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Supplemental Information

A Multicolor Split-Fluorescent Protein Approach to Visualize *Listeria* Protein Secretion in Infection

Dilara Batan, Esther Braselmann, Michael Minson, Dieu My Thanh Nguyen, Pascale Cossart, and Amy E. Palmer

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Supplementary Information

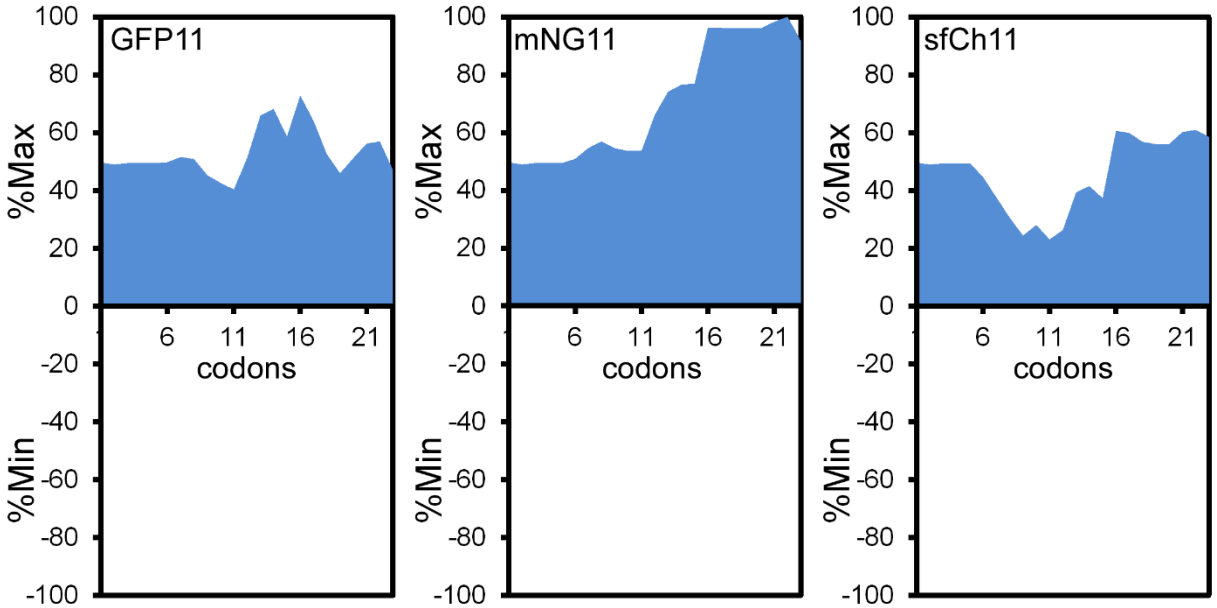


Figure S1: Codon usage for *Listeria monocytogenes* in split fluorescent protein tags. Codon usage in designed tags (see Table S3) were analyzed with the %MinMax algorithm (54), where codon usage is analyzed for a sliding window across the sequence. Positive values (%Max) indicate usage of common codons, whereas negative values (%Min) indicate rare codons.

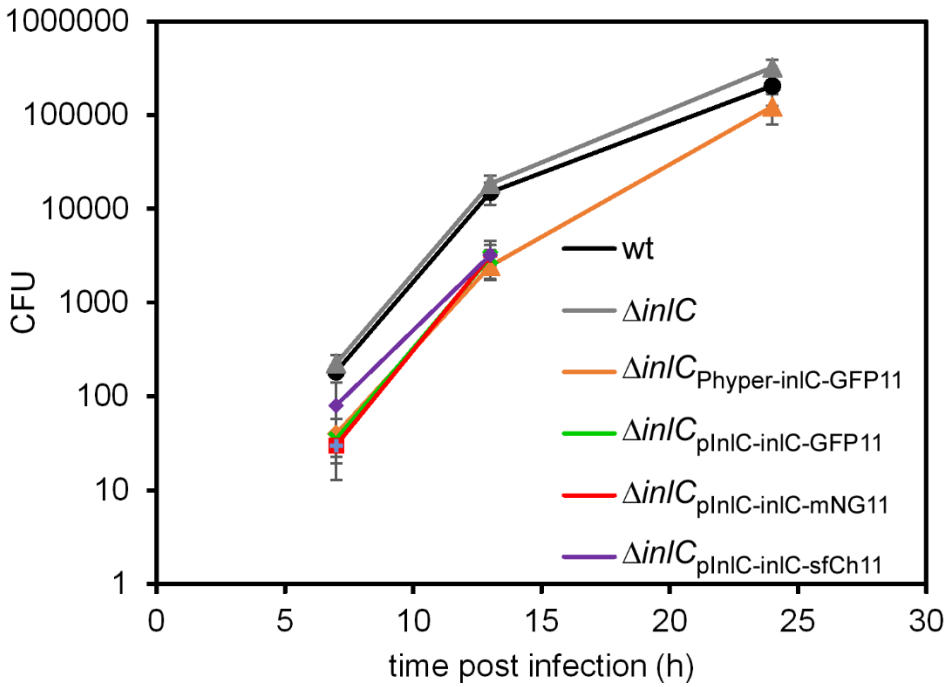


Figure S2: CFU analysis of HeLa cells infected for different lengths with the indicated strains (average from n=3 independent experiments, error bars indicate STD). 7 h time point: $p = 0.015$; 13 h time point: $p = 0.0035$, 24 h time point: $p = 0.0045$ (ANOVA test).

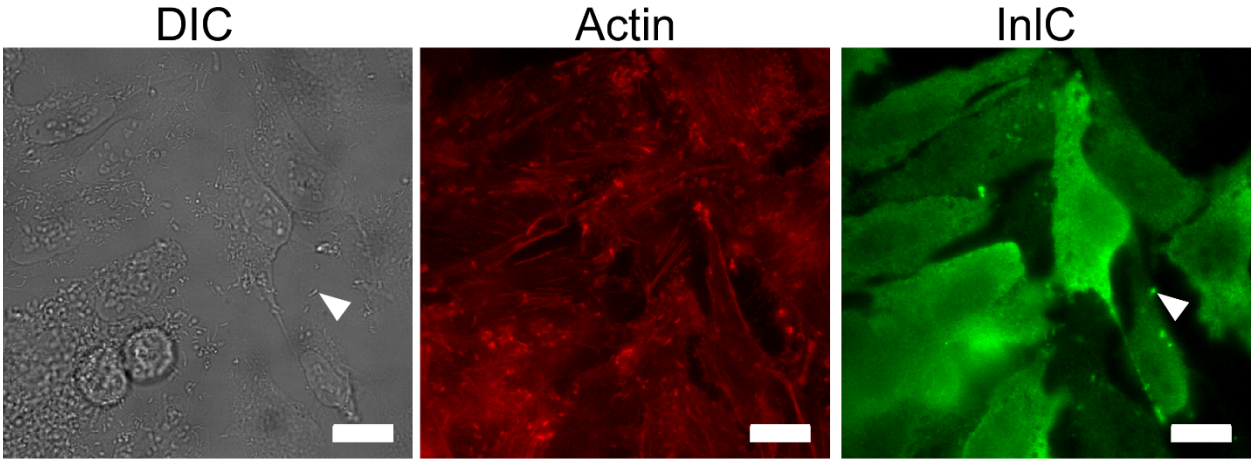


Figure S3: InlC occasionally localizes to puncta consistent with cell protrusions from *Listeria* spreading from cell to cell (white arrow). Shown is a select region of interest for infection of HeLa cells with Δ*inlC*$_{\text{Phyper-inlC-GFP11}}$ (see Fig. 3 for details). Scale bar = 20 μm.

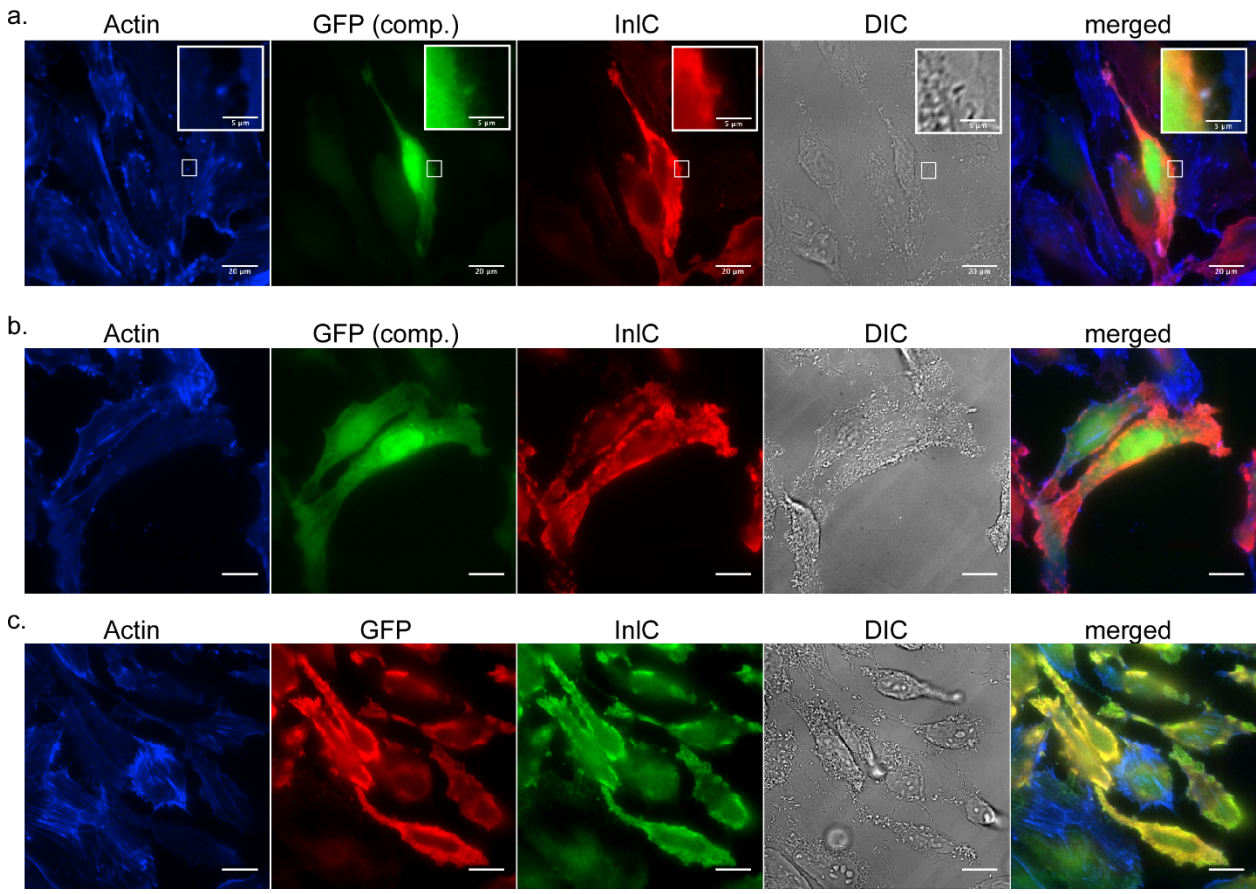


Figure S4: GFP signal after complementation colocalizes with fluorescence signal from anti-InlC immunofluorescence. (a, b) HeLa cells producing GFP1-10 were infected with Δ*inlC*$_{\text{pInlC-inlC-GFP11}}$ overnight and cells were fixed. After permeabilization, actin was stained with Coumarin-phalloidin and InlC was detected by immunofluorescence. The overall pattern and the insert in (a) demonstrate colocalization of complemented GFP signal and InlC. Note that the InlC signal in the nucleus appears brighter when detected by complemented GFP vs. anti-InlC immunofluorescence. This could be due to incomplete permeabilization of the nuclear envelope. Panels (a) and (b) show representative fields of view. (c) HeLa cells were infected with Δ*inlC*$_{\text{pInlC-inlC-GFP11}}$ overnight and cells were fixed. After permeabilization, actin was stained with Coumarin-phalloidin and InlC as well as GFP was detected by immunofluorescence. Scale bar = 20 µm (scale bar for insert in panel (a) = 5 µm).

Supplementary Tables

Strain Name	Resistance	Detailed genotype and source
wt	none	EGD-e wt (BUG 1600) (51)
Δ*inlC*	none	EGD-e Δ*inlC* (Cossart lab collection, BUG 2118)
Δ*inlC*$_{\text{Phyper-inlC-GFP11}}$	erythromycin	EGD-e Δ*inlC* pAT18_P*hyper_inlC_GFP11* (this study)
Δ*inlC*$_{\text{pInlC-inlC-GFP11}}$	erythromycin	EGD-e Δ*inlC* pAT18*_pInlC_inlC_GFP11* (this study)
Δ*inlC*$_{\text{pInlC-inlC-mNG11}}$	erythromycin	EGD-e Δ*inlC* pAT18*_pInlC_inlC_mNG11* (this study)
Δ*inlC*$_{\text{pInlC-inlC-sfCh11}}$	erythromycin	EGD-e Δ*inlC* pAT18*_pInlC_inlC_sfCh11* (this study)

Table S1: Names, genotypes and source of all strains used in this study along with antibiotic selection markers.

P*hyper*

5′**TCTAGA**AGACGAAAGGGCCTAATTTTGCAAAAAGTTGTTGACTTTATCTACAAGGTGTGGC

ATAATGTGTGTCGGCCATAAAGCAAGCATATAATATTGCGTTTCATCTTTAGAAGCGAATTTC

GCCAATATTATAATTATCAAAAGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAA

AATGTAGAAGGAGAGTGAAACCC **ATG TTG**…

p*inlC*

5′**TCTAGA**AGACGAAAGGGCCATTATTAACGCTTGTTAATTTAAACATCTCTTATTTTTGCTA

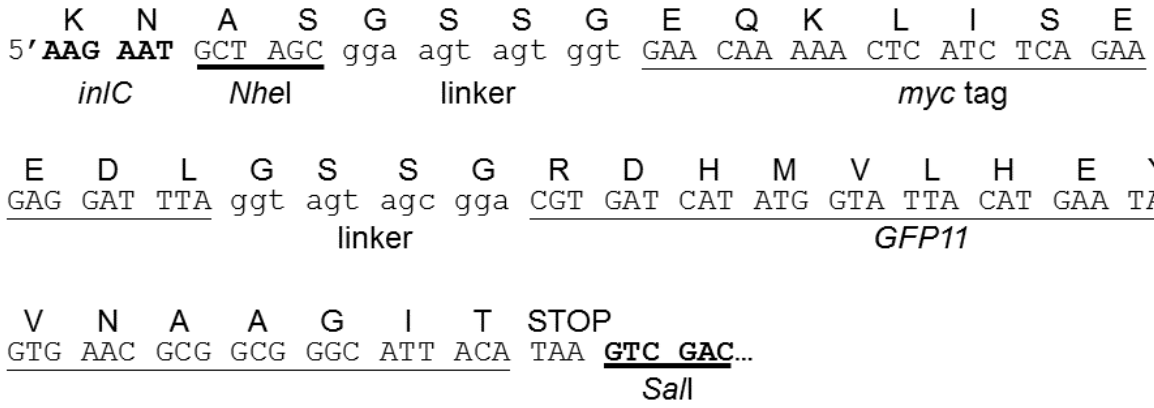
ACATATAAGTATACAAAGGGACATAAAAAGGTTAACAGCGTTTGTTAAATAGGAAGTATATGA

AAATCCTCTTTTGTGTTTCTAAATTTATTTTTAAGGAGTGGAGACGGTCCGCTAGC **ATG**

TTG…

Table S2: Sequences of P*hyper* and p*inlC*. The 5' *Xba*I restriction site flanking the *inlC* fusion genes is indicated in bold and underlined and the beginning of the *inlC* coding sequence (ATG TTG…) at the 3' end of the promoters is shown in bold. Top: the blue underlined region is the P*hyper* region and the *hly* 5'UTR is shown in yellow and underlined. Bottom: the green underlined region corresponds to the *inlC* promoter.

GFP11 tag



mNG11 tag

sfCh11 tag

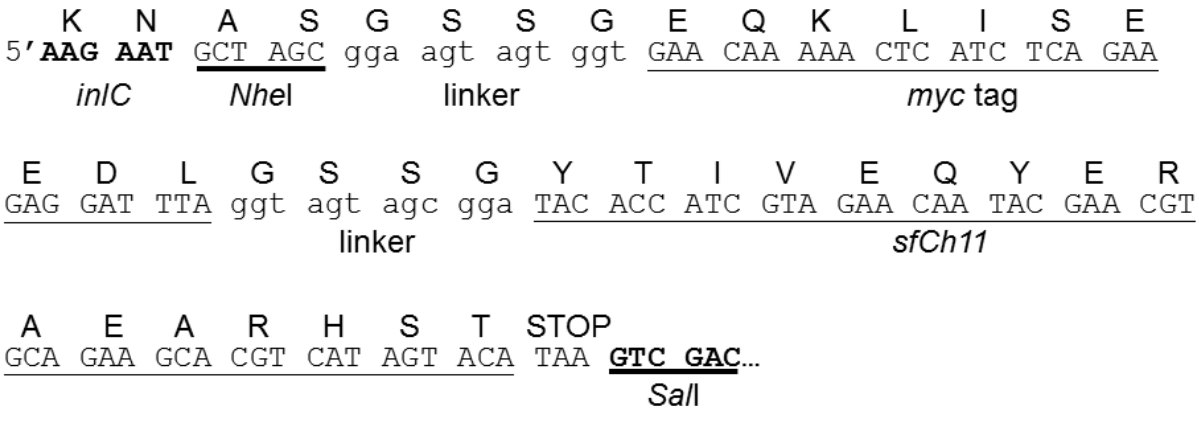


Table S3: DNA and protein sequences of split-FP tags at the 3' end of InlC used in this study.

The 3' end of the *inlC* gene and relevant sequence features are annotated.

GFP1-10 (34) MVSKGEELFT GVVPILVELD GDVNGHKFSV RGEGEGDATI GKLTLKFICT TGKLPVPWPT LVTTLTYGVQ CFSRYPDHMK RHDFFKSAMP EGYVQERTIS FKDDGKYKTR AVVKFEGDTL VNRIELKGTD FKEDGNILGH KLEYNFNSHN VYITADKQKN GIKANFTVRH NVEDGSVQLA DHYQQNTPIG DGPVLLPDNH YLSTQTVLSK DPNEK
mNG1-10 (52) MVSKGEEDNM ASLPATHELH IFGSINGVDF DMVGQGTGNP NDGYEELNLK STKGDLQFSP WILVPHIGYG FHQYLPYPDG MSPFQAAMVD GSGYQVHRTM QFEDGASLTV NYRYTYEGSH IKGEAQVMGT GFPADGPVMT NTLTAADWCM SKKTYPNDKT IISTFKWSYT TVNGKRYRST ARTTYTFAKP MAANYLKNQP MYVFRKTELK HSM
sfCh1-10 (52) MEEDNMAIIK EFMRFKVHME GSVNGHEFEI EGEGEGHPYE GTQTAKLKVT KGGPLPFAWD ILSPQFMYGS KAYVKHPADI PDYLKLSFPE GFTWERVMNF EDGGVVTVTQ DSSLQDGQFI YKVKLLGINF PSDGPVMQKK TMGWEASTER MYPEDGALKG EINQRLKLKD GGHYDAEVKT TYKAKKPVQL PGAYNVDIKL DITSHNED

Table S4: Protein sequences for fluorescent protein 1-10 (FP1-10) constructs used in this study. Each FP1-10 corresponds to the first 10 β-strands of the FP; the last and 11th strand represents the other fragment of the split-FP system (Table S3).

Antibody / stain (source)	**Dilution / incubation time**	**Purpose**
Alexa 594-Phalloidin (Fisher Scientific, Waltham, MA)	1:1,000 / 45 min	Actin staining in fixed cells
Coumarin-phalloidin (Sigma-Aldrich, St. Louis, MO)	1:1,000 / 45 min	Actin staining in fixed cells
Anti-GFP (mouse) (Thermo Fisher Scientific, Waltham, MA)	1:1,000 / 45 min	Immunofluorescence
Anti-InlC (rabbit) (Cossart lab (46))	Immunofluorescence: 1:500 / 45 min at room temperature Western blotting: 1:500 / overnight at 4°C	Immunofluorescence and Western blotting
Goat anti-rabbit HRP conjugated (Novus Biologicals, Littleton, CO)	1:2,000 / 1 h at room temperature	Western blotting
Rabbit anti-mouse IgG (Alexa Fluor 488) (Thermo Fisher Scientific, Waltham, MA)	1:1,000 / 45 min at room temperature	Immunofluorescence
Donkey anti-rabbit IgG H&L (Alexa Fluor 568) (Abcam, Cambridge, MA)	1:1,000 / 45 min at room temperature	Immunofluorescence

Donkey anti-rabbit IgG H&L (Alexa Fluor 488) (Abcam, Cambridge, MA)	1:1,000 / 45 min at room temperature	Immunofluorescence

Table S5: List of antibodies and stains used in this study.

Immunofluorescence of infections (Fig. 3, S2)	GFP: 287 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200, neutral density filter (25% emission)	mCherry: 90 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200, neutral density filter (25% emission)	DIC: 50 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 0, neutral density filter (25% emission)
Proof of principle live imaging of split-GFP complementation (Fig. 4a)	GFP: 50 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200, neutral density filter (25% emission)	Dapi: 287 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200, neutral density filter (25% emission)	DIC: 50 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 0, neutral density filter (25% emission)
GFP complementation time course (Fig. 4b)	GFP: 50 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200, neutral density filter (25% emission)	Dapi: 287 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200, neutral density filter (25% emission)	DIC: 50 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 0, neutral density filter (25% emission)
GFP complementation	GFP:	Dapi:	DIC:

promoter comparison end point (Fig. 8)	287 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200	287 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200	70 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 0
Super-folder Cherry complementation (Fig. 8)	mCherry: 287 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200	DIC: 50 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200	
mNeonGreen complementation (Fig. S3)	GFP: 287 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200	DIC: 50 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 0	
GFP complementation in macrophages (Fig. 7)	GFP: 287 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200	Dapi: 287 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 200	DIC: 90 ms exposure, EM gain 1 MHz at 16-bit readout mode, EM gain multiplier = 0

Table S6: Settings for fluorescence microscopy on Nikon Ti-E widefield fluorescence microscope.