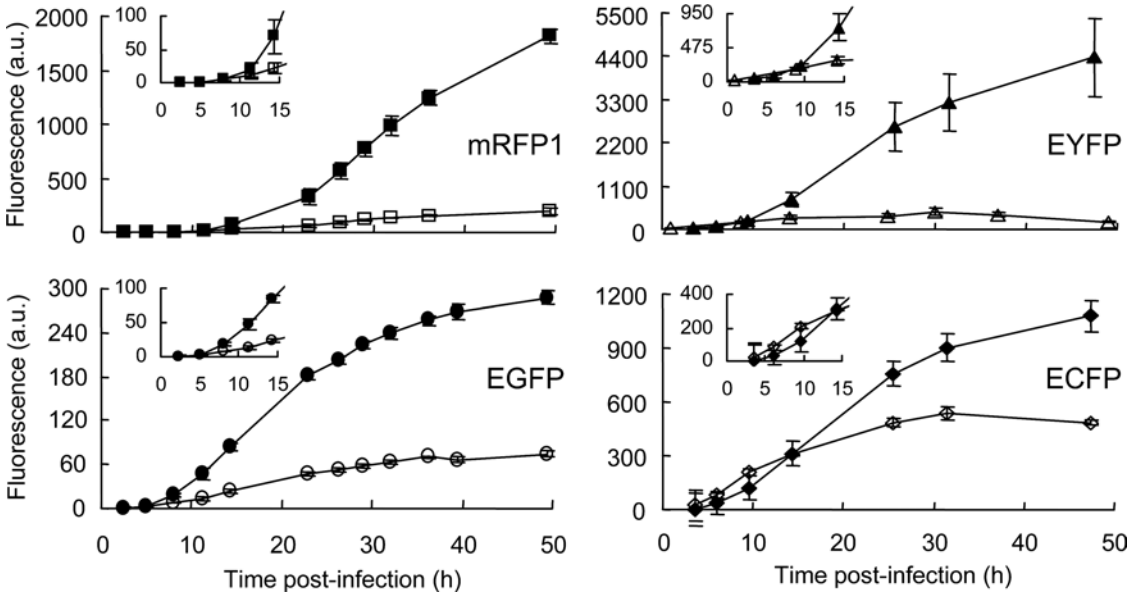

Figure 1 Transgene expression kinetics for wild-type SFV4 and the less cytopathic mutant SFV4(PD). BHK cells were infected with recombinant SFV4 (*open symbols*) or SFV4(PD) (*closed symbols*) encoding mRFP1, EYFP, EGFP, or ECFP, and the resulting fluorescence was analyzed at increasing time intervals post infection using a multiwell plate reader. Insets with fluorescence levels for early time points (<15 h) show that transgene expression also occurs for SFV4, but (later) remains at lower levels as compared to SFV4(PD). Data points represent the means and error bars indicate the standard deviations from quadruplicate samples. Similar results were obtained in two independent experiments for each fluorescent marker gene.

increasing transgene expression up to 50 h post infection, the longest time period examined. Most importantly, each of the four SFV4(PD) vectors resulted in significantly elevated peak expression compared to the SFV4 vectors, with 2.2- to 22-fold higher expression at 50 h post infection. At this time point, the SFV4(PD)-mediated expression level was so high that mRFP1 fluorescence was visible in bright field illumination (data not shown). These results confirm that SFV vectors are suitable for high-level transgene expression in cell culture.

We next determined how quickly the SFV vectors adsorb to cells under typical cell culture conditions, which is an important characteristic because the vectors will be used for sequential infections. BHK cells in 24-well plates were incubated for increasing time periods with SFV at 5×10^6 infectious particles/ml (multiplicity of infection [MOI] of ~20), which represented sufficient virus to yield >95% marker expression efficiency when the virus was continuously present on the cells. After the adsorption period (20 s to 2 h), BHK cells were washed and cultured for 1 day. Transgene expression was quantified by measuring fluorescence using a multiwell plate reader and directly visualized microscopically. As shown in Figure 2, both SFV4-mRFP1 and SFV4-ECFP demonstrated similar adsorption kinetics. When the fluorescence intensity was normalized to that obtained when the cells were continuously incubated with the virus, the maximum fluorescence intensity was 70% ± 16% ($n = 4$) for SFV4-mRFP1 and 66% ± 11% ($n = 4$) for SFV4-ECFP, obtained

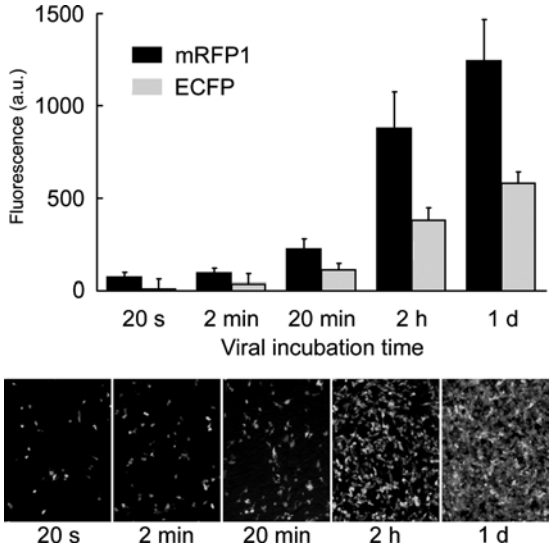

Figure 2 Marker expression depends on the cell exposure time to SFV. SFV4 particles encoding mRFP1 (*black bars*) or ECFP (*grey bars*) were added to BHK cells. After the indicated time (20 s to 2 h), the viral medium was removed, the cells were washed with PBS, and fresh culture medium was added. Fluorescence was measured using a multiwell plate reader and compared to cells continuously incubated with SFV for 1 day. The relative fluorescence intensity for mRFP1 was 6% ± 2%, 8% ± 2%, 18% ± 4%, and 70% ± 16% ($n = 4$) for 20 s, 2 min, 20 min, and 2 h virus incubation, and the relative intensity for ECFP was 1% ± 9%, 6% ± 9%, 19% ± 6%, and 66% ± 11% ($n = 4$) for the same incubation times. The bars represent the means and the error lines indicate the standard deviations from quadruplicate samples. The bottom panel shows fluorescence micrographs at 1 day post infection for BHK cell cultures treated with SFV4-mRFP1.

with a 2-h adsorption period. The fluorescence intensity did not significantly increase for adsorption periods between 2 h and 1 d, indicating that a 2-h incubation time is sufficient to transfect the majority of BHK cells under these conditions.

Infection of BHK cells with multiple vectors

To quantify the efficiency of SFV-mediated marker coexpression, we treated BHK cells in 24-well plates with either two SFV4 vectors or two less cytopathic SFV4(PD) vectors, one encoding mRFP1 and the other encoding ECFP or EGFP. These fluorophores were used because their spectral resolutions are distinct from mRFP1, which minimized fluorescence cross-contamination. The viruses were added either alone for 2 h (as a single-infection control), together at the same time for 2 h, or sequentially with a 2-h interval between them (as double-infections). For the experiments using two different viruses sequentially, EGFP or ECFP virus was adsorbed for 2 h and then removed before adding mRFP1 virus, which exhibited the highest fluorescence intensity and thus the highest signal-to-noise ratio. For both SFV4 and SFV4(PD), the simultaneous infection of BHK cells with two different SFV vectors (for EGFP, ECFP, or mRFP1) decreased each marker fluorescence by approx. half compared to the single-infected control cells (treated with SFV encoding either EGFP, ECFP, or mRFP1, respectively). In contrast, when the viral vectors were added sequentially at 2-h intervals, expression from the first vector was normal (100% ± 22% and 111% ± 25% of single-infected control for SFV4 and SFV4(PD), respectively; $n = 8$ determinations each), whereas expression of the second vector was significantly reduced (37% ± 11% and 30% ± 3%). Because the viral infection is likely to be in different stages in different cells, these results from heterogeneous populations do not provide information about infection of individual cells. We therefore analyzed individual cells after the infection.

Cells were treated simultaneously with two and three different SFV vectors at an MOI of ~20 each, and individual cell fluorescence was examined microscopically (Figure 3). For the double-infections, mRFP1 virus was combined with either EGFP or ECFP virus. For the triple-infections, virus encoding mRFP1, EYFP, and ECFP was combined. Both SFV4 and SFV4(PD) permitted the simultaneous expression of mRFP1 and ECFP in individual BHK cells, regardless of the total fluorescence intensity per cell (Figure 3A, arrows). Similar results were obtained using mRFP1 and EGFP (not shown), so the numbers of mRFP1/ECFP- and mRFP1/EGFP-fluorescent cells were combined for the quantitative analysis. Using SFV4, a relatively low percentage of doubly fluorescent cells was found when cells were infected at an MOI of ~20 for each virus (19%; Table 1). Use of the less cytopathic SFV4(PD) vectors led to a significantly higher (2.4-fold increase) fraction of doubly-fluorescent cells (46% versus 19%) and a concomitant decrease in the amount of singly-fluorescent cells compared to SFV4 (Table 1). In triple-infections (Figure 3B), use of SFV4(PD) resulted in a 2- to 19-fold increase in marker coexpression compared to SFV4, with 26% doubly and 21% triply fluorescent cells versus only 11% and 1% for SFV4 (Table 1). These results demonstrate that SFV4(PD) allows for a greater efficiency of transgene coexpression compared to SFV4 and is a significantly more effective vector for introducing multiple proteins into the same cell.

Table 1 Marker expression in BHK cells treated with multiple wild-type SFV4 and less cytopathic SFV4(PD) vectors

		Total cells		Singly fluorescent		Doubly fluorescent		Triply fluorescent	
Vector types	Interval	SFV4	SFV4(PD)	SFV4	SFV4(PD)	SFV4	SFV4(PD)	SFV4	SFV4(PD)
Two	0 s	725	573	81%	54%	19%	46%	N.D.	N.D.
	15 min	257	314	85%	74%	15%	26%	N.D.	N.D.
	2 h (cyan-red)	347	387	95%	77%	5%	23%	N.D.	N.D.
	2 h (red-cyan)	285	287	92%	64%	8%	36%	N.D.	N.D.
Three	0 s	361	525	88%	53%	11%	26%	1%	21%
P_{ANOVA}				.005		.004			
DF				1; 4		1; 4			
SS				1379; 176		946; 105			
MS				1379; 44		946; 26			
F value				31.2		36.0			

Note. BHK cells were treated with either two or three different SFV4 constructs or with two or three different SFV4(PD) constructs at an MOI of ~20, each. The different viral vectors were added concomitantly (0 s) or at 15-min or 2-h intervals. For the 2-h interval, the experiments were performed both with ECFP-virus first follwed by mRFP1-virus (cyan-red) and in the reverse order (mRFP1-virus first followed by ECFP-virus [red-cyan]). At 1 day post infection, fluorescence micrographs were taken for each marker gene. The number of all fluorescent cells was counted from the images (total cells), and the percentages of singly, doubly, and triply fluorescent cells calculated. The percentages for singly fluorescent cells include all cells expressing either fluorophore, whether it resulted from the initial or subsequent SFV addition. A one-way repeated measures ANOVA with Holm-Sidak correction demonstrated a significant difference between SFV4 and SFV4(PD) in the percentage of singly and doubly fluorescent cells. The respective percentage of fluorescent cells was set as the dependent variable and the viral type [SFV4 or SFV4(PD)] as the factor (independent variable). DF = degrees of freedom for treatment (before semicolon) and error (after semicolon); SS = sums of squares for treatment and error; MS = mean squares for treatment and errors. N.D. = not determined.

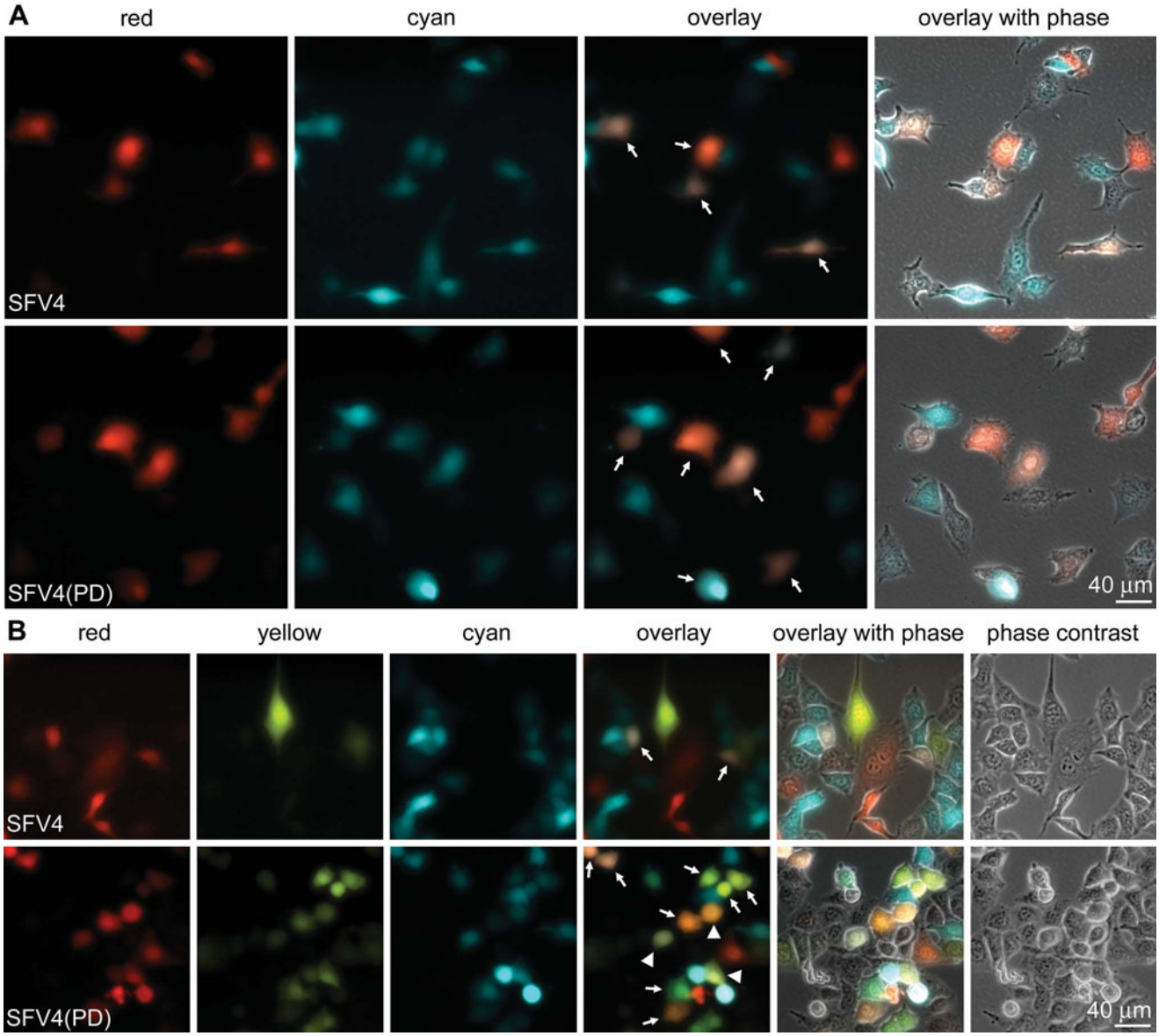

Figure 3 Marker coexpression in BHK cells. **A,** BHK cells were simultaneously infected with SFV4 expressing mRFP1 or ECFP (*top row*) or with less cytopathic SFV4(PD) expressing mRFP1 or ECFP (*bottom row*). An MOI of ~20 was used for each vector. At 21 to 23 h post infection, phase-contrast and fluorescence micrographs were obtained using specific filter sets for mRFP1 (*red*) and ECFP (*cyan*). Arrows point to doubly fluorescent cells. **B,** BHK cells were simultaneously treated with SFV4 expressing mRFP1, EYFP or ECFP (*top row*) or with less cytopathic SFV4(PD) expressing mRFP1, EYFP, or ECFP (*bottom row*). An MOI of ~20 was used for each vector. At 21 to 23 h post infection, phase-contrast and fluorescence micrographs were obtained using specific filter sets for mRFP1 (*red*), EYFP (*yellow*), and ECFP (*cyan*). Arrowheads point to triply fluorescent cells and arrows point to doubly fluorescent cells. Note that the overlay of the three separate fluorescence micrographs is more colorful for SFV4(PD) than SFV4, indicating more extensive marker coexpression.

Because the simultaneous infection of BHK cells with the less-cytopathic SFV4(PD) permitted a higher efficiency of multiple marker expression, we wanted to determine if the same effect would be observed when the viruses were added sequentially. It has previously been shown that a 15-min pretreatment with wild-type SFV can block subsequent SFV infection (Singh *et al*, 1997), so we used both a 15-min and a 2-h pretreatment interval for these experiments. Addition of a second SFV4 vector 15 min after the first one resulted in a significant percentage of doubly fluorescent cells when both treatments were carried out with SFV4 at an MOI of ~20 (Figure 4). These data demonstrate that superinfection exclusion for SFV4 is not complete after a 15-min interval under the conditions used in this study. However, there was a quantitative difference in the results for SFV4 and SFV4(PD). Applied at a 15-min interval, SFV4(PD) resulted in more doubly fluorescent cells compared to SFV4 (Table 1). The difference was

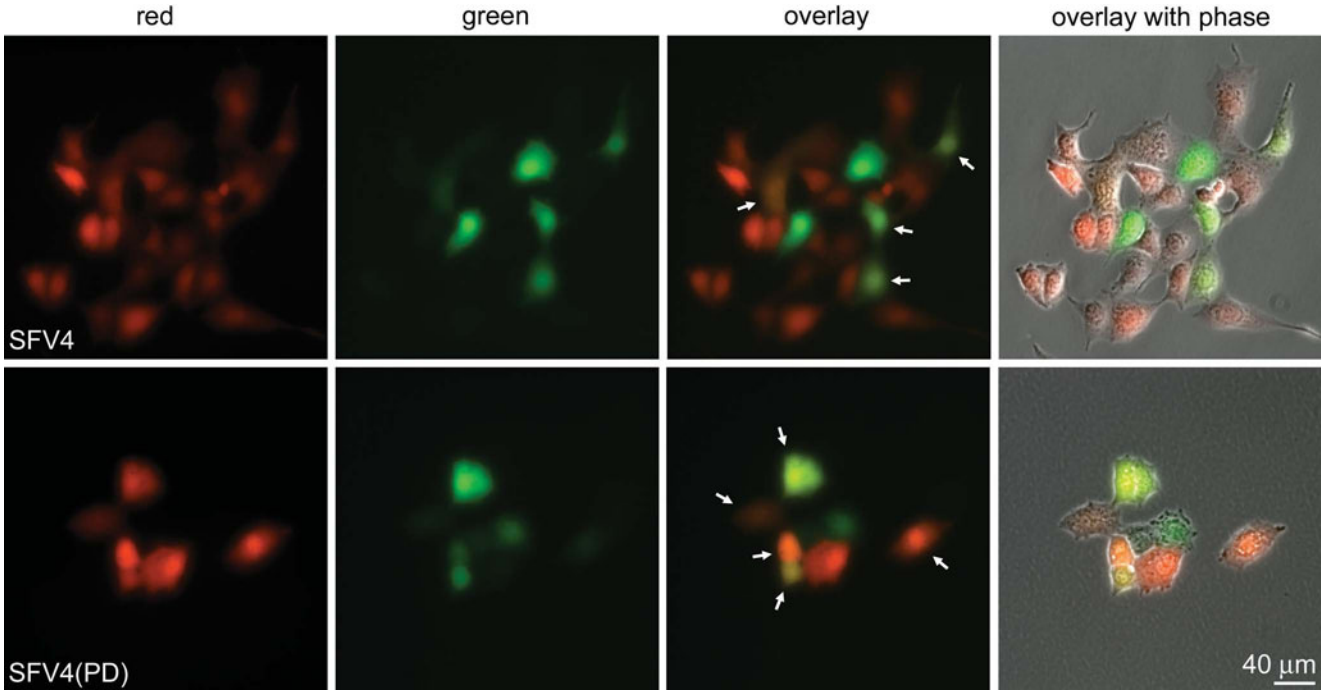

Figure 4 SFV4(PD) permits a higher frequency of marker coexpression than SFV4 at a 15-min interval. BHK cells were first treated with SFV4 (*top row*) or less cytopathic SFV4(PD) (*bottom row*) encoding mRFP1. At 15 min after the initial virus addition, SFV4 (*top row*) or SFV4(PD) (*bottom row*) encoding EGFP was added. An MOI of ~20 was used for each vector. Fluorescence micrographs of living cells were taken at 22 to 29 h after the initial virus addition. Arrows point to doubly fluorescent cells.

even larger when there was a 2-h interval between the first and second viral treatments. When the BHK cells were first infected with ECFP-expressing virus and 2 h later with mRFP1-expressing virus, SFV4 resulted in 5% doubly fluorescent cells, which is only a quarter of what was obtained by concomitant SFV4 application (Table 1). In contrast, the less cytopathic SFV4(PD) virus applied at a 2-h interval resulted in 23% doubly fluorescent cells, which is still more than half of what resulted from simultaneous SFV4(PD) addition (Table 1). In this experiment, the second virus encoded mRFP1, whose fluorescence intensities at ~24 h post infection were 6-fold higher for SFV4(PD) compared to SFV4 (see Figure 1). Thus, theoretically, at least part of the observed greater marker co-expression for SFV4(PD) might have arisen from the different mRFP1 expression levels caused by the second virus, with fewer doubly fluorescent cells being detectable for SFV4, which would appear as decreased expression of mRFP1. To address this possibility, we reversed the viral application order, first using mRFP1-encoding virus and 2 h later using virus expressing ECFP, for which SFV4 and SFV4(PD) cause similar fluorescence levels at ~24 h post infection (Figure 1). In this experiment, combining two different SFV4(PD) constructs resulted in 36% doubly fluorescent cells versus 8% doubly fluorescent cells for wild-type SFV4 (Table 1; Figure 5, rows 1 and 4). These results are similar to those obtained when the fluorophore markers were used in the reverse order, demonstrating that the less cytopathic SFV4(PD) is significantly more efficient than SFV4 for multiple marker expression in host cells, consistent with reduced superinfection exclusion for SFV4(PD).

To further demonstrate that the differences in coinfection efficiency did not result from differences in the sensitivity of detection for each fluorophore, we performed infection experiments in which BHK cells were (a) first treated with SFV4-mRFP1 and 2 h later with SFV4(PD)-ECFP, and (b) first treated with SFV4(PD)-mRFP1 and 2 h later with SFV4-ECFP. As expected from the previous infection experiments, initial infection with wild-type SFV4 precluded marker expression from the less cytopathic SFV4(PD) (Figure 5, row 2), causing only 12% doubly fluorescent BHK cells ($n = 252$ fluorescent cells). In contrast, initial infection with less cytopathic SFV4(PD) still permitted marker expression by wild-type SFV4 (Figure 5, row 3), leading to 38% doubly fluorescent cells ($n = 237$ fluorescent cells). These results are similar to the above infections for SFV4 and SFV4(PD) (Figure 5, rows 1 and 4), leading to 8% and 36% doubly fluorescent cells, respectively (Table 1), confirming that multiple marker expression in individual cells is more efficient for less cytopathic SFV4(PD) vectors than for wild-type SFV4 vectors.

Infection of neuronal cultures with multiple vectors

Both SFV4 and the less cytopathic SFV4(PD) demonstrate a high degree of neuronal specificity when used

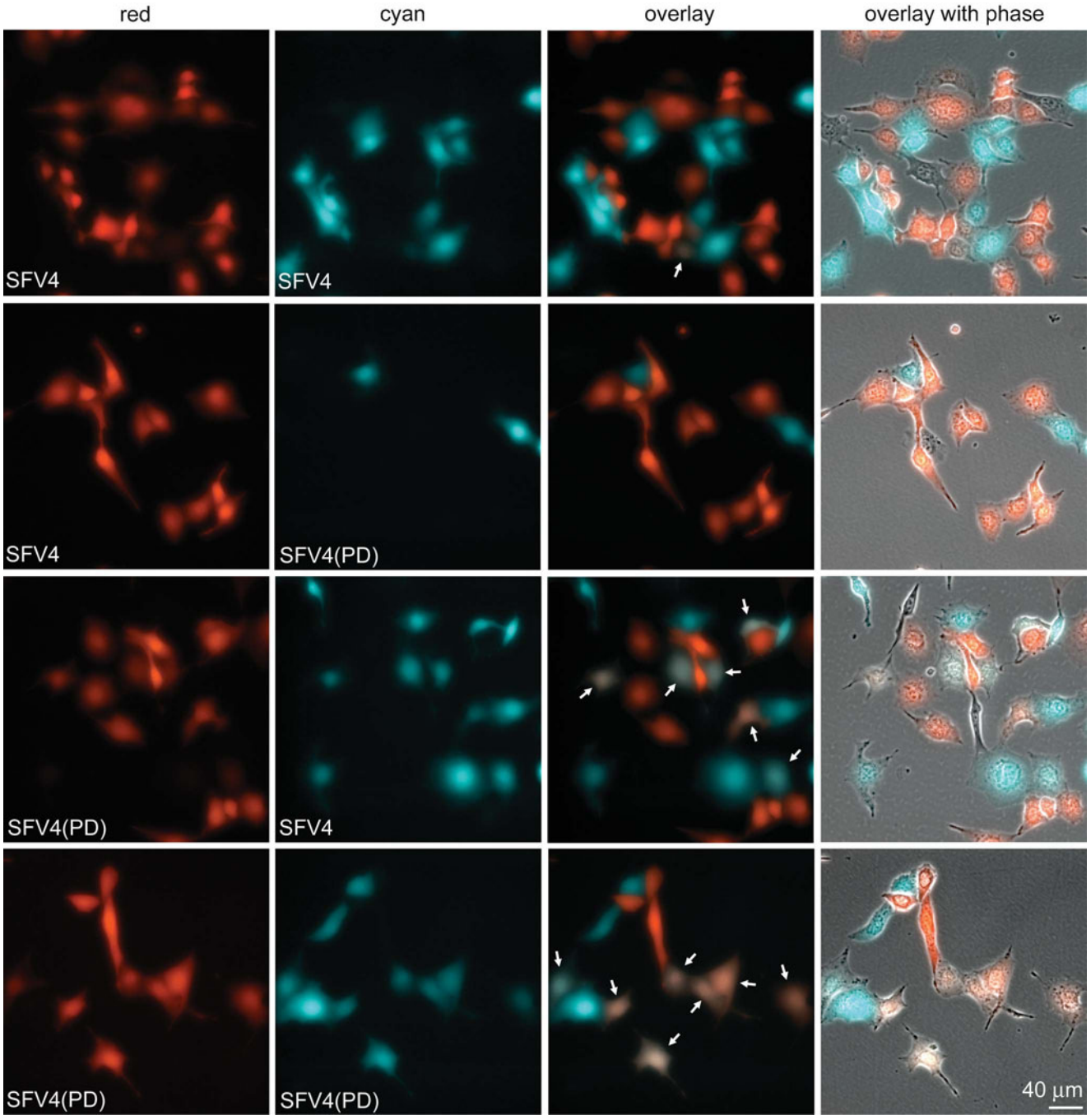

Figure 5 SFV4(PD) enables a higher frequency of marker coexpression than SFV4 at a 2-h interval. BHK cells were first treated with SFV4 (*rows 1 and 2*) or less cytopathic SFV4(PD) (*rows 3 and 4*) encoding mRFP1. At 2 h after the initial virus addition, the viral medium from the first infection was removed and cells were washed with PBS, after which SFV4 (*rows 1 and 3*) or SFV4(PD) (*rows 2 and 4*) encoding ECFP was added. An MOI of ~20 was used for each vector. Fluorescence micrographs of living cells were taken at 21 to 23 h after the initial virus addition. Arrows point to doubly fluorescent cells.

as vectors in CNS tissue preparations (Ehrengruber *et al*, 2001), and they have become the vectors of choice for a variety of neurobiological studies (Ehrengruber, 2002b). Because the major utility of SFV vectors is for expression in neurons, we examined the efficiency of multiple marker expression in primary mouse cortical neurons by both SFV4 and SFV4(PD). Mixed cultures of mouse cortical neurons and glial cells at 10 days in culture were treated with similar amounts of ECFP- and mRFP1-expressing virus (~3×10^5 particles/ml for each). At 19 to 22 h post infection, the number of ECFP- and/or mRFP1-fluorescent cells and the fraction of doubly fluorescent cells were determined (Figure 6). Infection

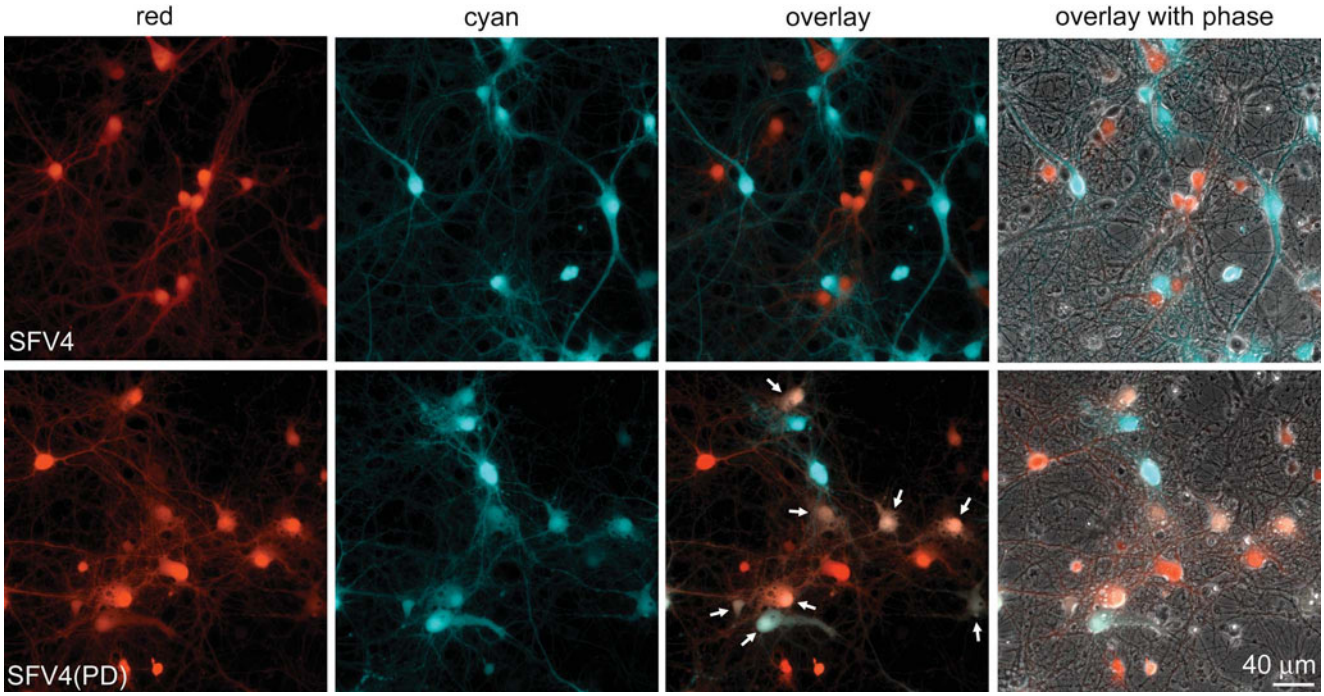

Figure 6 SFV4(PD) allows a higher frequency of marker coexpression in mouse cortical neurons than SFV4. Mixed cultures of mouse cortical neurons and glial cells at 10 days *in vitro* were simultaneously treated with comparable amounts ($\sim 3 \times 10^5$ particles/ml) of SFV4 (*top row*) or less cytopathic SFV4(PD) (*bottom row*) expressing mRFP1 and ECFP. Fluorescence micrographs of living cells were taken at 19 to 22 h post infection. Arrows point to doubly fluorescent cells.

with SFV4 resulted in 15% doubly fluorescent cells ($n = 869$ fluorescent cells), whereas infection with SFV4(PD) resulted in 67% doubly infected cells ($n = 732$ fluorescent cells). Therefore, multiple marker expression in cortical neurons with SFV4(PD) was 4.3-fold more efficient than with SFV4, consistent with the results for BHK cells.

To determine if SFV4(PD)-mediated marker coexpression is efficient for other types of neurons, we also infected rat hippocampal neurons at 16 days in culture with equal amounts of SFV4(PD) encoding mRFP1 and ECFP. We used a relatively high level of virus ($\sim 1 \times 10^6$ particles/ml for each) for these experiments, which is cytotoxic to neurons even for SFV4(PD) (Lundstrom *et al*, 2003), so we limited our examination to <1 days post infection. Extensive marker coexpression was observed in morphologically normal neurons at 19 h post infection (not shown). At this time, 78% of all fluorescent cells were expressing both fluorophores ($n = 145$). Similar data were obtained when SFV4(PD)-mRFP1 was combined with SFV4(PD)-EGFP rather than SFV4(PD)-ECFP, with 70% of the cells being doubly fluorescent ($n = 59$). These data demonstrate that SFV4(PD) can efficiently express transgenes from two different vectors in individual hippocampal and cortical neurons.

Discussion

This study examined multiple marker expression of SFV replicons that are derived from the wild-type SFV4 strain (Liljeström and Garoff, 1991; Tuittila *et al*, 2000) or that correspond to the less cytopathic SFV4(PD) variant (Lundstrom *et al*, 2003). Both SFV vectors were used in four different versions, with each one encoding a different fluorescent marker gene. For each marker, SFV4(PD) resulted in significantly higher expression compared to wild-type SFV (up to 22-fold for mRFP1). These data confirm our previously published data obtained with EGFP (Lundstrom *et al*, 2003) and demonstrate that the higher efficiency of expression is generalized to three additional fluorescent transgenes (mRFP1, EYFP, and ECFP).

When we analyzed marker coexpression on a macroscopic scale by recording fluorescence intensities from BHK cell populations, we found that the simultaneous addition of two different SFV vectors decreased transgene expression for each vector by ~50% compared to the respective single-infections. Because we used virus concentrations that infected the majority of BHK cells and the effect was similar for both SFV4 and SFV4(PD), this decrease in expression may result from both superinfection exclusion (in cells that had been infected during the initial infection round) and/or a limited cellular machinery that is available for SFV replication and expression.

By using different fluorescent marker genes, we were able to indirectly examine the superinfection of individual BHK cells, mouse cortical neurons, and rat hippocampal neurons. When two separate SFV4 vectors were applied simultaneously at an MOI

of ~20 for each, only 19% of all fluorescent BHK cells were doubly fluorescent. This number was even lower when the two infections were carried out with a 15-min interval between them. This result is consistent with a previous report that less than one third of cells were coinfected when equal amounts of wild-type SFV were applied at a 15-min interval, using immunocytochemical detection of infected cells (Singh *et al*, 1997). The same interval and viral load also prevents Sindbis virus superinfection (Johnston *et al*, 1974). In our experiments, coexpression of different markers was even lower with an interval of 2 h between addition of the first and second viruses, resulting in only 5% doubly fluorescent cells. Again, this result is consistent with the previously reported value of <1% super-infected cells for a 3-h interval (Singh *et al*, 1997). These data demonstrate that superinfection exclusion limits expression of multiple transgenes from different wild-type SFV vectors in single cells. It has been previously shown that superinfection exclusion occurs by blocking the superinfecting virus during viral replication *within* the cell rather than by blocking viral entry *into* the host cell (Johnston *et al*, 1974). Despite superinfection exclusion, however, wild-type alphaviral vectors have been used to functionally express complexes of adrenergic receptors and heterotrimeric G proteins in COS cells (Scheer *et al*, 1999), IgG-type antibodies in BHK cells (Liang *et al*, 1997), and an ionotropic glutamate receptor with its binding protein in hippocampal neurons (DeSouza *et al*, 2002). All of these studies were conducted at a relatively high viral concentration or with fully replication-competent virus, presumably to overcome superinfection exclusion. In agreement with these reports, we also found that infecting BHK cells with two different wild-type SFV4 vectors at an MOI of ~20 each in a fivefold lower volume, thus with a fivefold higher viral concentration, increased marker coexpression (data not shown).

The most important finding of this study is that the less cytopathic SFV4(PD) greatly increased the marker co-expression efficiency compared to SFV4. Simultaneous SFV4(PD) addition led to 43% to 47% multiply fluorescent BHK cells, 70% to 78% doubly fluorescent hippocampal neurons, and 67% doubly fluorescent cortical neurons. In addition, when an SFV4(PD) vector was added 2 h after the treatment with another SFV4(PD) replicon, 23% to 36% of the BHK cells were doubly fluorescent. As we used a 2-h incubation for each virus, which in single-infections resulted in 66% to 70% marker expression, a maximum of 44% to 49% doubly fluorescent cells would have occurred for two different viral vectors in the absence of superinfection exclusion. Our data match this theoretical number surprisingly well when the BHK cells were treated simultaneously, whereas they reach half to three quarters of the maximum value when SFV4(PD) was added with a 2-h interval. Thus, superinfection exclusion appears to be only partial for SFV4(PD). As this less cytopathic vector differs from SFV4 because of only two point mutations in nsP2 (Lundstrom *et al*, 2003), these data suggest that mutations in the nsP2 gene decrease superinfection exclusion. Because SFV4 and SFV4(PD) possess the same structural proteins and because nsP2 is an intracellular protein within infected cells, any reduced superinfection exclusion for SFV4(PD) most likely results from an alteration in viral replication. It has been shown for less cytopathic Sindbis virus variants that nsP2 mutations accelerate the proteolytic processing of the viral nsP1–4 polyprotein (Frolov *et al*, 1999), so accelerated nsP1–4 processing may enhance superinfection by SFV.

The nsP2 protein is a dual component of the alphaviral RNA replicase, containing both a predicted N-terminal RNA helicase domain and a C-terminal protease domain (Strauss and Strauss, 1994). It controls the shutoff of negative-strand RNA synthesis via proteolytic cleavage of the nonstructural polyprotein, regulates transcription of subgenomic RNA, and blocks host cell transcription (Strauss and Strauss, 1994; Kim *et al*, 2004; Sawicki *et al*, 2006; Garmashova *et al*, 2006). Mutations in nsP2 can thus lead to continuous negative-strand but reduced plus-strand viral RNA synthesis (Sawicki *et al*, 2006), and decreased alphaviral cytotoxicity (Rikkonen, 1996; Dryga *et al*, 1997; Agapov *et al*, 1998; Perri *et al*, 2000; Fazakerley *et al*, 2002; Lundstrom *et al*, 2003). Consistent with our results, it has been previously noted for Sindbis virus that such noncytopathic vectors are unable to establish superinfection exclusion (Frolov *et al*, 1999).

In summary, our results strongly suggest that superinfection exclusion is only partial for the less cytopathic SFV4(PD) vectors. This property, along with the fact that SFV4(PD) replicons retain the original advantages of wild-type alphaviral vectors (Schlesinger, 2000; Ehrengruber, 2002a; Lundstrom, 2007), makes SFV4(PD) particularly useful for expressing multiple proteins in individual neurons. This vector should be especially appropriate for the functional expression of heteromeric protein complexes such as multimeric receptors and channels, in which some cDNAs are too large to be combined into single viral particles because of the limited cloning capacity of the vector.

Materials and methods

Viral vectors

Sequencing primers for pSFV2gen (gift of Dr. Kenneth Lundstrom, Flamel Technologies, Vénissieux, France), resulting in overlapping nucleotide sequences, were designed on the basis of pSFV1 (Liljeström and Garoff, 1991) (Invitrogen). The program Megalign (Lasergene) was used for sequence analysis. The amino acid sequences of the nonstructural proteins 1 to 4 for pSFV2gen (GenBank EF535150) were identical to the ones for the

wild-type SFV4 strain (Tuittila *et al*, 2000) (GenBank AJ251359), but different from the pSFV1 vector (Liljeström and Garoff, 1991; 14 amino acid changes plus one insertion), from the prototype SFV strain (Takkinen, 1986; GenBank NC003215; 20 amino acid changes), and from the wild-type L10 strain (GenBank AY112987; 18 amino acid changes plus one insertion). In contrast to pSFV2gen, pSFV1 is more closely related to the prototype SFV. The following silent nucleotide changes occurred in pSFV2gen as compared to SFV4 (numbering according to the published SFV4 sequence; each change was confirmed in two different sequencing reactions): C59G (removing a Bgl I restriction site), C1627T, G1945A (destroying a Hind III site), C2029G (deleting an Rsr II site, which is also present in the multiple cloning region of pSFV2gen), G5308C (targeting a Xho I site; present in the multiple cloning region of pSFV2gen), and T6142C (removing a Not I site; also present in the multiple cloning region of pSFV2gen).

The cDNAs for mRFP1 (Campbell *et al*, 2002) (gift from Dr. Roger Y. Tsien, University of California, San Diego, CA), EYFP, and ECFP (both from Clontech) were inserted into the multiple cloning sites of the vector plasmids pSFV2gen and pSFV(PD) (Lundstrom *et al*, 2003). The previously described constructs were used for EGFP (Ehrengruber *et al*, 1999; Lundstrom *et al*, 2003). SFV replicons were generated by *in vitro* transcription and cotransfection with SFV-Helper2 RNA into baby hamster kidney 21 (BHK) cells according to a standard protocol (Ehrengruber and Lundstrom, 2002). The coelectroporated BHK cells were incubated at 37°C for 2 days for SFV4 and at 31°C for 2 days for SFV4(PD) to maximize the production of viral replicons.

To determine the viral titers, 100% confluent BHK cells in 6-well plates were infected in 1 ml culture medium (Dulbecco's modified eagle's medium [DMEM] supplemented with 10% heat-inactivated fetal bovine serum) per well with serial dilutions of SFV viral particles (typically 1 to 100 μl viral stock per well). The BHK cells were incubated without virus removal for 1 to 2 days at 37°C for both SFV4 and SFV4(PD), and the resulting number of fluorescent cells was determined using either an Axiovert 35 or 200M microscope with epifluorescent illumination. The viral titers were calculated from the viral dilutions achieving 30% to 70% fluorescent BHK cells, and were 4–9 $\times$ 10^8 SFV4 particles and 0.4–5 $\times$ 10^8 SFV4(PD) particles per milliliter.

Cell culture and viral infections

Completely confluent BHK cells in 24-well plates (Falcon) or ~20% confluent BHK cells in 35-mm plastic Petri dishes (Corning) were infected by adding 1 ml culture medium containing ~4 $\times$ 10^6 viral particles to each well and dish, respectively, corresponding to an MOI ~20. This quantity was the amount required to cause >95% fluorescent cells when the virus was continuously present. The cells were incubated without agitation at 37°C. In cases where the viral medium was removed after the indicated time periods (20 s to 2 h), the BHK cells were washed once with 1.5 to 2 ml phosphate-buffered saline (PBS) before adding back regular culture medium.

Dissociated cells from the cortex of postnatal day 1 to 3 mice and hippocampal neurons from embryonic day 18 rats were prepared as described (Bausch *et al*, 1995; Li *et al*, 1997) and cultured at ~250 cells/μm^2 in 35-mm glass bottom plastic Petri dishes (Mattek, Ashland, MA) or on 10-mm glass cover slips that had been coated with polyethyleneimine. The cortical neuron cultures were infected at 10 to 16 days *in vitro* in 1 ml culture medium by adding ~3 $\times$ 10^5 viral particles for each virus, as determined on BHK cells (MOI ~5–10). Cells were incubated with (cortical cultures) and without (hippocampal cultures) removing the viral medium after 2 to 3 h and one subsequent wash with glial conditioned medium.

Analysis of transgene expression

Transgene expression in living BHK cells in 24-well plates was quantified using a Cytofluor series 4000 multiwell plate reader (Perspective Biosystems) in quadruplicates using a gain of 40 (mRFP1) or 60 (EYFP, EGFP, ECFP) and the following band pass filter sets for excitation and emission, respectively: 545 $\pm$ 30 and 620 $\pm$ 40 nm (mRFP1), 485 $\pm$ 20 and 530 $\pm$ 25 nm (EYFP, EGFP), 450 $\pm$ 50 and 530 $\pm$ 25 nm (ECFP). Fluorescence and phase-contrast micrographs from living BHK and cortical cells in 35-mm Petri dishes were taken at 1 to 2 days post infection with an Axiovert 200M inverted microscope (Zeiss) using the corresponding filter sets and the software Axiovision 4.5 (Zeiss). Rat hippocampal cultures were fixed at 1 day post infection with 4% (*w*/*v*) paraformaldehyde (EMD Chemicals, Gibbstown, NJ) in PBS for the subsequent fluorescence microscopic examination.

Sigmastat 3.1 (Systat Software) was used for the statistical analysis. Fluorescence intensities obtained with the multiwell plate reader are expressed as means $\pm$ SD. Significance was evaluated using a nonpaired Student's *t* test. Significance in the percentages of fluorescent cells (Table 1) was analyzed with a one-way repeated measures analysis of variance (ANOVA) using the Holm-Sidak correction for multiple comparisons.

References

Agapov EV, Frolov I, Lindenbach BD, Pragai BM, Schlesinger S, Rice CM (1998). Non-cytopathogenic Sindbis RNA vectors for heterologous gene expression. *Proc Natl Acad Sci U S A* **95:** 12989–12994.

Bausch SB, Patterson TA, Ehrengruber MU, Lester HA, Davidson N, Chavkin C (1995). Colocalization of mu opioid receptors with GIRK1 potassium channels in rat brain: an immunocytochemical study. *Recept Channels* **3:** 221–241.

Bredenbeek PJ, Frolov I, Rice CM, Schlesinger S (1993). Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. *J Virol* **67:** 6439–6446.

Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, Zacharias DA, Tsien RY (2002). A monomeric red fluorescent protein. *Proc Natl Acad Sci U S A* **99:** 7877–7882.

DeSouza S, Fu J, States BA, Ziff EB (2002). Differential palmitoylation directs the AMPA receptor-binding protein ABP to spines or to intracellular clusters. *J Neurosci* **22:** 3493–3503.

Dryga SA, Dryga OA, Schlesinger S (1997). Identification of mutations in a Sindbis virus variant able to establish persistent infection in BHK cells: the importance of a mutation in the nsP2 gene. *Virology* **228:** 74–83.

Ehrengruber MU (2002a). Alphaviral gene transfer in neurobiology. *Brain Res Bull* **59:** 13–22.

Ehrengruber MU (2002b). Alphaviral vectors for gene transfer into neurons. *Mol Neurobiol* **26:** 183–201.

Ehrengruber MU, Hennou S, Büeler H, Naim HY, Déglon N, Lundstrom K (2001). Gene transfer into neurons from hippocampal slices: comparison of recombinant Semliki Forest virus, adenovirus, adeno-associated virus, lentivirus, and measles virus. *Mol Cell Neurosci* **17:** 855–871.

Ehrengruber MU, Lundstrom K (2002). Semliki Forest virus and Sindbis virus vectors. In: *Current protocols in human genetics*. Dracopoli NC, Haines JL, Korf BR, Morton CC, Seidman CE, Seidman JG, Smith DR (eds). New York: John Wiley & Sons, pp 12.2.1–12.2.23.

Ehrengruber MU, Lundstrom K, Schweitzer C, Heuss C, Schlesinger S, Gähwiler BH (1999). Recombinant Semliki Forest virus and Sindbis virus efficiently infect neurons in hippocampal slice cultures. *Proc Natl Acad Sci U S A* **96:** 7041–7046.

Fazakerley JK, Boyd A, Mikkola ML, Kääriäinen L (2002). A single amino acid change in the nuclear localization sequence of the nsP2 protein affects the neurovirulence of Semliki Forest virus. *J Virol* **76:** 392–396.

Frolov I, Agapov E, Hoffman TA, Prágai BM, Lippa M, Schlesinger S, Rice CM (1999). Selection of RNA replicons capable of persistent noncytopathic replication in mammalian cells. *J Virol* **73:** 3854–3865.

Garmashova N, Gorchakov R, Frolova E, Frolov I (2006). Sindbis virus nonstructural protein nsP2 is cytotoxic and inhibits cellular transcription. *J Virol* **80:** 5686–5696.

Graham A, Walker R, Baird B, Hahn CN, Fazakerley JK (2006). CNS gene therapy applications of the Semliki Forest virus 1 vector are limited by neurotoxicity. *Mol Ther* **13:** 631–635.

Johnston RE, Wan K, Bose HR (1974). Homologous interference induced by Sindbis virus. *J Virol* **14:** 1076–1082.

Kim KH, Rümenapf T, Strauss EG, Strauss JH (2004). Regulation of Semliki Forest virus RNA replication: a model for the control of alphavirus pathogenesis in invertebrate hosts. *Virology* **323:** 153–163.

Li Z, Massengill JL, O'Dowd DK, Smith MA (1997). Agrin gene expression in mouse somatosensory cortical neurons during development *in vivo* and in cell culture. *Neuroscience* **79:** 191–201.

Liang XH, Jiang HH, Levine B (1997). Expression of a biologically active antiviral antibody using a Sindbis virus vector system. *Mol Immunol* **34:** 907–917.

Liljeström P, Garoff H (1991). A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Bio/Technology* **9:** 1356–1361.

Lundstrom K (2007). Alphaviruses: Semliki Forest virus and Sindbis virus as gene delivery vectors. In: *Gene transfer: delivery and expression of DNA and RNA, a laboratory manual*. Friedmann T, Rossi J (eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 327–348.

Lundstrom K, Abenavoli A, Malgaroli A, Ehrengruber MU (2003). Novel Semliki Forest virus vectors with reduced cytotoxicity and temperature-sensitivity for long-term enhancement of transgene expression. *Mol Ther* **7:** 202–209.

Maletic-Savatic M, Malinow R, Svoboda K (1999). Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science* **283:** 1923–1927.

Perri S, Driver DA, Gardner JP, Sherrill S, Belli BA, Dubensky TW Jr, Polo JM (2000). Replicon vectors derived from Sindbis virus and Semliki Forest virus that establish persistent replication in host cells. *J Virol* **74:** 9802–9807.

Rikkonen M (1996). Functional significance of the nuclear-targeting and NTP-binding motifs of Semliki Forest virus nonstructural protein nsP2. *Virology* **218:** 352–361.

Sawicki DL, Perri S, Polo JM, Sawicki SG (2006). Role for nsP2 proteins in the cessation of alphavirus minus-strand synthesis by host cells. *J Virol* **80:** 360–371.

Scheer A, Björklöf K, Cotecchia S, Lundstrom K (1999). Expression of the α_{1b}-adrenergic receptor and G protein subunits in mammalian cell lines using the Semliki Forest virus expression system. *J Recept Signal Transduct Res* **19:** 369–378.

Schlesinger S (2000). Alphavirus expression vectors. *Adv Virus Res* **55:** 565–577.

Singh IR, Suomalainen M, Varadarajan S, Garoff H, Helenius A (1997). Multiple mechanisms for the inhibition of entry and uncoating of superinfecting Semliki Forest virus. *Virology* **231:** 59–71.

Strauss JH, Strauss EG (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* **58:** 491–562.

Takkinen K (1986). Complete nucleotide sequence of the nonstructural genes of Semliki Forest virus. *Nucleic Acids Res* **14:** 5667–5682.

Tuittila MT, Santagati MG, Röyttä M, Määttä JA, Hinkkanen AE (2000). Replicase complex genes of Semliki Forest virus confer lethal neurovirulence. *J Virol* **74:** 4579–4589.