Near-infrared fluorescent protein iRFP720 is optimal for in vivo fluorescence imaging of rabies virus infection

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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, despite its advantage over bioluminescence that no substrate administration is required, fluorescence imaging is not used because of severe autofluorescence. Recently, several far-red and near-infrared (NIR) fluorescent proteins (FPs) have been developed and 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 strain expressing iRFP720, the most red-shifted FP, was detected the 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 for RABV infection but also for other virus infections. Our findings will also be useful for developing dual-optical imaging of virus–host interaction dynamics using bioluminescence reporter mice for inflammation imaging.

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 titres 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 [nucleoprotein (N), phosphoprotein (P), matrix (M), glycoprotein (G) and large (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 green FP (GFP), red FP (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 Rhodopseudomonas palustris BphP (RpBphP2), is the brightest and most blue-shifted iRFP with Ex/Em maxima of 643/670 nm, and iRFP720, derived from RpBphP, is the most red-shifted iRFP with Ex/Em maxima of 702/720 nm [12]. Multicolour in vivo whole-body imaging was shown to be possible using mice injected with two tumours 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

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 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 (Ψ) 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 recombinant viruses were recovered from the genome plasmids, and those amplified in suckling mouse brains were used for further analyses.

FP expression by the recombinant viruses was evaluated by a fluorescence focus assay in mouse neuroblastoma NA cells using a fluorescence microscope (Fig. 2). Fluorescence foci visualized by the FP corresponded with foci visualized by N-antigen staining and were detected using filters appropriate for each FP. Fluorescence foci with nuclear staining are also shown in Fig. S2.

We also examined the virulence of the FP-expressing viruses by intracerebral (i.c.) inoculation into six-week-old ddY mice and found that the FP-expressing viruses were not significantly attenuated compared with the WT virus (Figs 3 and S3).

Comparison of i.c.-inoculated mice by live imaging

Before comparison by in vivo imaging, we evaluated the detectability of fluorescence in virus-infected NA cells using the Lumazone in vivo imaging system (Fig. S4). The imaging system could clearly detect all the FPs when appropriate filter sets were used; the Ka2S and E2Cr signals were detected using a filter set for 607/697 (Ex/Em) nm, the iRFP670 signal by either 607/697 or 655/732 nm, and the iRFP720 signal by either 655/732 or 710/785 nm.

Next, we evaluated the FPs for use in live imaging of i.c.-inoculated mice. Five-week-old nude mice were inoculated with 10^5 F.U. of each virus and imaged every day. We compared signal intensities detected from brains at 8 days post-inoculation (p.i.) using all three filter sets (Fig. 4a, b). Using the 607/697 nm filter set, the fluorescence signal from mice infected with 1088/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 titres determined from brain homogenates (Fig. 4d). Notably, the viral titre was not significantly higher for 1088/iRFP720 infection than for infections with 1088 (WT), 1088/E2Cr and 1088/iRFP670, although the viral titre 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 the brain began to be detectable in two out 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. 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/iRFP720. Six-week-old nude mice were inoculated with $5 \times 10^5$ 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 the 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 performed. The i.m.-inoculated mice were euthanized, and their skull, spine, ribs and thighs were isolated and imaged.

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*Fig. 1.* Construction of genome plasmids for (a) 1088 (WT) and (b) FP-expressing 1088 strains (see also Methods). (a) The cDNA of 1088, hammerhead ribozyme (HmRbz), and hepatitis delta virus ribozyme (HDVRbz) were inserted into the pCI vector using NheI and NotI sites in the multiple-cloning site by step-by-step subcloning of eight DNA fragments. The Sacl 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 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 AgeI and PacI sites.
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 analysing virus replication dynamics in small animals, but, unlike fluorescence imaging, it requires the administration of a substrate, which is burdensome and increases experimental costs. However, despite the potential advantage of fluorescence imaging, there have been few reports of successful live imaging to track infection dynamics using a pathogenic virus expressing an FP. Some attempts at fluorescence imaging of virus infection using far-red and NIR FPs have been reported, but all failed. Influenza A viruses expressing mCherry (a RFP with Ex/Em maxima 587/610 nm) [17] or TurboFP635 (a synonym of Katushka with Ex/Em maxima of 588/635 nm) [9] were shown to be pathogenic in mice and were successfully imaged *ex vivo* in infected lungs but not *in vivo* [18, 19]. An Influenza A virus expressing an NIR FP, iRFP713 (the Ex/Em maxima of 690/713 nm) [12] was generated and characterized, but failed in both *ex vivo* and *in vivo* imaging because of low expression of iRFP713 [19]. In the present study, we successfully observed virus replication dynamics in mice by *in vivo* fluorescence imaging using FP-expressing RABVs and found that the most red-shifted FP, iRFP720, was the best for imaging analysis of the four FPs tested.

A previous study reported that Ka2S was the best of the far-red FPs tested for *in vivo* whole-body imaging and that the S/N ratios for Ka2S and iRFP720 were comparable at their optimal wavelengths [8]. However, 1088/Ka2S had the worst S/N ratio of the four FP-expressing viruses examined in the present study. We considered that this result probably does not fully represent the capacity of Ka2S because the 1088/Ka2S titres in mouse brains were significantly lower than those of the other viruses (Fig. 4d), and the 1088/Ka2S-infected mice did not lose as much weight as the mice infected with the other viruses (Fig. 5b). This indicates that we imaged at an
earlier phase of infection for 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 vivo fluorescence imaging; even if a fluorescence signal is weak, the signal detection can be enhanced easily by extending the exposure time (EXP) and increasing the gain of the charge-coupled device (CCD) sensor if the autofluorescence is very low. In fact, although we imaged using a longer EXP 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). 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 Ex light reaches deeper and the Em light is detectable from deeper tissues, which contributed to the 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 p.i. 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 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 analyse primary infection precisely, it would be necessary to combine with ex vivo imaging, histology and flow cytometry using a bright FP 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 analyse 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\(^{-1}\) penicillin and 100 µg ml\(^{-1}\) 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. 1(a). 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 SacII site in the \(\Psi\) 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. 1(b). To generate the E2Cr expression cassette, the E2Cr gene was amplified by PCR using pCMV-E2Cr vector (Clontech) and primers to which restriction enzyme sites, transcription

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**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. Colour bars indicate relative signal intensities. (b) Mean changes in body weight of inoculated mice. Bars indicate SD.
stop and start sequences, and the Kozak sequence were added. Part of the Ψ 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 the pKatushka2S-N vector (Evrogen) as a template, and the codon-optimized iRFP genes (Fig. S1) with restriction enzyme sites were synthesized by Fasmac.

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-titre 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 titre was determined by the focus assay as described previously [14] and is expressed as f.f.u. ml⁻¹.

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 ddY mice (Japan SLC) were inoculated i.c. with 10⁵ 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-Foxn1nu/Crlj; Charles River Laboratories, Japan) were inoculated with 10⁵ 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 $5 \times 10^5$ 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, 655/40 and 710/40 nm for Ex, and 697/75, 732/68 and 785/62 nm for Em. Imaging conditions were as follows: EXP of 100 ms and EM gain (EMG) of 5 (1–1000) 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 p.i., 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 analysed 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 p.i. 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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**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. Greyscale bars indicate relative signal intensities. The arrow and arrowhead indicate the sciatic nerve and femoral nerve, respectively.
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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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