Supplementary Table 1. Amino acid sequence of superfolder GFP

Protein	Sequence
sfGFP*	$_{1}$MGKGEELFTGVVPILVELDGDVNGHKFSV**R**GEGEGDAT**N**GKLTLKFICTTGKLPV PWPTLVTT**LT**YGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTI**S**FKDDG**T**YKTR AEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYN**F**NSHNVYI**T**ADKQKNGIK**A**NFKI RHN**V**EDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQS**K**LSKDPNEKRDHMVLLE FVTAAGITHGMDELY$_{237}$

*Bolded amino acids are substitutions that enhance protein folding and limit aggregation (1, 2).

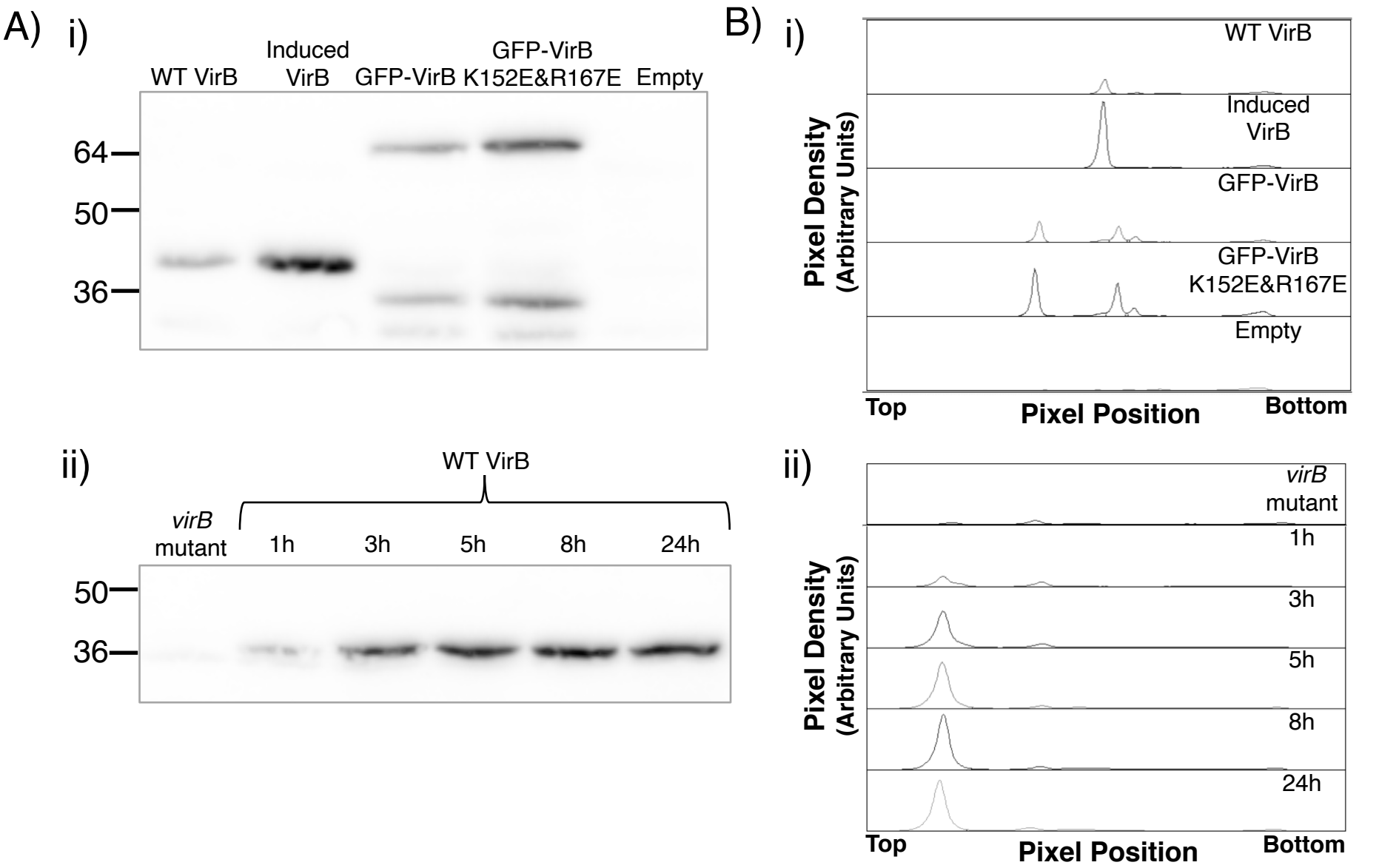
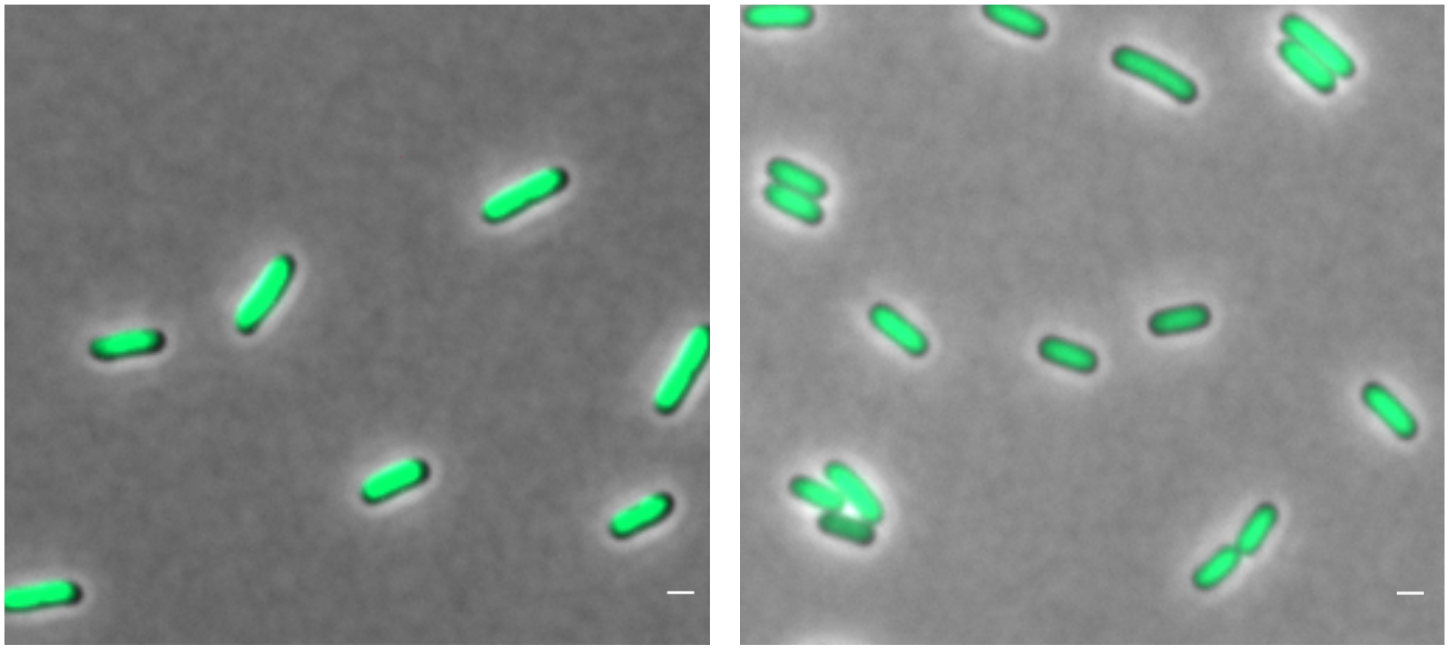


Fig S1. Western blots to compare WT protein levels to GFP-VirB fusion production and determine maximal expression of VirB protein. (A) Western blot analyses using an anti-VirB antibody to visualize (i) if fusion proteins are being produced at physiologically relevant levels and (ii) native levels of VirB in cell lysates collected at various timepoints over 24 hours. (B) Quantification of western blot analyses shown in panel A; (i) and (ii), respectively. Densitometry of bands detected in each lane (top to bottom) are represented (left to right). Note, the density of the WT VirB band (lane 1) is very similar to the GFP-VirB fusion (lane 3) and VirB protein production is close to maximal at the 5-hour timepoint, hence the rationale for growing cells for 5h in our assays.

***virB*::Tn5**

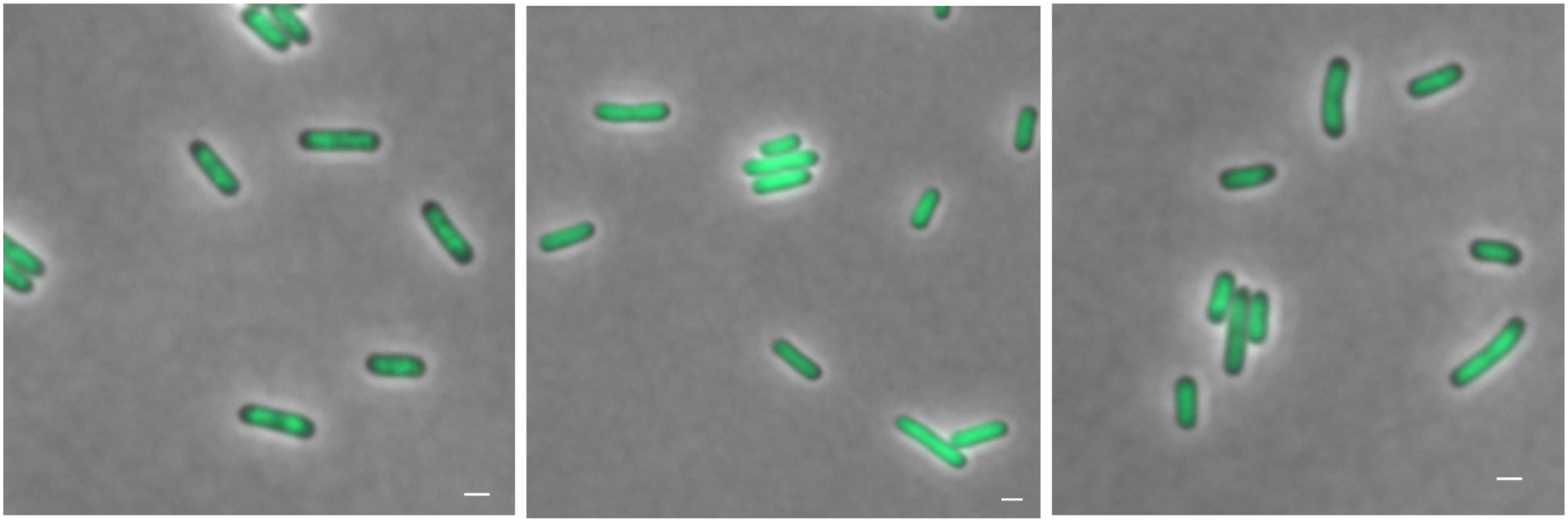


GFP

GFP-VirB
K152E&R167E

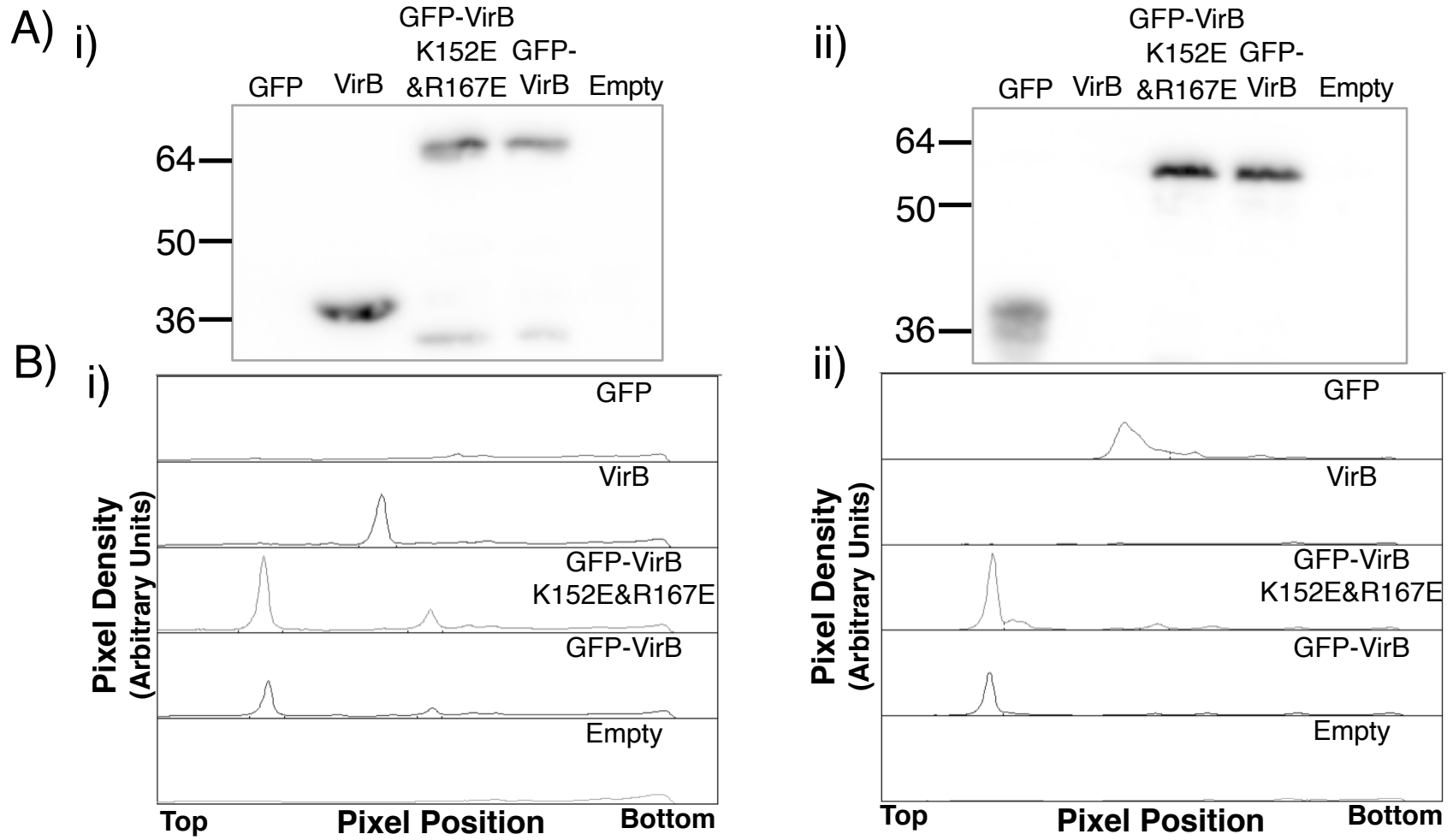
Fig S2. Live cell imaging of GFP & GFP-VirB K152E/R167E in a *virB* mutant strain of *S. flexneri*. Representative fields of view show diffuse signals for both the GFP control (left panel; 77 ms exposure) and GFP-VirB K152E&R167E (right panel; 348.8 ms exposure). Scale bar represents 1 μm in all images.

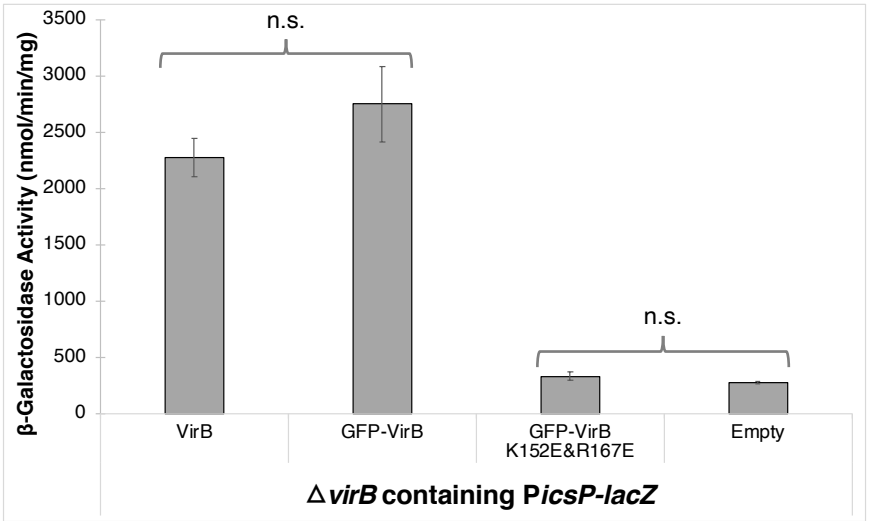
pINV-cured



GFP-VirB | GFP | GFP-VirB K152E&R167E

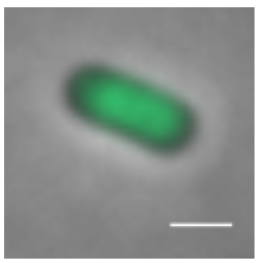
Fig S3. Live cell imaging of GFP-VirB, GFP, & GFP-VirB K152E/R167E in a pINV-cured strain of *S. flexneri*. Representative fields of view show either nucleoid-associated or diffuse signals for GFP-VirB (left panel; 217.5 ms exposure), the GFP control (middle panel; 51.6 ms exposure) and GFP-VirB K152E&R167E (right panel; 217.5 ms exposure). Scale bar represents 1 μm in all images.





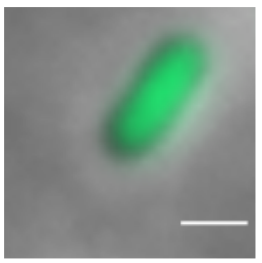
D)

WT **GFP-VirB K152E&R167E** (n = 738)



# of diffuse signals* per cell	Cell Length (in μm) 1.5 - 3.0	3.01 - 4.0
0	3%	<1%
1	74%	<1%
2	17%	5%
3	<1%	-

pINV-cured **GFP-VirB K152E&R167E** (n = 1387)



# of diffuse signals* per cell	Cell Length (in μm) 1.5 - 3.0	3.01 - 4.0
0	2%	<1%
1	65%	<1%
2	22%	10%
3	<1%	<1%
4	-	<1%

Fig. S4. Proof of principle experiments with GFP-VirB K152E/R167E & live cell imaging in wild-type and pINV-cured strains of *S. flexneri.* (A) Western blot analyses to detect (i) VirB or (ii) GFP to assess fusion protein stability. (B) Densitometry of western blots shown in panel A; (i) and (ii), respectively. Protein bands in each lane, top to bottom are represented left to right and labeled accordingly. (C) β-galactosidase assay used to compare the regulatory activity of GFP-VirB fusions at the VirB-dependent *icsP* promoter (P*icsP-lacZ*; pAFW04a). Student's *t*-tests were used to measure statistical significance, * $p < 0.05$. (D) Live cell imaging and quantification of diffuse fluorescence signal generated by GFP-VirB K152E/R167E in wild-type [219 ms exposure] and pINV-cured [223.1 ms exposure] *S. flexneri.* Scale bar represents 1 μm in all images. Within tables, (-) indicates no cells fell into this category in any of the images captured; *Maxima detected by MicrobeJ.

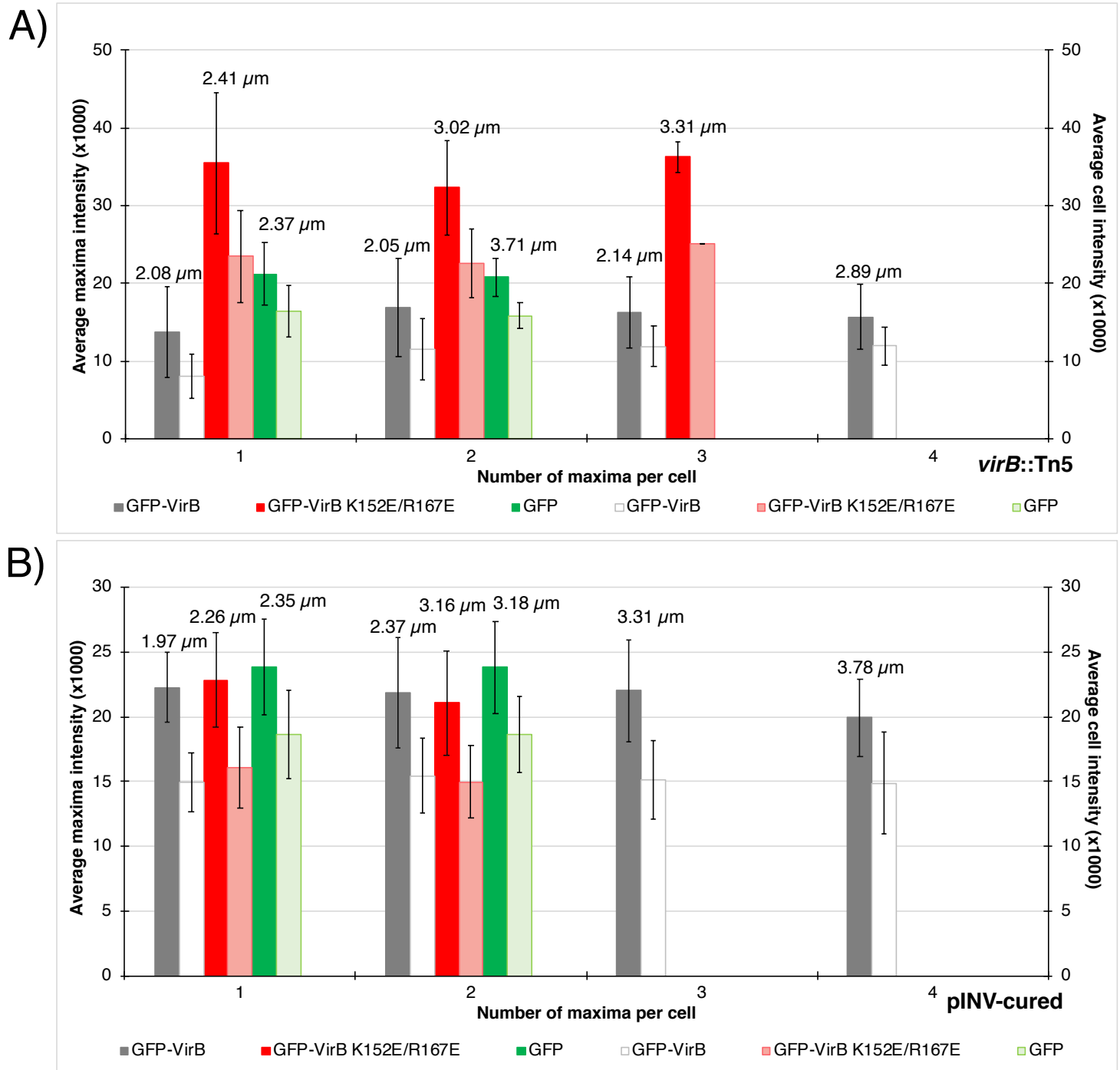


Fig. S5. Quantification of average fluorescence intensity of maxima/foci and average fluorescence intensity detected along medial axis of cells generated using MicrobeJ. Average fluorescence intensity of maxima/foci (dark bars; left axis) and average fluorescence intensity along medial axis of cells (light bars; right axis) is shown for GFP-VirB (grey hues), GFP-VirB K152E/R167E (red hues), and GFP (green hues). Average cell length (in μm) is shown for each data set. Data were sourced from one representative field of view for (A) the *virB* mutant and (B) the pINV-cured strain of *S. flexneri* expressing the aforementioned proteins (the same data sets were used to generate Figs. 4, 5, and Fig. S3). As expected, the average fluorescence intensity of foci (dark bars) was routinely higher than the average fluorescence intensity detected for the medial axis of cells (light bars), regardless of strain background. In panel A, the fluorescence intensity and length of cells expressing GFP-VirB was routinely lower than cells expressing GFP-VirB K152E/R167E. VirB is known to place a transcriptional and hence energetic burden on cells containing pINV (3). To further support this, in the absence of pINV (panel B), average fluorescence intensities for maxima/foci and along the medial axis of cells expressing GFP-VirB are more similar to cells expressing GFP-VirB K152E/R167E, where cell length is not as stunted. Note, in all analyses, GFP intensities appear artificially low because exposure time for cells expressing GFP was 5-fold (*virB* mutant; panel A) and 4-fold (pINV-cured; panel B) lower than for cells expressing either fusion protein during image capture.

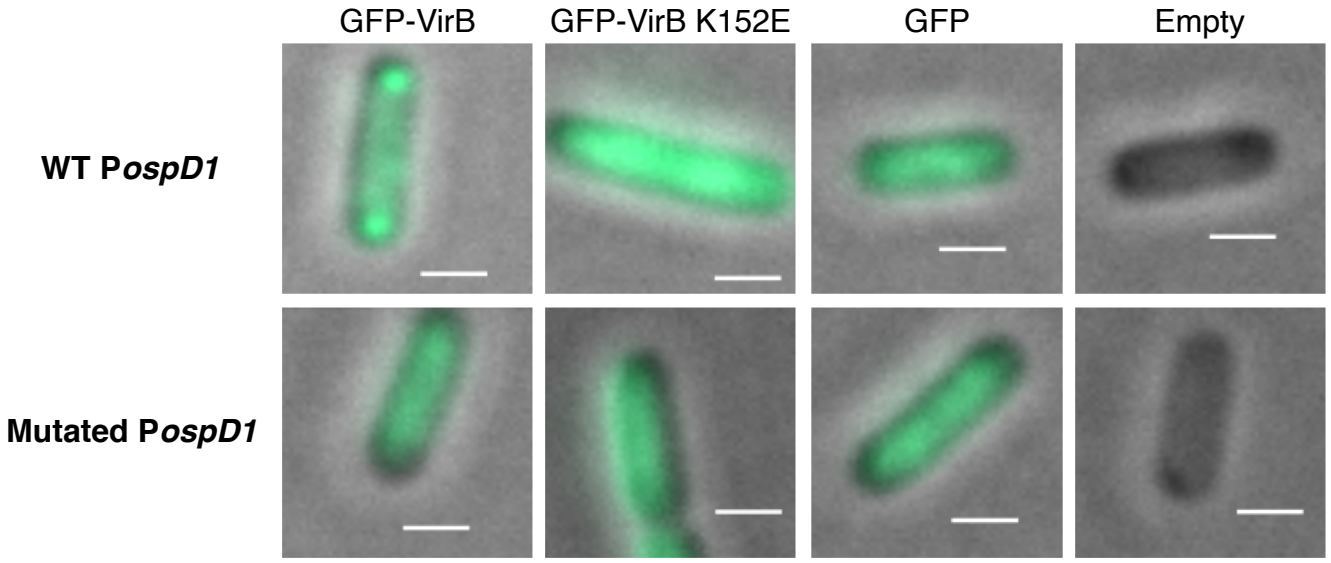


Fig. S6. Live cell imaging of GFP-VirB and controls in a pINV-cured strain of *S. flexneri* carrying a small plasmid bearing *PospD1*. Representative merged cell images of pINV-cured *S. flexneri* carrying pJAI28 (WT P*ospD1*) or pJAI36 (Mutated P*ospD1*) with either GFP-VirB, GFP-VirB K152E, GFP, or an empty plasmid control are shown [GFP, GFP-VirB, GFP-VirB K152E, & Empty = 4.8 s exposure] . Scale bar represents 1 μm in all images.

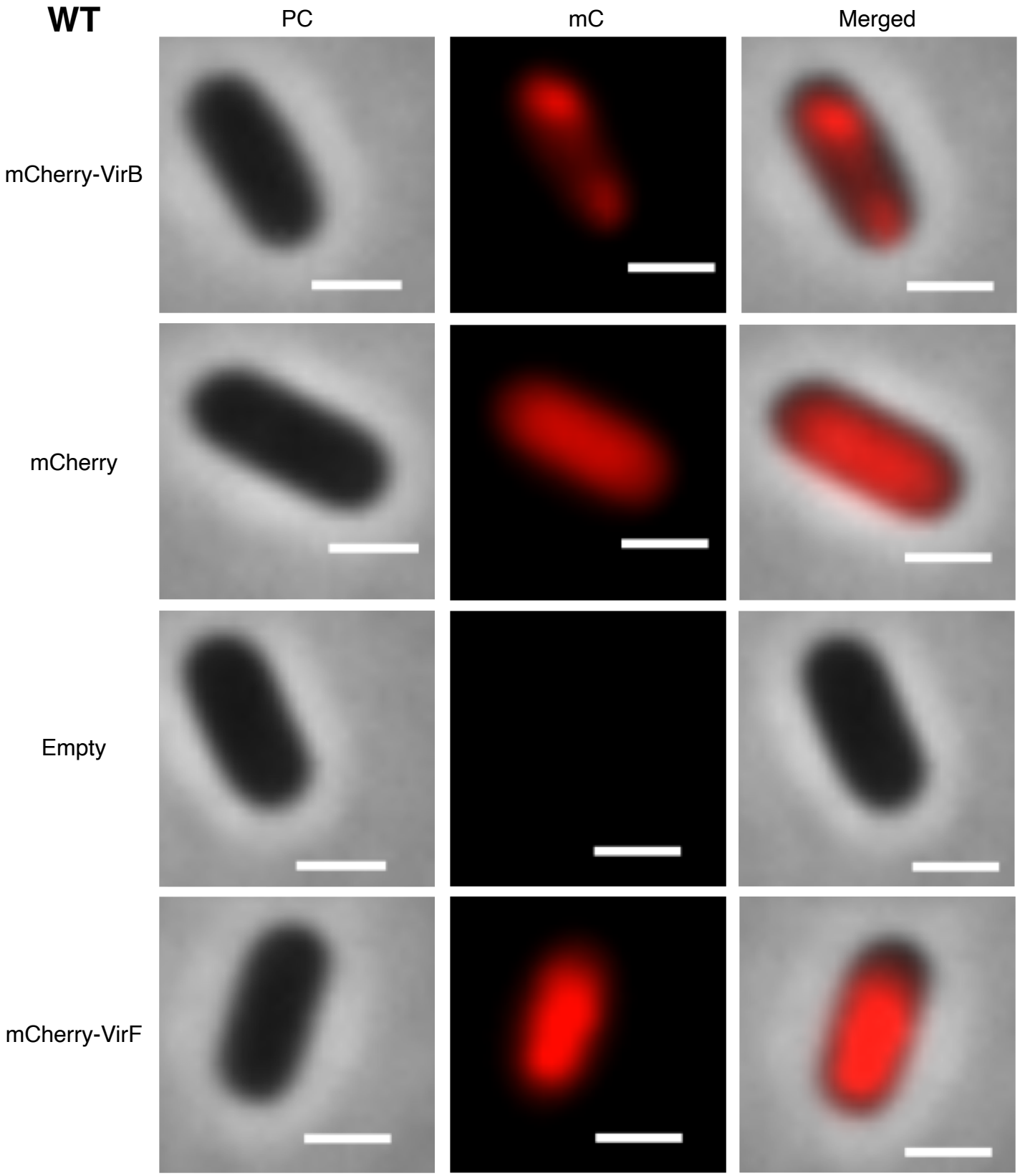


Fig. S7. Live cell imaging of mCherry-VirB & mCherry-VirF in a wild-type strain of *S. flexneri.* Representative cells are shown. Phase contrast – PC (left column), fluorescence – mC (middle column), and merged (right column) images are shown [mCherry, mCherry-VirB, mCherry-VirF, & Empty = 963.1 ms using 50% exposure]. Discrete foci are seen in cells producing mCherry-VirB (top row), but diffuse signals are observed in cells producing either mCherry alone (second row) or mCherry-VirF (bottom row). An empty plasmid control (third row) exhibits no fluorescence. Scale bar represents 1 μm in all images.

References

1. Pedelacq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS. 2006. Engineering and characterization of a superfolder green fluorescent protein. Nat Biotechnol 24:79-88.
2. Landgraf D, Okumus B, Chien P, Baker TA, Paulsson J. 2012. Segregation of molecules at cell division reveals native protein localization. Nat Methods 9:480-2.
3. Schuch R, Maurelli AT. 1997. Virulence plasmid instability in *Shigella flexneri* 2a is induced by virulence gene expression. Infect Immun 65:3686-92.