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Imaging Type-III Secretion reveals dynamics and spatial segregation of *Salmonella* effectors

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Abstract

The type-III secretion system (T3SS) enables gram-negative bacteria to inject effector proteins into eukaryotic host cells. Upon entry, T3SS effectors work cooperatively to reprogram host cells, enabling bacterial survival. Progress in understanding when and where effectors localize within host cells has been hindered by a dearth of tools to study these proteins in the native cellular environment. We report a method to label and track T3SS effectors during infection using a split-GFP system. The breadth of this technique is demonstrated by labeling three effectors from *Salmonella* (PipB2, SteA, and SteC) and characterizing their localizations within host cells. PipB2 displays highly dynamic behavior on tubules emanating from the *Salmonella* containing vacuole labeled with both endo- and exocytic markers. SteA is preferentially enriched on tubules localizing with Golgi markers. This segregation suggests effector targeting and localization may play a functional role during infection.

Introduction

Salmonella enterica causes acute gastroenteritis and systemic typhoid fever in humans. This gram-negative bacterial pathogen establishes a replicative niche within the host, enabled by type-III secretion systems (T3SS) encoded within the *Salmonella* genome. The T3SS is a multi-subunit protein complex capable of injecting proteins, termed effectors, directly from bacterial cytosol into host cells 1. Upon translocation, effector proteins, which possess an array of biochemical functions, commandeer host cell signaling to enable bacterial entry and replication. *Salmonella* effectors are categorized in two ways, those translocated into the host cell by the T3SS, encoded on *Salmonella* pathogenicity island-1 (SPI-1) which allow bacteria to gain entry into host cells and aid in the biogenesis of the nascent *Salmonella* Containing Vacuole (SCV), and those secreted after internalization of the bacteria by the T3SS encoded within *Salmonella* pathogenicity island-2 (SPI-2) which are required for SCV maturation, trafficking, and intracellular replication, 2. Over 60 effectors have been characterized as *Salmonella* T3SS substrates and biochemical functions have been ascribed

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to a subset of these proteins. Emerging evidence suggests that T3SS effector activity is regulated both spatially within host cells and temporally by injection/degradation to coordinate the hijacking of host cell signaling machinery 3. Further diversification of effector targeting is achieved by posttranslational modification of effectors within the host cell 4-6. This complexity of effector regulation underscores the importance of being able to directly localize and monitor dynamics of individual effectors in living infected host cells. Numerous studies have localized T3SS effectors in host cells by immunofluorescence at a single (or small subset) of time points. Unfortunately, immunofluorescence studies require fixation of infected cells which has been shown to perturb effector localization 7,8, thus confounding some studies of effector function. A widely used technique for tracking the movement of proteins in living cells is to genetically fuse the protein of interest to a fluorescent protein, such as GFP. However, fusion of effector proteins to GFP perturbs secretion, perhaps because GFP cannot be unfolded to fit through the T3SS needle complex 9. Effector proteins fused to GFP have been transiently transfected into host cells to attempt to overcome these limitations 7. However, host cell expressed effectors often display different localizations compared with bacterially derived and T3SS delivered effectors 2, highlighting the importance of studying translocated effectors in the presence of the entire effector cohort at a given time post-infection.

Recently, methods developed by our laboratory 10 and others 11,12 have enabled the measurement of SPI-1 type-III translocation by time-lapse microscopy. These approaches are not easily adaptable for SPI-2 effectors which are upregulated in the environment of the SCV and only secreted after bacterial internalization. Several techniques exist to measure accumulation of T3SS effectors within infected host cells at distinct time points 13,14. However, these methods necessitate host cell lysis and bulk measurement of effector populations which precludes their use in monitoring subcellular dynamics of effectors in individual cells. Currently, no technique exists to directly label and visualize the dynamics of T3SS injected SPI-2 effectors required for intracellular survival within living host cells. To address this need we have adapted a split GFP system 15 to tag *Salmonella* T3SS SPI-2 effectors and detect their localization and dynamics in living host cells. We tag three effectors, SteA, PipB2 and SteC, with GFP11 and track their localization upon translocation.

Results

Tagging effectors with split GFP

To exploit the split GFP system for effector tagging, the small 13 amino acid 11th strand of the GFP β -barrel (GFP-11) is genetically fused to *Salmonella* effectors and the complementary fragment corresponding to the first ten strands of GFP (GFP1-10) is expressed *in trans* within the host cell. Upon T3SS effector translocation, spontaneous complementation of the two fragments (GFP_{comp}) occurs resulting in fluorescent tagging of the effector population within the host cell (Fig. 1a). The SPI-2 effector PipB2 was selected as an initial target as this effector is well characterized and has been shown to directly bind the molecular motor kinesin-1 16-18. We developed a bicistronic bacterial expression platform for PipB2-GFP11 and the fluorescent marker mCherry under the control of the endogenous *pipB2* promoter and the ribosomal promoter *rpsM* respectively. *Salmonella*

enterica serovar Typhimurium strain SL1344 null for the *pipB2* allele was restored with the low-copy plasmid pmCherry-PipB2-GFP11 (SL1344 PipB2-GFP11). The dual expression platform does not affect *Salmonella* growth rates *in vitro*, compromise the ratio of secreted SPI-2 effectors, perturb expression or translocation of another randomly selected SPI-2 effector, and impact invasion and replication in HeLa cells (Supplementary Fig. 1). Expression of non-fluorescent GFP1-10 in HeLa cells followed by infection with SL1344 PipB2-GFP11 led to robust PipB2-GFP_{comp} signals which labeled the SCV and associated membrane tubules (Fig. 1b), consistent with immunofluorescence studies at the same time post-invasion 18. Reliable fluorescence complementation could be detected in cells containing as few as 4-5 internalized bacteria. To address whether this system could be used to detect chromosomally expressed effectors, PipB2-GFP11 was integrated into the genome at its normal locus. Fluorescence complementation led to illumination of PipB2 on the SCV (Supplementary Fig. 2) however the membrane tubules were too dim to be resolved. As a control, we verified GFP fluorescence was not detected in *Salmonella* infected HeLa cells upon omission of any split-GFP component or deletion of the type-III secretion system (Supplementary Fig. 3).

Next we verified that tagging did not disrupt the function or localization of PipB2. PipB2 has been shown to be required for the dynamic positioning of the SCV during late stages (14 hours post-infection) of infection 19. Tagging of PipB2 with GFP11 did not significantly perturb the localization of SCVs and hence the infection-associated phenotype ascribed to PipB2 (Fig. 1c **and** Supplementary Fig. 4). Biochemically, PipB2 has been shown to bind kinesin, a plus-end directed microtubule motor protein 17 .Transient transfection of PipB2-GFP11 with GFP1-10, resulted in significant overlay with kinesin, (average Manders' correlation coefficient = 0.75 vs. 0.22 for overlap between free GFP and kinesin, *P* value < 0.05) (Fig. 1d) suggesting that the biochemical function of PipB2 is not affected by tagging. Although there are no antibodies to native PipB2, localization of PipB2-GFP_{comp} was analogous to that observed for chromosomally integrated PipB2-3xFLAG (Supplementary Fig. 5).

An important consideration for validating the utility of the split GFP system for tracking effectors is the kinetics of fluorescence complementation. Under our conditions fluorescence signals were detected as early as 4 hrs post invasion and up to the latest time point measured (24 hrs post-invasion) (Supplementary Fig. 6). Because PipB2 is a SPI2 effector, it is not translocated into the host immediately upon invasion. Instead, studies have demonstrated that epitope tagged PipB2 is faintly detected at 1-2 hours post-infection by Western blotting, but expression increases from 4 - 24 hours after invasion 19. Our data suggest fluorescence complementation occurs within 2 hrs of effector translocation and we are now testing this with effectors that are secreted immediately upon invasion. The GFP1-10 fragment is expressed at higher levels than the effector-GFP11 which should not limit the rate of complementation and enables illumination of secreted effectors throughout the course of infection (Supplementary Fig 7).

To demonstrate the broad scope of this methodology, two additional effectors were tagged with GFP11 and detected upon invasion of host cells. SteA, a poorly characterized effector reported to localize to the *trans*-Golgi Network (TGN) upon transient transfection 20,

resulted in fluorescent signals on the SCV and associated membrane tubules (Fig. 2a). To determine if SteA-GFP_{comp} co-localized with Golgi cisternae in the context of the entire effector cohort, we infected host cells containing the *trans*-Golgi marker GalT-mCherry. Indeed, we observed partial co-localization of SteA-GFP_{comp} with Golgi derived membranes (Fig. 2a). We also tagged SteC, an effector known to be essential for formation of SCV-associated actin nests 21. SteC-GFP_{comp} displayed fluorescent signal on the SCV and filamentous protrusions which colocalized with the actin marker coumarin-phalloidin and *Salmonella* (Fig. 2b). Together, our data indicate the split GFP system can be used on a diverse set of T3SS effectors for direct tracking of effector populations within live host cells.

Ectopic expression versus T3SS-dependent translocation

An advantage of this method is that it allows us to evaluate the importance of the mode of effector delivery (ectopic expression vs. translocation by *Salmonella*) and the role of the remaining effector cohort on PipB2 localization and dynamics. Transient transfection of PipB2-GFP11 and GFP1-10 gave rise to fluorescence at the cell periphery and perinuclear region, consistent with previous reports 16 and resulted in subtle movement of transfected PipB2-GFP_{comp}, particularly in ruffles at the cell periphery (Figure 1d and Supplementary Movie 1). Conversely, when PipB2-GFP11 was translocated by the T3SS along with other effectors during *Salmonella* infection, PipB2-GFP_{comp} localized to highly dynamic tubules projecting from the SCV in both HeLa and macrophage-like RAW264.7 cells (Fig. 3 and Supplementary Movies 2 and 3). These results confirm recent reports that *Salmonella* induced tubules are highly dynamic and present in both cell types 8,22. PipB2-GFP_{comp} dynamics closely match previously observed tubule dynamics, including multidirectional movement, growth, and retraction of pre-formed and nascent tubules. Likewise, PipB2 tubules appeared to move along microtubules (Supplementary Fig S8). PipB2-GFP_{comp} tubules were highly dynamic in the majority of infected cells at 4-12 hours post-invasion and motility decreased 12-24 hours post-invasion. However we observed variability in tubule dynamics and highly motile tubules were observed in a subset of cells > 12 hours post-infection, as previously reported 8,22. While previous studies detected tubules using fluid-phase tracers and host cell markers, our approach involves direct labeling of a specific effector which aids in the formation of these tubules. This enabled us to make the intriguing observation that PipB2-GFP_{comp} *itself* appears to move in both directions along microtubules suggesting a dynamic regulation of the kinesin-PipB2 interaction over the course of infection.

Infection of HeLa cells with a *Salmonella* strain null for *sifA*, an effector responsible for stabilizing SCVs and membrane tubules 23, resulted in loss of PipB2-GFP_{comp} tubules emanating from SCVs. However, we observed transient tubulation of PipB2-GFP_{comp} structures which collapsed or fragmented back to the origin of formation (Fig. 3c and Supplementary Movie 4), suggesting that perhaps SifA is not explicitly required for tubulation but rather stabilizes these transient structures.

Precise localization of effectors

To further characterize PipB2-GFP_{comp} tubules, we used two live-cell endocytic markers 8,22. As shown in Supplementary Fig. 9, Lysosome Associated Membrane Protein 1

(LAMP1-mCherry) and Alexa595 labeled Dextran co-localized with PipB2-GFP_{comp} tubules as previously reported 24. LAMP1-mCherry positive tubules co-localized with PipB2-GFP_{comp} in $65 \pm 5.5\%$ of cells. However, we routinely observed PipB2-GFP_{comp} tubules which did not produce any discernable signal for either LAMP1-mCherry or Alexa595-Dextran endosomal markers (Fig. 4; Supplementary Movies 5 and 6).

To gain insight into the origin of PipB2-GFP_{comp} tubules which were not co-localized with endocytic markers, we tested for the presence of Golgi markers which are typically segregated from endocytic membranes. As shown in Fig. 5, tubulated compartments positive for the *trans*-Golgi marker GalT-mCherry co-localized with at least one PipB2-GFP_{comp} tubule in $44 \pm 1.2\%$ of cells. We further found that SteA-GFP_{comp} co-localized with GalT-mCherry tubules in $52 \pm 16\%$ of infected HeLa cells (Fig. 5c). Conversely, SteA-GFP_{comp} tubules rarely ($3.3 \pm 2.8\%$) co-localized with the endocytic marker LAMP1-mCherry (Fig. 5; Supplementary Movie 7). Moreover, approximately 45% of SteA-GFP_{comp} tubules contained neither GalT-mCherry nor the LAMP1-mCherry marker. To demonstrate that some SteA-GFP_{comp} tubules lacked both endo- and exocytic markers tested, we performed three color live cell imaging analysis of SteA-GFP_{comp}, GalT-mCherry, and LAMP1-CFP. With both compartments labeled, we observed SteA-GFP_{comp} tubules emanate from the SCV which did not contain either marker (Supplementary Fig. 10). These results suggest SteA-GFP_{comp} is specifically recruited to GalT-mCherry positive membranes and is spatially segregated from LAMP1 positive endocytic membranes. Additionally, a subpopulation of SteA-GFP_{comp} tubules did not co-localize with either endocytic or Golgi markers highlighting the importance of directly following T3SS effectors themselves to explore how effectors mediate the formation of distinct subpopulations of these tubules. These studies show the unexpected result that some bacterial effectors segregate onto a subset of *Salmonella*-induced membrane tubules, with SteA found on membrane tubules containing a *trans*-Golgi marker but largely excluded from tubules containing endolysosomal markers; and PipB2 found on all tubules, regardless of “origin”. This demonstration of differential localization highlights that effectors can mediate subcompartmentalization of *Salmonella*-induced structures.

Tracking effector protein diffusion by FRAP

PipB2 has been shown to partition into detergent resistant microdomains and is predicted to possess membrane spanning regions 24. To explore the mechanism of PipB2-GFP_{comp} movement, we determined whether PipB2-GFP11 diffuses along tubules using fluorescence recovery after photobleaching (FRAP). Experiments have shown that tubules become less dynamic 12-16 hours post-invasion^{8,22}, although the differences between static and dynamic tubules is poorly understood. A stable tubule (at 16 hrs post-infection) was selected to prevent possible artifacts from the tubule moving out of the observation window. Upon photobleaching of PipB2-GFP_{comp}, directional recovery of fluorescence along pre-formed tubules was observed suggesting PipB2 protein diffuses laterally along membrane tubules (Fig. 6). These results support biochemical experiments suggesting PipB2 contains or is strongly associated with an integral membrane domain 24.

Discussion

To our knowledge, no methods currently exist to directly label SPI-2 effectors and track these effector populations over the course of infection at the single cell level. Yet recent studies highlight the intricate connection between effector localization and function 4,25,26. Moreover, localization of effectors may be dynamic and may change throughout the course on infection, highlighting the importance tracking these populations in living cells. The advantage of the split-GFP method described here is that it enables direct tracking of translocated effectors in the context of infection. Although we apply this method to SPI-2 effectors from *Salmonella*, it should be widely applicable to other gram negative pathogens possessing T3SSs and even other injection devices, such as the Type-IV and Type-VI Secretion Systems. However the method does have limitations. The most significant relates to the kinetics of complementation. Because reconstitution of fluorescence is not instantaneous, the method would not be appropriate for effectors whose actions are manifested within a short time frame (minutes) after translocation. Instead the biarsenical/tetracycline labeling system, which has been demonstrated to monitor translocation in *Shigella flexneri* 11 and *Salmonella* 10, might be a more appropriate system. We see split-GFP and biarsenical/tetracycline labeling technologies as complementary; combined they may enable researchers to track effectors throughout the infection cycle. Another potential limitation of the split-GFP system is that more robust fluorescence was observed when the effector and its endogenous promoter were incorporated into a low copy plasmid than when the tag was appended onto the genomically expressed effector. Although we demonstrate that the plasmid-based system does not perturb infection, it may be important to verify such controls for each labeled effector. Lastly, some effectors may be more amenable to tagging than others and in all cases it will be important to evaluate whether tagging perturbs effector function.

Ectopically expressed *Salmonella* effectors have been shown to localize to many subcellular compartments including the nucleus 27, plasma membrane 4, and endosomal compartments 7. SteA, an effector of unknown function which is required for full virulence in mice, has been shown to localize to the TGN of uninfected cells 20. However, our data indicate *Salmonella* derived and T3SS injected SteA localizes predominantly to the SCV and tubules, enriched in host *trans*-Golgi marker. These results suggest that SteA contains a cryptic Golgi targeting domain and may play a role in enabling *Salmonella* to hijack exocytic-derived membrane cargo to the SCV and tubules. It was recently demonstrated that the TGN associated host cell protein SCAMP3 was involved in the tubulation of post-Golgi vesicles 28 and contributed to the formation of *Salmonella*-induced tubules. Although in their studies the canonical TGN marker TGN46 did not co-localize with tubulated Golgi membranes 28 in *Salmonella* infected cells, immuno-electron microscopy demonstrates partial segregation of the two markers, TGN46 and GalT 29. Additional studies will be required to establish the nature of distinct Golgi and TGN cargo seized by *Salmonella* and used to potentially supply host factors required for virulence, membrane, and nutrients to the SCV. While many T3SS effectors have been shown to localize with the SCV and tubules when examined by immunofluorescence, it is not clear how specific biochemical functions can be performed in a directed fashion when antagonistic proteins are also present. Our data suggest one possible

mechanism for sorting effectors to ensure accurate timing of signaling events may be the selective targeting to specific populations of internal membrane signaling scaffolds.

\We hope this method will find widespread use in laboratories studying other gram-negative pathogens and symbionts possessing a T3SS. Furthermore, this method may be applicable to effector substrates from other secretion systems with structural requirements preventing canonical GFP tagging.

Methods

Methods and associated references are available online at <http://www.nature.com/naturemethods/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Online Methods

Plasmids

Construction of the effector GFP11 expression plasmids can be found in Supplementary Information. Plasmid pCMV-mGFP1-10 encoding the GFP1-10 fragment with mammalian optimized codons was purchased from TheraNostech (Albuquerque, NM). Plasmid pCLNCX was purchased from Imgenex (San Diego, CA). Plasmids pKD46, pKD3, and pKD4 were obtained from B. Wanner (Purdue University, West Lafayette, IN) by means of C. Detweiler (University of Colorado, Boulder, CO) and used as described for chromosomal recombination 30. Marker plasmid pLAMP1-mCherry (C-terminal FP fusion) was created using the Gateway system (Invitrogen) from human LAMP1 cDNA containing attB recombination sequences and pmCherry-DEST (pCDNA3.1(+) parent). Marker plasmid pGalT-mCherry encodes the targeting amino acids (1-60) of human galactosyltransferase and was obtained from J. Lippincott-Schwartz (NIH, Bethesda, MD) 31. We have described the generation of plasmid pAmCh previously 10.

Bacterial Strains, Culture Conditions, and Mutagenesis

Salmonella enterica serovar Typhimurium derived from the strain LT2, harboring plasmid pKD46, was used for all recombination exchanges described (λ red). Strain SL1290 was used for creation of P22 bacteriophage. The *Salmonella enterica* serovar Typhimurium wild-type strain SL1344 (Str^R) was used for P22 transduction of antibiotic resistance markers for gene knockout. Where indicated, strains carrying null mutations in the structural T3SS gene *ssaK* or the T3SS effector *pipB2* were created using recombinant exchanges with the

chloramphenicol resistance gene (CAT) as previously described 30. Gene deletions and insertions were confirmed by PCR using flanking gene specific primers. *S. Typhimurium* destined for mammalian cell infection assays were cultured in LB (EMD Chemicals, Gibbstown, NJ) supplemented with 300 mM NaCl (Sigma, St. Louis, MO) at 37°C. Ampicillin ($100 \mu\text{g}\times\text{mL}^{-1}$), chloramphenicol ($20 \mu\text{g}\times\text{mL}^{-1}$), kanamycin ($50 \mu\text{g}\times\text{mL}^{-1}$), and streptomycin ($50 \mu\text{g}\times\text{mL}^{-1}$) were used as needed.

Constructing the GFP11 and mCherry Expression Platform

The linker (GSSGGSSG) and GFP11 (RDHMETVLHEYVNAAGIT) 15 coding sequence with attB recombination sites were used for PCR of the kanamycin resistance gene from plasmid pKD4. The resulting amplicon was cloned into the vector pDONR221 by recombination using the Gateway system (Invitrogen, Carlsbad, CA). The resulting vector, pGFP11, encoded a flexible linker fused to the GFP11 peptide followed by the kanamycin resistance gene. The template vector pGFP11 was used for PCR with primers encoding 40-nt homology regions flanking the stop codon of the desired T3SS effector gene. Recombination of the effector stop codon with the linker and GFP11 coding sequence was performed by the λ red system as previously described 32. Transformants were selected with kanamycin and lysates were used directly in PCR reactions amplifying the coupled effector promoter, coding sequence, and GFP11 tag. The resulting amplicons were cloned directly into plasmid pAmCh using HindIII (NEB, Ipswich, MA) restriction sites. The resulting bicistronic expression vector yielded dual expression of mCherry under the control the strong ribosomal promoter (*PrpsM*) and a given T3SS effector fused at the C-terminus with the GFP11 epitope under control of the respective effectors native promoter. Plasmids were chosen with opposing mCherry and effector-GFP11 open reading frames eliminate potential read-through of the mCherry transcription platform. The resulting mCherry/effector-GFP11 expression vectors were electroporated into *S. Typhimurium* strain SL1344 (Str^R) and transformants were selected with ampicillin.

Growth Measurements

For comparison of the metabolic burden imposed by the dual expression mCherry and PipB2-GFP11 expression plasmid (above), growth in LB media was monitored by OD₆₀₀ measurements. For comparison between strains, SL1344 (WT), SL1344 (WT) mCherry, and SL1344 (WT) *pipB2* pmCh-PipB2-GFP11 were grown under antibiotic selection in LB media for 6 hours at 37° C with shaking. OD₆₀₀ measurements were recorded at the indicated time points. The growth measurements were performed in triplicate for each genotype.

SDS-PAGE SPI-2 Secretome

Salmonella strains: wild-type SL1344 and pmCh-PipB2-GFP11 *pipB2* SL1344 were cultured in Low Phosphate and Low Mg²⁺ media (LPLM) at 37°C with shaking to induce *Salmonella* Pathogenecity Island-2 T3SS expression 33. Culture supernatants were collected by centrifugation and 0.2 μm filtration to remove intact bacteria. Effector containing supernatants were concentrated using a 3 KDa molecular weight cutoff Amicon filter device

(Millipore, Billarica, MA). Protein samples were further concentrated by TCA precipitation and processed for 4-20% SDS-PAGE analysis. The gel was then visualized using SYPRO Ruby protein gel stain (Molecular Probes, Eugene, OR) with UV illumination according to the manufacturer's protocol.

Invasion and Replication Assay

HeLa cells were seeded into 12-well plates for each genotype for both invasion and replication assays. *Salmonella* strains were cultured under SPI-1 T3SS inducing conditions (0.3 M NaCl, LB pH 7.6, Low O₂) for the following genotypes: SL1344, SL1344 pmCh-PipB2-GFP11 *pipB2*, and SL1344 pmCh-PipB2-GFP11 *ssaK* (SPI-2 T3SS knock-out). HeLa cells at ~70% confluence were infected at a Multiplicity of Infection of 50 at 37 °C and 5% CO₂. The invasion was allowed to proceed for 15 minutes and extracellular bacteria were removed by vigorous washing followed by overlaying 100 µg × mL⁻¹ gentamycin in DMEM for 45 minutes at 37 °C and 5% CO₂. For measurements of invasiveness, each well was washed thrice with PBS prior to addition of 1% Triton X-100 for 5 minutes at room temperature to lyse host cells and liberate intracellular bacteria. For measurements of replication, each well was washed thrice with PBS and overlaid with 10 µg × mL⁻¹ gentamycin in DMEM and 10% FBS for 18 hours prior to lysis using the aforementioned conditions. Each genotype/replicate was chilled in PBS and dilutions were plated to determine the cfu × mL⁻¹. For comparison of the invasiveness of each genotype the cfu × mL⁻¹ was normalized to the bacterial inputs. Comparisons of the replication efficiency at 18 hours post-infection were normalized to their respective genotype means for invasion 1 hour post-infection. For both invasion and replication assays, all experiments were performed in triplicate for each genotype.

SCV Positioning Assay

The position of individual LAMP-1⁺ SCVs was determined as described previously 19. Briefly, HeLa cells were seeded onto coverslips 24 hours prior to infection. HeLa cells were infected at an MOI of 100 with the indicated *S. Typhimurium* strains expressing the mCherry bacterial marker. Twenty minutes post-infection, the media was replaced with fresh media containing 100 µg × mL⁻¹ gentamicin (Sigma) and after 1 hour this solution was replaced with 10 µg × mL⁻¹ gentamicin for the remainder of the infection. Infections were quenched at 8 and 24 hours post-inoculation with 3.0% paraformaldehyde. Cells were permeabilized with 0.1% saponin (Sigma) and immunolabeled with mouse anti-LAMP1 antibody H4A3 (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-mouse conjugated Alexa Fluor 488 as secondary (Invitrogen). Cells were also counterstained with Hoechst 3325 (2-4 µg × mL⁻¹) to label HeLa nuclei (Sigma). Coverslips were mounted with Fluoromount G (Electron Microscopy Sciences, Hatfield, PA) and imaged at 100×. Images were background subtracted and processed in ImageJ (NIH, Bethesda, MD) for overlay. LAMP1⁺ SCV distances from the nearest edge of the host cell nuclei were measured in ImageJ (NIH) with the standard line selection tool and calibrated by known pixel distances using a stage micrometer. Each genotype was scored in triplicate (n > 20 HeLa cells; n > 100 LAMP1⁺ SCVs per replicate) for each time point. The non-Gaussian data sets were analyzed by Kruskal-Wallis One-way ANOVA with Dunn's multiple comparisons posttest

and used to determine the statistical significance of mean SCV distances for each genotype and timed interval. Two-way ANOVA with Bonferroni posttest was used for determining the significance of binned SCV distances. *P* values of < 0.01 were considered significant.

Effector-3×FLAG Immunofluorescence and Western Blotting

Salmonella strains containing chromosomal 3×FLAG tagged effectors PipB2 and SseJ were generated as described previously 34. Strain PipB2-3×FLAG SL1344 expressing mCherry was used to infect confluent HeLa cell monolayers on coverslips at an MOI of 50. Cells were fixed at 16 hours post-infection with 3.7% formaldehyde, permeabilized with 0.25% Triton X-100 (Sigma), blocked with 10% BSA, incubated with mouse anti-FLAG M2 antibody (1:500; Sigma), and goat anti-mouse (Fab)₂-conjugated Qdot 800 secondary (1:100; Invitrogen) according to the manufacturers protocols respectively. Images were acquired in the mCherry channel as described above. Images in the Qdot 800 channel were acquired using excitation: 340/26 nm BP and emission: 700 nm LP filters (100 ms exposures). Strains containing the chromosomal SseJ-3×FLAG gene in the indicated strain genotypes were grown under invasion inducing conditions and used to infect fully confluent HeLa monolayers in 10 cm dishes (MOI = 100). After 10 hours of infection, cells were harvested in selective lysis buffer: 150 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% TritonX-100, and protease inhibitor cocktail (Roche) (Under these conditions, only the mammalian cell is lysed and intracellular bacteria remain intact). Lysates were clarified by centrifugation and the supernatant was filtered (0.2 µm PES membranes) to remove any residual bacteria. SPI-2 induced SseJ-3×FLAG bacterial lysates were prepared as described above. All lysates were concentrated by TCA precipitation and proteins were resolved by 4-20% SDS-PAGE. The samples were transferred to PVDF membrane for Western blotting analysis (anti-FLAG M2, 1:500 (Sigma); rabbit anti-mouse HRP conjugate, 1:5000 (Zymed)). Loading was either normalized using OD₆₀₀ (SPI-2 induced bacterial lysates) or measured using mouse anti-β-actin antibody (Sigma; 1:7,500) after stripping the anti-FLAG antibody.

Time-Lapse and Immunofluorescence Microscopy

Fluorescence imaging of bacterial effector localization and trafficking was performed using an Axiovert 200M wide-field (Zeiss), equipped with Lambda 10-3 filter changer (Sutter Instruments). This system is configured to allow rapid acquisition of fluorescence images in separate channels during live-cell imaging. Images were acquired with a Cascade 512B CCD camera at 5 MHz transfer speed (Roper Scientific) using METAFLUOR software (Universal Imaging). All images were obtained using either a 1.4 NA 40× or 100× PlanAPO objective and the following filter combinations: Coumarin/Hoechst 3325: 350/20 (excitation), 470/40 (emission) 430 (dichroic); GFP/Alexa Fluor 488: 480/20 (excitation), 520/10 (emission), 515 (dichroic); mCherry: 577/20 (excitation), 630/60 (emission), 595 (dichroic). A neutral density filter of 1.0 was used unless otherwise stated. Immunofluorescence colocalization of transfected PipB2-GFP_{comp} and Kinesin-1 Heavy-Chain was performed as previously described using mouse anti-Kinesin-1-HC (KN-01; Abcam) 16. Images were background subtracted, thresholded, and correlation coefficients were calculated using the ImageJ plugin: Colocalization Indices 35.

Live-Cell Effector Tracking Assay

SL1344 strains carrying the effector-GFP11 expression constructs were grown under invasion-associated conditions as described previously 36. HeLa epithelial and RAW264.7 macrophage-like cells were seeded onto 3.5 cm imaging dishes at $\sim 3 \times 10^5$ cells. HeLa cells were transfected 24 hours later with the GFP1-10 expression plasmid pCMV-mGFP1-10 using Transit LT1 (MirusBio). RAW264.7 cells were transduced 24 hours later with retrovirus derived from the GFP1-10 viral expression plasmid pCL-GFP1-10 and $5 \mu\text{g} \times \text{mL}^{-1}$ hexadimethrine bromide (Sigma) according to the manufacturers protocol (Imigenex). 24-36 hours post-transfection/-transduction, the media was replaced with complete media containing no antibiotic. Cells were infected with invasion induced SL1344 carrying the effector-GFP11 expression platform at an MOI of 20 to 100. The MOI for individual experiments is listed in each Figure caption. Invasions were allowed to proceed for 30-60 minutes before addition of $100 \mu\text{g} \times \text{mL}^{-1}$ gentamicin (Sigma). Under these conditions the typical number of internalized bacteria per mammalian cell was 4-8 and we did not observe significant host cell death at the time points tested. Killing of non-internalized bacteria was allowed to proceed for 45-60 minutes before addition of $10 \mu\text{g} \times \text{mL}^{-1}$ gentamicin for the remainder of the infection. At the indicated hour post-infection, infected cells were washed with Hank's Balanced Salt Solution with 10 mM HEPES pH 7.4 (HBSS; Gibco) which was subsequently used for imaging. Infected cells were imaged at room temperature (approximately 25°C). Because the PipB2 dynamics closely matched the tubule dynamics from prior studies conducted at 37°C 8,22, we do not believe the temperature difference has a significant effect on nature of tubule dynamics. Images were acquired every 5-10 s with exposures of 800 ms for the GFP and mCherry channels. When using mCherry endomembrane markers, exposures were reduced to 300-400 ms. For three color imaging experiments employing simultaneous GalT and LAMP1 imaging, LAMP1-CFP and GalT-mCherry were cotransfected with GFP1-10 into HeLa cells. Cells were infected 24-40 hours and imaged 48 hours post transfection. The cyan and green fluorescent protein channels were deconvoluted to correct for bleedthrough using cell samples lacking the respective components in separate experiments.

GFP1-10 Visualization

The coding sequence for GFP1-10 was PCR amplified and using a 5' primer containing a Kozak translation initiation site and coding sequence for the biarsenical binding tetracycline optimized motif (N-terminal to GFP1-10: MVSFLNCCPGCCMEPGS) 37. The resulting amplicon was subcloned into pCDNA3.1(+) using BamHI and EcoRI restriction sites resulting in the vector pTetCys-GFP1-10. About $3 \mu\text{g}$ of pTetCys-GFP1-10 vector was used for transfection of HeLa cells using Trans-IT LT1 according to the manufacturers protocol (MirusBio). Twenty-four hours post-transfection, cells were infected with invasion induced *Salmonella* strain PipB2-GFP11 (MOI = 50). After 7 hours of infection, cells were stained with $0.5 \mu\text{M}$ ReAsH (Invitrogen) in HBSS containing $10 \mu\text{M}$ 1,2-ethanedithiol (Sigma) at 37°C and 5% CO_2 . After one hour of staining, cells were washed thoroughly with 0.25 mM 2,3-dimercaptopropanol. The infected cell dish was then aspirated and replaced with complete media for 8 hours. Cells were imaged at 16 hours post-infection.

PipB2-GFP_{comp} fluorescence was collected as described above and ReAsH fluorescence wash collected using the aforementioned mCherry filter set and camera settings.

Fluorescence Recovery After Photobleaching Analysis

Complemented PipB2-GFP11 in *Salmonella* infected HeLa cells were prepared as described above. *Salmonella* induced filaments exhibiting no movement over 5 minutes were selected for bleaching analysis. The microscope iris was closed to the size of the filament to be bleached, neutral density filters were removed, and the filament was illuminated for 10 minutes with GFP excitation wavelength. Fluorescence recovery was then monitored in the GFP channel with 10 second acquisition intervals. LAMP1-mCherry was used to monitor the bleached filaments for movement over the duration of the experiment.

Abbreviations

SCV	<i>Salmonella</i> -Containing Vacuole
T3SS	type-III secretion system
GFP	green fluorescent protein
SPI-1 and SPI-2	<i>Salmonella</i> pathogenicity island-1 and -2

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Figure 1.

Split-GFP can be used to detect *Salmonella* effectors upon translocation into host cells. **(a)** General strategy for detecting T3SS delivered effectors in live cells. **(b)** PipB2-GFP11 is detected in HeLa cells 16 hours post-infection and co-localizes with mCherry expressing *Salmonella*, (arrows and overlay) (MOI = 50). **(c)** Average distance of SCVs from infected host cell nuclei Error bars represent s.e.m. of three independent experiments ($n = 100$ SCVs per replicate; $P < 0.0001$, ns = not significant) **(d)** Transfected PipB2-GFP_{comp} colocalizes with kinesin-1 by immunofluorescence in HeLa cells (arrows). Scale bars represent 10 μm .

Figure 2. *Salmonella* effectors SteA and SteC tagged with GFP11 are efficiently detected in host HeLa cells upon translocation by T3SS. **(a)** SteA-GFP_{comp} partially localizes with tubulated *trans*-Golgi membranes (GalT-mCherry). **(b)** SteC-GFP_{comp} co-localizes with coumarin-phalloidin labeled actin foci and the SCV. Both proteins were imaged at 16 hours post-infection with an MOI = 50. Scale bars represent 10 μm.

Figure 3.

Time-lapse microscopy of PipB2-GFP_{comp} reveals highly dynamic tubules in *Salmonella* infected host cells. **(a)** HeLa cells displaying PipB2-GFP_{comp} (green) and *Salmonella* (red) overlay. Time-lapse microscopy 16 hours post-infection (MOI = 50) shows a PipB2-GFP_{comp} tubule (white) emanate from the SCV with multidirectional movement, bifurcation, and temporary lariat formation. **(b)** Highly dynamic PipB2-GFP_{comp} tubules are also observed in macrophage-like RAW264.7 cells (abbreviated Mφ) at 14 hours post-infection (MOI = 20). **(c)** Transient PipB2-GFP_{comp} tubules in HeLa cells infected for 16 hours with SifA null *Salmonella* (MOI = 50). The overlay shows PipB2_{comp} (green) and LAMP1-mCherry (magenta). Scale bars represent 10 μm.

Figure 4.

PipB2-GFP_{comp} tubules do not always co-localize with endocytic markers. **(a)** Infected HeLa cells (MOI = 50; 14 hours post-infection) displaying PipB2-GFP_{comp} and LAMP1-mCherry 48 hours post-transfection. Time-lapse microscopy reveals a nascent PipB2-GFP11 tubule which excludes the late-endosomal/lysosomal marker LAMP1-mCherry (arrow). **(b)** Infected HeLa cell displaying PipB2-GFP_{comp} and the fluid-phase endocytic marker Alexa 568-dextran. Cells were loaded with labeled dextran for 24 hrs and imaged 18 hrs post-infection. PipB2-GFP_{comp} labels a dynamic tubule which does not co-localize with the dextran endocytic marker (arrowheads). Scale bars represent 10 μ m.

Figure 5.

Tagged *Salmonella* effectors co-localize with the *trans*-Golgi and the effector SteA is selectively segregated from the endocytic system. **(a)** PipB2-GFP_{comp} co-localizes with the *trans*-Golgi marker GalT-mCherry (arrowheads) and highlights tubulation of GalT-mCherry⁺ membranes. **(b)** SteA-GFP_{comp} co-localizes with LAMP1-mCherry on the SCV, but LAMP1-mCherry is excluded from SteA-GFP_{comp} tubules (arrowheads). **(c)** Proportion of tubules labeled with PipB2- and SteA-GFP_{comp} co-localizing with endocytic or *trans*-Golgi derived membranes. Imaging experiments were performed at 12-14 hours post-infection (MOI = 50). Error bars represent s.d. from 3 independent experiments (n = 20 cells per replicate; *P* < 0.001). Scale bars represent 10 μm.

Figure 6. Fluorescence recovery after photobleaching (FRAP) analysis of a representative PipB2-GFP_{comp} tubule. FRAP analysis shows directional recovery along a pre-formed filament. Similar results were obtained in 4 independent experiments. As control, LAMP1-mCherry (Magenta) shows bleached tubules remain static for the duration of the experiment. Image acquisition was performed at 16 hours post-infection (MOI = 50). Scale bar represent 10 μ m.