Research Article

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Near-Infrared Fluorescent Protein iRFP720 is Optimal for *In Vivo* Fluorescence Imaging of Rabies Virus Infection

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Abbreviations: BphPs, Bacterial phytochrome photoreceptors; CCD, charge-coupled device; E2Cr, E2-Crimson; EMEM, Eagle's minimal essential medium; EM, electron multiplying; EMG, electron-multiplying gain; Em, emission; Ex, excitation; EXP, exposure time; FP, fluorescent protein; G, glycoprotein; i.c., intracerebral; i.m., intramuscular; Ka2S, Katushka2S; L, large; M, matrix; N, nucleoprotein; NIR, near-infrared; P, phosphoprotein; Ψ, pseudogene; RABV, rabies virus; RFP, red fluorescent protein; ROI, region of interest; S/N, signal to noise; WT, wild type.

ABSTRACT

In vivo imaging is a noninvasive method that enables real-time monitoring of viral infection dynamics in a small animal, which allows a better understanding of viral pathogenesis. *In vivo* bioluminescence imaging of virus infection is widely used, but fluorescence imaging is not used because of severe autofluorescence, despite its advantage over bioluminescence that no substrate administration is required. Recently, several far-red and near-infrared (NIR) fluorescent proteins (FPs) have been developed that have been shown to be useful for whole-body fluorescence imaging. Here, we report comparative testing of far-red and NIR FPs in the imaging of rabies virus (RABV) infection. Using the highly neuroinvasive 1088 strain, we generated recombinant RABV that expressed FPs such as Katushka2S, E2-Crimson, iRFP670, or iRFP720. After intracerebral inoculation to nude mice, the 1088 expressing iRFP720, the most red-shifted FP, was detected earliest with the highest signal-to-noise ratio using a filter set for >700 nm, in which the background signal level was very low. Furthermore, we could also track viral dissemination from the spinal cord to the brain in nude mice after intramuscular inoculation of iRFP720-expressing 1088 into the hind limb. Hence, we conclude that the NIR FP iRFP720 used with a filter set for >700 nm is useful for *in vivo* fluorescence imaging not only of RABV infection but also of other virus infections. Our findings will also be useful for developing dual-optical imaging of virus–host interaction dynamics using bioluminescence reporter mice for imaging of inflammation.

241 words

INTRODUCTION

In vivo imaging is now widely used in the field of virology. This noninvasive method has advantages for the understanding of viral pathogenesis [1, 2], because the dynamics of viral replication and spread can be monitored semiquantitatively and longitudinally throughout the body of the same animal before any signs of disease appear, and the number of animals subjected to experiments can be reduced dramatically because the harvesting of tissues and organs to determine viral titers at multiple time points is not required. It is also possible that whole-body *in vivo* imaging can identify unexpected but important sites of viral replication that would be missed using a traditional approach [1, 2]. Furthermore, multimodal (a combination of fluorescence, bioluminescence, and positron emission tomography/computed tomography) imaging allows us to monitor simultaneously viral replication and host immune responses and to improve our understanding of virus–host interaction dynamics [2, 3].

Rabies virus (RABV) is the causative agent of rabies, a fatal encephalitis, and belongs to the genus *Lyssavirus* in the family *Rhabdoviridae*. RABV has a nonsegmented, negative-sense RNA genome of approximately 12 kb in length, which encodes five structural proteins (N, P, M, G, and L). RABV is usually transmitted by a bite from an infected animal and causes encephalitis after a long and variable incubation period, a significant feature of RABV infection. It is not fully understood where and how RABV persists in a host during the incubation period, which makes it a good example to study using *in vivo* imaging.

In general, recombinant viruses expressing a reporter gene are utilized for *in vivo* optical imaging of virus infection, and luciferase genes have been widely used as the

reporter for *in vivo* bioluminescence imaging of virus infections in small mammals [2]. However, despite fluorescence imaging having the advantage over bioluminescence imaging that no substrate administration is required, there are few reports of successful *in vivo* fluorescence imaging using a virus expressing a fluorescent protein (FP). The main reason for this is that conventional reporter FPs, such as GFP, red fluorescent protein (RFP), and their derivatives, are detected using light in the visible wavelength region, in which autofluorescence (nonspecific background fluorescence from tissues) is also strong and interferes with *in vivo* imaging by severely limiting the signal-to-noise (S/N) ratio [4].

It is well known that wavelengths around 650–900 nm, the so-called 'near-infrared (NIR) window' or 'biological window,' are preferable for deep-tissue optical imaging, because photon scattering, light absorption, and tissue autofluorescence are significantly lower in this region than in the visible light region [4-7]. Recently, a number of far-red and NIR FPs have been developed and assessed for utility in *in vivo* imaging. Katushka2S (Ka2S) is a bright far-red FP that is a modification of Katushka derived from a sea anemone (*Entacmaea quadricolor*), and has excitation/emission (Ex/Em) maxima of 588/633 nm [8, 9]. Ka2S was found in a comparative study to be a better FP for whole-body fluorescence imaging [8]. E2-Crimson (E2Cr) is a bright far-red FP derivative of DsRed-Express2 with Ex/Em maxima of 611/646 nm [10]. It was reported that E2Cr was one of the most favorable FPs for imaging of bacterial infections [11]. NIR FPs, known as iRFPs, have also been engineered from bacterial phytochrome photoreceptors (BphPs) and have Ex/Em maxima within the NIR window [12]. Among these iRFPs, iRFP670, which is derived from the *Rhodopseudomonas palustris* BphP (RpBphP2), is the brightest and most blue-shifted iRFP with Ex/Em maxima of 643/670 nm, and iRFP720, derived

from RpBphP6, is the most red-shifted iRFP with Ex/Em maxima of 702/720 nm [12]. Multicolor *in vivo* whole-body imaging was shown to be possible using mice injected with two tumors expressing iRFP670 and iRFP720 [12], and the performance of iRFP720 for imaging was shown to be comparable to that of Ka2S [8].

In this study, to identify the optimal FP for *in vivo* fluorescence imaging of virus infection, we performed a comparative study using Ka2S, E2Cr, iRFP670, and iRFP720. To this end, we generated RABV expressing each FP and assessed the fluorescence signals from inoculated mice. We found that iRFP720 was optimal for the imaging of RABV infection, and should be applicable to other viruses and to multimodal imaging of virus infection dynamics.

RESULTS

Generation of FP-expressing RABVs. The street RABV strain 1088 was originally isolated from a rabid woodchuck in North America [13] and is highly pathogenic even after intramuscular (i.m.) inoculation [14]. For the present study, we constructed genome plasmids for wild-type (WT) 1088 and four recombinant 1088 viruses expressing different FPs, i.e., 1088/Ka2S, 1088/E2Cr, 1088/iRFP670, and 1088/iRFP720 (Fig. 1). The expression cassette for the FP was inserted into the pseudogene region (Fig. 1b) because previous studies demonstrated that a foreign gene inserted in this region was stably expressed and did not affect viral replication and pathogenicity [15, 16]. The Ka2S and E2Cr genes were derived from commercial expression vectors for mammalian cells, whereas the iRFP genes that were used were synthesized to be codon-optimized for mammalian cells (Fig. S1, available in the online Supplementary Material). The

123 recombinant viruses were recovered from the genome plasmids, and those amplified in
124 suckling mouse brains were used for further analyses.
125 FP expression by the recombinant viruses was evaluated by a fluorescence focus
126 assay in mouse neuroblastoma (NA) cells using a fluorescence microscope (Fig. 2).
127 Fluorescence foci visualized by the FP corresponded with foci visualized by N-antigen
128 staining and were detected using filters appropriate for each FP. Fluorescence foci with
129 nuclear staining are also shown in Fig. S2, available in the online Supplementary
130 Material.
131 We also examined the virulence of the FP-expressing viruses by intracerebral (i.c.)
132 inoculation into six-week-old ddY mice and found that the FP-expressing viruses were
133 not significantly attenuated compared with the WT virus (Fig. 3 and Fig. S3, available in
134 the online Supplementary Material).
135 **Comparison of i.c.-inoculated mice by live imaging.** Before comparison by *in vivo*
136 imaging, we evaluated the detectability of fluorescence in virus-infected NA cells using
137 the Lumazone *in vivo* imaging system (Fig. S4, available in the online Supplementary
138 Material). The imaging system could clearly detect all the FPs when appropriate filter
139 sets were used; the Ka2S and E2Cr signals were detected using a filter set for 607/697
140 (Ex/Em) nm, the iRFP670 signal by either 607/697 nm or 655/732 nm, and the iRFP720
141 signal by either 655/732 nm or 710/785 nm.
142 Next, we evaluated the FPs for use in live imaging of i.c.-inoculated mice. Five-
143 week-old nude mice were inoculated with 10^4 f.f.u. of each virus and imaged every day.
144 We compared signal intensities detected from brains at 8 days postinoculation using all
145 three filter sets (Fig. 4a and b). Using the 607/697 nm filter set, the fluorescence signal

from mice infected with1088/E2Cr exhibited the highest S/N ratio of 3.76 (mock infection as 1). The signals for infections with 1088/iRFP670 and 1088/iRFP720 showed higher S/N ratios (4.42 and 4.89, respectively) using the 655/732 nm filter set. However, the signal from the 1088/iRFP720 infection demonstrated the best S/N ratio (16.27) using the 710/785 nm filter set. Of note, fine brain images with high contrast and high resolution could be obtained from the 1088/iRFP720-infected mice using the 710/785 nm filter set (Fig. 4c). After live imaging at day 8, mice were euthanized, their brains isolated, and viral titers determined from brain homogenates (Fig. 4d). Notably, the viral titer was not significantly higher for 1088/iRFP720 infection than for infections with 1088 (WT), 1088/E2Cr, and 1088/iRFP670, although the viral titer for 1088/Ka2S infection was significantly lower than the others. Fig. 5 shows time-course imaging of the i.c.-inoculated mice using the optimal filter set. In 1088/iRFP720 infection, the signal from brain began to be detectable in two of four mice at day 4, when mice were asymptomatic, and was clearly detected in all of the mice after day 5 when the mice began to lose weight. The clinical progression of the infected mice is also illustrated in Fig. S5, available in the online Supplementary Material. For the other infections, the signal began to be detectable later than that for the 1088/iRFP720 infection, and the 1088/Ka2S infection was only detectable the day after weight loss began.

***In vivo* imaging of mice inoculated i.m. with 1088/iRFP720.** As shown above, comparison analysis indicated that of the FPs examined in this study, iRFP720 was optimal for *in vivo* fluorescence imaging of virus infection. Therefore, we performed *in vivo* whole-body imaging of mice inoculated i.m. with 1088/iRP720. Six-week-old nude mice were inoculated with 5×10^5 f.f.u. of the virus in the right hind limb (triceps surae

muscle) and then imaged every day using the Lumazon system with the 710/785 nm filter set (Fig. 6). In inoculated mouse no. 5, a weak fluorescence signal was detectable from the spinal cord on day 6 before the mouse began to lose weight and was clearly detectable at day 7. At that time, a spot of signal was detected from the brain, and the mouse started losing weight. The signals from the brain and spinal cord continued to increase until day 12 when the mouse was euthanized. In the other infected mice (nos. 4 and 6), a weak signal from the spinal cord was detectable at day 7, one day later than in mouse no. 5. Thereafter, these mice showed a similar pattern to mouse no. 5. Although we also imaged the ventral side of the mouse during the observation period, we could not detect any signal even at day 12, except for signals thought to be autofluorescence from something attached to the skin.

After live imaging at day 12, *ex vivo* imaging was also done. The i.m.-inoculated mice were euthanized, and their skull, spine, ribs, and thighs were isolated and imaged under the same conditions as the live imaging (Fig. 7). In addition to the central nervous system, peripheral nerves (e.g., intercostal, sciatic, and femoral nerves) could also be clearly visualized by their fluorescence signal. Notably, a strong signal was detected from the terminals of the spinal cord, the signal was stronger on the right side than on the left, and we could detect signals from the sciatic and femoral nerves of the right hind limb. These observations were considered to reflect the route of virus inoculation.

DISCUSSION

In vivo bioluminescence imaging is very useful for observing and analyzing virus replication dynamics in small animals, but, unlike fluorescence imaging, it requires the

192 administration of a substrate, which is burdensome and increases experimental costs.
193 However, despite the potential advantage of fluorescence imaging, there have been few
194 reports of successful live imaging to tracking of infection dynamics using a pathogenic
195 virus expressing an FP. Some attempts at fluorescence imaging of virus infection using
196 far-red and NIR FPs have been reported, but all failed. Influenza A viruses expressing
197 mCherry (a RFP with Ex/Em maxima 587/610 nm) [17] or TurboFP635 (a synonym of
198 Katushka with Ex/Em maxima of 588/635 nm) [9] were shown to be pathogenic in mice
199 and were successfully imaged *ex vivo* in infected lungs but not *in vivo* [18, 19]. An
200 Influenza A virus expressing an NIR FP, iRFP713 (the Ex/Em maxima of 690/713 nm)
201 [12] was generated and characterized, but failed in both *ex vivo* and *in vivo* imaging
202 because of low expression of iRFP713 [19]. In the present study, we successfully
203 observed virus replication dynamics in mice by *in vivo* fluorescence imaging using FP-
204 expressing RABVs and found that the most red-shifted FP, iRFP720, was the best for
205 imaging analysis of the four FPs tested.
206 A previous study reported that Ka2S was the best of the far-red FPs tested for *in vivo*
207 whole-body imaging and that the S/N ratios for Ka2S and iRFP720 were comparable at
208 their optimal wavelengths [8]. However, 1088/Ka2S had the worst S/N ratio of the four
209 FP-expressing viruses examined in the present study. We considered that this result
210 probably does not fully represent the capacity of Ka2S because the 1088/Ka2S titers in
211 mouse brains were significantly lower than those of the other viruses (Fig 4d), and the
212 1088/Ka2S-infected mice did not lose as much weight as the mice infected with the other
213 viruses (Fig. 5b). This indicates that we imaged at an earlier phase of infection for
214 1088/Ka2S; therefore, the S/N ratio obtained for 1088/Ka2S infection is likely to improve

when mice are imaged at a later stage. However, even at a later stage, we expect that 1088/Ka2S could only achieve an S/N ratio comparable to that for 1088/E2Cr because the *in vitro* S/N ratios of these two strains were similar at every time point when using the 607/697 nm filter set (Fig. S4). It is also possible that the replication of 1088/Ka2 might be somewhat affected *in vivo* but not *in vitro*. The viral G gene is known to be the major determinant of neurovirulence [20-24], but no mutations were detected in the G gene of 1088/Ka2S (data not shown). However, a mutation in the other regions or expression of Ka2S might interfere with replication in the brain.

The NIR window has also been defined as the 'biological transparency NIR window' because, in this range, tissues show reduced photon scattering, light absorption, and autofluorescence, which can allow deeper tissue optical imaging with an improved S/N ratio [7]. In particular, tissue autofluorescence caused by endogenous chromophores can severely limit the S/N ratio, but it has been shown that use of the NIR filter set can dramatically reduce autofluorescence [4, 7]. Elimination of autofluorescence is the most important factor for successful in *in vivo* fluorescence imaging: even if a fluorescence signal is weak, the signal detection can be enhanced easily by extending the exposure time and increasing the gain of the CCD sensor if the autofluorescence is very low. In fact, although we imaged using a longer exposure time and enhanced gain when using the 710/785 nm filter set (see Methods), these imaging conditions still produced a lower background signal than those using the other filter sets. Therefore, because only iRFP720 can generate fluorescence efficiently in the range of the 710/785 nm filter set and thereby avoid autofluorescence, 1088/iRFP720 produced the highest S/N ratio *in vivo* (Fig. 4), although it showed the lowest S/N ratio *in vitro* (Fig. S4). It is possible that the lower

light scattering and absorbance in the NIR window could also contribute to improving the S/N ratio when using the 710/785 nm filter set for detection of fluorescence from iRFP720 expressed in the brain. However, the signal permeability of the head using this filter set was comparable to that using the 655/732 nm filter set and slightly higher (by approximately 20%) than that using the 607/697 nm filter set (Fig. S6, available in the online Supplementary Material). Indeed, the skin and cranial bone are thin and highly transparent tissues within the spectrum used in this study [7]. The brain has a higher scattering coefficient at shorter wavelengths, but this declines markedly with increasing wavelength [7]. Therefore, it is also likely that when using the 710/785 nm filter set, the excitation light reaches deeper and the emission light is detectable from deeper tissues, which would contributed to improved signal intensity.

To achieve truly real-time fluorescence imaging of RABV infection dynamics in mice, it is necessary to improve the detection sensitivity. This is important to allow us to address where and how RABV persists in a host during its long and variable incubation period. In this study, we found by live imaging of mice inoculated i.m. with 1088/iRFP720 that the fluorescence signal was first detected from the spinal cord and then from the brain, suggesting that RABV replication dynamics can be tracked in mice. However, we could not detect the primary site of infection and viral spread to the connected peripheral nerves by live imaging, although a signal was detected from the sciatic and femoral nerves by *ex vivo* imaging. Moreover, we could not detect a specific signal from mice inoculated i.c. with 1088/iRFP720 until 3 days postinoculation. One simple way to improve detectability would be to enhance the level of iRFP720 expression in infected cells. Previous studies reported that insertion of the reporter cassette between

the N and P genes led to higher gene expression compared with insertion between the G and L genes [25, 26]. Hence, we are now attempting to generate and assess a recombinant 1088 strain encoding the iRFP720 gene inserted between the N and P genes. However, it is likely that although this recombinant virus might allow live imaging to detect replication sites or regions macroscopically at an early phase of infection, it would still be difficult to identify the tissue and cellular tropisms of primary infection. The amounts of the reporter protein produced in small numbers of initially infected cells are unlikely to be sufficient to allow tracing by live imaging, but should be detectable by fluorescent microscopy. Therefore, to analyze primary infection precisely, it would be necessary to combine with *ex vivo* imaging, histology, and flow cytometry using a bright fluorescent protein as previously reported for measles virus [27-29].

In conclusion, we consider that using iRFP720 with a filter set for > 700 nm is useful for *in vivo* fluorescence imaging of not only RABV infection but also other virus infections. Our findings would also be useful for establishing a dual-optical (combination of fluorescence and bioluminescence) imaging, which would be a cost-effective way to analyze virus–host interaction dynamics using bioluminescence reporter mice for imaging of inflammation [30].

METHODS

Cells. Human neuroblastoma SK-N-SH cells, which were kindly provided by Dr. Morita (Nagasaki University, Japan), and mouse neuroblastoma NA cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% (vol/vol) FCS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). BHK cells that stably

express T7 RNA polymerase (BHK/T7-9 cells) [31] were kindly provided by Drs. Ito and Sugiyama (Gifu University, Japan) and maintained in EMEM supplemented with 5% (vol/vol) FCS, 10% (vol/vol) tryptose phosphate broth solution, and antibiotics.

Plasmid construction. A genome plasmid for the 1088 strain, pCI-1088 (WT), was constructed as shown in Fig. 1a. The cDNA template was synthesized from a virus solution stored as 10% (wt/vol) brain homogenate [14]. DNA fragments that were amplified by PCR using the template and the desired primers were cloned into a pT7Blue T-vector (Millipore), and we confirmed that the nucleotide sequences of the cloned fragments were identical to the genomic sequence of 1088 (GenBank accession no. AB645847), except for two mutations to introduce a *Sac*II site in the pseudogene region. All fragments including ribozyme sequences were assembled into a pCI vector (Promega). Subsequently, we constructed a genome plasmid for 1088/E2Cr (pCI-1088/E2Cr) as shown in Fig. 1b. To generate the E2Cr expression cassette, the E2Cr gene was amplified by PCR using pCMV-E2-Crimson vector (Clontech) and primers to which restriction enzyme sites, transcription stop and start sequences, and the Kozak sequence were added. Part of the pseudogene region of pCI-1088 (WT) was removed and replaced by the E2Cr expression cassette. Based on pCI-1088/E2Cr, genome plasmids for 1088/Ka2S, 1088/iRFP670, and 1088/iRFP720 were constructed by replacing the E2Cr gene with the Ka2S, iRFP670, and iRFP720 genes, respectively. The Ka2S gene with restriction enzyme sites was amplified by PCR using pKatushka2S-N vector (Evrogen) as a template, and the codon-optimized iRFP genes (Fig. S1) with restriction enzyme sites were synthesized by Fasmac Co., Ltd.

Recovery of recombinant viruses. To generate recombinant viruses, each genome plasmid was transfected into BHK/T7-9 cells together with helper plasmids pCI-RG, pT7IRES-RN, -RP, and -RL (kindly provided by Drs. Ito and Sugiyama) [31, 32] using TransIT-LT1 transfection reagent (Mirus Bio). The culture supernatant was collected after incubation for several days and the recovered virus was amplified twice in SK-N-SH cells. To obtain high-titer virus stocks, 10% (wt/vol) brain homogenate in PBS was prepared from i.c.-inoculated suckling mice and stored in aliquots at –80 °C until use.

Virus titration. The virus titer was determined by the focus assay as described previously [14] and is expressed as f.f.u./ml.

Immunofluorescence focus assay. NA cell monolayers were inoculated with each virus and incubated at 37 °C for 1 h. After removal of the inoculum, the cells were overlaid with medium consisting of EMEM supplemented with 5% FCS, antibiotics, and 1% methylcellulose and incubated at 37 °C for 4 days. The inoculated cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 solution. Then, cells were stained with the anti-N monoclonal antibody 10-41-F2 [33] and Alexa Fluor 488-conjugated goat anti-mouse IgG (Thermo Fisher Scientific). Fluorescence images were obtained using an EVOS FL fluorescence microscope with Light Cubes for GFP (470/22 nm Ex; 510/40 nm Em), Texas Red (585/29 nm; 624/40 nm), Cy5 (628/40 nm; 692/40 nm), or Cy5.5 (655/46 nm; 794/160 nm).

Virulence in mice. Groups of ten six-week-old female *dd*Y mice (Japan SLC) were inoculated i.c. with 10^2 f.f.u. of each virus (0.03 ml) and monitored daily for 14 days. Mice were humanely euthanized when they showed severe neurological signs such as opisthotonus or were moribund (i.e., in a deep coma).

***In vivo* and *ex vivo* fluorescence imaging analyses.** For i.c. inoculation, groups of four five-week-old female nude mice (CAnN.Cg-*Foxn1*^{nu}/CrlCrlj; Charles River Laboratories Japan) were inoculated with 10^4 f.f.u. of each virus or medium (0.03 ml). In addition, groups of three nude mice were fed a chlorophyll-free diet, D10001 (Research Diets), for a week and then inoculated i.m. (right hind limb triceps surae muscle) with 5×10^5 f.f.u. of 1088/iRFP720 or medium (0.05 ml). Inoculated mice were monitored, weighed, and fed D10001 every day. Furthermore, mice were imaged every day under inhalation anesthesia (2% isoflurane) using the Lumazone imaging system (Nippon Roper) equipped with an X-Cite 200DC illumination system (Lumen Dynamics, Excelitas Technologies), an Evolve 512 electron-multiplying (EM) CCD camera (Photometrics), and Techspec fluorescence bandpass filters (Edmund Optics) for 607/36 nm, 655/40 nm, and 710/40 nm for Ex, and 697/75 nm, 732/68 nm, and 785/62 nm for Em. Imaging conditions were as follows: exposure time (EXP) of 100 ms and EM gain (EMG) of 5 (1–1,000) for the 607/697 nm filter set; EXP of 200 ms and EMG of 5 for the 655/732 nm filter set; EXP of 400 ms and EMG of 10 for the 710/785 nm filter set. At 12 days postinoculation, the i.m.-inoculated mice were euthanized after live imaging and dissected to isolate skull, spine, ribs, and thighs. The isolated samples were imaged using the Lumazone imaging system. All images were acquired as 16-bit TIFF files and processed and analyzed using ImageJ software [34]. In addition, 20% (wt/vol) brain homogenates were prepared from the i.c.-inoculated mice after live imaging at 8 days postinoculation and titrated using the focus assay as described above.

Statistical analysis. Log-rank (Mantel–Cox) test and Tukey's multiple comparisons test were performed using GraphPad Prism (version 6.0, GraphPad software).

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

ETHICAL STATEMENT

All animal experiments were approved by the Oita University Animal Ethics Committee (approval nos. R010001, 1610001, and 1610002).

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Figure Legends

Fig. 1. Construction of genome plasmids for (a) 1088 (WT) and (b) FP-expressing 1088 strains (see also the Methods section). (a) The cDNA of 1088, hammerhead ribozyme (HmRbz), and hepatitis delta virus ribozyme (HDVRbz) were inserted into the pCI vector using *Nhe*I and *Not*I sites in the multiple cloning site by step-by-step subcloning of eight DNA fragments. The *Sac*II site was introduced just downstream of the stop codon of the G gene by two nucleotide substitutions. Restriction enzyme sites used for construction are also shown. (b) First, the genome plasmid pCI-1088/E2Cr was constructed as follows: the E2Cr expression cassette was inserted into the pseudogene (Ψ) region and located upstream of the transcription stop sequence for the G gene. Based on pCI-1088/E2Cr, genome plasmids for 1088/Ka2S, 1088/iRFP670, and 1088/iRFP720 were constructed using *Age*I and *Pac*I sites.

Fig. 2. Fluorescence focus assay of recombinant viruses. Each virus was inoculated to NA cells, and then cells were incubated with overlay medium containing methylcellulose. After 4 days of incubation, cells were fixed, permeabilized and stained with an anti-N monoclonal antibody (10-41-F2) and secondary antibody labeled with Alexa Fluor 488. Images were obtained using an EVOS FL fluorescence microscope with Light Cubes, which integrate LEDs and filters, for GFP (470/22 nm excitation; 510/40 nm emission), Texas Red (585/29 nm; 624/40 nm), Cy5 (628/40 nm; 692/40 nm), or Cy5.5 (655/46 nm; 794/160 nm), under the same conditions (exposure time and gain) for each Light Cube. Bars indicate 100 μm.

Fig. 3. Kaplan–Meier survival curves of mice inoculated with recombinant viruses. Adult mice (n = 10) were inoculated i.c. with 10^2 f.f.u. of each virus indicated and monitored for 14 days. There was no significant difference in survival (Bonferroni-corrected $P \geq 0.05$ by log-rank test; each *P*-value obtained was multiplied by four) between mice inoculated with WT virus and mice inoculated with an FP-expressing virus.

Fig. 4. *In vivo* fluorescence imaging of nude mice inoculated i.c. with 10^4 f.f.u. of recombinant viruses. (a) Fluorescence images of the brains were obtained at 8 days postinoculation using the Lumazone imaging system with the filter sets 607/697 (607/36 nm for excitation; 697/75 nm for emission), 655/732 (655/40 nm; 732/68 nm), and 710/785 (710/40 nm; 785/62 nm). Representative mouse images are shown. The asterisk indicates the inoculation site. Color bars indicate relative signal intensities. (b) Comparison of fluorescence intensities in the brain region, which is indicated as a region of interest (ROI) in (a). S/N ratios (test/mock infected) were calculated for each filter set and are presented as means and standard deviations (SD); n = 4. (c) The fluorescence image obtained from mice inoculated with 1088/iRFP720 using the 710/785 nm filter set is shown as an unsaturated grayscale image. The grayscale bar indicates the relative signal intensity. (d) Virus titers in the brains of nude mice at 8 days postinoculation. Pairs that are not significantly different (n.s., $P \geq 0.05$ by Tukey's multiple comparisons test) are indicated; pairs not indicated are significantly different ($P < 0.05$).

Fig. 5. Time-course imaging of nude mice inoculated i.c. with 10^4 f.f.u. of FP-expressing viruses. (a) Images obtained using the optimal filter set are shown from days 3 to 7

postinoculation. Color bars indicate relative signal intensities. (b) Mean changes in body weight of inoculated mice. Bars indicate SD.

Fig. 6. Time-course imaging of nude mice inoculated i.m. with 5×10^5 f.f.u. of 1088/iRFP720. (a) Images of mock- or virus-inoculated mice were obtained daily using the 710/785 filter set, and dorsal images from 5 to 12 days post-inoculation are shown. Ventral images at day 12 are also shown. Color and grayscale bars indicate relative signal intensities. (b) Changes in body weight of individual inoculated mice. Mouse ID numbers indicated correspond to those in (a).

Fig. 7. *Ex vivo* imaging of nude mice inoculated i.m. with 1088/iRFP720. After live imaging at day 12, their skull, spine, ribs, and thighs were isolated and imaged under bright-field and fluorescence (the 710/785 filter set) modes. Representative sample images are shown, and the ID numbers indicated correspond to those in Fig. 6. For dorsal images of the sample from the 1088/iRFP720-inoculated mouse, both low- and high-saturated (enhanced) images are presented. Grayscale bars indicate relative signal intensities. The arrow and arrowhead indicate the sciatic nerve and femoral nerve, respectively.