Figure S1

(a)

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KC991142      1 ATGGCGCGTAAGGTCGATCTCACCTCCTGCGATCGCGAGCCGATCCACATCCCCGGCAGCATTCAGCCGTGCGGCTGCCTGCTAGCCTGCGACGCGCAGG
optimized       .....T..G..A..G..C..G..A..........G..A.....A.....T..T..GTCT........A..T.....T.....C.....T..T.....A.

KC991142    101 CGGTGCGGATCACGCGCATTACGGAAAATGCCGGCGCGTTCTTTGGACGCGAAACTCCGCGGGTCGGTGAGCTACTCGCCGATTACTTCGGCGAGACCGA
optimized       .C..TA....T..C..A..A..C..G.....A..A..A........C..T.....C...A.A..G..C.....T..T..G..C........G..A.....

KC991142    201 AGCCCATGCGCTGCGCAACGCACTGGCGCAGTCCTCCGATCCAAAGCGACCGGCGCTGATCTTCGGTTGGCGCGACGGCCTGACCGGCCGCACCTTCGAC
optimized       ...A.....C...A.A..T........T...AG.AGT..C......A.G..T..C..C.....T..C........T..A..C..A..T.....T......

KC991142    301 ATCTCACTGCATCGCCATGACGGTACATCGATCATCGAGTTCGAGCCTGCGGCGGCCGAACAGGCCGACAATCCGCTGCGGCTGACGCGGCAGATCATCG
optimized       ........C..C..T..C.....A...AGC..............A..A..A..C..............T..C..TT..A.......A...........A.

KC991142    401 CGCGCACCAAAGAACTGAAGTCGCTCGAAGAGATGGCCGCACGGGTGCCGCGCTATCTGCAGGCGATGCTCGGCTATCACCGCGTGATGTTGTACCGCTT
optimized       .A..G..T..G..G......AGCT.G...........T.....A..A..C...........A..T........G........A......C....T.....

KC991142    501 CGCGGACGACGGCTCCGGGATGGTGATCGGCGAGGCGAAGCGCAGCGACCTCGAGAGCTTTCTCGGTCAGCACTTTCCGGCGTCGCTGGTCCCGCAGCAG
optimized       T..C.....T..AAGT..C........T........T..A...TC...TT.G........C..T..G.....T..C..T.....CT....T..C.....A

KC991142    601 GCGCGGCTACTGTACTTGAAGAACGCGATCCGCGTGGTCTCGGATTCGCGCGGCATCAGCAGCCGGATCGTGCCCGAGCACGACGCCTCCGGCGCCGCGC
optimized       ..T..T..G......C..........C...A.A..C..G..T...AGTA.A..G...TC.TCTA....T..............T..T..T..A..T..T.

KC991142    701 TCGATCTGTCGTTCGCGCACCTGCGCAGCATCTCGCCCTGCCATCTCGAATTTCTGCGGAACATGGGCGTCAGCGCCTCGATGTCGCTGTCGATCATCAT
optimized       .T..C.....C..T..C..T..C..GTCA..T..T...........G..G..C...A..........T........AAGC...AGC.....A.....T..

KC991142    801 TGACGGCACGCTATGGGGATTGATCATCTGTCATCATTACGAGCCGCGTGCCGTGCCGATGGCGCAGCGCGTCGCGGCCGAAATGTTCGCCGACTTCTTA
optimized       C........T..C.....TC..........C..C...........TA.G.....C..A.....C...A.A..A..T.....G.....T.........C.G

KC991142    901 TCGCTGCACTTCACCGCCGCCCACCACCAACGCTAA
optimized       AGT........T..G..T..A.....T..GA.A.G.
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(b)

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KC991145      1 ATGGCGGAAGGATCCGTCGCCAGGCAGCCTGACCTCTTGACCTGCGACGATGAGCCGATCCATATCCCCGGTGCCATCCAACCGCATGGACTGCTGCTCG
optimized       .....C.....G..A..G..TC.A........T..GC....G.....T........C.....C..A..G..A..T..T..G..A.....C..T...T.G.

KC991145    101 CCCTCGCCGCCGACATGACGATCGTTGCCGGCAGCGACAACCTTCCCGAACTCACCGGACTGGCGATCGGCGCCCTGATCGGCCGCTCTGCGGCCGATGT
optimized       ..T.G..T.....T.....C..T........T.....T.....C..T..G..G..T..C..T.....A..G..A........G..A.....T.....C..

KC991145    201 CTTCGACTCGGAGACGCACAACCGTCTGACGATCGCCTTGGCCGAGCCCGGGGCGGCCGTCGGAGCACCGATCACTGTCGGCTTCACGATGCGAAAGGAC
optimized       G..T...AGC.....A......A.G..C..C..T..AC.T.....A..A..A..T..G..A.....C........A..T..T.....C.....G..A...

KC991145    301 GCAGGCTTCATCGGCTCCTGGCATCGCCATGATCAGCTCATCTTCCTCGAGCTCGAGCCTCCCCAGCGGGACGTCGCCGAGCCGCAGGCGTTCTTCCGCC
optimized       ..T..G..T......AGT.....CA.A..C........G........G..A.....A..A.....A........A.....A..T.....T......A.GA

KC991145    401 GCACCAACAGCGCCATCCGCCGCCTGCAGGCCGCCGAAACCTTGGAAAGCGCCTGCGCCGCCGCGGCGCAAGAGGTGCGGAAGATTACCGGCTTCGATCG
optimized       .G......TC......T..TA.AT....A.....T..G..TC....GTCT..T..T..A..T..A..A..G.....CA.A.....C..T..G..T.....

KC991145    501 GGTGATGATCTATCGCTTCGCCTCCGACTTCAGCGGGTCCGTGATCGCAGAGGATCGGTGCGCCGAGGTCGAGTCAAAACTAGGCCTGCACTATCCTGCC
optimized       ............C..A.....A........TTC...CAGT.....T..C......A.......A.....G..AAGC..G..G..A.............G

KC991145    601 TCATTCATCCCGGCGCAGGCCCGTCGGCTCTATACCATCAACCCGGTACGGATCATTCCCGATATCAATTATCGGCCGGTGCCGGTCACCCCAGACCTCA
optimized       AGC.....T..A..A..A...A.A..T..G..C..G..T..T..C..GA.A.....C.....C.....C.....C..T..T..A.....T..T.....G.

KC991145    701 ATCCGGTCACCGGGCGGCCGATTGATCTTAGCTTCGCCATCCTGCGCAGCGTCTCGCCCAACCATCTGGAGTTCATGCGCAACATAGGCATGCACGGCAC
optimized       ....T..G..A..A.....A..A..C..G..T........T..C..GTCT.....A.....T..C........T...................T..T..

KC991145    801 GATGTCGATCTCGATTTTGCGCGGCGAGCGACTGTGGGGATTGATCGTTTGCCATCACCGAACGCCGTACTACGTCGATCTCGATGGCCGCCAAGCCTGC
optimized       C...AGC.....C..CC.CA.G..A..A..CT.......CC.......G........T..C..A..C.....T.....C..T..C..TA.G..G.....T

KC991145    901 GAGCTAGTCGCCCAGGTTCTGGCCTGGCAGATCGGCGTGATGGAAGAGTGA
optimized       ..A..C..T..A.....C.....T........T...........G......
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Fig. S1. DNA sequences of codon-optimized (a) iRFP670 and (b) iRFP720 genes. KC991142 and KC991145 are the GenBank accession nos. for iRFP670 and iRFP720, respectively. Dots indicate identical nucleotides.

Figure S2

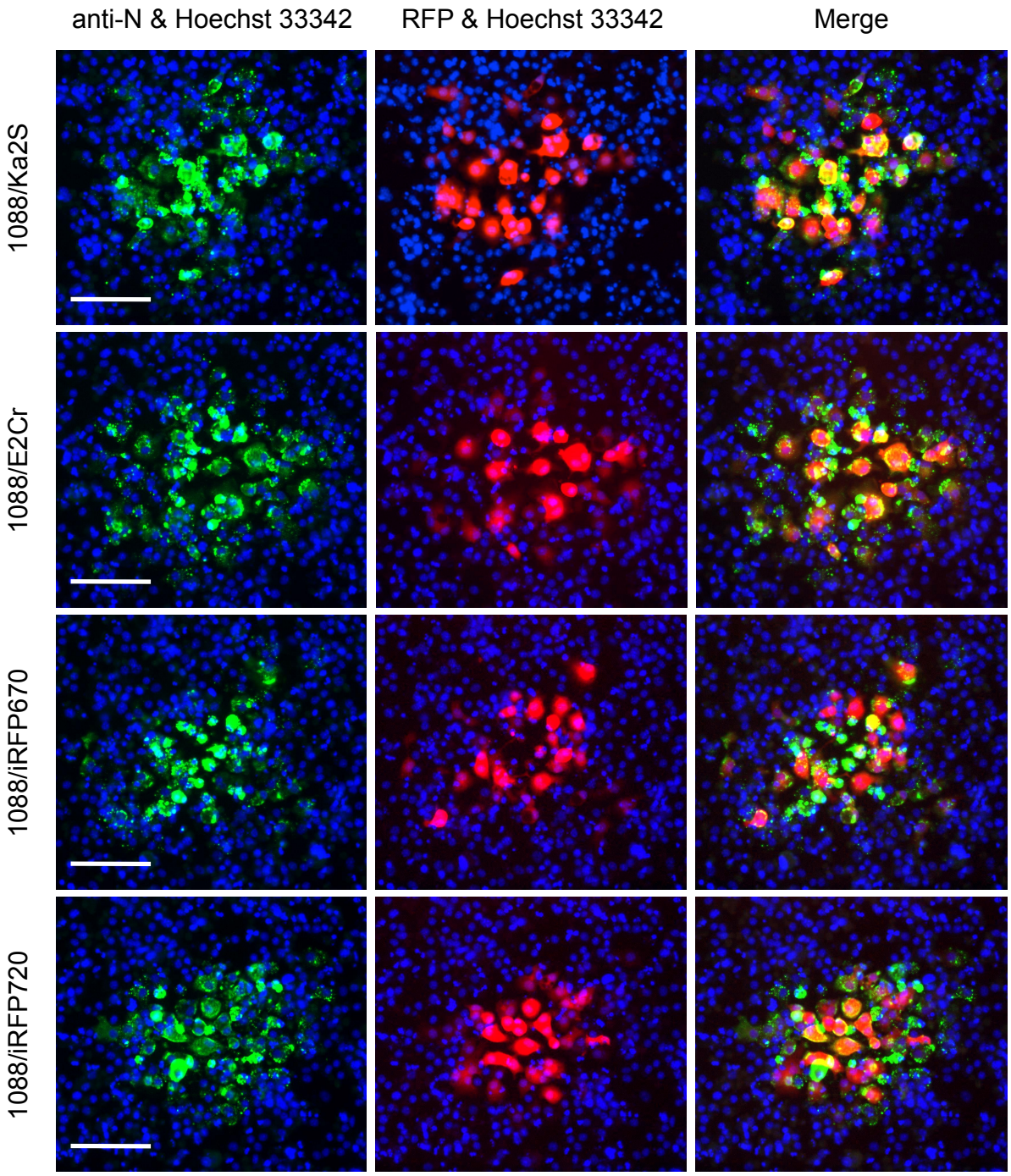

Fig. S2. Fluorescence focus assay of recombinant viruses was performed as illustrated in Fig. 2. In addition, nuclei were stained with Hoechst 33342. Images were obtained using the EVOS FL fluorescence microscope with Light Cubes for DAPI (Hoechst 33342), GFP (the N protein), Texas Red (Ka2S and E2Cr), and Cy5.5 (iRFP670 and iRFP720). Bars indicate 100 μm.

Figure S3

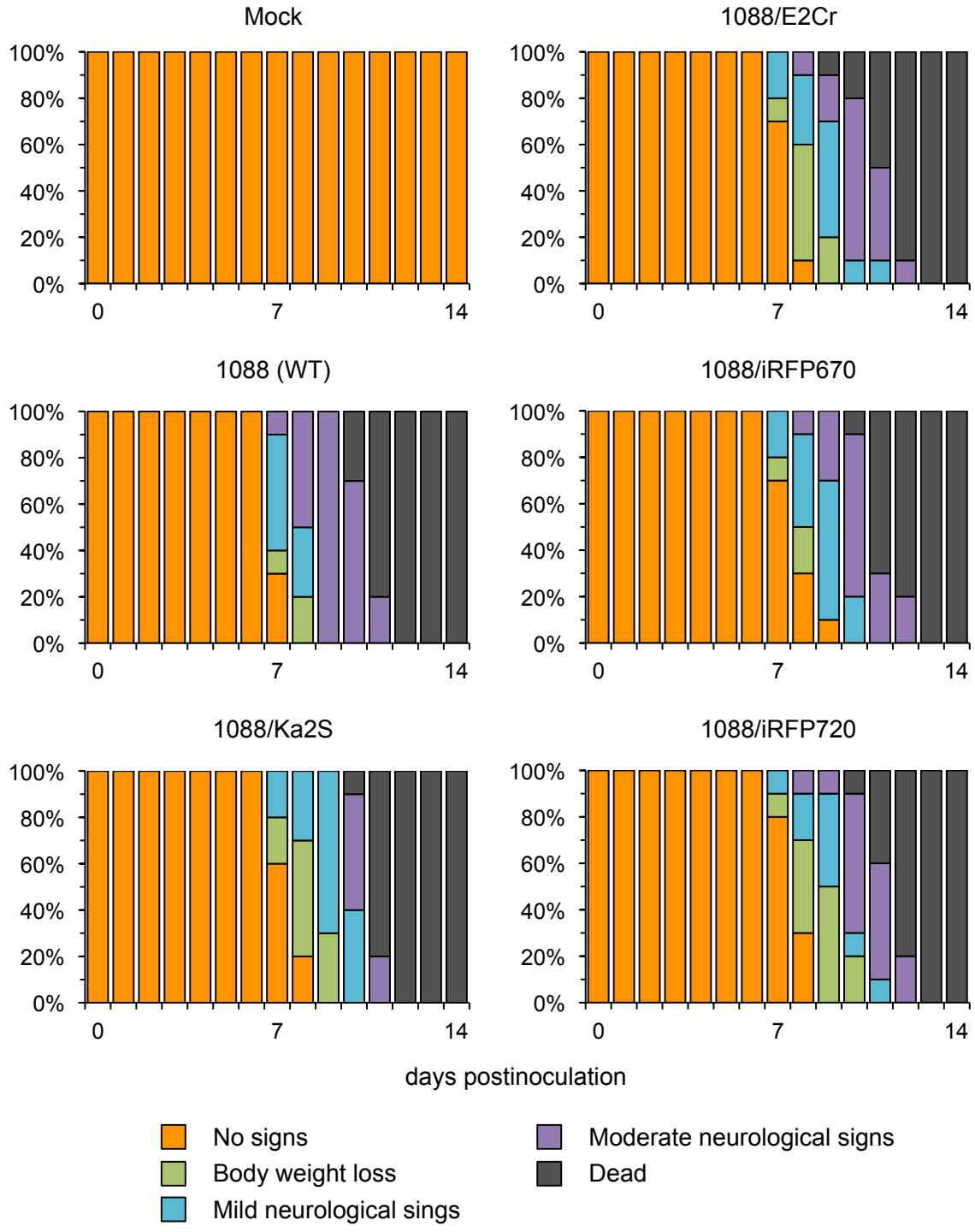

Fig. S3. Progression of disease in ddY mice inoculated i.c. with 10^2 f.f.u. of each virus shown in Fig. 3. "Mild neurological signs" indicates that mice showed a foot slip on a stainless steel wire top clip of a mouse cage without paralysis. "Moderate neurological signs" indicates that mice showed paralysis of the hind limb(s). Mice that showed severe neurological signs such as opisthotonus or were moribund (i.e., in a deep coma) were humanely euthanized and classified as "dead".

Figure S4

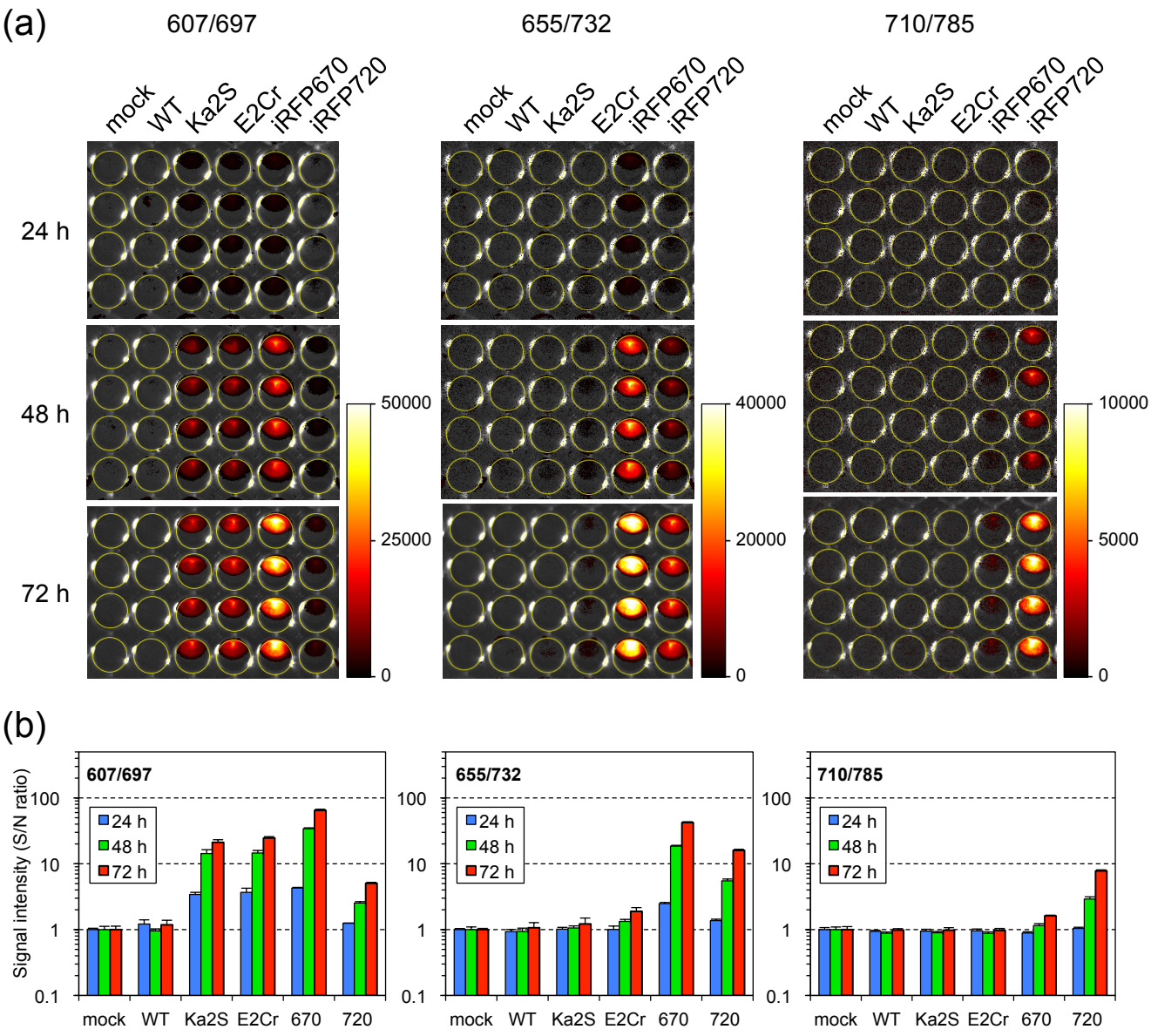

Fig. S4. Detection of fluorescence signals from NA cells infected with recombinant viruses using the Lumazone imaging system. (a) Each virus solution was mixed with NA cells (4×10^4 cells/well) at a m.o.i. of 3, and the mixture was seeded on Sumilon 96-well black plates (Sumitomo Bakelite). After 24, 48, or 72 h of incubation, cells were fixed with 4% paraformaldehyde and then imaged using 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). Because the source of the excitation light was obliquely placed, signals were detected only from the upper half of the well. Color bars indicate relative signal intensities. (b) S/N ratios (test well/mock well) were calculated for each filter set and time point and are presented as means and standard deviations (SD); n = 4.

Figure S5

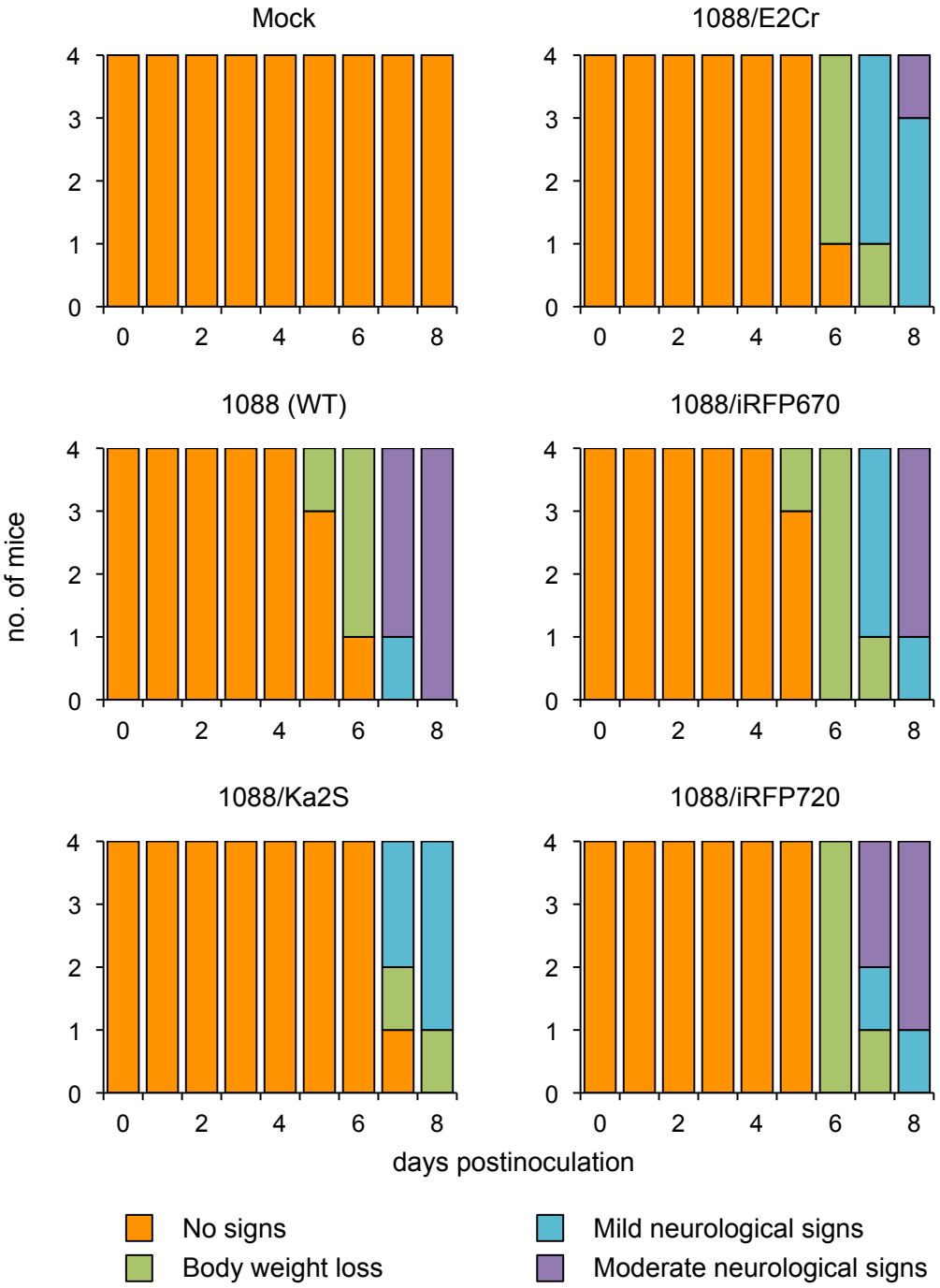

Fig. S5. Progression of disease in nude mice inoculated i.c. with 10^4 f.f.u. of each virus as illustrated in Figs. 4 and 5. As described in the legend for Fig. S3, "mild neurological signs" indicates that mice showed a foot slip without paralysis, and "moderate neurological signs" indicates that mice showed paralysis of the hind limb(s).

Figure S6

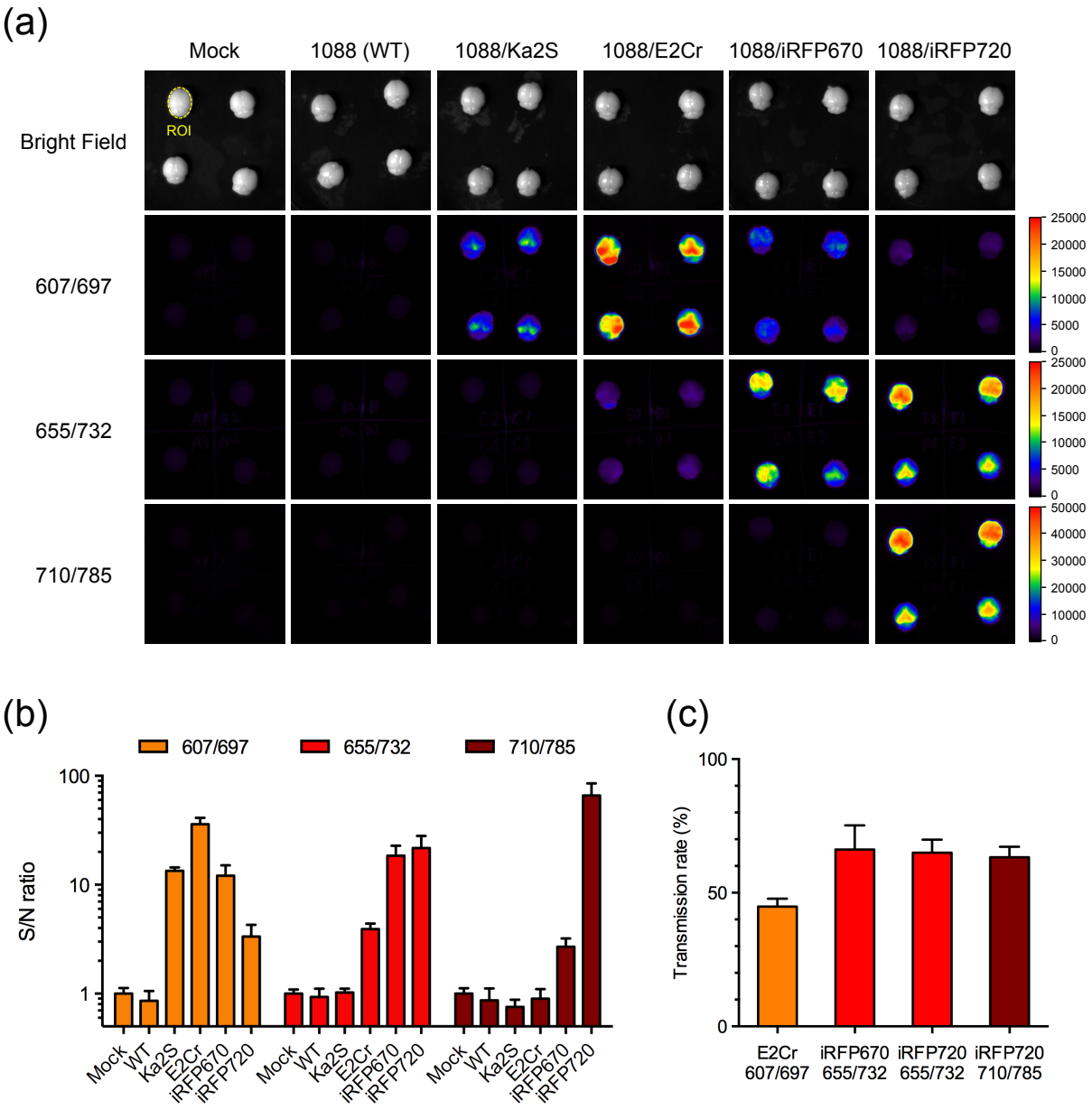

Fig. S6. *Ex vivo* fluorescence imaging of nude mice inoculated i.c. (a) After live imaging at 8 days postinoculation (as shown in Fig. 4), all mice were euthanized, and their brains were isolated. Before being subjected to titration, these brains were imaged using the Lumazone imaging system with the filter sets 607/697, 655/732, and 710/785. Color bars indicate relative signal intensities. (b) S/N ratios (test/mock at the region of interest, ROI) were calculated for each filter set and are presented as means and SD (n = 4). (c) The transmission rate [(signal intensity with subtraction of mock signal at *in vivo* imaging)/(signal intensity with subtraction of the mock signal at *ex vivo* imaging)] were calculated for selected combinations that showed a clear signal in live imaging (see Fig. 4), and are presented as means and SD (n = 4).