Supplemental Materials

Molecular Biology of the Cell

Bastounis et al.

SUPPORTING INFORMATION CAPTIONS

Supplemental Figure S1. Lm infection of VECs residing on hydrogels of varying stiffness. (A) Young's moduli of hydrogels. Plot showing the expected Young's modulus of the hydrogels given the amount of acrylamide and bis-acrylamide used versus the Young's modulus measured through AFM (N=6). Stiffness of the 0.6 kPa could not be measured, as the hydrogels were very soft and adhered to the AFM tip. (B) Fraction of cells passed through the flow cell in 20 s for HMEC-1 residing on varying stiffness substrates. HMEC-1 were seeded on 0.6 kPa, 3 kPa, 20 kPa and 70 kPa PA hydrogels as well as TC polystyrene substrates for 24 h at a starting concentration of $4x10^5$ cells per well for 24 h. Cells were then detached from their matrix and number of cells per well passed through the flow cell in 20 s was measured using a flow cytometry for cells coming from N=5-6 wells for each substrate stiffness. Results from 3 independent experiments were combined and for each experiment values were normalized relative to the mean number of cells passed through the flow cell for cells residing on TC polystyrene substrates. No significant differences were detected. (C) Boxplots of normalized fraction of HMEC-1 infected with Δ*actA* Lm for cells residing on PA hydrogels of varying stiffness or TC polystyrene. Stiffness (kPa) of each substrate is indicated. Boxplots refer to data from 3 independent experiments, with N = 5-6 replicates per experiment. For each experiment values have been normalized relative to the mean infection level of cells residing on polystyrene substrates. (D-F) HMEC-1 residing on collagen I-coated glass substrates were infected with Lm (constitutively expressing GFP) or Δ*atcA* Lm at an MOI of 2.7. 30 min post-infection samples were fixed, immunostained and infection was analyzed by microscopy followed by image processing. Boxplots showing: (D) total bacteria per cell; (E) internalized bacteria per cell; (F) invasion efficiency (ratio of internalized bacteria to total bacteria). For each condition, 500-600 cells were analyzed in total. Representative data come from 1 of 2 independent experiments. Circles represent outliers, and the boxplots' notched sections show the 95% confidence interval around the median (Wilcoxon–Mann–Whitney test, for details about boxplots see Materials and

Methods). One or two asterisks denote statistically significant differences between the medians of two distributions (<0.05 or <0.01, respectively; Wilcoxon rank sum test).

Supplemental Figure S2. FAK-dependent decrease in bacterial uptake. (A) HMEC-1 treated with 20 nM of non-targeting siRNA (siNT, first row) or FAK siRNA (siFAK, second row) were stained for FAK using an anti-FAK antibody. Representative phase image of cells (left column), superimposed with the image of the nuclei (blue) and anti-FAK antibody fluorescence (purple, right column) are shown. Scale bar shown in white is 23 μm. (B) Relative with respect to GAPDH gene expression of FAK obtained by RT-qPCR. The levels relative levels of expression in each siRNA treated sample (#1-#3) are expressed relative to the control siNT treated sample #2 (normalized relative quantity, NRQ). N=3 replicates are shown for each group treated with either siNT or siVIM. (C-D) Histograms of the logarithm of Lm fluorescence intensity per cell for HMEC-1 treated with 20 nM of non-targeting siRNA (siNT) (C) or FAK siRNA (siFAK) (D). HMEC-1 were infected with *ΔactA* Lm (actAp::mTagRFP) and infection was analyzed by flow cytometry, 7–8 h after infection. MOI is 45. Histograms for N=6 replicates are shown in different colors. Control uninfected cells' histogram is shown in purple. Based on the autofluorescence of the control group a gate is defined (see black and red lines) showing what is considered uninfected (left, black line) and infected (right, red line). (E) Increase in bacterial uptake by Angiotensin II. Angiotensin II or vehicle control was added 2 h before addition of bacteria to HMEC-1 residing on polystyrene substrates. Percentage of HMEC-1 infected with Δ*actA* Lm (actAp::mTagRFP) as a function of inhibitor concentration (mean +/- SD, N = 4 replicates). Infection was analyzed by flow cytometry, 7–8 h after infection. MOI is 70. (F) Western blots from whole HMEC-1 lysates showing expression of phosphorylated FAK (Tyr397) and total FAK for cells residing on TC polystyrene substrates and treated for varying amount of time with 100 nM Angiotensin-II. In each Western blot, equal quantities of protein were loaded and equal loading was confirmed in relation to glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

expression. The Western blots shown are representative of 3 independent experiments. (G) Boxplots of percentage of HMEC-1 infected with Δ*actA* Lm (actAp::mTagRFP) for cells residing on soft (3 kPa) or stiff (70 kPa) hydrogels and pre-treated for 2 h either with vehicle control or 100 nM Angiotensin-II (N=6 replicates). MOI is 62.5. Two asterisks denote statistically significant differences between the medians of two distributions (<0.01; Wilcoxon rank sum test).

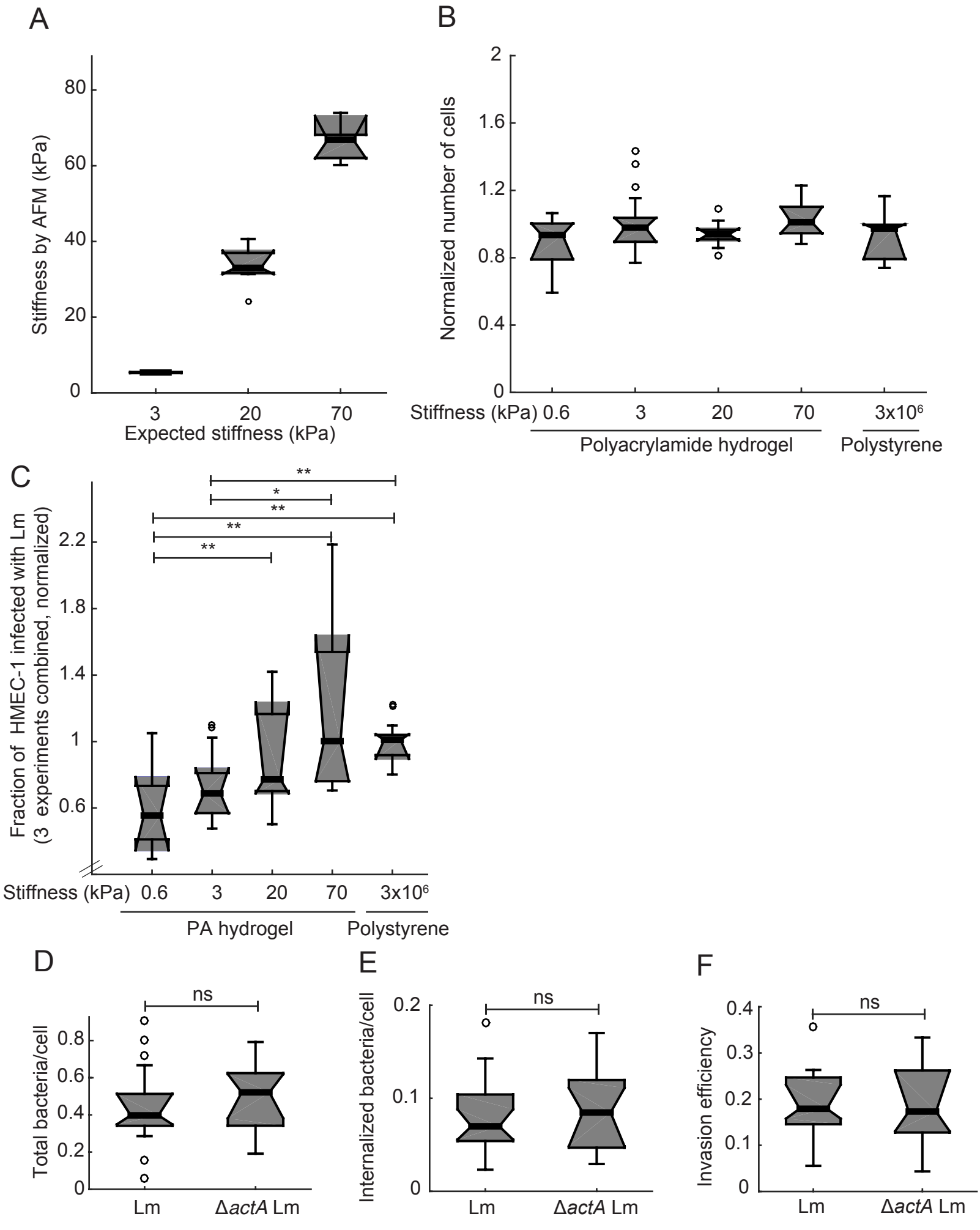
Supplemental Figure S3. Met-dependent decrease in bacterial uptake. (A) Western blot from whole HMEC-1 lysates showing expression of Met for cells seeded on soft (3 kPa), stiff (70 kPa) and TC polystyrene substrates treated or not with 2 μM PF537228 FAK inhibitor. In each case, equal quantities of protein were loaded and equal loading was also confirmed in relation to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. (B-C) Histograms of the logarithm of Lm fluorescence intensity per cell for HMEC-1 treated with 20 nM of non-targeting siRNA (siNT) (C) or Met siRNA (siMet) (D). HMEC-1 were infected with *ΔactA* Lm (actAp::mTagRFP) and infection was analyzed by flow cytometry, 7–8 h after infection. MOI is 45. Histograms for N=6 replicates are shown in different colors. Control uninfected cells' histogram is shown in purple. Based on the autofluorescence of the control group a gate is defined (see black and red lines) showing what is considered uninfected (left, black line) and infected (right, red line). (D) Relative with respect to GAPDH gene expression of Met obtained by RT-qPCR. The levels relative levels of expression in each siRNA treated sample (#1-#3) are expressed relative to the control siNT treated sample #2 (normalized relative quantity, NRQ). N=3 replicates are shown for each group treated with either siNT or siVIM. (E) Boxplots of percentage of HMEC-1 infected with Δ*actA*/Δ*inlB* Lm (actAp::mTagRFP) for cells HMEC-1 treated with siNT or siMet (N=4 replicates). MOI is 60.

Supplemental Figure S4. Knocking down vimentin decreases Lm uptake. (A) HMEC-1 treated with 20 nM of non-targeting siRNA (siNT, first row) or VIM siRNA (siVIM, second row) were stained for VIM using the H-84 anti-vimentin antibody. Representative phase image of cells (Phase, left column), superimposed with the image of the nuclei (blue) and anti-vimentin antibody fluorescence (green, right column) are shown. Scale bar shown in white is 35 μm. (B) Relative with respect to GAPDH gene expression of vimentin obtained by RT-qPCR. The levels relative levels of expression in each siRNA treated sample (#1-#3) are expressed relative to the control siNT treated sample #2 (normalized relative quantity, NRQ). N=3 replicates are shown for each group treated with either siNT or siVIM (C-D) Histograms of the logarithm of Lm fluorescence intensity per cell for HMEC-1 treated with 20 nM of non-targeting siRNA (siNT) (C) or vimentin siRNA (siVIM) (D). HMEC-1 were infected with *ΔactA* Lm (actAp::mTagRFP) and infection was analyzed by flow cytometry, 7–8 h after infection. MOI is 34. Histograms for N=6 replicates are shown in different colors. Control uninfected cells' histogram is shown in purple. Based on the autofluorescence of the control group a gate is defined (see black and red lines) showing what is considered uninfected (left, black line) and infected (right, red line). (E) Number of HMEC-1 passed through the flow cell in 20 s, for N = 4 samples coming from wells treated with either siNT or siVIM. (F) Boxplots of percentage of HMEC-1 infected with *ΔactA* Lm for the data shown in panels C-D. One or two asterisks denote statistically significant differences between the medians of two distributions (<0.05 or <0.01, respectively; Wilcoxon rank sum test). (F) HMEC-1 residing on collagen I-coated glass substrates, treated with siNT or siVIM were also blocked for 1 h with anti-vimentin antibodies prior to infection. Cells were infected with Lm (constitutively expressing GFP) at an MOI of 1.25. 30 min post-infection samples were fixed, immunostained and infection was analyzed by microscopy followed by image processing. Boxplots shows total bacteria per cell for an average of N=650 cells analyzed per condition (F) HMEC-1 residing on collagen I-coated glass substrates, treated with siNT or siVIM were also treated for 30 min with vehicle control or 5 μM withaferin prior to infection. Cells were infected

with Lm (constitutively expressing GFP) at an MOI of 1.25. 30 min post-infection samples were fixed, immunostained and infection was analyzed by microscopy followed by image processing. Boxplots shows total bacteria per cell for an average of N=630 cells analyzed per condition.

Supplemental Figure S5. Fewer Lm localize at cell-cell junctions for siVIM treated HMEC-1. (A-B) HMEC-1 treated with 20 nM of non-targeting siRNA (siNT, A) or vimentin siRNA (siVIM, B) were infected with Lm at an MOI of 3. 30 min post-infection samples were fixed, stained for VE-cadherin that localizes all throughout the cell-cell junctions using an anti-VE-cadherin antibody. Representative phase image of cells (left column), superimposed with the image of the nuclei (blue) and anti-VE-cadherin antibody fluorescence (white, right column) superimposed with fluorescence of Lm (red). Scale bar is 23 μm. Two representative examples are shown for each condition. Yellow arrows point at cell-cell junctions were bacteria are co-localized.

Suppl. Figure 1



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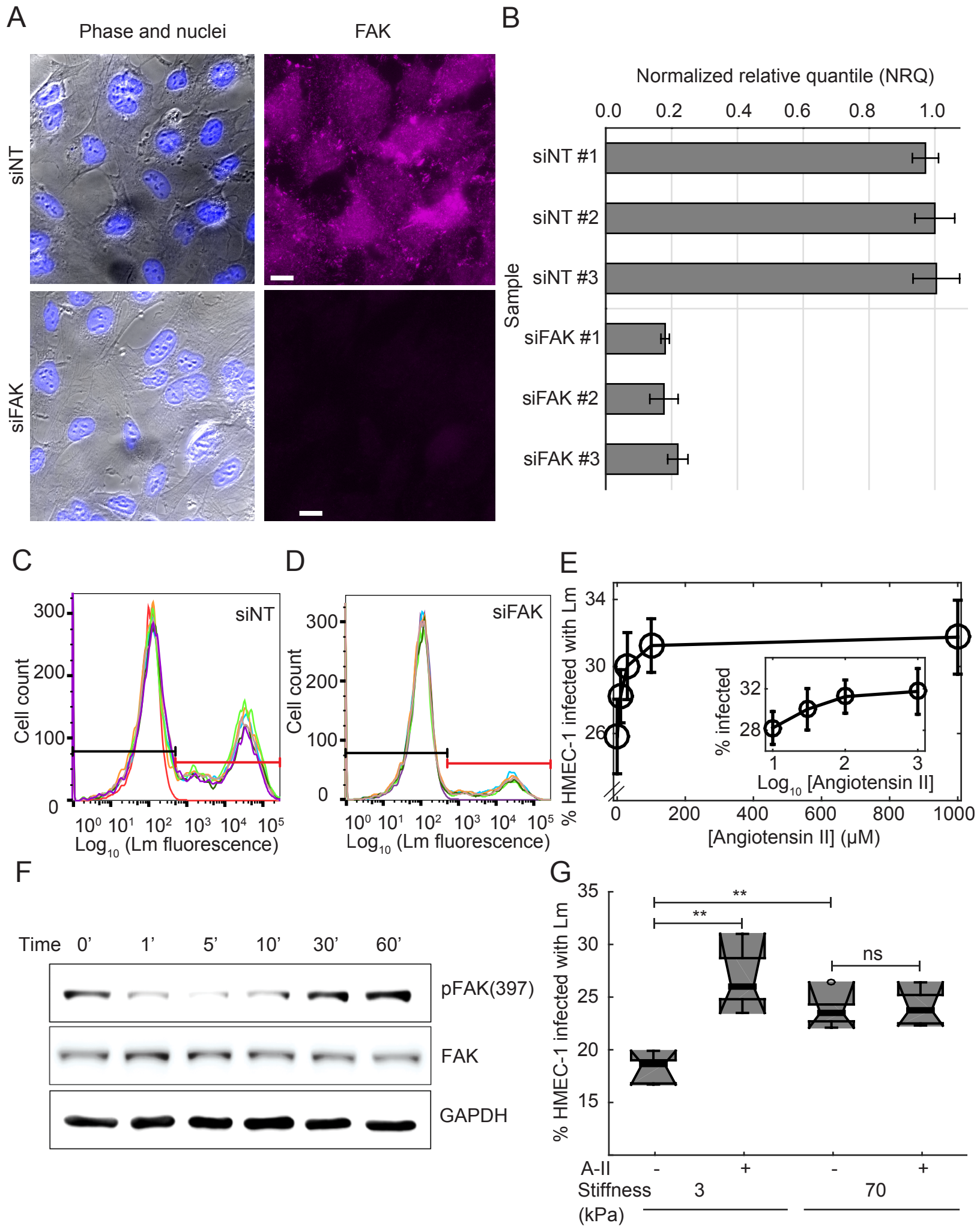

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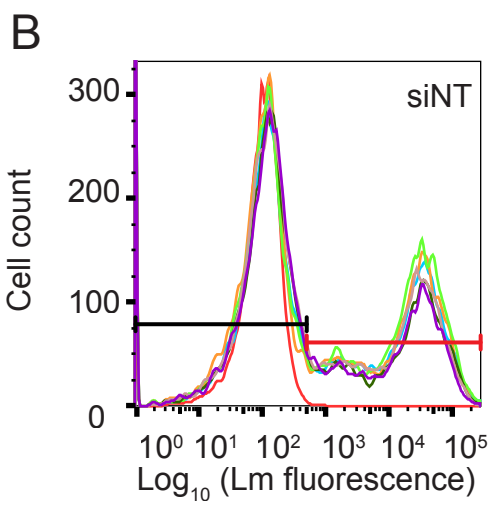

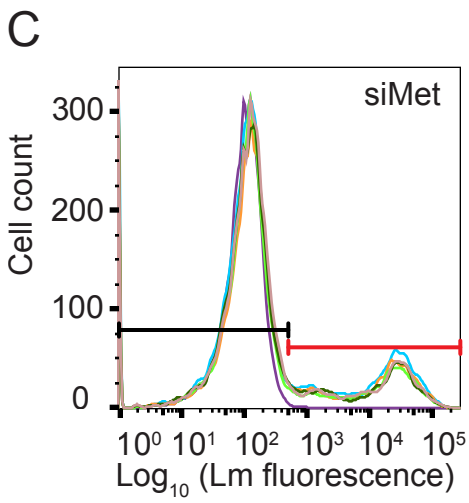

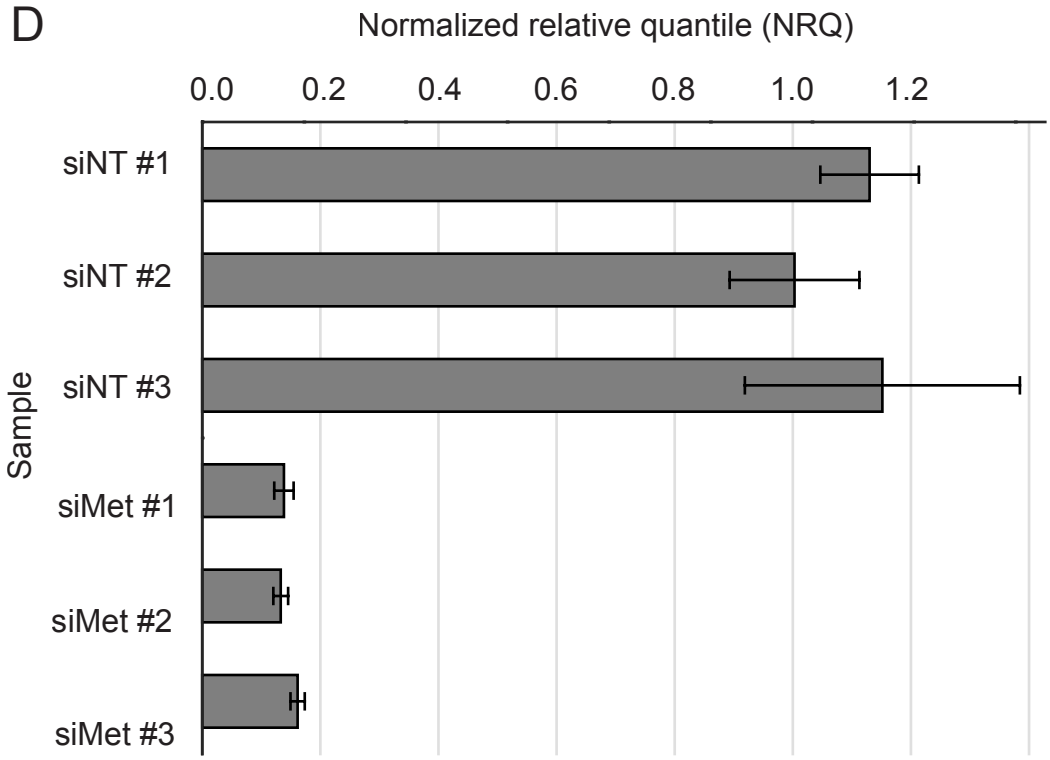

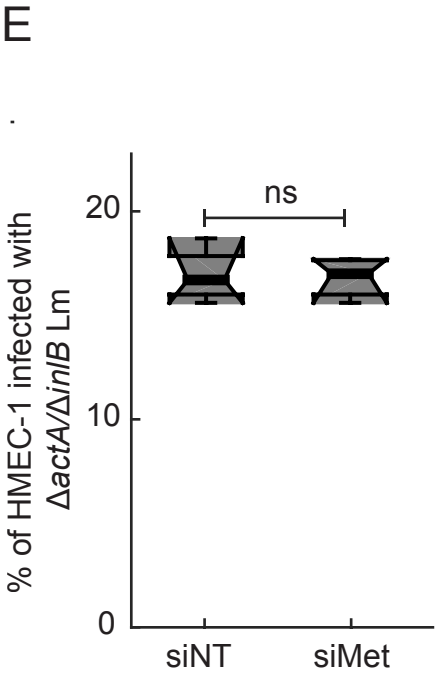

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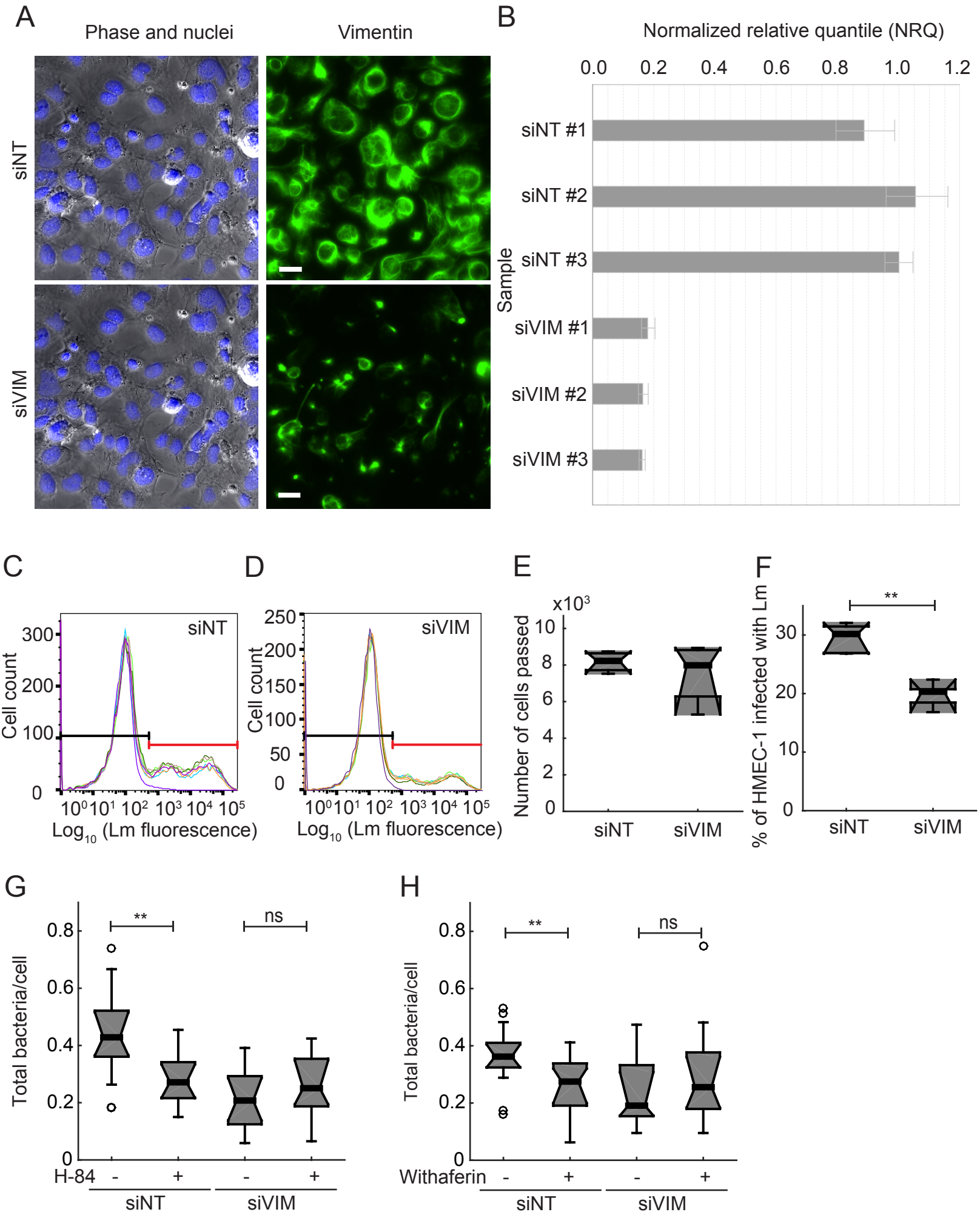

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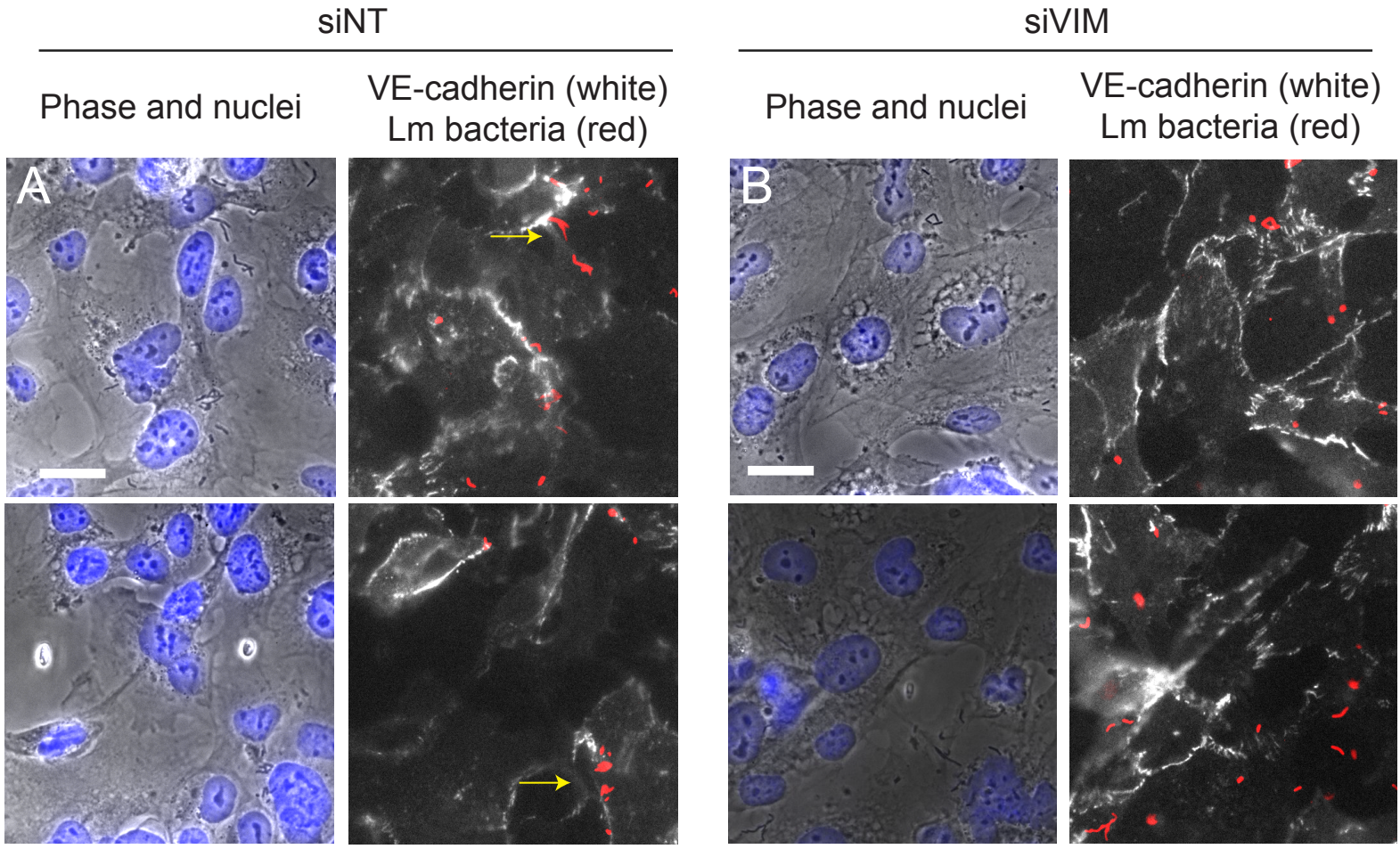

Supplementary Table 1: Bacterial strains used in this study

Name and source	Species	Genotype/Description
JAT983 (100)	*L. monocytogenes* 10403S	LLO^{G486D} actAp::mTagRFP
JAT985 (100)	*L. monocytogenes* 10403S	LLO^{G486D} *ΔactA* actAp::mTagRFP
JAT1045 (100)	*L. monocytogenes* 10403S	LLO^{G486D} Consitutive sGFP
JAT1046 (100)	*L. monocytogenes* 10403S	LLO^{G486D} *ΔactA* Consitutive sGFP
JAT1063 (100)	*L. monocytogenes* 10403S	LLO^{G486D} *ΔactA*/*ΔinlB* actAp::mTagRFP
JAT1060 (100)	*L. monocytogenes* 10403S	LLO^{G486D} *ΔinlB* Consitutive sGFP
JAT1051 (100)	*L. monocytogenes* 10403S	LLO^{G486D} *ΔinlA* actAp::mTagRFP
JAT1062 (100)	*L. monocytogenes* 10403S	LLO^{G486D} *ΔinlB* actAp::mTagRFP
JAT1115 (100)	*L. monocytogenes* 10403S	LLO^{G486D} *ΔinlF* actAp::mTagRFP
JAT638 (DP-L392) (85)	*L. innocua*	

Supplementary Table 2: Synthetic siRNA pools used in this study

Accession number	Gene (Protein)	Product Name	Catalog Number
	Negative control	ON-TARGETplus Non-Targeting Pool	D-001810-10
NM_000942	siGLO	ON-TARGETplus siGLO Control Reagent	D-001610-01
NM_004523.3	Kif11	ON-TARGETplus Human KIF11 (3832) siRNA	L-003317-00-0005
NM_003380.3	VIM	ON-TARGETplus Human VIM	L-003551-00-0005
NM_000245.3	MET	ON-TARGETplus Human MET	L-003156-00-0005
NM_001199649.1	PTK2 (FAK)	ON-TARGETplus Human PTK2	L-003164-00-0005