A NanoLuc luciferase-based assay enabling the real-time analysis of protein secretion and injection by bacterial type III secretion systems

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Abstract
The elucidation of the molecular mechanisms of secretion through bacterial protein secretion systems is impeded by a shortage of assays to quantitatively assess secretion kinetics. Also the analysis of the biological role of these secretion systems as well as the identification of inhibitors targeting these systems would greatly benefit from the availability of a simple, quick and quantitative assay to monitor principle secretion and injection into host cells. Here, we present a versatile solution to this need, utilizing the small and very bright NanoLuc luciferase to assess the function of the type III secretion system encoded by Salmonella pathogenicity island 1. Type III secretion substrate–NanoLuc fusions are readily secreted into the culture supernatant, where they can be quantified by luminometry after removal of bacteria. The NanoLuc-based secretion assay features a very high signal-to-noise ratio and sensitivity down to the nanolitre scale. The assay enables monitoring of secretion kinetics and is adaptable to a high throughput screening format in 384-well microplates. We further developed a split NanoLuc-based assay that enables the real-time monitoring of type III secretion-dependent injection of effector–HiBiT fusions into host cells stably expressing the complementing NanoLuc–LgBiT.

KEYWORDS
effector proteins, Luciferase, protein injection, protein secretion, Salmonella, Type III secretion system

1 | INTRODUCTION
The ability to secrete proteins to the bacterial cell surface, to the extracellular environment or even into target cells is one of the foundations of interbacterial as well as pathogen–host interaction. Protein export is particularly challenging for Gram-negative bacteria as two membranes of the bacterial cell envelope have to be passed. So far, nine different protein secretion systems, named type I–IX secretion systems (T1SS–T9SS), have been discovered in Gram-negative bacteria (Costa et al., 2015; Lauber, Deme, Lea, & Berks, 2018). Three of these systems, T3SS, T4SS and T6SS, provide direct application of effector proteins into target cells of either prokaryotic or eukaryotic origin (Galán & Waksman, 2018).

Due to its form and function, the type III secretion machine, as used by many enteric pathogens like Salmonella, Shigella, Yersinia or enteropathogenic Escherichia coli, is referred to as injectisome (Wagner...
et al., 2018). It is composed of a base that anchors the machine to the inner and outer membranes of the bacterial cell envelope (Marlovits et al., 2004), cytoplasmic components that serve in targeting and receiving of substrates (Diepold, 2019; Lara-Tejero, 2019), an inner membrane-localized export apparatus performing substrate unfolding and export (Minamino, Kawamoto, Kinoshita, & Namba, 2019) and a needle filament through which secreted substrates reach the host cell (Kubori et al., 1998; Figure 1a). Injection itself is mediated by a needle tip complex and by hydrophobic translocators forming pores in the host cell's target membrane (Picking & Barta, 2019). Type III secretion tip complex and by hydrophobic translocators forming pores in the bacterial inner membrane (Renault, Guse, & Erhardt, 2019). Secretion of substrates follows a strict hierarchy with early substrates building up the needle filament, intermediate substrates forming the needle tip and translocon pore and late substrates that serve as effectors inside the target cell.

While great progress has been made in elucidating assembly and structure of the type III secretion injectisome (Goessweiner-Mohr et al., 2019; Hu et al., 2018; Singh & Wagner, 2019), our understanding of its secretion mechanism lags behind, not least because of the challenge to quantitatively assess secretion function. Traditionally, T3SS function is assessed by SDS PAGE, western blotting and immunodetection of secreted substrates, either acid precipitated from the bacterial culture supernatant, or analysed in lysates of eukaryotic target cells (Collazo & Galan, 1997). This approach is time-consuming, at best semi-quantitative and lacks sensitivity. To facilitate a simplified analysis of principle secretion, injection and intracellular localization, several enzyme-linked and fluorescent reporters have been developed (Maffei, Francetic, & Subtil, 2017).

Ampicillin resistance conferred by β-lactamase fusions secreted into the periplasm was used to monitor the function of flagellar T3SS, which are closely related to T3SS of injectisomes (Lee & Hughes, 2006). Secretion into the periplasm through partially assembled injectisomes was assessed using PhoA fusions, instead (Diepold, Wiesand, Amstutz, & Cornelis, 2012). While these assays proved very valuable to address some specific questions, monitoring of secretion into the periplasm is only sensible for early substrates as switching to the secretion of later substrates does not occur without an assembled needle. High-throughput (HTP) assays for screening of T3SS inhibitors exploited the turnover of the fluorogenic substrate PED6 by a secreted phospholipase fusion (Fellise et al., 2008), the turnover of the chromogenic cephalosporin nitrocefin by a secreted β-lactamase fusion (Aiello et al., 2010) and the enzymatic uncaging of the fluorogenic substrate Glu-CyFur by a secreted carboxypeptidase fusion (Tsou et al., 2016; Yount et al., 2010).

Likewise, several reporter assays have been developed to assess the injection of T3SS effectors into eukaryotic host cells. Pioneering work by the Cornelis lab exploited the specific increase in intracellular cAMP levels upon injection of effectors fused to a calmodulin-activated adenylate cyclase (Cya; Sory & Cornelis, 1994). Later, this assay was also adapted to assay injection of effectors by T4SS (Nagai et al., 2008). While the Cya assay showed a very good signal to noise ratio (S/N) of several logs, it was not suitable to monitor injection kinetics or to be adapted for HTP screening because of a tedious cAMP analysis protocol. Widely used to assay injection of effector proteins in T3SS and T4SS is an assay that utilizes the enzymatic cleavage of the FRET-reporter cephalosporin CCF2 by injected β-lactamase fusions (Charpentin & Oswald, 2004). The CCF2 assay facilitated the analysis of injection kinetics and of intracellular accumulation levels of effectors (Mills, Baruch, Charpentin, Kobi, & Rosenshine, 2008). It was also successfully used for HTP high content screening of T3SS inhibitors (Harmon, Davis, Castillo, & Mecsas, 2010). Real-time observation of injection was achieved by direct fluorescent labelled of tetracycline motif-tagged effectors (Enninga, Mounier, Sanonsonetti, & Tran Van Nhieu, 2005). However, since this approach requires multidimensional time-lapse microscopy, it is not feasible for routine analysis of effector injection or HTP. Split GFP technology (Van Engelenburg & Palmer, 2010) and self-labeling enzyme tags (Göser, Kommnick, Liss, & Hensel, 2019) were successfully used to monitor intracellular localization of effector proteins but both techniques are not optimal for the analysis of translocation kinetics: split GFP because of a low sensitivity and the slow kinetics of GFP complementation, and the self-labelling enzyme tags because labelling can only be done with effectors that have already been translated before host cell contact.

We aimed to develop a T3SS assay based on effector–luciferase fusions to enable a simple, quantitative and HTP-compatible assessment of principle secretion and injection. The advantage of luciferase-reporters is a very high S/N and sensitivity. In addition, luciferase-based assays benefit from the lack of product (light) accumulation, simplifying the analysis of secretion and injection rates. We developed a secretion assay utilizing NanoLuc (NLuc) luciferase, an engineered 19 kDa glow-type luciferase from the deep-sea shrimp Ophlophorus graciliros-tris that converts furimazine, emitting blue light (Hall et al., 2012). The NLuc-based secretion assay allowed quantification of minute amounts of secreted effectors either in the supernatant of the bacterial culture or within eukaryotic host cells. The assay’s ultra-high sensitivity, its wide dynamic range and quick response dynamics qualify it as an enabling technology to elucidate the mechanisms of secretion and injection of T3SS and is likely adaptable to assay secretion through other bacterial secretion systems.

2 | RESULTS

2.1 | Assessment of effector–luciferase fusion proteins as reporters for type III secretion

In order to identify a luciferase compatible with type III secretion through the T3SS encoded by Salmonella pathogenicity island 1 (SPI-1, T3SS-1), we evaluated six different commercially available luciferases as effector-fused secretion reporters: Cypripedina luciferase (CLuc), Guassia princeps luciferase (GLuc), Guassia dura luciferase (GDLuc), NLuc, Renilla luciferase (RLuc) and Red Firefly luciferase (RFLuc; Fan & Wood, 2007; Hall et al., 2012; Lorenz, McCann, Longiaru, & Cormier, 1991; Tannous, Kim, Fernandez, Weissleder, & Breakfield, 2005; Thompson, Nagata, & Tsuji, 1989). We generated translational fusions of the effectors SipA and SopE, respectively, coupled at their C-termi
Assessing different luciferases as reporters for type III secretion. Cartoon of the T3SS injectisome. Names or proteins mentioned herein are shown according to the unified nomenclature. The figure is adapted from reference (Wagner et al., 2018).

(a) Proteins of whole cell lysates and culture supernatants of S. Typhimurium expressing the indicated SipA–Luc and SopE–Luc fusions were analysed by SDS PAGE, western blot and Immunodetection with an anti-myc antibody. The calculated masses for the tested luciferase fusions are: SipA–NLuc: 94 kDa, SipA–RFLuc: 137 kDa, SipA–GDLuc: 96 kDa, SipA–GLuc: 96 kDa, SipA–RLuc: 111 kDa, SipA–CLuc: 137 kDa, SopE–NLuc: 47 kDa, SopE–RFLuc: 90 kDa, SopE–GDLuc: 50 kDa, SopE–GLuc: 50 kDa, SopE–RLuc: 64 kDa, SopE–CLuc: 90 kDa.

(b) Signal to noise ratios (wt/ΔsctV) of luciferase activities of secreted SipA–Luc and SopE–Luc fusions were graphed. Bar graphs represent the mean S/N of three independent measurements.

(c) Immunodetection of SipA–NLuc myc and SctE on western blot of SDS-PAGE separated culture supernatants and whole cell lysates, either expressing SipA–NLuc myc from a plasmid or from the chromosome.

(d) Signal to noise ratios (wt/ΔsctV) of luciferase activities of secreted SipA–NLuc either expressed from a plasmid or from the chromosome, each with or without flagella (flhD) were graphed. Bar graphs represent the mean S/N of three independent measurements.

(e) SipA–NLuc myc secretion in S. Typhimurium Para hilA and in S. Typhimurium ΔsctDFIJ, Para hilA with and without flagella (flhD) respectively. Bar graphs represent mean (±standard deviation) of three technical replicates. Asterisks indicate statistical significance of SipA–NLuc myc secretion assessed by Student's t test. *p ≤ .05.

CLuc, Cypridinia Luciferase; GDLuc, Gaussia Dura Luciferase; GLuc, Gaussia princeps Luciferase; NLuc, Nanoluc; ns, nonsignificant; RFLuc, Red Firefly luciferase; RLuc, Green Renilla Luciferase; S/N, signal to noise [Colour figure can be viewed at wileyonlinelibrary.com]
to a luciferase and a myc epitope-tag. The effector–luciferase fusions were expressed from a rhamnose-inducible promoter on a low-copy number plasmid in wild-type S. Typhimurium and in a secretion deficient mutant (ΔsctV). The expression and type III-dependent secretion of the effector luciferase fusions was assessed by SDS PAGE, western blotting and immunodetection of the myc epitope tag in whole bacterial cells and culture supernatants, respectively, after 5 hr of growth. All effector–luciferase fusions could be detected at the expected molecular mass in whole cells and in culture supernatants, indicating their productive expression and secretion (Figure 1b). CLuc and RFLuc showed additional bands likely corresponding to the cleaved luciferase-myc. In general, SipA–luciferase fusions were secreted more efficiently than SopE fusions. SipA and SopE fusion with CLuc as well as SopE fusions with NLuc and RLuc could only be detected in very low levels in the culture supernatants (Figure 1b).

The activity of the secreted luciferases in filtered culture supernatants of the S. Typhimurium wild type and of the ΔsctV mutant, respectively, was assessed by luminometry using the specified conditions for each luciferase. The S/N (wild type vs. ΔsctV) was highest with effector–NLuc fusions (SipA–NLuc S/N = 45, SopE–NLuc S/N = 22), and, with the exception of GDLuc, always higher for SipA-luciferase fusions (Figure 1c).

Since the SipA–NLuc fusion showed the best S/N, we introduced SipA–NLuc–myc into the chromosome of a S. Typhimurium wild-type strain and of a ΔsctV mutant for further analysis. First, we compared the expression and secretion of plasmid and chromosome-encoded SipA–NLuc, respectively, and as a reference also of the secreted translocator SctE, by SDS PAGE, western blotting and immunodetection. SipA–NLuc was expressed well from the chromosome even though, not unexpectedly, at lower levels compared to its expression from the plasmid (Figure 1d). The extent of T3SS-dependent secretion of plasmid and chromosome-encoded SipA–NLuc was indistinguishable (Figure 1d).

We next evaluated the S/N of the secreted SipA–NLuc fusion when expressed from plasmid or chromosome by measuring the NLuc activity in filtered culture supernatants of the wild-type and the ΔsctV mutant. While plasmid-based expression resulted in a S/N = 45, plasmid-based expression even achieved a S/N = 200 (Figure 1e). The stronger plasmid-based expression may lead to a greater liberation of SipA–NLuc upon occasional cell lysis, compromising the S/N.

Both, injectisomes and flagella possess T3SS for the export of proteins and it has been shown that substrates of one system may be secreted by the other one to a limited degree (Ehrbar, Winnen, & Hardt, 2006; Young & Young, 2002). In order to test the versatility of NLuc as secretion reporter, we, also constructed fusions with the early T3SS substrate SctP (needle length regulator) and with the intermediate substrate SctA (tip protein). While NLuc compromised secretion and function of SctP when fused to its C-terminus (Figure S1aB), SctA–NLuc fusions were readily secreted, even when NLuc was placed at different positions within the polypeptide chain of SctA (Figure S1cD). To overcome the limitation of NLuc in supporting secretion of SctP, we utilized a split NLuc approach. Split NLuc is composed of a large fragment (LgBiT, 18 kDa) comprising most of the protein’s beta barrel and of a small fragment with a high affinity to the LgBiT (HiBiT, 1.3 kDa), comprising only one beta strand (Schwinn et al., 2018). SctP–HiBiT fusions were successfully secreted into the culture supernatant and strong luminescence was detected when complementing SctP–HiBiT with LgBiT (Figure S1a,b), showing that split NLuc can serve as a secretion reporter when NLuc fails.

In summary, we could show that luciferases are versatile reporters for T3SS and that effector–NLuc fusions report on secretion with a very high S/N, even in the absence of plasmid-based overexpression.

2.2 | Assessment of the sensitivity of the NLuc-based secretion assay

One handicap of the traditional, western blot-based secretion assay is its low sensitivity that impedes analysing low culture volumes as required for the analysis of secretion kinetics or for the development of HTP screens.

In order to compare the sensitivity of the western blot- and the SipA–NLuc-based secretion assays, we made a serial dilution of the filtered supernatant of wild-type and ΔsctV S. Typhimurium cultures grown for 5 hr. In the western blot-based assay, we could detect the intermediate substrate SctE down to a supernatant volume of 113 µl and the early substrate SctP as well as the late substrate SipA–NLuc down to 225 µl (Figure 2a). In contrast, using the SipA–NLuc assay, we were able to obtain a stable S/N = 200 down to 195 µl.
supernatant volume. The S/N even remained above 50 when assaying an equivalent of only 24 nl (Figure 2b).

Next, we assessed the performance of the SipA–NLuc assay in monitoring the onset kinetics of type III secretion, which requires very high sensitivity due to the small amounts of secreted material that is initially present. To this end, we grew S. Typhimurium harbouring arabinose-controlled HilA to an $A_{\text{600}} = 0.9$, after which expression of the pathogenicity island was induced by the addition of 0.02% (w/v) arabinose. Bacterial cells and culture supernatants were collected every 10 min and kept on ice until reading at the end of the experiment. Induction of SPI-1 was monitored by western blot and immunodetection of the base component SctJ in whole cells. It was first observed 30 min after the addition of arabinose (Figure 2c). Only 10 min later, luminescence of SipA–NLuc was detected in the culture supernatant, then luminescence increased steadily to the end of the measurement after 120 min (Figure 2c). This increase in luminescence correlates directly with SipA–NLuc secretion and is not influenced by NLuc maturation or turnover as the activity of

**FIGURE 2** Assessment of the sensitivity of the NLuc secretion reporter. (a) Immunodetection of the T3SS substrates SctP, SctE and SipA–NLuc\textsuperscript{myc} on a western blot of SDS-PAGE separated, serially diluted culture supernatants. (b) Luminescence of secreted SipA–NLuc\textsuperscript{myc} in serially diluted culture supernatants of the S. Typhimurium wild type and a ΔsctV mutant. The technical noise of 96 RLU was subtracted for each measurement. Triangles show the calculated signal to noise ratios for each dilution. Data represent the mean (±standard deviation) of three technical replicates. (c) Normalized luminescence of secreted SipA–NLuc\textsuperscript{myc} at different time points after induction of hilA with 0.02% arabinose. Experiments were normalized by setting the maximum luminescence of each experiment to 1. The data points represent mean (±standard deviation) of four independent measurements. At each time point, samples of whole cell lysates were taken for immunodetection of SctJ.
NLuc remains stable in the culture supernatant over extended periods of time (Figure S2).

Both experiments, serial dilution and secretion kinetics, prove the superior sensitivity of the NLuc-based over the traditional secretion assay. While the detection of secreted substrate proteins using the traditional assay requires larger volumes and accumulation of substrates in the culture supernatant for an extended period of time, the NLuc assay allows detection of secretion in very small volumes, in brief intervals and with very short handling times (10 min after collection of supernatant). Our results also show that induction and assembly of the megadalton injectisome is a very quick process that gets bacteria rapidly armed for attack.

2.3 Application example: Harnessing the NLuc secretion assay for high throughput screening

The high sensitivity and the short handling time of the SipA–NLuc-based secretion assay provided an excellent basis to develop a HTP assay for drug screening in a 384-well microplate format.

Centrifugation or filtering is not feasible for separation of bacterial cells and culture supernatant in a microplate format. In order to overcome this problem, we made use of the high-protein binding capacity of the microplates and tested whether secreted substrates would specifically bind to the plate wall after being secreted (Figure 3a). To this end, 50 µl of S. Typhimurium wild-type and ΔsctV mutant cultures were grown in white high protein binding 384-well plates. Bacteria were washed out of the wells after 5 hr of growth using a microplate washer. Then, PBS, NLuc buffer and NLuc substrate were supplied to each well and the luciferase activity was measured. Using this set-up, a S/N = 37 could be achieved (Z’ = 0.8), which is excellent for HTP screening (Figure 3b).

To assess the robustness of this assay and the variation across the plate, we filled an entire 384-well plate with 50 µl of a S. Typhimurium, SipA–NLuc culture and allowed it to grow for 5 hr at 37°C. Luminescence of secreted, wall-bound SipA–NLuc was assessed after washing out bacteria as described above. The assay proved very robust with a coefficient of variation of 7% over the entire plate and with little edge effects (Figure 3c, Table S1). We then performed a proof-of-concept inhibitor screen by assessing the

**FIGURE 3** Development of a SipA–NLuc-based HTP secretion. (a) Cartoon of the assay set-up. S. Typhimurium expressing SipA–NLuc was grown in a 384-well microplate format. Secreted SipA–NLuc bound to the wall of the high protein-binding microplate. Bacteria were washed out and luminescence was measured. (b) Luminescence and signal to noise ratio of secreted SipA–NLuc. Bacteria were grown in white high protein-binding 384-well plates. Bacteria were washed out and luminescence was measured. (c) Signal variation of SipA–NLuc secretion assayed over an entire 384-well microplate as shown in (a). The grayscale shows the luminescence of each well relative to the average luminescence of all wells of the plate. (d) SipA–NLuc secretion in response to treatment with 37 different bioactive compounds, assayed as shown in (a). Positive and negative controls (wild type and ΔsctV mutant, both treated with 1% DMSO) are not shown on this image but where set to 100% and 0% respectively. The grayscale shows the luminescence of each well relative to the DMSO-treated positive control. The layout of the plate is shown in Table S1. (e) Comparison of the results of two independent compound screens as in (d). The R² value was calculated from a linear regression [Colour figure can be viewed at wileyonlinelibrary.com]
effect of a range of 37 different bioactive compounds on the activity of the T3SS in the 384-well format (Table S2, Figure 3d). The bioactive compounds were selected to represent a wide range of biological activities, including several antibiotics and membrane active substances, to ensure detection of positive and negative results in a range of different biological assays (more details can be found in Table S2). Each well of the plate was printed with 0.5 µl of a compound in 100% DMSO, to which 50 µl of a S. Typhimurium, SipA–NLuc culture was added. Again, the culture was allowed to grow for 5 hr, after which secretion of SipA–NLuc was assessed by luminometry. The assay showed a highly dynamic response from 10% to 120% secretion activity compared to the DMSO-treated wild-type control (Figure 3d). Detection of SipA–NLuc was most strongly reduced by the flavonoids quercetin (30 µg/ml, 90% reduction) and scutellarin (10 µg/ml, 75% reduction), which confirms the previously reported observation that flavonoids target T3SS (Tsou et al., 2016). Also treatment with the 3-hydroxy-3-methylglutaryl (HMG) coenzyme A reductase-blocker simvastatin reduced detection of SipA–NLuc by 44%. Replication of the screen proved a high reproducibility of the assay with a R² of 0.915 (Figure 3e).

Over all, the SipA–NLuc assay proved to be highly adaptable to a high throughput screening format in 384-well plates, featuring a high S/N, a low error across the plate, a great reproducibility and requiring only short hands-on time.

2.4 | Application example: Assessment of the PMF-dependence of type III secretion by the NLuc secretion assay

It has been known for long that secretion through T3SS depends on two sources of energy, on the hydrolysis of ATP by the system’s ATPase (FliI in flagella, SctN in injectisomes) and on the PMF (Akedo & Galán, 2005; Minamino & Namba, 2008; Paul, Erhardt, Hirano, Blair, & Hughes, 2008; Radics, Königsmaier, & Marlowsits, 2014; Shen and Blocker, 2016), which itself is composed of the ∆pH, that is, the proton concentration gradient across the membrane, and the ∆Ψ, the electric potential difference between the periplasm and cytoplasm. The contribution of these two PMF components to T3SS function can be dissected with specific inhibitors. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is a MF uncoupler (ionophore) and discharges both the ∆pH and the ∆Ψ by transporting protons through the membrane (Heytler & Prichard, 1962). At acidic pH, potassium benzoate is a weak acid and can enter the membrane and discharge the ∆pH (Kihara & Macnab, 1981). Valinomycin can shuttle potassium ions across the membrane, which collapses the electric potential difference ∆Ψ (Pressman, Harris, Jagger, & Johnson, 1967). Evaluating the contribution of each PMF component to T3SS function requires the careful analysis of secretion kinetics, for which the classical, semi-quantitative western blot-based secretion assay is not well suited, but for which the NLuc-based secretion assay proved very powerful. To further show this, CCCP, potassium benzoate and valinomycin, respectively, were added to the bacterial culture at different concentrations, 60 min after induction of SPI-1 (for experimental details, please refer to the methods section), while samples of culture supernatants were taken every 10 min for subsequent analysis of the luminescence of secreted SipA–NLuc. While SipA–NLuc secretion progressed over time in the control sample (Figure 4), addition of the inhibitors lead to sudden changes in secretion kinetics. CCCP blocked secretion instantly, even at concentrations of 5 µM, showing the critical relevance of the PMF for type III secretion (Figure 4a). Discharging the ∆pH by potassium benzoate resulted in a concentration-dependent instant reduction of secretion (Figure 4b). At 20 mM potassium benzoate, secretion was completely abolished while it proceeded at 60% of the untreated control in the presence of 5 mM and at 10% in the presence of 10 mM potassium benzoate. Collapsing the electric potential by valinomycin lead to a concentration-dependent reduction of secretion (Figure 4c): in the presence of 20 µM valinomycin, no significant change in secretion rate was observed, while 40 µM and 60 µM valinomycin, respectively, lead to 70% and 40% secretion of the untreated control.

These results show that both components of the PMF, ∆pH and ∆Ψ, contribute to energizing secretion in the SPI-1-encoded T3SS of S. Typhimurium. As the PMF-compromising compounds took effect so quickly after treatment, it is highly unlikely that the PMF-dependence of type III secretion is the consequence of a secondary effect of PMF reduction like ATP or nutrient depletion – an issue that could only be resolved with the sensitive and highly time-resolved NLuc secretion assay. These results open the door for further experiments dissecting the role of the different T3SS components in utilizing the PMF.

2.5 | Development of NLuc-based host cell injection assays

Assessment of secretion of T3SS substrates into the culture supernatant is very useful for investigating the basic secretion mechanism of T3SS, however the intended biological function of T3SS injectisomes is the injection of effector proteins into host cells. Since the SipA–NLuc-based secretion assay proved to be very sensitive and simple, we attempted to adapt the assay to monitoring the injection of SipA–NLuc into host cells.

In a first and simple approach, we infected HeLa cells in 96-well plates at an MOI = 50 with SipA–NLuc-expressing S. Typhimurium, using wild-type bacteria and secretion-deficient ΔstcV mutants. After infection for 60 min, attached bacteria were gently washed off with PBS using a microplate washer and subsequently, the HeLa cell-associated luminescence was measured using live cell buffer (Figure 5a). The non-secreting ΔstcV mutants (Figure 5b) showed a HeLa cell-associated luminescence of 8% of the wild type, corresponding to a S/N = 12 (Figure 5c). To determine whether the HeLa cell-associated signal was truly resulting from injected SipA–NLuc, we assessed injection in a set of mutants that are capable of secreting SipA but incapable of injecting it into host cells: a needle tip-deficient ΔstcA, a translocon-deficient ΔstcEBA and a
gatekeeper-deficient ΔsctW mutant. While secretion of SipA–NLuc into the culture supernatant was increased between two- and five-folds in ΔsctA, ΔsctEBA and ΔsctW mutants (Figure 5b), which are reportedly unlocked for secretion of late substrates like SipA (Kubori & Galán, 2002; Lara-Tejero, Kato, Wagner, Liu, & Galán, 2011), the HeLa cell-associated luminescence was strongly reduced to 9%–24% of the wild type when infecting with these mutants (Figure 5c). From these results we can conclude that the luminescence signal obtained from infection with wild-type S. Typhimurium resulted to more than 90% from injected SipA–NLuc and that only little signal may stem from bacteria remaining attached to HeLa cells or to the plate even after washing. Over all, this NLuc-based injection assay proved very useful for the quick and simple assessment of translocation of effectors into host cells by an end-point measurement, however the kinetics of injection cannot be assessed by this assay due to the required washing step.

To gain a higher specificity for the signal of injected SipA and enable analysis of injection kinetics, we employed the split version of the NLuc luciferase. To this end, SipA was fused to HiBiT, while LgBiT was expressed stably by the HeLa cell line. Complementation of LgBiT with HiBiT to a functional luciferase should only occur inside the HeLa cells after translocation of SipA–HiBiT (Figure 5d). We first tested the secretion of SipA–HiBiT into the culture supernatant by providing LgBiT to the assay buffer. Similar to what was observed for SipA–NLuc, secretion of SipA–HiBiT into the culture supernatant was increased between two- and sixfolds in ΔsctA, ΔsctEBA and ΔsctW mutants respectively (Figure 5e). However, in contrast to the SipA-NLuc-based injection assay, none of the T3SS mutant strains yielded any detectable luminescence in the split NLuc assay (Figure 5f), making this assay highly suitable for monitoring the specific injection of T3SS effectors into host cells. This set-up even allowed us to follow the kinetics of SipA–HiBiT injection over time directly in a microplate reader (Figure 5g).

3 | DISCUSSION

The elucidation of the molecular mechanisms of secretion through T3SS and other bacterial protein secretion systems is impeded by a shortage of assays to quantitatively assess secretion kinetics. Also the analysis of the biological role of these secretion systems as well as the identification of inhibitors targeting these systems would greatly benefit from the availability of a simple, quick and quantitative assay to monitor principle secretion and injection into host cells. Here, we present a versatile solution to this need, utilizing the small and very bright NLuc luciferase to assess secretion and injection through the T3SS encoded by SPI-1 of S. Typhimurium. Secretion of a SipA–NLuc fusion showed a very high S/N and sensitivity down to the nanolitre scale, making it exquisitely suited for the assessment of secretion kinetics. In addition, the NLuc-based secretion assay proved highly adaptable to a HTP screening format in 384-well
**FIGURE 5** Development of NLuc-based host cell injection assays. (a) Cartoon showing set-up of NLuc injection assay. S. Typhimurium expressing SipA–NLuc was allowed to infect HeLa cells for 60 min. SipA–NLuc was injected into HeLa cells by use of the T3SS injectisome. Bacteria were washed away using a microplate washer and subsequently NLuc luminescence was measured. (b) Luminescence of SipA–NLuc secreted by the S. Typhimurium wild type and indicated mutants in the absence of host cells. The measurement was done after 3.5 hr of growth, just before infection of HeLa cells. The luminescence of the wild type was set to 100%. (c) Luminescence of SipA–NLuc injected into HeLa cells by the S. Typhimurium wild type and indicated mutants. The experimental set-up was as shown in (a). The luminescence of the wild type was set to 100%. (d) Cartoon showing set-up of split NLuc (HiBiT) injection assay. S. Typhimurium expressing SipA–HiBiT was allowed to infect HeLa cells (expressing LgBiT) for 60 min. SipA–HiBiT was injected into HeLa cells by use of the T3SS injectisome. Luminescence of the complemented split NLuc was measured. (e) Luminescence of LgBiT-complemented SipA–HiBiT secreted by the S. Typhimurium wild type and indicated mutants in the absence of host cells. The luminescence of the wild type was set to 100%. (f) Luminescence of SipA–HiBiT injected into LgBiT-expressing HeLa cells by the S. Typhimurium wild type and indicated mutants. The experimental set-up was as shown in (d). The luminescence of the wild type was set to 100%. (g) Luminescence of SipA–HiBiT injected into LgBiT-expressing HeLa cells by the S. Typhimurium wild type and the ∆sctV mutant. At timepoint zero, HeLa cells were infected with S. Typhimurium after which cells were incubated inside a microplate reader at 37°C (w/o CO₂) in the presence of NLuc substrate. Luminescence was followed in 2 min intervals. Values of the ∆sctV mutant were set to zero for each time point. The results show the mean of technical triplicates. Bar graphs represent mean (± standard deviation) of three independent measurements. Asterisks indicate statistical significance between wild type and mutant strains assessed by a Students t test, *** p ≤ .001; ** p ≤ .01 [Colour figure can be viewed at wileyonlinelibrary.com]
microplates. We further developed NLuc and split NLuc-based assays that enable the monitoring of T3SS-dependent injection of effector proteins into host cells.

An optimal assay to monitor protein secretion would feature: (a) A lack of signal from the un-secreted reporter, resulting in a high S/N. (b) A small reporter that does not interfere with secretion through the secretion system of interest. In case of T3SS, this also includes a not too fast and tight folding inside bacteria as only unfolded protein can be secreted and as the unfolding capacity of the system is not very high. (c) A fast and efficient folding of the reporter outside of the bacterium, guaranteeing fast response dynamics. (d) An intrinsic signal of the reporter, not necessitating an enzyme substrate. (e) A high sensitivity. (f) A lack of accumulation of product of the reporter's reaction. And (g) Be quick, simple and needing only short hands-on time.

While fluorescent proteins would be desirable secretion reporters as they benefit from an intrinsic signal (and thus do not come with the problem of accumulation of product of the reporter's reaction), they often suffer from a very slow maturation time and/or insufficient brightness. In addition, fluorescent proteins tend to form very stable β-barrels that block secretion through T3SS (Radics et al., 2014), excluding them as secretion reporters, at least for T3SS. While the use of split GFP can overcome the limitation associated with tight folding, slow complementation and maturation of GFP compromise its use. The NLuc-based secretion assay as presented herein matches most of the needs listed above. While NLuc lacks an intrinsic signal and requires the addition of a substrate, the analysis of secretion by this assay is not complicated by the overlay of the kinetics of the reporting enzyme and the kinetics of secretion, as it is in other enzyme-linked secretion assays. Instead, the measured signal of the NLuc assay is directly proportional to the amount of accumulated secreted protein. This advantage, together with the superior sensitivity, yield a very high dynamic range of the NLuc secretion assay.

We demonstrated that the NLuc secretion assay is highly suited to study the kinetics of secretion due to its superior sensitivity. Our simple assay set-up only allowed deduction of secretion kinetics from the accumulation of NLuc in the culture supernatant but culturing bacteria in a microfluidics system could enable the direct and online reading of secretion into the medium flow through and by this facilitate an even better resolved analysis of the mechanism of secretion.

Our experiments show that secretion of NLuc is supported by fusion to a range of intermediate and late T3SS substrates, even within a polypeptide chain, but fails to be secreted when fused to the early substrate SctP. It is conceivable that the mode of early substrate secretion does not provide a sufficient unfolding capacity to support secretion of NLuc, while this seems not a problem when NLuc is fused to intermediate and late substrates. Interestingly, a Yersinia SctP–PhoA fusion was secreted (Diepold et al., 2012), pointing either to a higher unfolding capacity of the Yersinia T3SS or to a weaker fold of PhoA. We could overcome the limited use of NLuc as secretion reporter for early substrates using the split NLuc technology instead.

The 11 amino acid-long HiBiT was accommodated well by SctP and it is conceivable that this very small piece allows secretion in most circumstances.

In its current form, the NLuc secretion assay requires the separation of bacteria and supernatant to achieve a good S/N because of the membrane-permeating properties of the NLuc substrate. A membrane impermeant NLuc substrate could overcome this limitation, would make NLuc-based secretion assays even more simple and versatile, and increase their robustness due to less steps of handling.

In addition to the points important for a secretion assay, an optimal injection assay would also: (a) Feature a high specificity for injected effectors as opposed to secreted but not injected ones. (b) Allow the analysis of injection kinetics. And (b) Allow localization of the injected protein, at best at single molecule resolution.

While fluorescence-based assays proved highly suitable to study the localization dynamics of injected effectors inside host cells, they are very limited in their use to study injection kinetics and are always instrumentation-demanding. The CCF2-based injection assay features simple handling, instead, and proves very useful for the analysis of injection, but suffers from high costs of CCF2 and a low dynamic range. In addition, the product accumulation resulting from the enzymatic activity of the injected β-lactamase complicates the analysis of injection kinetics. The herein-presented NLuc-based injection assays offer very quick and simple analysis, even of injection kinetics, and feature a high dynamic range and sensitivity. While a high-resolution analysis of the localization of the effector–NLuc fusions inside host cells is not supported by these assays, microscopic set-ups exist that utilize luminescence for long-duration monitoring of single cells (Horibe et al., 2018). Long-duration monitoring of luminescence is facilitated by the recently introduced NanoLuc substrates Endurazine and Vivazine that exhibit an extended life-time of up to 72 hr. Unfortunately, the brightness of these novel substrates is up to 10-fold lower in the first hour compared to the herein used furimazine, making them less suitable for monitoring the initial phase of injection.

As performed herein, cytoplasmic expression of LgBiT will only generate luminescence if the HiBiT of the injected effector also localizes to the cytoplasm. However, the split NLuc injection assay may also be utilized to analyse the localization and topogenesis of effector proteins inside host cells by targeting LgBiT to specific organelles instead (Figure 6). Furthermore, complementation of LgBiT by the low-affinity SmBiT instead of the high-affinity HiBiT may provide a useful tool to investigate effector–host protein interactions in vivo by bimolecular complementation (Dixon et al., 2016).

In summary, our data show that NLuc fusions of secreted substrate proteins can be used as a robust, versatile, cheap, simple and quick reporter for T3SS secretion and injection that will enable future in-depth elucidation of T3SS function (Figure 6). Since the NanoLuc reaction follows a Michaelis–Menten kinetics (Hall et al., 2012), use of this assay does even allow estimating the actual concentration of secreted or injected proteins. The herein described set-up has been successfully used for assessing the function of the
Yersinia T3SS (Lindner, Milne-Davies, Langenfeld, & Diepold, 2019) and it was shown that a similar set-up also works for monitoring secretion through the Sec-translocon (Pereira et al., 2019). Thus, NLuc fusions have a high potential to also enable the functional analysis of other bacterial secretion systems in the future.

4 | EXPERIMENTAL PROCEDURES

4.1 | Materials

Chemicals were from Sigma-Aldrich unless otherwise specified. SERVAGel™ TG PRIME™ 8%–16% precast gels were from Serva. Primers, listed in Table S3, were synthesized by Eurofins and Integrated DNA Technologies. Monoclonal anti-c-myc antibody was from Roche (11-667-149-001). Secondary antibodies goat anti-mouse IgG DyLight 800 conjugate were from Thermo-Fisher (SA5-35571).

4.2 | Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S3. All Salmonella strains were derived from Salmonella enterica serovar Typhimurium strain SL1344 (Hoiseth and Stocker, 1981) and created by allelic exchange as previously described (Kaniga, Bossio, & Galan, 1994). S. Typhimurium strains were cultured with low aeration at 37°C in Lennox broth (LB) supplemented with 0.3 M NaCl to induce expression of SPI-1. As required, bacterial cultures were supplemented with tetracycline (12.5 µg/ml), streptomycin (50 µg/ml) or kanamycin (25 µg/ml). Plasmids were generated by Gibson cloning (Gibson et al., 2009) using KOD (Novagen) or Q5 polymerase (NEB). Expression of pT10-based plasmids was induced by the addition of 100 µM of rhamnose to the culture medium.

4.3 | Western blot-based secretion assay

Western blot-based analysis of type III-dependent secretion of proteins into the culture medium was carried out as described previously (Monjarás Feria et al., 2015). S. Typhimurium was cultured at 37°C for 5 hr. For separation of whole cells and cell culture supernatant, the bacterial suspensions were centrifuged at 10,000 × g for 2 min at 4°C. Whole cells were directly resuspended in SDS PAGE loading buffer. The supernatant was filtered through a 0.22 µm pore size filter, sodium deoxycholic acid was added to a final concentration of 0.1% (w/v) and proteins were precipitated by addition of 10% trichloroacetic acid (v/v; final concentration) for 30 min at 4°C. After
pelleting by centrifugation at 20,000 g for 20 min at 4°C, precipitated proteins were washed with acetone and subsequently resuspended in SDS PAGE loading buffer.

4.4 | Luciferase assays

To measure NLuc, RFLuc, Gluc, GDLuc, Rluc and Cluc activity of secreted translational fusions, bacteria were grown under SPI-1-inducing conditions for 5 hr. Culture supernatants were separated from whole bacterial cells by centrifugation for 2 min at 10,000 g. The following buffers were prepared with their substrates according to the manufacturers’ protocols: For NLuc, 25 µl of Nano-glo assay buffer containing furimazine (NLuc working solution, Promega) was added to 25 µl of the culture supernatant. For RFLuc, 30 µl of constituted One-glo assay buffer containing luciferin (Promega) was added to 30 µl of the culture supernatant. For Gluc and GDLuc, 50 µl of the assay buffer containing coelenterazine (Thermo Fisher) was added to 20 µl of culture supernatant. For RLuc, 25 µl of the constituted assay buffer (Promega), in which the substrate was 1:100 diluted, was added to 25 µl of the culture supernatant. For CLuc, a working solution was prepared containing assay buffer and 1:100 of the substrate vargulin (Thermo Fisher). 30 µl of the working solution was added to 10 µl of the supernatant. The luciferase activities were measured in white 384-well plates (MaxiSorp, Nunc), with acquisition settings as recommended by the manufacturers.

4.5 | NLuc assay for wall-bound protein

In order to measure wall-bound protein, overnight cultures of S. Typhimurium were back-diluted to an A$_{600}$ = 0.1 and 50 µl of the bacterial suspension was transferred to a 384-well microplate (MaxiSorp, Nunc) and grown at 37°C for 5 hr. The plate was washed with water using a microplate washer (Tecan Hydrospeed) and the NLuc working solution was diluted in PBS (30 µl PBS + 10 µl NLuc working solution) and added to each well to measure luminescence according to the manufacturer’s protocols: For NLuc, 25 µl of Nano-glo assay buffer containing furimazine (NLuc working solution, Promega) was added to 25 µl of the culture supernatant. For RFLuc, 30 µl of the constituted assay buffer (Promega), in which the substrate was 1:100 diluted, was added to 25 µl of the culture supernatant. For CLuc, a working solution was prepared containing assay buffer and 1:100 of the substrate vargulin (Thermo Fisher). 30 µl of the working solution was added to 10 µl of the supernatant. The luciferase activities were measured in white 384-well plates (MaxiSorp, Nunc), with acquisition settings as recommended by the manufacturers.

4.6 | SDS PAGE, western blotting and immunodetection

For protein detection, samples were separated by SDS PAGE using SERVAGel™ TG Prime™ 8%–16% precast gels and transferred to a PVDF membrane (Bio-Rad) by standard protocols. Membranes were probed with primary antibodies α-SctP (Monjarás Feria et al., 2015), α-SctE (Monjarás Feria et al., 2015), α-c-Myc and α-SctJ (Monjarás Feria et al., 2015). Secondary antibodies were goat anti-mouse IgG DyLight 800 conjugate. Detection was performed using the Odyssey imaging system (Li-Cor).

4.7 | MBP–NLuc and MBP–HiBiT expression and purification

NLuc and HiBiT, respectively, were cloned into a pMal-c5X vector to yield a translational fusion with maltose-binding protein (MBP). E. coli BL21 was transformed with the plasmids. Bacterial cultures were grown overnight at 37°C in LB broth and back-diluted in Terrific Broth (TB) the next day to an A$_{600}$ = 0.1. They were grown to an A$_{600}$ = 0.6–0.8 at 37°C. Subsequently, expression of MBP–NLuc/HiBiT was induced by addition of IPTG to a final concentration of 0.5 mM, after which bacteria were further grown at 37°C for 4 hr. Bacterial cells were harvested by centrifugation (6,000 x g, 15 min, 4°C) and resuspended in column binding buffer (CB) containing 200 mM NaCl, 20 mM Tris HCl (pH 7.4), 1 mM EDTA, Protease inhibitor (P8849, 1:100; Sigma-Aldrich), DNase 10 µg/ml, 1 mM MgSO$_4$ and lysozyme (10 µg/ml) and lysed with a French press. The obtained solution containing cell lysate and cell debris was two times centrifuged at 15,000 x g for 20 min at 4°C. MBP–NLuc/HiBiT in the clear lysate was bound to an amylose resin (NEB), washed with CB and eluted by 10 mM maltose in the same buffer. Buffer was exchanged to PBS by using the Amicon Ultra system (Merck).

4.8 | Stability test of NLuc

40 µl Purified MBP–NLuc was added (2 µg, final concentration) to 1 ml LB/ 0.3 M NaCl and to 1 ml culture supernatant of wild-type S. Typhimurium. Samples were kept either at 37°C, at room temperature, or on ice for up to 4 hr. Aliquots were removed over time and transferred to a 384-well plate, 25 µl of the NLuc working solution was added and luminescence was directly measured in a microplate reader (Tecan Spark).

4.9 | Kinetic measurement

SipA–NLuc was introduced into the chromosome of S. Typhimurium, $P_\text{ara}$-hilA by allelic exchange. The resulting strain was grown overnight at 37°C in LB/0.3 M NaCl, and was back-diluted the following day to an A$_{600}$ = 0.1. Bacterial cultures grew to an A$_{600}$ = 0.9 in an Erlenmeyer flask in a 37°C water bath, stirred with a magnet stirrer. Expression of SPI-1 was induced by addition of arabinose to a final concentration of 0.02% (v/v) and samples were taken at different time points thereafter for assessment of the luminescence of secreted SipA–NLuc or for immunodetection of SctJ. For testing the role of PMF inhibitors, bacterial cells were washed twice after reaching an A$_{600}$ = 0.9 in LB/0.3 M NaCl containing either 120 mM Tris HCl, pH 7.0 for CCCP (Sigma) or 120 mM Tris HCl, pH 7.0 and 150 mM KCl for valinomycin (Sigma). For potassium benzoate, cells at the same growth stage (A$_{600}$ = 0.9) were
harvested and then washed twice with LB/0.3 M NaCl containing 80 mM MES buffer, pH 6.8. The cultures in the different media (without inhibitor, with 0.02% (v/v) arabinose) were kept in the water bath at 37°C and 200 µl of samples were taken at different time points and kept on ice. The inhibitors were added to the bacterial culture 60 min after hilA induction. Cultures were kept in the water bath and samples were taken every 10 min. Samples were centrifuged to separate whole cells and supernatant. 25 µl of the supernatant was transferred to a white 384-well plate and luminescence was measured upon addition of the Nluc working solution in a luminometer.

4.10  Generation of stable HeLa cell line expressing LgBiT

LgBiT was cloned into the MCS of pLVX-EF1α-IRES-Puro (Takara) resulting in pLVX-EF1α-LgBiT-IRES-Puro by Gibson assembly. 24 hr prior to transfection, three 10 cm cell culture plates containing each 4 x 10^5 HEK 293T cells in 8 ml DMEM supplemented with 10% FCS (v/v) and sodium pyruvate were incubated at 37°C, 5% CO2 overnight. The next day, 7 µg DNA of pLVX-EF1α-LgBiT-IRES-Puro in 600 µl sterile water was added to Lenti-X Packaging Single Shot (Takara). The containing pellet was completely resuspended and the solution incubated for 10 min at room temperature to allow formation of nanoparticle complexes. Finally the DNA/nanoparticle solution was added dropwise to the HEK 293T cells. After 4 hr of incubation at 37°C, 6 ml growth medium was added and cell supernatant was harvested after 48 hr and sterile filtered. In total 42 ml supernatant were reduced to a total volume of 4.2 ml used Lentix-Concentrator (Takara) exactly according to the protocol of the manufacturer. The viral suspension was aliquoted and stored at ~80°C. The virus titer was determined using the QuickTiter Lentivirus Titer Kit (Cell Biolabs) according to the manufacturers’ protocol. The viral supernatant was then diluted to a final MOI of 2–10 in 10% FCS-VLE RPMI, supplemented with polybrene (4 µg/ml final concentration) and added to HeLa cells (5 x 10^5 cell in 500 µl medium in six well plates). After overnight culture, medium was exchanged and cells were cultured for another day. The cells were then split, transferred to cell culture plates and 2 µg/ml puromycin was supplemented. After outgrowth of stably transduced cells, single cell clones were generated by single cell dilution. Various cell clones were tested and verified for LgBiT expression by lysing the cells and performing a luciferase assay by the addition of purified MBP-HiBiT in the HiBiT Lytic Buffer from the HiBiT Lytic Detection Kit. Buffer and substrate was added in 1:50 ratio as described in the manufacturer’s protocol, MBP–HiBiT (2 mg/ml) was added in 1:100 ratio to the buffer-substrate mixture.

4.11  Injection assay and injection kinetics

1 x 10^4 HeLa cells and HeLa LgBiT cells were seeded out in white 96 well plates with glass bottom 24 hr before infection in 100 µl DMEM + 10% FCS (GIBCO). S. Typhimurium was washed and resuspended in HBSS to infect the cells at a MOI = 50 for 60 min. After infection, cells were gently washed with a microplate washer (Tecan Hydrospeed, 5 cycles dispensing and aspirating [speed: 70 µl/sec]) using 1 x PBS (GIBCO). A final wash volume of 100 µl was used together with 25 µl of Nanoglo live cell assay buffer (Promega) containing substrate for luminescence measurement in a Tecan Spark reader with the following settings: attenuation: auto, settle time: 0 ms, integration time: 1,000 ms. For monitoring the injection kinetics, HeLa LgBiT cells were seeded out and S. Typhimurium bacteria in HBSS were added to the cells as described above. Directly upon addition of the bacteria, 25 µl of the reconstituted Nanoglo live cell buffer was added to the infection culture and luminescence reading was carried out for 90 min with a 2 min reading interval in the Tecan Spark with the same settings as for the injection assay.

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AUTHOR CONTRIBUTIONS

S. Westerhausen, M.N, C.E.T.-V. and I.G. performed experiments. S. Westerhausen, C.E.T.-V., I.G. and S. Wagner analysed data. U.B. developed and provided the compound test plate. E.B. generated the stable LargeBiT-expressing HeLa cell line. S. Westerhausen and S. Wagner developed the study and wrote the paper.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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