Supplementary Information

Visualizing the RNP content of single bunyavirus virions reveals more efficient genome packaging in the arthropod host

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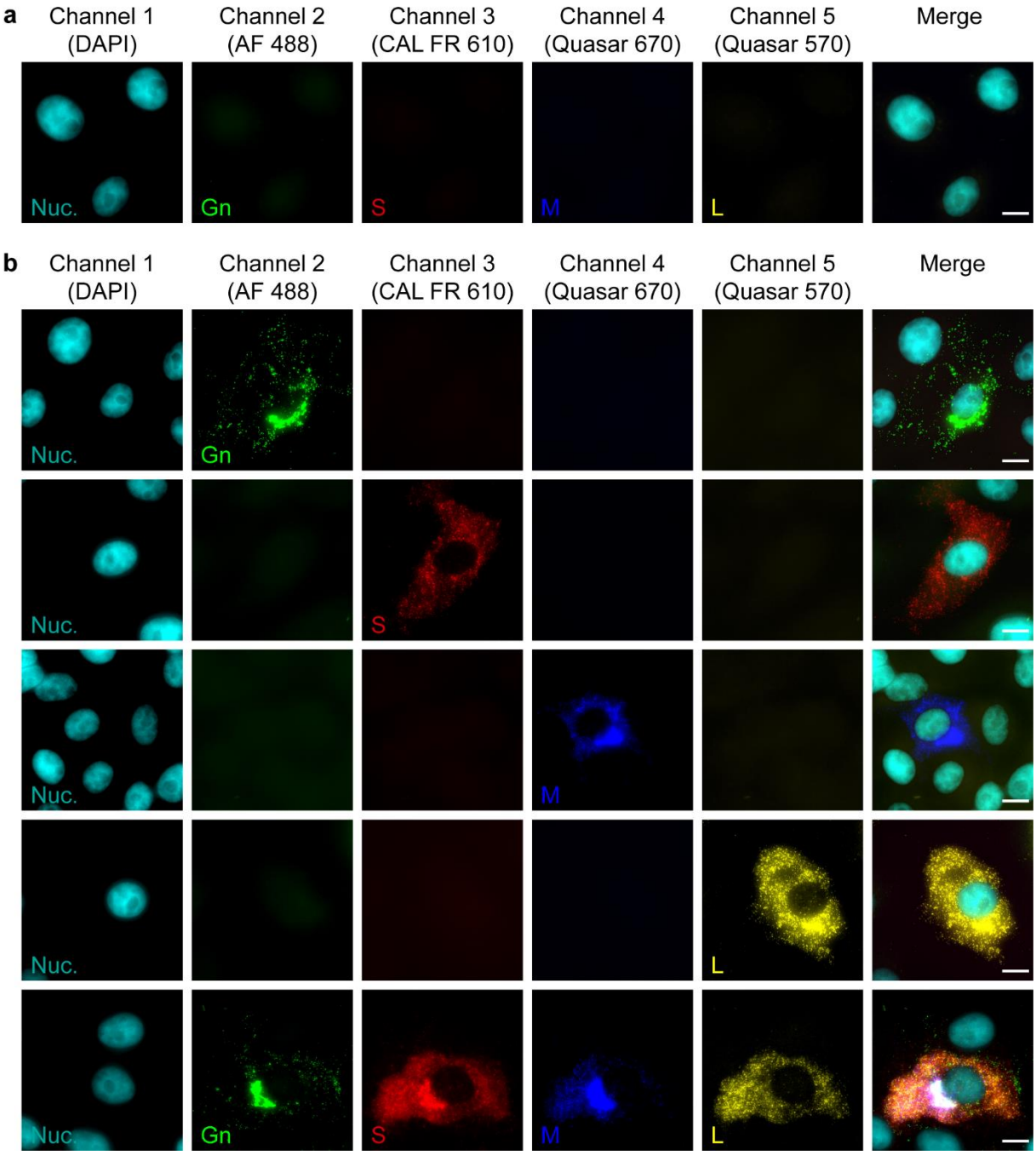
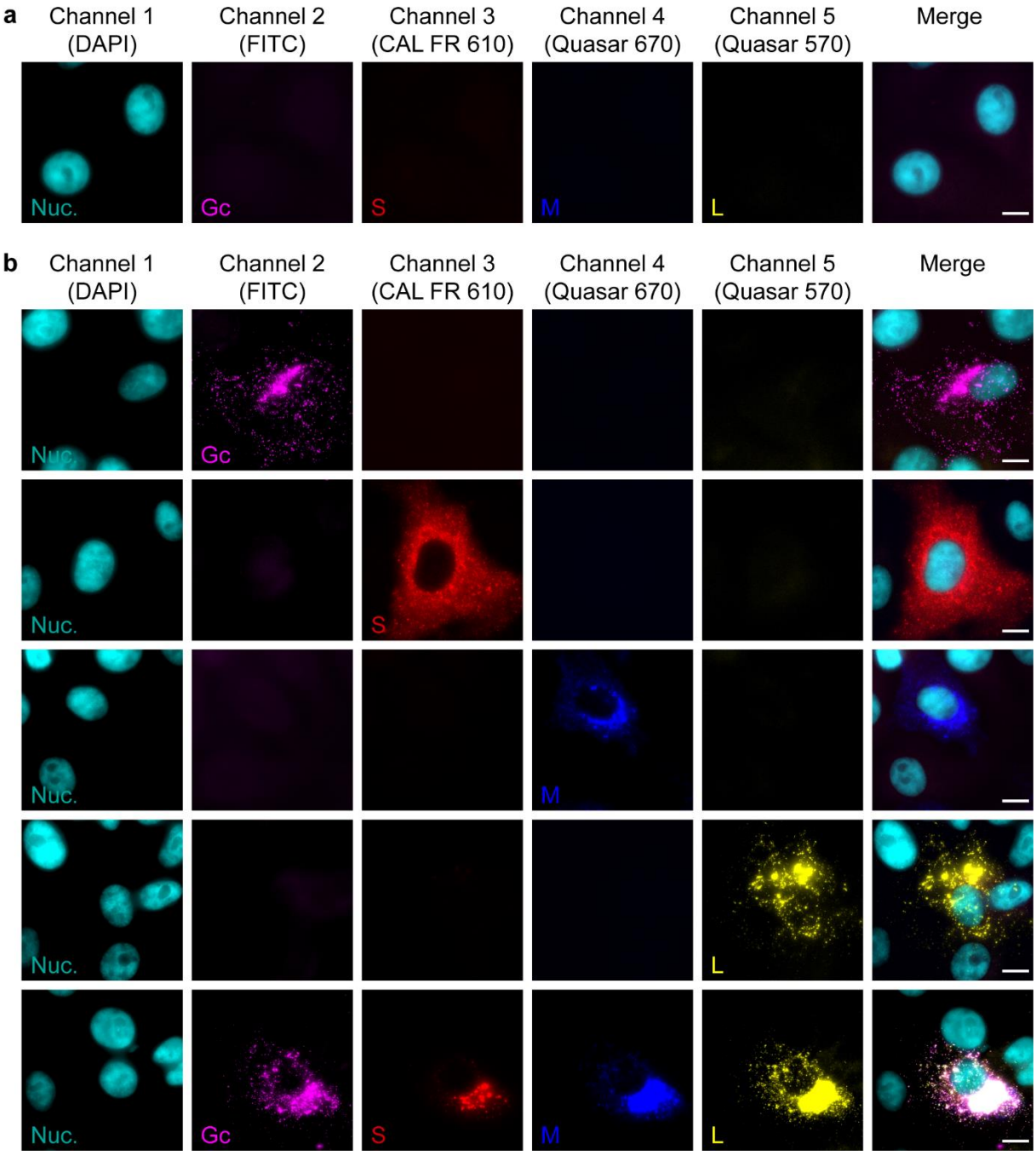
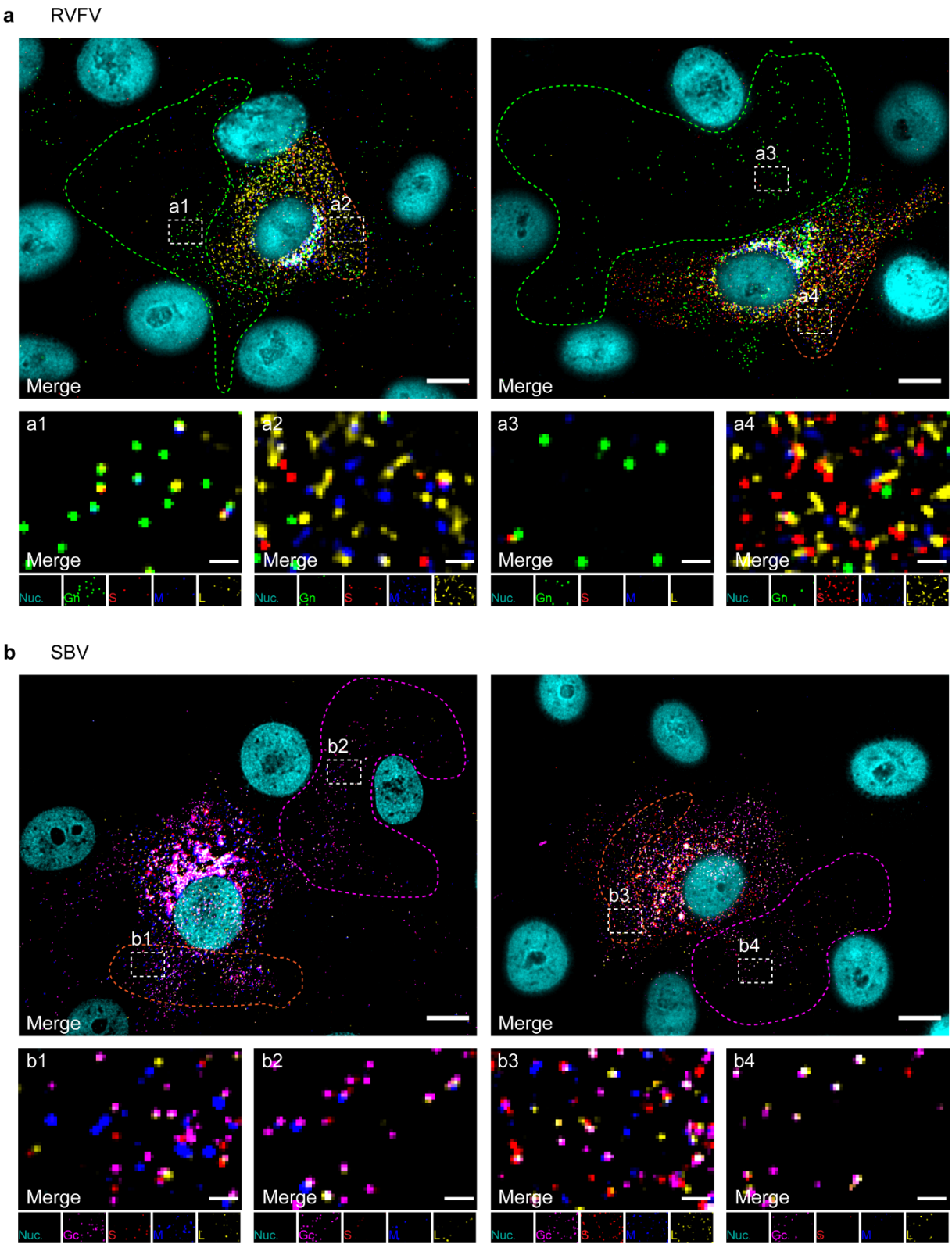


Supplementary Fig. 1 Specificity of RVFV FISH probe sets and antibodies. **a, b** Vero E6 cells were mock-infected (**a**) or infected with RVFV (MOI 0.75) (**b**) and cells were fixed at 8 h post-infection. The S segment (N gene; red), M segment (polyprotein gene; blue) and L segment (RdRp gene; yellow) were hybridized using probe sets labelled with CAL Fluor Red 610, Quasar 670 and Quasar 570, respectively. Progeny RVFV particles (green) were detected with antibody 4-D4[1] targeting the Gn glycoprotein in combination with Alexa Fluor 488-conjugated secondary antibodies. Cell nuclei (cyan) were visualized with DAPI. Mock-infected cells (**a**) were treated simultaneously with the three probe sets and antibodies. RVFV infected cells (**b**) were treated with either one probe set or antibodies at a time as single-color controls (first four rows) or simultaneously with the three probe sets and antibodies (last row). Scale bars, 10 µm.

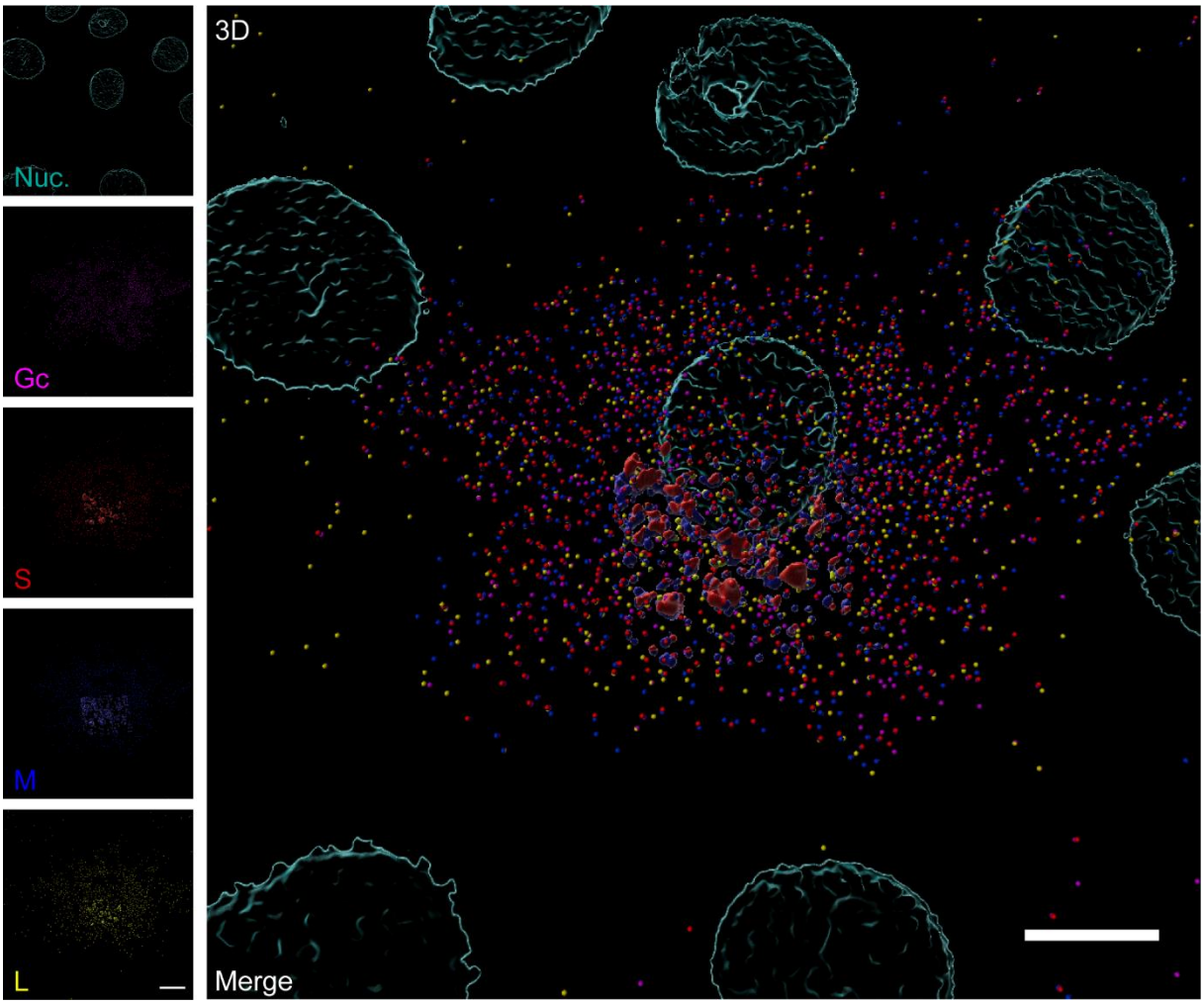


Supplementary Fig. 2 Specificity of SBV FISH probe sets and antibodies. **a, b** Vero E6 cells were mock-infected (**a**) or infected with SBV (MOI 0.33) (**b**) and cells were fixed at 8 h post-infection. The S segment (N gene; red), M segment (polyprotein gene; blue) and L segment (RdRp gene; yellow) were hybridized using probe sets labelled with CAL Fluor Red 610, Quasar 670 and Quasar 570, respectively. Progeny SBV particles (magenta) were detected with serum from an immunized rabbit[2] targeting the Gc glycoprotein in combination with FITC-conjugated secondary antibodies. Cell nuclei (cyan) were visualized with DAPI. Mock-infected cells (**a**) were treated simultaneously with the three probe sets and antibodies. SBV infected cells (**b**) were treated with either one probe set or antibodies at a time as single-color controls (first four rows) or simultaneously with the three probe sets and antibodies (last row). Scale bars, 10 µm.

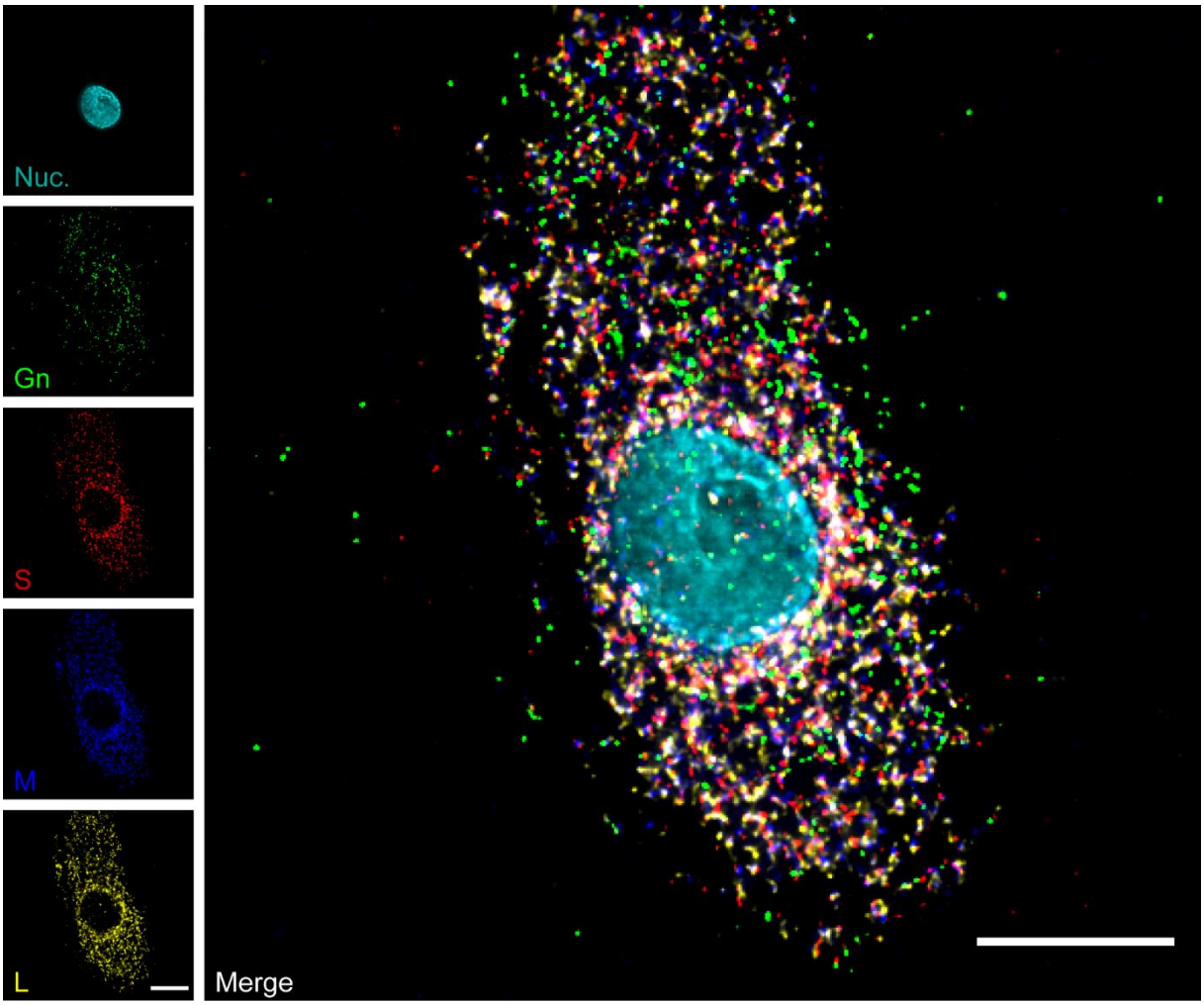


Supplementary Fig. 3 Representative regions of interest for the analysis of bunyavirus infected mammalian cells and their progeny virions using single-molecule vRNA FISH-immunofluorescence. **a, b** Vero E6 cells were infected with RVFV (MOI 0.50-0.75) (**a**) or SBV (MOI 0.33) (**b**) and cells were fixed at 8 h post-infection. The S segment (N gene; red), M segment (polyprotein gene; blue) and L segment (RdRp gene; yellow) were hybridized using probe sets labelled with CAL Fluor Red 610, Quasar 670 and Quasar 570, respectively. Progeny RVFV

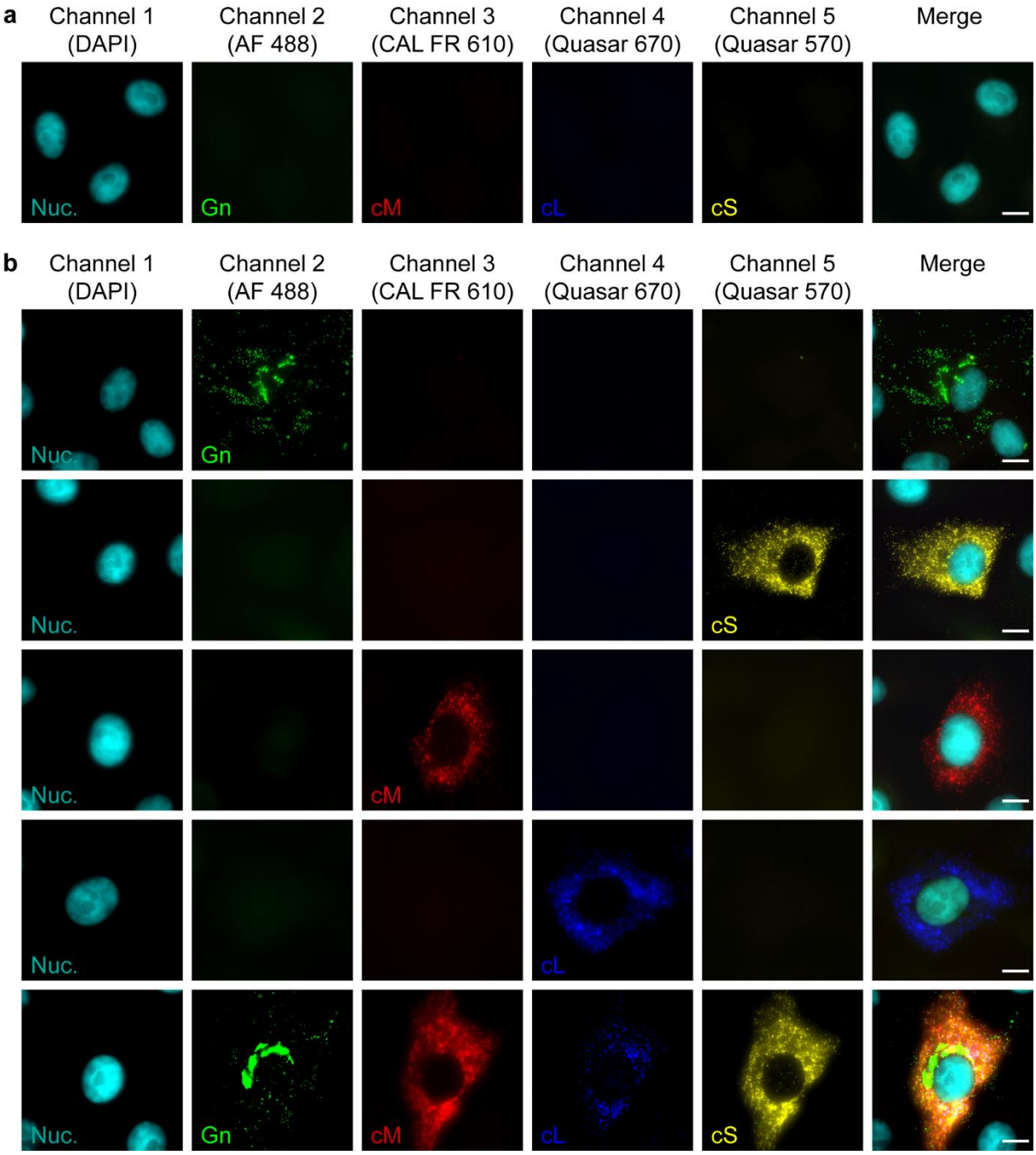
particles (green) were detected with antibody 4-D4[1] targeting the Gn glycoprotein in combination with Alexa Fluor 488-conjugated secondary antibodies. Progeny SBV particles (magenta) were detected with serum from an immunized rabbit[2] targeting the Gc glycoprotein in combination with FITC-conjugated secondary antibodies. Cell nuclei (cyan) were visualized with DAPI. The orange dashed contours depict representative regions of interest selected for quantification of cytoplasmic vRNPs. The green (**a**) and magenta (**b**) dashed contours depict representative regions of interest selected for determining the genome composition of RVFV and SBV extracellular virions, respectively, through co-localization analysis. Middle rows of **a** and **b** show magnifications of smaller regions of interest within the dashed contours (indicated in top rows as white dashed boxes and labelled as a1-4 and b1-4). Bottom rows of **a** and **b** show individual channels of the magnified regions of interest. Main images are merged maximum intensity projections of five channels. Due to a higher fluorescence intensity of the green and magenta channels compared to the other channels, spots co-localizing with the glycoprotein may sometimes appear masked and not entirely evident in merged images. Scale bars, 10 µm (top rows) and 1 µm (middle rows).



Supplementary Fig. 4 Single-molecule vRNA FISH-immunofluorescence of SBV infected mammalian cells. Vero E6 cells were infected with SBV (MOI 0.33) and cells were fixed at 8 h post-infection. The S segment (N gene; red), M segment (polyprotein gene; blue) and L segment (RdRp gene; yellow) were hybridized using probe sets labelled with CAL Fluor Red 610, Quasar 670 and Quasar 570, respectively. Progeny SBV particles were detected with serum from an immunized rabbit[2] targeting the Gc glycoprotein in combination with FITC-conjugated secondary antibodies. Cell nuclei (cyan) were visualized with DAPI. The three-dimensional representation showing the spatial distribution of vRNPs and virions was created with Imaris using the Surfaces and Spots modes. Accumulation of vRNPs in a perinuclear region shows active vRNP recruitment to the site of virion assembly. Co-localization of vRNPs and virions is depicted by merged spheres. Scale bars, 10 µm.



Supplementary Fig. 5 Single-molecule vRNA FISH-immunofluorescence of a RVFV infected insect cell. C6/36 cells were infected with RVFV (MOI 0.75) and cells were fixed at 32 h post-infection. The S segment (N gene; red), M segment (polyprotein gene; blue) and L segment (RdRp gene; yellow) were hybridized using probe sets labelled with CAL Fluor Red 610, Quasar 670 and Quasar 570, respectively. Progeny RVFV particles (green) were detected with antibody 4-D4[1] targeting the Gn glycoprotein in combination with Alexa Fluor 488-conjugated secondary antibodies. Cell nuclei (cyan) were visualized with DAPI. The main image merges maximum intensity projections of five channels (shown on the left). Due to a higher fluorescence intensity of the green channel compared to the other channels, spots co-localizing with the glycoprotein may sometimes appear masked and not entirely evident in merged images. Scale bars, 10 µm.



Supplementary Fig. 6 Specificity of RVFV complementary RNAs (cRNAs) FISH probe sets and antibodies. **a, b** Vero E6 cells were mock-infected (**a**) or infected with RVFV (MOI 0.75) (**b**) and cells were fixed at 8 h post-infection. The S segment cRNA (N gene; yellow), M segment cRNA (polyprotein gene; red) and L segment cRNA (RdRp gene; blue) were hybridized using probe sets labelled with Quasar 570, CAL Fluor Red 610, Quasar 670, respectively. Progeny RVFV particles (green) were detected with antibody 4-D4[1] targeting the Gn glycoprotein in combination with Alexa Fluor 488-conjugated secondary antibodies. Cell nuclei (cyan) were visualized with DAPI. Mock-infected cells (**a**) were treated simultaneously with the three probe sets and antibodies. RVFV infected cells (**b**) were treated with either one probe set or antibodies at a time as single-color controls (first four rows) or simultaneously with the three probe sets and antibodies (last row). Scale bars, 10 µm.

Supplementary Table 1 Oligonucleotide sequences of RNA FISH probe sets.

Provided as an individual .xlsx file due to its large size.

Supplementary Table 2 Primers for cDNA synthesis of viral genome segments.

Target	Name	Sequence
RVFV-Clone 13-S and RVFV-35/74-S	JR860-For	ACAAAGCTCCCTAGAGATACA
RVFV-Clone 13-M and RVFV-35/74-M	JR861-For	GACACAAAGACGGTGCATTA
RVFV-Clone 13-L and RVFV-35/74-L	JR890-For	GACACAAAGGCGCCCAATC
SBV-NL-F6-S	JR875-For	GTGAACTCCACTATTAACTACAGA
SBV-NL-F6-M	JR891-For	GAGTAGTGAACTACCACAATCAA
SBV-NL-F6-L	JR892-For	GTAGTGTACCCCTAATTACAATCAC

Supplementary Table 3 Primers for RT-qPCR amplifications of viral genome fragments.

Target	Name	Sequence
RVFV-Clone 13-S and RVFV-35/74-S	JR907-For	TCCAGTTTGCTGCTCAA
	JR908-Rev	CTGCTTTAAGAGTTCGATAACC
RVFV-Clone 13-M and RVFV-35/74-M	JR909-For	GCTGATGGCTTGAACAAC
	JR910-Rev	GTCTCTCACACCGAACTATC
RVFV-Clone 13-L and RVFV-35/74-L	JR911-For	TCGATAGATGTGGAAGATATGG
	JR912-Rev	CGTCATTCATCATGGGAAAC
SBV-NL-F6-S	JR878-For	CGGGTATGTGGCATTTATTG
	JR879-Rev	GACCATCTTGGCCTTCTT
SBV-NL-F6-M	JR882-For	CGACGTGGATTGAAGATAATG
	JR883-Rev	GAGGCTCTGTGAATTGTTAAG
SBV-NL-F6-L	JR886-For	CCCTGGATTGATGAGGATAC
	JR887-Rev	GACTCATGGAATGTCAGTTTAG

Supplementary Movie 1 Three-dimensional representation of individual virus particles released by a RVFV infected Vero E6 cell as shown in Fig. 2d. Progeny virions (green) were detected with antibody 4-D4[1] targeting the Gn glycoprotein in combination with Alexa Fluor 488-conjugated secondary antibodies. Cell nuclei (cyan) were visualized with DAPI. Gn accumulates in a perinuclear region, the site of virion assembly. The spatial distribution of virions and cell nuclei was created with Imaris using the Surfaces and Spots modes. Dynamic scale bar.

Supplementary Movie 2 Three-dimensional representation of individual virus particles released by a SBV infected Vero E6 cell as shown in Fig. 2e. Progeny virions (magenta) were detected with serum from an immunized rabbit[2] targeting the Gc glycoprotein in combination with FITC-conjugated secondary antibodies. Cell nuclei (cyan) were visualized with DAPI. The spatial distribution of virions and cell nuclei was created with Imaris using the Surfaces and Spots modes. Dynamic scale bar.

Supplementary Movie 3 Three-dimensional representation of vRNPs and individual virus particles in a RVFV infected Vero E6 cell detected by single-molecule vRNA FISH-immunofluorescence as shown in Fig. 3c. S segment (N gene; red), M segment (polyprotein gene; blue), L segment (RdRp gene; yellow), progeny RVFV particles (green) and cell nuclei (cyan). Accumulation of vRNPs and co-localization to the same perinuclear region as Gn show active vRNP recruitment to the site of virion assembly. Co-localization of vRNPs and virions is depicted by merged spheres. The spatial distribution of virions and cell nuclei was created with Imaris using the Surfaces and Spots modes. Dynamic scale bar.

Supplementary Movie 4 Three-dimensional representation of vRNPs and individual virus particles in a SBV infected Vero E6 cell detected by single-molecule vRNA FISH-immunofluorescence as shown in Supplementary Fig. 4. S segment (N gene; red), M segment (polyprotein gene; blue), L segment (RdRp gene; yellow), progeny SBV particles (magenta) and cell nuclei (cyan). Accumulation of vRNPs in a perinuclear region shows active vRNP recruitment to the site of virion assembly. Co-localization of vRNPs and virions is depicted by merged spheres. The spatial distribution of virions and cell nuclei was created with Imaris using the Surfaces and Spots modes. Dynamic scale bar.

Supplementary References

1. Keegan, K. & Collett, M. S. Use of bacterial expression cloning to define the amino acid sequences of antigenic determinants on the G2 glycoprotein of Rift Valley fever virus. *Journal of Virology* **58**, 263–270 (1986).
2. Oymans, J. *et al.* Reverse Genetics System for Shuni Virus, an Emerging Orthobunyavirus with Zoonotic Potential. *Viruses* **12**, 455 (2020).