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Supplemental Information

Visualizing Oncolytic Virus-Host Interactions in Live Mice Using Intravital Microscopy

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Supplementary Material

Figure S1. *In vitro* and *in vivo* optimization of VSV labeling with AF555. VSV was labeled with variable dilutions of AF555 using a fixed incubation time (20 min) and analyzed using flow cytometry. Representative analysis of labeled virus gating on FSC-A/SSC-A **(a),** followed by gating on FSC-H/FSC-W **(b)** and SSC-H/SSC-W **(c)** to exclude doublets followed by measurement of percentage of events positive in AF555 channel **(d)** and fluorescence intensity **(f).** A reference sample of PBS as a control **(e)** demonstrates lack of false events using this flow virometry protocol. Values for each labeling condition are reported as percentage of labeled events **(g)** and the Mean fluorescence intensity of labeled particles **(h)** ±SD (n=3). VSV labeled with 30 μg/ml AF555 (red) was injected i.v. while imaging skin blood vessels (gray, delineated by white dashed lines) using resonant scanning confocal **(i)** or multiphoton **(j)** microscopy (see also **Movie S2**). Blue, CD11b; green, Ly6g; cyan, collagen; scale bar=50 μm, representative images of two independent experiments. VSV-AF555 injection (red) was followed by injection of VSV-AF647 (cyan) **(k)**. Intravital imaging of skin blood vessels (gray, delineated by white dashed lines). Arrow indicates a CD11b+/Ly6g- cell co-localized with both AF555 and AF647 labeled virus. Blue, CD11b; green, Ly6g; scale bar=50 μm, representative images of two independent experiments. VSV was labeled with AF555 (30 ug/ml; 20 min) and particle size analyzed by dynamic light scattering. Representative intensity distribution plot (**l**) and particle diameters (**m**) are shown (representative plots of three independent experiments).

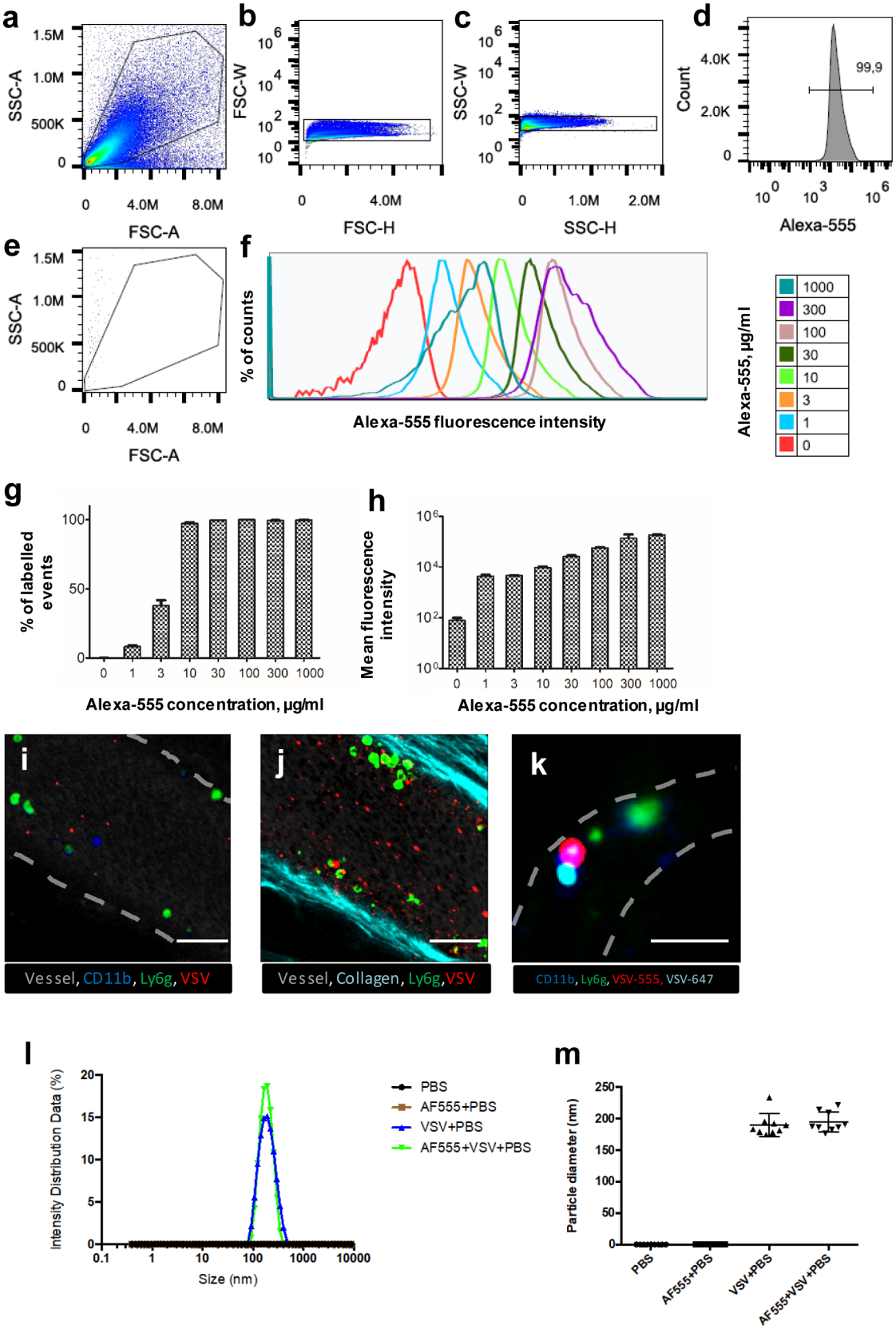

a
SSC-A
FSC-A
b
FSC-W
FSC-H
c
SSC-W
SSC-H
d
Count
99,9
Alexa-555
e
f
% of counts
Alexa-555 fluorescence intensity
Alexa-555, µg/ml
1000
300
100
30
10
3
1
0
g
% of labelled events
Alexa-555 concentration, µg/ml
h
Mean fluorescence intensity
Alexa-555 concentration, µg/ml
i
Vessel, CD11b, Ly6g, VSV
j
Vessel, Collagen, Ly6g, VSV
k
CD11b, Ly6g, VSV-555, VSV-647
l
Intensity Distribution Data (%)
Size (nm)
PBS
AF555+PBS
VSV+PBS
AF555+VSV+PBS
m
Particle diameter (nm)
PBS
AF555+PBS
VSV+PBS
AF555+VSV+PBS

Figure S2. *In vitro* optimization of Maraba labeling with AF647. Maraba was labeled with serial dilutions of AF647 using a fixed incubation time of 20 min **(a-h)** or with 30 μg/ml AF647 for variable incubation times **(i-k)**. Labeled virions were then analyzed by flow cytometry gating on FSC-A/SSC-A **(a),** and gating on FSC-H/FSC-W **(b)** and SSC-H/SSC-W **(c)** to exclude doublets followed by measurement of percentage of events positive in AF647 channel **(d)** and fluorescence intensity **(e)**. Values are reported as percentage of labeled events **(f, i)** and Mean fluorescence intensity of labeled particles **(g, j)** ±SD (n=3). The ability of labeled viruses to infect and replicate was measured *in vitro* and are reported as $TCID_{50}$ **(h, k).** Values represent mean±SD (n=3); * $p<0.05$, ns=non-significant in comparison to unlabeled control.

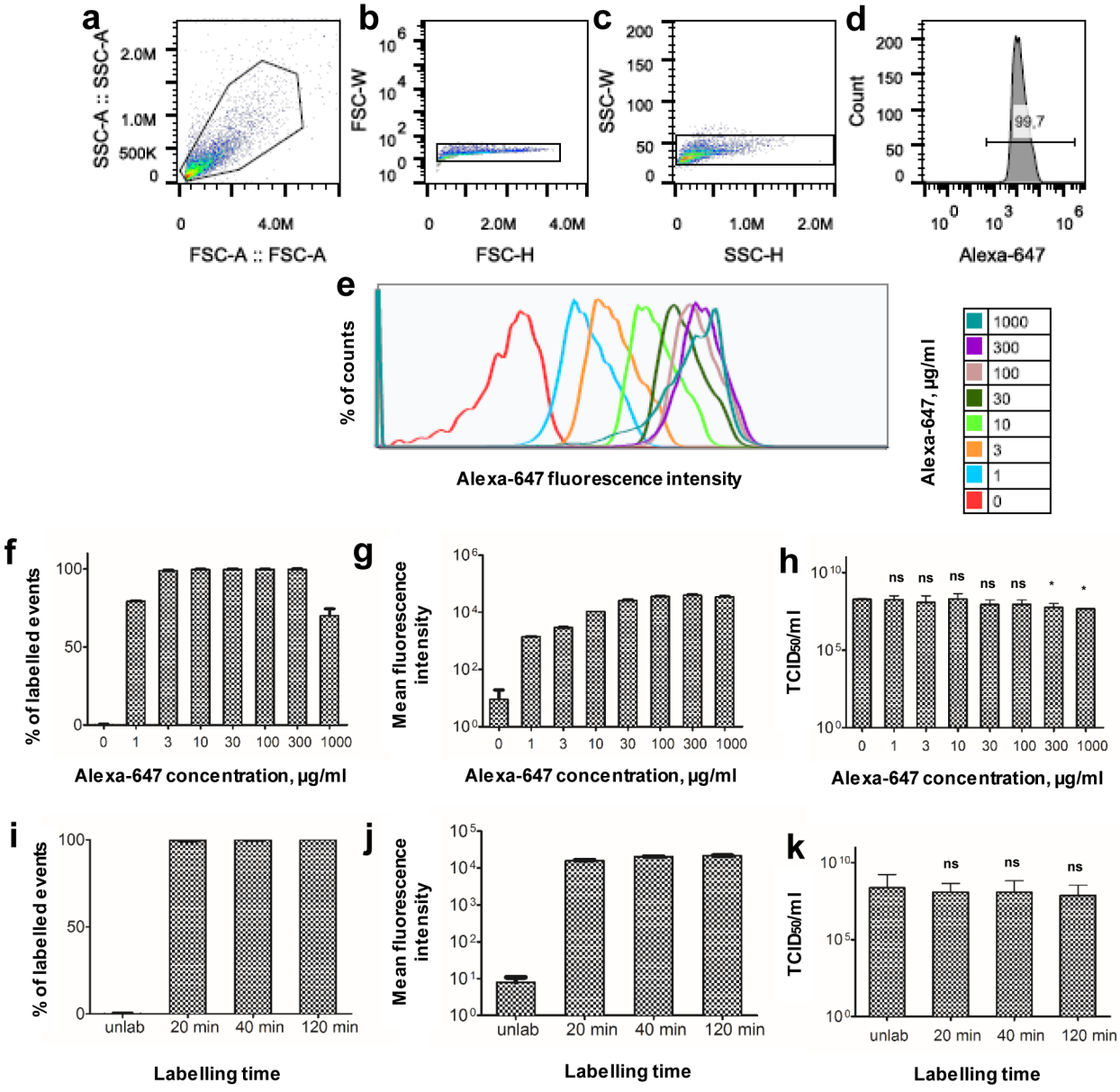

a
SSC-A :: SSC-A
2.0M
1.0M
500K
0
0
4.0M
FSC-A :: FSC-A
b
FSC-W
10^6
10^4
10^2
10^0
0
2.0M
4.0M
FSC-H
c
SSC-W
200
150
100
50
0
0
1.0M
2.0M
SSC-H
d
Count
200
150
100
50
0
99.7
10^0
10^3
10^6
Alexa-647
e
% of counts
Alexa-647 fluorescence intensity
Alexa-647, µg/ml
1000
300
100
30
10
3
1
0
f
% of labelled events
100
50
0
0 1 3 10 30 100 300 1000
Alexa-647 concentration, µg/ml
g
Mean fluorescence intensity
10^6
10^4
10^2
10^0
0 1 3 10 30 100 300 1000
Alexa-647 concentration, µg/ml
h
TCID50/ml
10^{10}
10^5
10^0
ns ns ns ns ns * *
0 1 3 10 30 100 300 1000
Alexa-647 concentration, µg/ml
i
% of labelled events
100
50
0
unlab
20 min
40 min
120 min
Labelling time
j
Mean fluorescence intensity
10^5
10^4
10^3
10^2
10^1
10^0
unlab
20 min
40 min
120 min
Labelling time
k
TCID50/ml
10^{10}
10^5
10^0
ns ns ns
unlab
20 min
40 min
120 min
Labelling time

Figure S3. *In vitro* optimization of reovirus labeling with AF647. Reovirus was labeled with serial dilutions of AF647 using a fixed incubation time of 20 min **(a-h)** or with 3 μg/ml AF647 for variable incubation times **(i-k)**. Labeled virions were then analyzed by flow cytometry gating on FSC-A/SSC-A **(a),** and gating on FSC-H/FSC-W **(b)** and SSC-H/SSC-W **(c)** to exclude doublets, followed by measurement of percentage of events positive in AF647 channel **(d)** and fluorescence intensity **(e)**. Values are reported as percentage of labeled events **(f, i)** and Mean fluorescence intensity of labeled particles **(g, j)** ±SD (n=3). The ability of labeled viruses to infect and replicate was measured *in vitro* and are reported as the number of PFU recovered in culture supernatant **(h, k).** Values represent mean±SD (n=3); * $p<0.05$, ns=non-significant in comparison to unlabeled control.

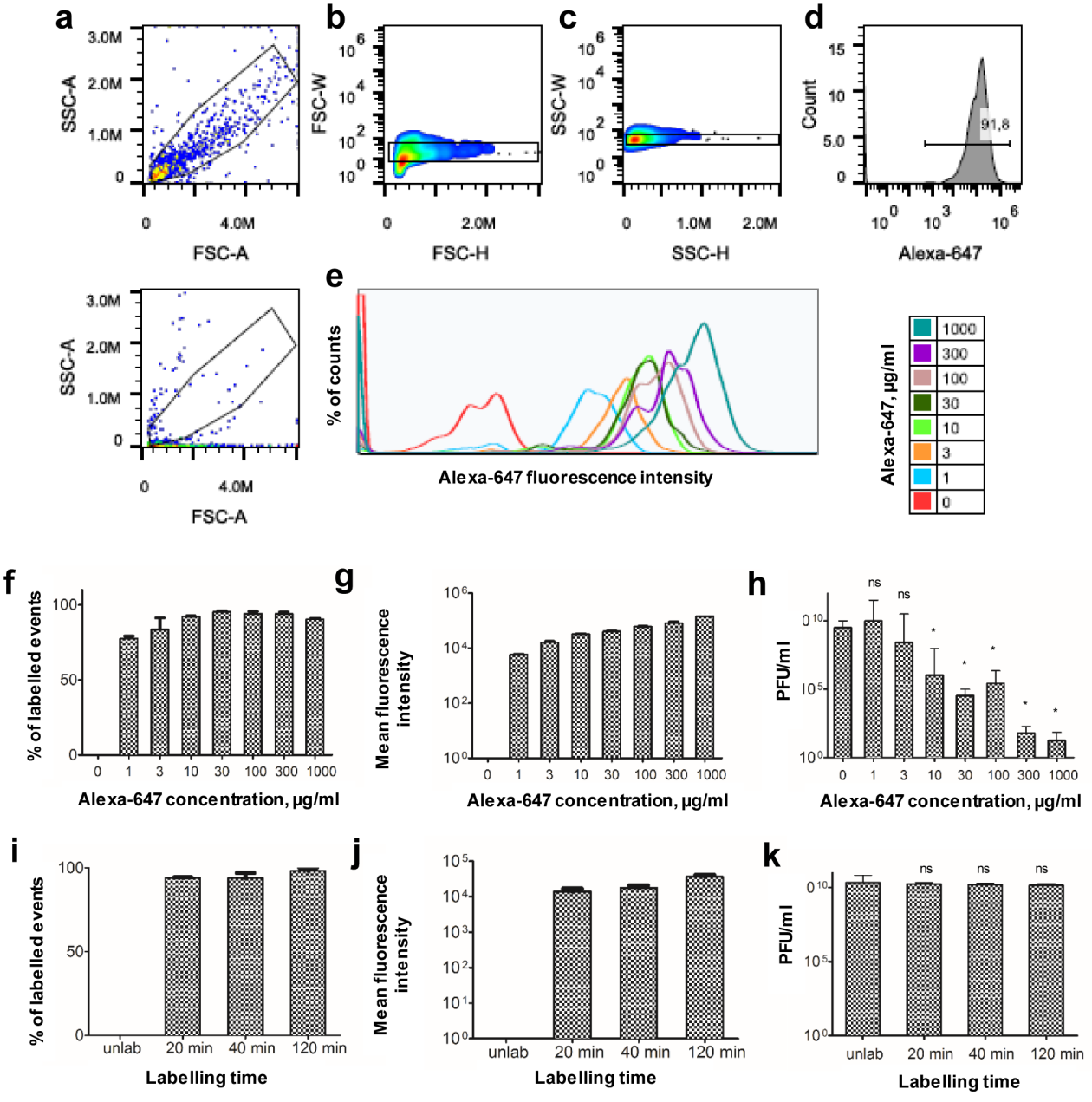

a
SSC-A
FSC-A
b
FSC-W
FSC-H
c
SSC-W
SSC-H
d
Count
91,8
Alexa-647
e
% of counts
Alexa-647 fluorescence intensity
Alexa-647, µg/ml
1000
300
100
30
10
3
1
0
f
% of labelled events
Alexa-647 concentration, µg/ml
g
Mean fluorescence intensity
Alexa-647 concentration, µg/ml
h
PFU/ml
ns
Alexa-647 concentration, µg/ml
i
% of labelled events
unlab
20 min
40 min
120 min
Labelling time
j
Mean fluorescence intensity
Labelling time
k
PFU/ml
ns
Labelling time

Figure S4. IVM of Maraba-AF647 and reovirus-AF647. IVM of skin vasculature following i.v. injection of 5×10^8 PFU Maraba labelled with 30 μg/ml AF647 **(a)** or 5×10^8 PFU reovirus labeled with 3 μg/ml AF647 **(b)**. Vessels are delineated by white dashed lines, arrows indicate virus particles (blue); red, CD11b; green, Ly6g; scale bar=25 μm, representative images of three independent experiments.

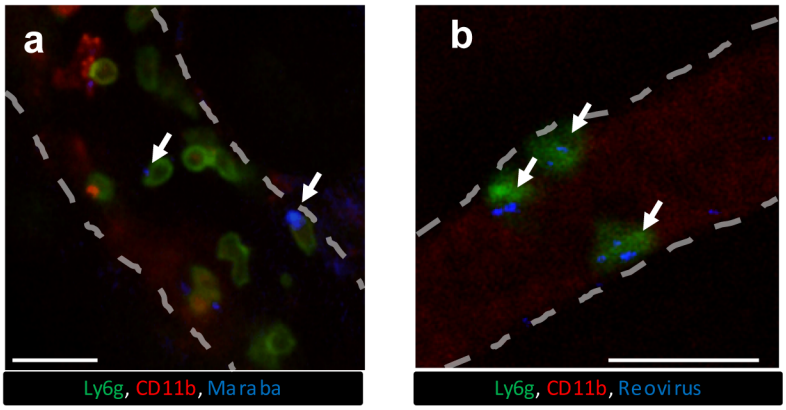
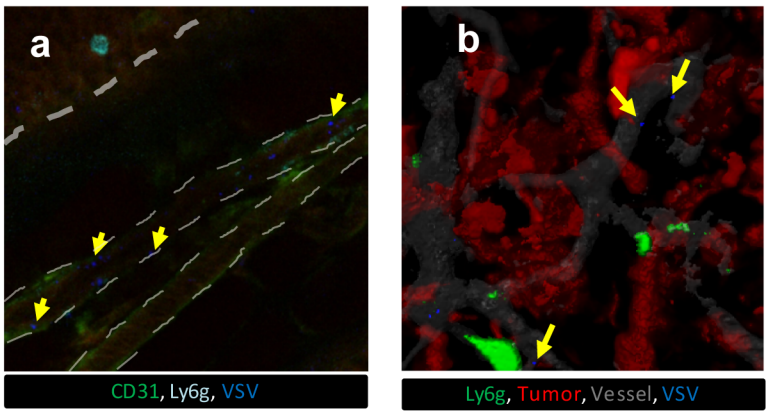

a
Ly6g, CD11b, Maraba
b
Ly6g, CD11b, Reovirus

Figure S5. IVM of VSV binding to vascular endothelial cells in CT-26 tumours. 5×10^8 PFU VSV labeled with 30 μg/ml AF647 were injected i.v. while visualizing CT-26 tumour vessels by confocal IVM. Virus particles (blue, indicate by arrows) were found binding to endothelial cells (green) in tumor veins **(a)**. Green, CD31; cyan, Ly6g; blue, VSV; vessels are delineated by white dashed lines. Scale bar=25 μm, representative images of three independent experiments. Virus particles (blue, indicate by arrows) were found binding to endothelial cells in tumor capillaries (gray) **(b)**. Green, Ly6g; red, CT-26 tumour cells; blue, VSV. Scale bar=25 μm, representative images of three independent experiments.


a
CD31, Ly6g, VSV
b
Ly6g, Tumor, Vessel, VSV

Movie S1. Visualization of VSV labeled with 10 µg/ml AF647 (white) on the surface of Vero cells immediately after inoculation with MOI=50. Arrows indicate co-localization of multiple virus particles on the cell surface. Images are captured at 1 frame/s for a 420s interval **(associated with Fig. 2m)**.

Movie S2. IVM of VSV labeled with 30 µg/ml AF555 (red) in a skin blood vessel (gray) using resonant scanning confocal microscopy followed by imaging the same vessel with multiphoton microscopy. Blue, CD11b; green, Ly6g; cyan, collagen **(associated with Fig. S1i-j)**.

Movie S3. IVM of VSV-AF647 (white) binding to intravascular leukocytes (shadows) following i.v. injection. Arrows indicate examples of leukocytes bound by many virus particles, forming a "halo" around the cell surface. Images are captured at 1 frame/6 s for a 420 s interval **(associated with Fig. 4a-b)**.

Movie S4. IVM of monocytes (CD11b+, red) "capturing" VSV-AF647 (blue) bound in CT-26 tumour vessels following i.v. injection. Arrow indicates multiple virus particles co-localizing on the cell surface. Images are captured at 1 frame/6s for a 420 s interval. Green, Ly6g (**associated with Fig. 4**).

Movie S5. Tracking of individual VSV-AF647 particles (blue) on the surface of leukocytes (red, CD11b; green, Ly6g) followed by tracking virions within the blood vessel (gray) using IVM 5 minutes after i.v. injection of virus **(associated with Fig. 4h).**

Movie S6. IVM of VSV-AF647-bound leukocytes, demonstrating stationary, probing and crawling behavior within CT-26 tumour vessels. Prominent examples are indicated by arrows.

Red, CD11b; Blue, VSV-AF647; Green, Ly6g. Each of 3 videos were captured with 1 frame/6s acquisition rate for a 300 s interval. (**associated with Fig. 4i**).

Movie S7. IVM of VSV-AF647 (blue) transfer from monocyte (red) to neutrophil (green) within a blood vessel (gray) 30 minutes after virus injection **(associated with Fig. 4j)**.

Movie S8. Multiphoton IVM z-stack (171 images, 1 μm step) of VSV^{GFP} (green) infection of CD169+ macrophages (red) surrounding a splenic follicle 8 hours following i.v. injection. Cyan, Ly6g. **(associated with Fig. 5)**.

Movie S9. 3D reconstruction of multiphoton IVM z-stack. VSV^{GFP} (green) infection of CD169+ macrophages (red) surrounding a splenic follicle 8 hours following i.v. injection. Cyan, Ly6g/second harmonic. **(associated with Fig. 5)**.

Movie S10. 4D intravital imaging of B220+ cells (blue) infected by VSV^{GFP} (green) in spleen, 8 hpi. Z-stack (22 images, 1 μm step) is captured by at a rate of 1 frame/6s for a 360s interval. Red, Gr-1; cyan, F4/80 **(associated with Fig. 5)**.

Movie S11. Confocal IVM of VSV^{GFP} (green) infection of splenic DC. Red, F4/80; Blue, B220. **(associated with Fig. 5).**

Movie S12. IVM of VSV-AF647 (blue) capture in liver (red, F4/80; green, CD11b) and lung (red, CD45; green, Ly6g; white, CD49b) after i.v. injection of virus **(associated with Fig. 5l-o)**. Arrow indicates multiple viruses forming aggregate on cell surface.

Abbreviations

ACK, Ammonium-Chloride-Potassium

AF, Alexa Fluor

ANOVA, analysis of variance

APC, antigen presenting cell

CRIg, complement receptor of the immunoglobulin family

DC, dendritic cell

DIC, differential interference contrast

DMEM, Dulbecco's Minimum Essential Medium

EDAC, 1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide

FCS, fetal calf serum

FITC, fluorescein isothiocyanate

Fluc, firefly luciferase

FP, fluorescent protein

FSC, forward scatter

FSL, Function-Spacer-Lipid

GFP, green fluorescent protein

Hpi, hours post infection

i.v., intravenous

IVM, intravital microscopy

LDLR, low density lipoprotein receptor

LN, lymph node

MFI, mean fluorescent intensity

OV, oncolytic virus

OVT, oncolytic virus therapy

PBS, phosphate-buffered saline

PE, phycoerythrinPD

PFU, plaque forming units

QDots, quantum dots

RFP, red fluorescent protein

SSC, side scatter

SD, standard deviation

VSV, vesicular stomatitis virus