

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

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13 Running Head: NanoLuc-based T3SS secretion and injection assay

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19 **Abstract**

20 The elucidation of the molecular mechanisms of secretion through bacterial protein secretion
21 systems is impeded by a lack of assays to quantitatively assess secretion kinetics. Also the
22 analysis of the biological role of these secretion systems as well as the identification of
23 inhibitors targeting these systems would greatly benefit from the availability of a simple, quick
24 and quantitative assay to monitor principle secretion and injection into host cells. Here we
25 present a versatile solution to this need, utilizing the small and very bright NanoLuc luciferase
26 to assess secretion and injection through the type III secretion system encoded by *Salmonella*
27 pathogenicity island 1. The NanoLuc-based secretion assay features a very high signal-to-noise
28 ratio and sensitivity down to the nanoliter scale. The assay enables monitoring of secretion
29 kinetics and is adaptable to a high throughput screening format in 384-well microplates. We
30 further developed NanoLuc and split-NanoLuc-based assays that enable the monitoring of type
31 III secretion-dependent injection of effector proteins into host cells.

32

33 **Importance**

34 The ability to secrete proteins to the bacterial cell surface, to the extracellular environment, or
35 even into target cells is one of the foundations of interbacterial as well as pathogen-host
36 interaction. While great progress has been made in elucidating assembly and structure of
37 secretion systems, our understanding of their secretion mechanism often lags behind, not last
38 because of the challenge to quantitatively assess secretion function. Here, we developed a
39 luciferase-based assay to enable the simple, quick, quantitative, and high throughput-
40 compatible assessment of secretion and injection through virulence-associated type III secretion
41 systems. The assay allows detection of minute amounts of secreted substrate proteins either in
42 the supernatant of the bacterial culture or within eukaryotic host cells. It thus provides an
43 enabling technology to elucidate the mechanisms of secretion and injection of type III secretion
44 systems and is likely adaptable to assay secretion through other bacterial secretion systems.

45 Introduction

46 The ability to secrete proteins to the bacterial cell surface, to the extracellular environment, or
47 even into target cells is one of the foundations of interbacterial as well as pathogen-host
48 interaction. Protein export is particularly challenging for Gram-negative bacteria as two
49 membranes of the bacterial cell envelope have to be passed. So far, nine different protein
50 secretion systems, named type I – IX secretion systems (T1SS – T9SS), have been discovered
51 in Gram-negative bacteria (1, 2). Three of these systems, T3SS, T4SS, and T6SS, serve the
52 direct application of effector proteins into target cells of either prokaryotic or eukaryotic origin
53 (3).

54 Due to its form and function, the type III secretion machine, as used by many enteric pathogens
55 like *Salmonella*, *Shigella*, *Yersinia*, or enteropathogenic *Escherichia coli*, is called injectisome
56 (4). It is composed of a base that anchors the machine to the inner and outer membranes of the
57 bacterial cell envelope (5), of cytoplasmic components that serve in targeting and receiving of
58 substrates (6, 7), of an inner membrane-localized export apparatus performing substrate
59 unfolding and export (8), and of a needle filament through which secreted substrates reach the
60 host cell (9) (Fig. 1A). Injection itself is mediated by a needle tip complex and by hydrophobic
61 translocators forming pores in the host cell's target membrane (10). Type III secretion is
62 energized by ATP hydrolysis of the system's ATPase and by the proton motive force (PMF)
63 across the bacterial inner membrane (11). Secretion of substrates follows a strict hierarchy with
64 early substrates building up the needle filament, intermediate substrates forming the needle tip
65 and translocon pore, and late substrates that serve as effectors inside the target cell.

66 While great progress has been made in elucidating assembly and structure of the type III
67 secretion injectisome (12-14), our understanding of its secretion mechanism lags behind, not
68 last because of the challenge to quantitatively assess secretion function. Traditionally, T3SS
69 function is assessed by SDS PAGE, Western blotting, and immunodetection of secreted
70 substrates, either acid precipitated from the bacterial culture supernatant, or analyzed in lysates
71 of eukaryotic target cells (15). This approach is time-consuming, at best semi-quantitative, and
72 lacks sensitivity. To facilitate a simplified analysis of principle secretion, injection, and
73 intracellular localization, several enzyme-linked and fluorescent reporters have been developed
74 (16).

75 Ampicillin resistance conferred by β -lactamase-fusions secreted into the periplasm was used to
76 monitor the function of flagellar T3SS, which are closely related to T3SS of injectisomes (17).
77 Secretion into the periplasm through partially assembled injectisomes was assessed by using

78 PhoA-fusions, instead (18). While these assays proved very valuable to address some specific
79 questions, monitoring of secretion into the periplasm is only sensible for early substrates as
80 switching to the secretion of later substrates does not occur without an assembled needle. High
81 throughput (HTP) assays for screening of T3SS inhibitors exploited the turn-over of the
82 fluorogenic substrate PED6 by a secreted phospholipase fusion (19), the turn-over of the
83 chromogenic cephalosporine nitrocefin by a secreted β -lactamase fusion (20), and the
84 enzymatic uncaging of the fluorogenic substrate Glu-CyFur by a secreted carboxypeptidase
85 fusion (21, 22).

86 Likewise, several reporter assays have been developed to assess the injection of T3SS effectors
87 into eukaryotic host cells. Pioneering work by the Cornelis lab exploited the specific increase
88 in intracellular cAMP levels upon injection of effectors fused to a calmodulin-activated
89 adenylate cyclase (Cya) (23). Later, this assay was also adapted to assay injection of effectors
90 by T4SS (24). While the Cya assay showed a very good signal to noise ratio (S/N) of several
91 logs, it was not suitable to monitor injection kinetics or to be adapted for HTP screening because
92 of a tedious cAMP analysis protocol. Widely used to assay injection of effector proteins in T3SS
93 and T4SS is an assay that utilizes the enzymatic cleavage of the FRET-reporter cephalosporin
94 CCF2 by injected β -lactamase-fusions (25). The CCF2 assay facilitated the analysis of injection
95 kinetics and of intracellular accumulation levels of effectors (26). It was also successfully used
96 for HTP high content screening of T3SS inhibitors (27). Real-time observation of injection was
97 achieved by direct fluorescent labeling of tetracysteine motif-tagged effectors (28). However,
98 since this approach requires multidimensional time-lapse microscopy, it is not feasible for
99 routine analysis of effector injection or HTP. Split-GFP technology (29) and self-labelling
100 enzyme tags (30) were successfully used to monitor intracellular localization of effector
101 proteins but both techniques are not optimal for the analysis of translocation kinetics: split-GFP
102 because of a low sensitivity and the slow kinetics of GFP complementation, and the self-
103 labelling enzyme tags because labelling can only be done with effectors that have already been
104 translated before host cell contact.

105 We aimed to develop a T3SS assay based on effector-luciferase fusions to enable a simple,
106 quantitative, and HTP-compatible assessment of principle secretion and injection. The
107 advantage of luciferase-reporters is a very high S/N and sensitivity. In addition, luciferase-based
108 assays benefit from the lack of product (light) accumulation, simplifying the analysis of
109 secretion and injection rates. We developed a secretion assay utilizing NanoLuc (NLuc)
110 luciferase, an engineered 19 kDa glow-type luciferase from the deep-sea shrimp *Oplophorus*
111 *gracilirostris* that converts furimazine, emitting blue light (31). The NLuc-based secretion assay

112 allowed quantification of minute amounts of secreted effectors either in the supernatant of the
113 bacterial culture or within eukaryotic host cells. The assay's ultra-high sensitivity, its wide
114 dynamic range and quick response dynamics qualify it as an enabling technology to elucidate
115 the mechanisms of secretion and injection of T3SS and is likely adaptable to assay secretion
116 through other bacterial secretion systems.

117

118 **Results**

119 *Assessment of effector-luciferase fusion proteins as reporters for type III secretion*

120 In order to identify a luciferase compatible with type III secretion through the T3SS encoded
121 by *Salmonella* pathogenicity island 1 (SPI-1, T3SS-1), we evaluated six different commercially
122 available luciferases as effector-fused secretion reporters: Cypridinia luciferase (CLuc),
123 *Gaussia princeps* luciferase (GLuc), *Gaussia dura* luciferase (GDLuc), NLuc, Renilla luciferase
124 (RLuc), and Red Firefly luciferase (RFLuc) (31-35). We generated translational fusions of the
125 effectors SipA and SopE, respectively, coupled at their C-termini to a luciferase and a myc
126 epitope-tag. The effector-luciferase fusions were expressed from a rhamnose-inducible pro-
127 moter on a low-copy number plasmid in wild type *S. Typhimurium* and in a secretion deficient
128 mutant ($\Delta sctV$). The expression and type III-dependent secretion of the effector luciferase fu-
129 sions was assessed by SDS PAGE, Western blotting and immunodetection of the myc epitope
130 tag in whole bacterial cells and culture supernatants, respectively, after 5 h of growth. All ef-
131 fector-luciferase fusions could be detected at the expected molecular mass in whole cells and
132 in culture supernatants, indicating their productive expression and secretion (Fig. 1A). CLuc
133 and RFLuc showed additional bands likely corresponding to the cleaved luciferase-myc. In
134 general, SipA-luciferase fusions were secreted more efficiently than SopE fusions. SipA and
135 SopE fusion with CLuc as well as SopE fusions with NLuc and RLuc could only be detected in
136 very low levels in the culture supernatants (Fig.1B).

137 The activity of the secreted luciferases in filtered culture supernatants of the *S. Typhimurium*
138 wild type and of the $\Delta sctV$ mutant, respectively, was assessed by luminometry using the speci-
139 fied conditions for each luciferase. The S/N (wild type vs. $\Delta sctV$) was highest with effector-
140 NLuc fusions (SipA-NLuc S/N = 45, SopE-NLuc S/N = 22), and, with the exception of GDLuc,
141 always higher for SipA-luciferase fusions (Fig. 1C).

142 Since the SipA-NLuc fusion showed the best S/N, we introduced SipA-NLuc-myc into the
143 chromosome of a *S. Typhimurium* wild type strain and of a $\Delta sctV$ mutant for further analysis.
144 First, we compared the expression and secretion of plasmid and chromosome-encoded SipA-

145 NLuc, respectively, and as a reference also of the secreted translocator SctE, by SDS PAGE,
146 Western blotting and immunodetection. SipA-NLuc was expressed well from the chromosome
147 even though, not unexpectedly, at lower levels compared to its expression from the plasmid
148 (Fig. 1D). The extent of T3SS-dependent secretion of plasmid and chromosome-encoded SipA-
149 NLuc was indistinguishable (Fig. 1D).

150 We next evaluated the S/N of the secreted SipA-NLuc fusion when expressed from plasmid or
151 chromosome by measuring the NLuc activity in filtered culture supernatants of the wild type
152 and the $\Delta sctV$ mutant. While plasmid-based expression resulted in a S/N = 45, chromosome-
153 based expression even achieved a S/N = 200. The stronger plasmid-based expression may lead
154 to a greater liberation of SipA-NLuc upon occasional cell lysis, compromising the S/N.

155 Both, injectisomes and flagella possess T3SS for the export of proteins and it has been shown
156 that substrates of one system may be secreted by the other one to a limited degree (36, 37). In
157 order to assess the contribution of the flagellar T3SS to the S/N of SipA-NLuc secretion, we
158 blocked expression of flagella by deleting the gene of the flagellar master regulator FlhD. In
159 the absence of flagella, the S/N of SipA-NLuc secretion increased to 140 when SipA-NLuc was
160 expressed from the plasmid and to 1000 when it was expressed from the chromosome (Fig. 1E).
161 FlhD contributes to the induction of the SPI-1-encoded T3SS by indirectly regulating the ex-
162 pression of the the major SPI-1 regulator HilA (38), which results in a strongly decreased ex-
163 pression of T3SS-1 and its effectors in the absence of FlhD. To determine whether the improved
164 S/N of SipA-NLuc secretion in the *flhD* mutant resulted from an overall lower expression of
165 the reporter or from preventing secretion through flagella, we also tested SipA-NLuc secretion
166 in a strain expressing chromosome-encoded HilA from an arabinose-inducible promoter (39),
167 thus uncoupling its expression from control by FlhD. In this strain, T3SS-1-dependent SipA-
168 NLuc secretion was identical in the wild type and in the *flhD* mutant (Fig. 1F). However, in the
169 absence of a functional T3SS-1 ($\Delta sctDFIJ$), 150-fold lower levels of SipA-NLuc were detecta-
170 ble in the culture supernatant of the strain lacking flagella. These results indicate that about 1%
171 of the SipA-NLuc secretion signal in the wild type strain stems from secretion through the
172 flagellar T3SS (Fig. 1E) and that the increased S/N in the absence of FlhD results from prevent-
173 ing secretion through flagella. Despite the increased S/N in the absence of flagella, we used
174 *flhD* wild type bacteria for most of the work presented herein because of the higher overall
175 signal and because motility appeared to promote growth in a microplate format.

176 In order to test the versatility of NLuc as secretion reporter, we also constructed fusions with
177 the early T3SS substrate SctP (needle length regulator) and with the intermediate substrate SctA

178 (tip protein). While NLuc compromised secretion and function of SctP when fused to its C-
179 terminus (Fig. S1AB), SctA-NLuc fusions were readily secreted, even when NLuc was placed
180 at different positions within the polypeptide chain of SctA (Fig. S1CD). To overcome the limi-
181 tation of NLuc in supporting secretion of SctP, we utilized a split-NLuc approach. Split-NLuc is
182 composed of a large fragment (LgBiT, 18 kDa) comprising most of the protein's beta barrel and
183 of a small fragment with a high affinity to the LgBiT (HiBiT, 1.3 kDa), comprising only one
184 beta strand (40). SctP-HiBiT fusions were successfully secreted into the culture supernatant and
185 strong luminescence was detected when complementing SctP-HiBiT with LgBiT (Fig. S1AB),
186 showing that split-NLuc can serve as a secretion reporter when NLuc fails.

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

190

191 *Assessment of the sensitivity of the NLuc-based secretion assay*

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

195 In order to compare the sensitivity of the Western blot- and the SipA-NLuc-based secretion
196 assays, we made a serial dilution of the filtered supernatant of wild type and $\Delta sctV$ *S. Typhi-*
197 *murium* cultures grown for 5 h. In the Western blot-based assay, we could detect the intermedi-
198 ate substrate SctE down to a supernatant volume of 113 μ l and the early substrate SctP as well
199 as the late substrate SipA-NLuc down to 225 μ l (Fig. 2A). In contrast, using the SipA-NLuc
200 assay, we were able to obtain a stable S/N = 200 down to 195 nl supernatant volume. The S/N
201 even remained above 50 when assaying an equivalent of only 24 nl (Fig. 2B).

202 Next, we assessed the performance of the SipA-NLuc assay in monitoring the onset kinetics of
203 type III secretion, which requires very high sensitivity due to the small amounts of secreted
204 material that is initially present. To this end, we grew *S. Typhimurium* harboring arabinose-
205 controlled HilA to an $A_{600} = 0.9$, after which expression of the pathogenicity island was induced
206 by the addition of 0.02% (w/v) arabinose. Bacterial cells and culture supernatants were col-
207 lected every 10 min and kept on ice until reading at the end of the experiment. Induction of SPI-
208 1 was monitored by Western blot and immunodetection of the base component SctJ in whole
209 cells. It was first observed 30 min after the addition of arabinose (Fig. 2C). Also luminescence
210 of SipA-NLuc was detected in the culture supernatant for the first time 30 min after induction

211 of SPI-1 and then luminescence increased steadily to the end of the measurement after 120 min
212 (Fig. 2C). This increase in luminescence correlates directly with SipA-NLuc secretion and is
213 not influenced by NLuc maturation or turn-over as the activity of NLuc remains stable in the
214 culture supernatant over extended periods of time (Fig. S2).

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

222

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

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

227 Centrifugation or filtering is not feasible for separation of bacterial cells and culture supernatant
228 in a microplate format. In order to overcome this problem, we made use of the high-protein
229 binding capacity of the microplates and tested whether secreted substrates would specifically
230 bind to the plate wall after being secreted (Fig. 3A). To this end, 50 μ l of *S. Typhimurium* wild
231 type and Δ *sctV* mutant were grown in white high protein binding 384-well plates. Bacteria were
232 washed out of the wells after 5 h of growth using a microplate washer. Then, PBS, NLuc buffer,
233 and NLuc substrate were supplied to each well and the luciferase activity was measured. Using
234 this setup, a S/N = 37 could be achieved ($Z' = 0.8$), which is excellent for HTP screening (Fig.
235 3B).

236 To assess the robustness of this assay and the variation across the plate, we filled an entire 384-
237 well plate with 50 μ l of a *S. Typhimurium*, SipA-NLuc culture and allowed it to grow for 5 h at
238 37°C. Luminescence of secreted, wall-bound SipA-NLuc was assessed after washing out bac-
239 teria as described above. The assay proved very robust with a coefficient of variation of 7%
240 over the entire plate and with little edge effects (Fig. 3C, Table S1). We then performed a proof-
241 of-concept inhibitor screen by assessing the effect of a range of 37 different bioactive com-
242 pounds on the activity of the T3SS in the 384-well format (Table S2, Fig. 3D). Each well of the
243 plate was printed with 0.5 μ l of a compound in 100% DMSO, to which 50 μ l of a *S.*

244 Typhimurium, SipA-NLuc culture was added. Again, the culture was allowed to grow for 5 h,
245 after which secretion of SipA-NLuc was assessed by luminometry. The assay showed a highly
246 dynamic response from 10 % to 120 % secretion activity compared to the DMSO-treated wild
247 type control (Fig. 3D). Detection of SipA-NLuc was most strongly reduced by the flavonoids
248 quercetin (30 $\mu\text{g}/\text{ml}$, 90% reduction) and scutellarin (10 $\mu\text{g}/\text{ml}$, 75% reduction), which con-
249 firms the previously reported observation that flavonoids target T3SS (22). Also treatment with
250 the 3-hydroxy-3-methylglutaryl (HMG) coenzyme A reductase-blocker simvastatin reduced de-
251 tection of SipA-NLuc by 44%. Replication of the screen proved a high reproducibility of the
252 assay with a R^2 of 0.95 (Fig. 3E).

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

256

257 *Application example: Assessment of the PMF-dependence of type III secretion by the NLuc*
258 *secretion assay*

259 It has been known for long that secretion through T3SS depends on two sources of energy, on
260 the hydrolysis of ATP by the system's ATPase (FliI in flagella, SctN in injectisomes) and on the
261 PMF (41-43), which itself is composed of the ΔpH , i.e., the proton concentration gradient across
262 the membrane, and the $\Delta\Psi$, the electric potential difference between the periplasm and cyto-
263 plasm. The contribution of these two PMF components to T3SS function can be dissected with
264 specific inhibitors. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is a PMF uncoupler
265 (ionophore) and discharges both the ΔpH and the $\Delta\Psi$ by transporting protons through the mem-
266 brane (44). At acidic pH, potassium benzoate is a weak acid and can enter the membrane and
267 discharge the ΔpH (45). Valinomycin can shuttle potassium ions across the membrane which
268 collapses the electric potential difference $\Delta\Psi$ (46). Evaluating the contribution of each PMF
269 component to T3SS function requires the careful analysis of secretion kinetics, for which the
270 classical, semi-quantitative Western blot-based secretion assay is not well suited, but for which
271 the NLuc-based secretion assay proved very powerful. To further show this, CCCP, potassium
272 benzoate, and valinomycin, respectively, were added to the bacterial culture at different con-
273 centrations, 60 min after induction of SPI-1 (for experimental details, please refer to the meth-
274 ods section), while samples of culture supernatants were taken every 10 min for subsequent
275 analysis of the luminescence of secreted SipA-NLuc. While SipA-NLuc secretion progressed
276 over time in the control sample (Fig. 4), addition of the inhibitors lead to sudden changes in

277 secretion kinetics. CCCP blocked secretion instantly, even at concentrations of 5 μ M, showing
278 the critical relevance of the PMF for type III secretion (Fig. 4A). Discharging the Δ pH by
279 potassium benzoate resulted in a concentration-dependent instant reduction of secretion (Fig.
280 4B). At 20 mM potassium benzoate, secretion was completely abolished while it proceeded at
281 60% of the untreated control in the presence of 5 mM and at 10% in the presence of 10 mM
282 potassium benzoate. Collapsing the electric potential by valinomycin lead to a strongly reduced
283 luciferase signal after 10 min, after which secretion proceeded in a concentration-dependent
284 manner (Fig. 4C): in the presence of 20 μ M valinomycin, no significant change in secretion
285 rate was observed, while 40 μ M and 60 μ M valinomycin, respectively, lead to 70% and 40%
286 secretion of the untreated control.

287 These results show that both components of the PMF, Δ pH and Δ Ψ , contribute to energizing
288 secretion in the SPI-1-encoded T3SS of *S. Typhimurium*. As the PMF-compromising com-
289 pounds took effect so quickly after treatment, it is highly unlikely that the PMF-dependence of
290 type III secretion is the consequence of a secondary effect of PMF reduction – an issue that
291 could only be resolved with the sensitive and highly time-resolved NLuc secretion assay. These
292 results open the door for further experiments dissecting the role of the different T3SS compo-
293 nents in utilizing the PMF.

294

295 *Development of NLuc-based host cell injection assays*

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

301 In a first and simple approach, we infected HeLa cells in 96-well plates at an MOI = 50 with
302 SipA-NLuc-expressing *S. Typhimurium*, using wild type bacteria and secretion-deficient Δ *sctV*
303 mutants. After infection for 60 min, attached bacteria were gently washed off with PBS using a
304 microplate washer and subsequently, the HeLa cell-associated luminescence was measured us-
305 ing live cell buffer (Fig. 5A). The non-secreting Δ *sctV* mutants (Fig. 5A) showed a HeLa cell-
306 associated luminescence of 8% of the wild type, corresponding to a S/N = 12 (Fig. 5C). To
307 determine whether the HeLa cell-associated signal was truly resulting from injected SipA-
308 NLuc, we assessed injection in a set of mutants that are capable of secreting SipA but incapable
309 of injecting it into host cells: a needle tip-deficient Δ *sctA*, a translocon-deficient Δ *sctEBA*, and

310 a gatekeeper-deficient $\Delta sctW$ mutant. While secretion of SipA-NLuc into the culture superna-
311 tant was increased between 2 and 5-fold in $\Delta sctA$, $\Delta sctEBA$, and $\Delta sctW$ mutants (Fig. 5B),
312 which are reportedly unlocked for secretion of late substrates like SipA (47, 48), the HeLa cell-
313 associated luminescence was strongly reduced to 9-24% of the wild type when infecting with
314 these mutants (Fig. 5C). From these results we can conclude that the luminescence signal ob-
315 tained from infection with wild type *S. Typhimurium* resulted to more than 90% from injected
316 SipA-NLuc and that only little signal may stem from bacteria remaining attached to HeLa cells
317 or to the plate even after washing. Over all, this NLuc-based injection assay proved very useful
318 for the quick and simple assessment of translocation of effectors into host cells by an end-point
319 measurement, however the kinetics of injection cannot be assessed by this assay.

320 To gain a higher specificity for the signal of injected SipA and enable analysis of injection
321 kinetics, we employed the split version of the NLuc luciferase. To this end, SipA was fused to
322 HiBiT while LgBiT was expressed stably by the HeLa cell line. Complementation of LgBiT
323 with HiBiT to a functional luciferase should only occur inside the HeLa cells after translocation
324 of SipA-HiBiT (Fig. 5D). We first tested the secretion of SipA-HiBiT into the culture superna-
325 tant by providing LgBiT to the assay buffer. Similar to what was observed for SipA-NLuc,
326 secretion of SipA-HiBiT into the culture supernatant was increased between 2 and 6-fold in
327 $\Delta sctA$, $\Delta sctEBA$, and $\Delta sctW$ mutants, respectively (Fig. 5E). However, in contrast to the SipA-
328 NLuc-based injection assay, none of the T3SS mutant strains yielded any detectable lumines-
329 cence in the split NLuc assay (Fig. 5F), making this assay highly suitable for monitoring the
330 specific injection of T3SS effectors into host cells. This setup even allowed us to follow the
331 kinetics of SipA-HiBiT injection over time directly in a microplate reader (Fig. 5G).

332

333 Discussion

334 The elucidation of the molecular mechanisms of secretion through T3SS and other bacterial
335 protein secretion systems is impeded by a lack of assays to quantitatively assess secretion
336 kinetics. Also the analysis of the biological role of these secretion systems as well as the
337 identification of inhibitors targeting these systems would greatly benefit from the availability
338 of a simple, quick and quantitative assay to monitor principle secretion and injection into host
339 cells. Here we present a versatile solution to this need, utilizing the small and very bright NLuc
340 luciferase to assess secretion and injection through the T3SS encoded by SPI-1 of *S.*
341 *Typhimurium*. Secretion of a SipA-NLuc fusion showed a very high S/N and sensitivity down
342 to the nanoliter scale, making it exquisitely suited for the assessment of secretion kinetics. In

343 addition, the NLuc-based secretion assay proved highly adaptable to a HTP screening format
344 in 384-well microplates. We further developed NLuc and split-NLuc-based assays that enable
345 the monitoring of T3SS-dependent injection of effector proteins into host cells.

346 A perfect assay to monitor protein secretion would feature: i) A lack of signal from the un-
347 secreted reporter, resulting in a high S/N. ii) A small reporter that does not interfere with
348 secretion through the secretion system of interest. In case of T3SS, this also includes a not too
349 fast and tight folding inside bacteria as only unfolded protein can be secreted and as the
350 unfolding capacity of the system is not very high. iii) A fast and efficient folding of the reporter
351 outside of the bacterium, guaranteeing fast response dynamics. iv) An intrinsic signal of the
352 reporter, not necessitating an enzyme substrate. v) A high sensitivity. vi) A lack of accumulation
353 of product of the reporter's reaction. And vii) Be quick, simple, and needing only short hands-
354 on time.

355 While fluorescent proteins would be desirable secretion reporters as they benefit from an
356 intrinsic signal (and thus do not come with the problem of accumulation of product of the
357 reporter's reaction), they often suffer from a very slow maturation time and/or insufficient
358 brightness. In addition, fluorescent proteins tend to form very stable β -barrels that block
359 secretion through T3SS (49), excluding them as secretion reporters, at least for T3SS. While
360 the use of split GFP can overcome the limitation associated with tight folding, slow
361 complementation and maturation of GFP compromise its use. The NLuc-based secretion assay
362 as presented herein matches most of the needs listed above. While NLuc lacks an intrinsic signal
363 and requires the addition of a substrate, the analysis of secretion by this assay is not complicated
364 by the overlay of the kinetics of the reporting enzyme and the kinetics of secretion, as it is in
365 other enzyme-linked secretion assays. Instead, the measured signal of the NLuc assay is directly
366 proportional to the amount of accumulated secreted protein. This advantage, together with the
367 superior sensitivity, yield a very high dynamic range of the NLuc secretion assay.

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

374 Our experiments show that secretion of NLuc is supported by fusion to a range of intermediate
375 and late T3SS substrates, even within a polypeptide chain, but fails to be secreted when fused

376 to the early substrate SctP. It is conceivable that the mode of early substrate secretion does not
377 provide a sufficient unfolding capacity to support secretion of NLuc while this seems not a
378 problem when NLuc is fused to intermediate and late substrates. Interestingly, a *Yersinia* SctP-
379 PhoA fusion was secreted (18), pointing either to a higher unfolding capacity of the *Yersinia*
380 T3SS or to a weaker fold of PhoA. We could overcome the limited use of NLuc as secretion
381 reporter for early substrates by using the split-NLuc technology instead. The 11 amino acid-
382 long HiBiT was accommodated well by SctP and it is conceivable that this very small piece
383 allows secretion in most circumstances.

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

389 In addition to the points important for a secretion assay, a perfect injection assay would also: i)
390 Feature a high specificity for injected effectors as opposed to secreted but not injected ones. ii)
391 Allow the analysis of injection kinetics. And iii) Allow localization of the injected protein, at
392 best at single molecule resolution.

393 While fluorescence-based assays proved highly suitable to study the localization dynamics of
394 injected effectors inside host cells, they are very limited in their use to study injection kinetics
395 and are always instrumentation-demanding. The CCF2-based injection assay features simple
396 handling, instead, and proves very useful for the analysis of injection, but suffers from high
397 costs of CCF2 and a low dynamic range. In addition, the product accumulation resulting from
398 the enzymatic activity of the injected β -lactamase complicate the analysis of injection kinetics.
399 The herein-presented NLuc-based injection assays offer very quick and simple analysis, even
400 of injection kinetics, and feature a high dynamic range and sensitivity. While a high-resolution
401 analysis of the localization of the effector-NLuc-fusions inside host cells is not supported by
402 these assays, microscopic setups exist that utilize luminescence for long-duration monitoring
403 of single cells (50), which may become useful for studying the role of individual effectors in
404 bacterial persistence.

405 As performed herein, cytoplasmic expression of LgBiT will only generate luminescence if the
406 HiBiT of the injected effector also localizes to the cytoplasm. However, the split-NLuc injection
407 assay may also be utilized to analyze the localization and topogenesis of effector proteins inside
408 host cells by targeting LgBiT to specific organelles instead (Fig. 6). Furthermore,

409 complementation of LgBiT by the low-affinity SmBiT instead of the high-affinity HiBiT may
410 provide a useful tool to investigate effector-host protein interactions *in vivo* by bimolecular
411 complementation (51).

412 In summary, our data show that NLuc-fusions of secreted substrate proteins can be used as a
413 robust, versatile, cheap, simple and quick reporter for T3SS secretion and injection that will
414 enable future in-depth elucidation of T3SS function (Fig. 6). The NLuc reporter is likely to be
415 adaptable to other bacterial secretion systems as well.

416

417 **Materials and methods**

418 *Materials*

419 Chemicals were from Sigma-Aldrich unless otherwise specified. SERVAGel™ TG PRiME™
420 8–16% precast gels were from Serva. Primers, listed in Table S3, were synthesized by Eurofins
421 and Integrated DNA Technologies. Monoclonal anti-c-myc antibody was from Roche (11-667-
422 149-001). Secondary antibodies goat anti-mouse IgG DyLight 800 conjugate were from
423 Thermo-Fisher (SA5-35571).

424

425 *Bacterial strains, plasmids and growth conditions*

426 Bacterial strains and plasmids used in this study are listed in Table S3. All *Salmonella* strains
427 were derived from *Salmonella enterica* serovar Typhimurium strain SL1344 (Hoise and
428 Stocker, 1981) and created by allelic exchange as previously described (52). *S. Typhimurium*
429 strains were cultured with low aeration at 37°C in Lennox broth (LB) supplemented with 0.3 M
430 NaCl to induce expression of SPI-1. As required, bacterial cultures were supplemented with
431 tetracycline (12.5 µg/ml), streptomycin (50 µg/ml), or kanamycin (25 µg/ml). Plasmids were
432 generated by Gibson cloning (53) using KOD (Novagen) or Q5 polymerase (NEB). Expression
433 of pT10-based plasmids was induced by the addition of 100 µM of rhamnose to the culture
434 medium.

435

436 *Western-blot-based secretion assay*

437 Western-blot-based analysis of type III-dependent secretion of proteins into the culture medium
438 was carried out as described previously (39). *S. Typhimurium* was cultured at 37°C for 5 h. For
439 separation of whole cells and cell culture supernatant, the bacterial suspensions were

440 centrifuged at $10,000 \times g$ for 2 min at 4°C . Whole cells were directly resuspended in SDS PAGE
441 loading buffer. The supernatant was filtered through a $0.22 \mu\text{m}$ pore size filter, sodium deoxy-
442 cholic acid was added to a final concentration of 0.1% (w/v), and proteins were precipitated by
443 addition of 10% trichloroacetic acid (v/v; final concentration) for 30 min at 4°C . After pelleting
444 by centrifugation at $20,000 \times g$ for 20 min at 4°C , precipitated proteins were washed with ace-
445 tone and subsequently resuspended in SDS PAGE loading buffer.

446

447 *Luciferase assays*

448 To measure NLuc, RFLuc, Gluc, GDLuc, RLuc and CLuc activity of secreted translational fu-
449 sions, bacteria were grown under SPI-1-inducing conditions for 5 h. Culture supernatants were
450 separated from whole bacterial cells by centrifugation for 2 min at $10,000 \times g$. The following
451 buffers were prepared with their substrates according to the manufacturers' protocols: For NLuc,
452 $25 \mu\text{l}$ of Nano-glo assay buffer containing furimazine (NLuc working solution, Promega) was
453 added to $25 \mu\text{l}$ of the culture supernatant. For RFLuc, $30 \mu\text{l}$ of constituted One-glo assay buffer
454 containing luciferin (Promega) was added to $30 \mu\text{l}$ of the culture supernatant. For Gluc and
455 GDLuc, $50 \mu\text{l}$ of the assay buffer containing coelenterazine (Thermo Fisher) was added to 20
456 μl of culture supernatant. For RLuc, $25 \mu\text{l}$ of the constituted assay buffer (Promega), in which
457 the substrate was 1:100 diluted, was added to $25 \mu\text{l}$ of the culture supernatant. For CLuc, a
458 working solution was prepared containing assay buffer and 1:100 of the substrate vargulin
459 (Thermo Fisher). $30 \mu\text{l}$ of the working solution was added to $10 \mu\text{l}$ of the supernatant. The
460 luciferase activities were measured in white 384-well plates (MaxiSorp, Nunc), with acquisition
461 settings as recommended by the manufacturers.

462

463 *NLuc assay for wall-bound protein*

464 In order to measure wall-bound protein, overnight cultures of *S. Typhimurium* were back-di-
465 luted to an $A_{600} = 0.1$ and $50 \mu\text{l}$ of the bacterial suspension was transferred to a 384-well micro-
466 plate (MaxiSorp, Nunc) and grown at 37° for 5 h. The plate was washed with water using a
467 microplate washer (Tecan Hydrospeed) and the NLuc working solution was diluted in PBS (30
468 μl PBS + $10 \mu\text{l}$ NLuc working solution) and added to each well to measure luminescence using
469 the Tecan Spark reader with following settings: attenuation: auto, settle time: 0 ms, integration
470 time: 100 ms.

471 For the inhibitor screen, 0.5 μ l of each compound (Table S2) was added to 50 μ l bacterial culture
472 prior to incubation at 37°C for 5 h, and the plate was processed as described above.

473

474 *SDS PAGE, Western blotting and immunodetection*

475 For protein detection, samples were separated by SDS PAGE using SERVAGel™ TG Prime™
476 8-16% precast gels and transferred to a PVDF membrane (Bio-Rad) by standard protocols.
477 Membranes were probed with primary antibodies α -SctP (39), α -SctE (39), α -c-Myc and α -SctJ
478 (39). Secondary antibodies were goat anti-mouse IgG DyLight 800 conjugate. Detection was
479 performed using the Odyssey imaging system (Li-Cor).

480

481 *MBP-NLuc and MBP-HiBiT expression and purification*

482 NLuc and HiBiT, respectively, were cloned into a pMal-c5X vector to yield a translational fu-
483 sion with maltose-binding protein (MBP). *E. coli* BL21 was transformed with the plasmids.
484 Bacterial cultures were grown overnight at 37°C in LB broth and back-diluted in Terrific Broth
485 (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,
486 expression of MBP-NLuc/ HiBiT was induced by addition of IPTG to a final concentration of
487 0.5 mM, after which bacteria were further grown at 37°C for 4 h. Bacterial cells were harvested
488 by centrifugation (6,000 x g, 15 min, 4°C) and resuspended in column binding buffer (CB)
489 containing 200 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, Protease inhibitor (Sigma-
490 Aldrich, P8849, 1:100), DNase 10 μ g/ml, 1 mM MgSO₄ and lysozyme (10 μ g/ml) and lysed
491 with a French press. The obtained solution containing cell lysate and cell debris was two times
492 centrifuged at 15,000 x g for 20 min at 4°C. MBP-NLuc/HiBit in the clear lysate was bound to
493 an amylose resin (NEB), washed with CB and eluted by 10 mM maltose in the same buffer.
494 Buffer was exchanged to PBS by using the Amicon Ultra system (Merck).

495

496 *Stability test of NLuc*

497 40 μ l Purified MBP-NLuc was added (2 μ g, final concentration) to 1 ml LB/ 0.3 M NaCl and
498 to 1 ml culture supernatant of wild type *S. Typhimurium*. Samples were kept either at 37°C, at
499 room temperature, or on ice for up to 4 h. Aliquots were removed over time and transferred to
500 a 384-well plate, 25 μ l of the NLuc working solution was added and luminescence was directly
501 measured in a microplate reader (Tecan Spark).

502

503 *Kinetic measurement*

504 SipA-NLuc was introduced into the chromosome of *S. Typhimurium*, $P_{ara-hilA}$ by allelic ex-
505 change. The resulting strain was grown overnight at 37°C in LB/0.3 M NaCl, and was back-
506 diluted the following day to an $A_{600} = 0.1$. Bacterial cultures grew to an $A_{600} = 0.9$ in an Erlen-
507 meyer flask in a 37°C water bath, stirred with a magnet stirrer. Expression of SPI-1 was induced
508 by addition of arabinose to a final concentration of 0.02% (v/v) and samples were taken at
509 different time points thereafter for assessment of the luminescence of secreted SipA-NLuc or
510 for immunodetection of SctJ. For testing the role of PMF inhibitors, bacterial cells were washed
511 twice after reaching an $A_{600} = 0.9$ in LB/0.3 M NaCl containing either 120 mM Tris-HCl, pH
512 7.0 for CCCP (Sigma) or 120 mM Tris-HCl, pH 7.0 and 150 mM KCl for valinomycin (Sigma).
513 For potassium benzoate, cells at the same growth stage ($A_{600} = 0.9$) were harvested and then
514 washed twice with LB/0.3 M NaCl containing 80 mM MES buffer, pH 6.8. The cultures in the
515 different media (without inhibitor, with 0.02% (v/v) arabinose) were kept in the water bath at
516 37°C and 200 μ l of samples were taken at different time points and kept on ice. The inhibitors
517 were added to the bacterial culture 60 min after *hilA*-induction. Cultures were kept in the water
518 bath and samples were taken every 10 min. Samples were centrifuged to separate whole cells
519 and supernatant. 25 μ l of the supernatant was transferred to a white 384-well plate and lumi-
520 nescence was measured upon addition of the Nluc working solution in a luminometer.

521

522 *Generation of stable HeLa cell line expressing LgBiT*

523 LgBiT was cloned into the MCS of pLVX-EF1 α -IRES-Puro (Takara) resulting in pLVX-EF1 α -
524 LgBiT-IRES-Puro by Gibson assembly. 24 h prior to transfection, three 10 cm cell culture plates
525 containing each 4 x 10⁶ HEK 293T cells in 8 ml DMEM supplemented with 10% FCS (v/v)
526 and sodium pyruvate were incubated at 37°C, 5% CO₂ overnight. The next day, 7 μ g DNA of
527 pLVX-EF1 α -LgBiT-IRES-Puro in 600 μ l sterile water was added to Lenti-X Packaging Single
528 Shot (Takara). The containing pellet was completely resuspended and the solution incubated
529 for 10 min at room temperature to allow formation of nanoparticle complexes. Finally the
530 DNA/nanoparticle solution was added dropwise to the HEK 293T cells. After 4 h of incubation
531 at 37°C, 6 ml growth medium was added and cell supernatant was harvested after 48 h and
532 sterile filtered. In total 42 ml supernatant were reduced to a total volume of 4.2 ml used Lentix-
533 Concentrator (Takara) exactly according to the protocol of the manufacturer. The viral suspen-
534 sion was aliquoted and stored at -80°C. The virus titer was determined using the QuickTiter

535 Lentivirus Titer Kit (Cell Biolabs) according to the manufacturers protocol. The viral superna-
536 tant was then diluted to a final MOI of 2-10 in 10% FCS-VLE RPMI, supplemented with
537 polybrene (4 $\mu\text{g/ml}$ final concentration) and added to HeLa cells (5×10^5 cell in 500 μl medium
538 in six well plates). After overnight culture, medium was exchanged and cells were cultured for
539 another day. The cells were then split, transferred to cell culture plates, and 2 $\mu\text{g/ml}$ puromycin
540 was supplemented. After outgrowth of stably transduced cells, single cell clones were generated
541 by single cell dilution. Various cell clones were tested and verified for LgBiT expression by
542 lysing the cells and performing a luciferase assay by the addition of purified MBP-HiBiT in the
543 Hibit Lytic Buffer from the Hibit Lytic Detection Kit. Buffer and substrate was added in 1:50
544 ratio as described in the manufacturer's protocol, MBP-Hibit (2 mg/ml) was added in 1:100
545 ratio to the buffer-substrate mixture.

546

547 *Injection assay and injection kinetics*

548 1×10^4 HeLa cells and HeLa LgBiT cells were seeded out in white 96 well plates with glass
549 bottom 24 h before infection in 100 μl DMEM + 10% FCS (GIBCO). *S. Typhimurium* was
550 washed and resuspended in HBSS to infect the cells at a MOI = 50 for 60 min. After infection,
551 cells were gently washed with a microplate washer (Tecan Hydrospeed, 5 cycles dispensing and
552 aspirating (speed: 70 $\mu\text{l/sec}$)) using 1 x PBS (GIBCO). A final wash volume of 100 μl was
553 used together with 25 μl of Nanoglo live cell assay buffer (Promega) containing substrate for
554 luminescence measurement in a Tecan Spark reader with the following settings: attenuation:
555 auto, settle time: 0 ms, integration time: 1,000 ms. For monitoring the injection kinetics, HeLa
556 LgBiT cells were seeded out and *S. Typhimurium* bacteria in HBSS were added to the cells as
557 described above. Directly upon addition of the bacteria, 25 μl of the reconstituted Nanoglo live
558 cell buffer was added to the infection culture and luminescence reading was carried out for 90
559 min with a 2 min reading interval in the Tecan Spark with the same settings as for the injection
560 assay.

561

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567

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700

701 **Figure Legends**

702 **Fig 1** Assessing different luciferases as reporters for type III secretion.

703 (A) Cartoon of the T3SS injectisome. Names or proteins mentioned herein are shown according
704 to the unified nomenclature. The figure is adapted from reference (4).

705 (B) Proteins of whole cell lysates and culture supernatants of *S. Typhimurium* expressing the
706 indicated SipA-Luc and SopE-Luc fusions were analyzed by SDS PAGE, Western blot and
707 Immunodetection with an anti-myc antibody.

708 (C) Signal to noise ratios (wt/ Δ sctV) of luciferase activities of secreted SipA-Luc and SopE-
709 Luc fusions were graphed. Bar graphs represent the mean S/N of three independent
710 measurements.

711 (D) Immunodetection of SipA-NLuc^{myc} and SctE on Western blot of SDS PAGE-separated
712 culture supernatants and whole cell lysates, either expressing SipA-NLuc^{myc} from a
713 plasmid or from the chromosome.

714 (E) Signal to noise ratios (wt/ Δ sctV) of luciferase activities of secreted SipA-NLuc either
715 expressed from a plasmid or from the chromosome, each with or without flagella (*flhD*)
716 were graphed. Bar graphs represent the mean S/N of three independent measurements.

717 (F) SipA-NLuc^{myc} secretion in *S. Typhimurium* P_{ara}*hilA* and in *S. Typhimurium* Δ sctDFIJ,
718 P_{ara}*hilA* with and without flagella (*flhD*), respectively. Bar graphs represent mean (\pm
719 standard deviation) of three technical replicates. Asterisks indicate statistical significance
720 of SipA-NLuc^{myc} secretion assessed by Student's *t*-test, *: $p \leq 0.05$,

721 Abbreviations: Nluc: Nanoluc, RFLuc: Red Firefly luciferase, GDLuc: Gaussia Dura
722 Luciferase, GLuc: Gaussia princeps Luciferase, RLuc: Green Renilla Luciferase, CLuc:
723 Cypridinia Luciferase, S/N: signal to noise, ns: non-significant

724 **Fig 2** Assessment of the sensitivity of the NLuc secretion reporter

725 (A) Immunodetection of the T3SS substrates SctP, SctE and SipA-NLuc^{myc} on a Western blot
726 of SDS PAGE-separated, serially diluted culture supernatants.

727 (B) Luminescence of secreted SipA-NLuc^{myc} in serially diluted culture supernatants of the
728 *S. Typhimurium* wild type and a Δ sctV mutant. Triangles show the calculated signal to
729 noise ratios for each dilution. Data represent the mean (\pm standard deviation) of three
730 technical replicates.

731 (C) Normalized luminescence of secreted SipA-NLuc^{myc} at different time points after induction
732 of *hilA* with 0.02% arabinose. Experiments were normalized by setting the maximum
733 luminescence of each experiment to 1. The data points represent mean (\pm standard
734 deviation) of five independent measurements. At each time point, samples of whole cell
735 lysates were taken for immunodetection of SctJ.

736 **Fig 3** Development of a SipA-NLuc-based HTP secretion

737 (A) Cartoon of the assay setup. *S. Typhimurium* expressing SipA-NLuc was grown in a 384-
738 well microplate format. Secreted SipA-NLuc bound to the wall of the high protein-
739 binding microplate. Bacteria were washed out and luminescence was measured.

740 (B) Luminescence and signal to noise ratio of secreted SipA-NLuc. The experimental setup
741 was as shown in (A). Bars represent the mean (\pm standard deviation) of three technical
742 replicates.

743 (C) Signal variation of SipA-NLuc secretion assayed over an entire 384-well microplate as
744 shown in (A).

745 (D) SipA-NLuc secretion in response to treatment with 37 different bioactive compounds,
746 assayed as shown in (A). The DMSO-treated control was set to 100%. The layout of the
747 plate is shown in Table S1.

748 (E) Comparison of the results of two independent compound screens as in (D). The R² value
749 was calculated from a linear regression.

750

751

752 **Fig 4** Assessment of the PMF-dependence of type III secretion by the NLuc secretion assay

753 (A) Normalized secretion of SipA-NLuc in *S. Typhimurium* P_{ara}*hila* after induction of SPI-
754 1 by addition of 0.02% arabinose. CCCP was added to a final concentration of 0, 5, 10
755 and 15 μ M, respectively, 60 min after induction of SPI-1.

756 (B) As in (A) but addition of K⁺ benzoate to final concentration of 0, 5, 10 and 20 mM,
757 respectively.

758 (C) As in (A) but addition of Valinomycin to a final concentration of 0, 20, 40 and 60 μ M,
759 respectively.

760 All data represent means (\pm standard deviation) of three independent measurements.

761

762 **Fig 5** Development of NLuc-based host cell injection assays

763 (A) Cartoon showing setup of NLuc injection assay. *S. Typhimurium* expressing SipA-NLuc
764 was allowed to infect HeLa cells for 60 min. SipA-NLuc was injected into HeLa cells
765 by use of the T3SS injectisome. Bacteria were washed away using a microplate washer
766 and subsequently NLuc luminescence was measured.

767 (B) Luminescence of SipA-NLuc secreted by the *S. Typhimurium* wild type and indicated
768 mutants in the absence of host cells. The luminescence of the wild type was set to 100%.

769 (C) Luminescence of SipA-NLuc injected into HeLa cells by the *S. Typhimurium* wild type
770 and indicated mutants. The experimental setup was as shown in (A). The luminescence
771 of the wild type was set to 100%.

772 (D) Cartoon showing setup of split-NLuc (HiBiT) injection assay. *S. Typhimurium*
773 expressing SipA-HiBiT was allowed to infect HeLa cells (expressing LgBiT) for
774 60 min. SipA-HiBiT was injected into HeLa cells by use of the T3SS injectisome.
775 Luminescence of the complemented split-NLuc was measured.

776 (E) Luminescence of LgBiT-complemented SipA-HiBiT secreted by the *S. Typhimurium*
777 wild type and indicated mutants in the absence of host cells. The luminescence of the
778 wild type was set to 100%.

779 (F) Luminescence of SipA-HiBiT injected into LgBiT-expressing HeLa cells by the *S.*
780 *Typhimurium* wild type and indicated mutants. The experimental setup was as shown in
781 (D). The luminescence of the wild type was set to 100%.

782 (G) Luminescence of SipA-HiBiT injected into LgBiT-expressing HeLa cells by the *S.*
783 Typhimurium wild type and the $\Delta sctV$ mutant. At timepoint zero, HeLa cells were
784 infected with *S. Typhimurium* after which cells were incubated inside a microplate
785 reader in the presence of NLuc substrate. Luminescence was followed in 2 min intervals.
786 Values of the $\Delta sctV$ mutant were set to zero for each time point. The results show the
787 mean of technical triplicates.

788 Bar graphs represent mean (\pm standard deviation) of three independent measurements. Asterisks
789 indicate statistical significance between wt and mutant strains assessed by a Students *t*-test,
790 ***: $p \leq 0.001$ **: $p \leq 0.01$

791

792 **Fig 6** Cartoon summarizing the utilization of the NLuc-based T3SS secretion and injection
793 assays

794

795 **Supplemental Material**

796 **Fig S1** Expression and secretion of SctP-NLuc, SctP-HiBiT, and SctA-NLuc fusions

797 **Fig S2** Stability of NLuc in LB and in culture supernatant

798 **Table S1** Statistics of the reproducibility assessment of the 384-well microplate format NLuc secretion
799 assay

800 **Table S2** Layout of compound screening test plate incl. SipA-NLuc secretion of one measurement

801 **Table S3** Strains, plasmids, primers

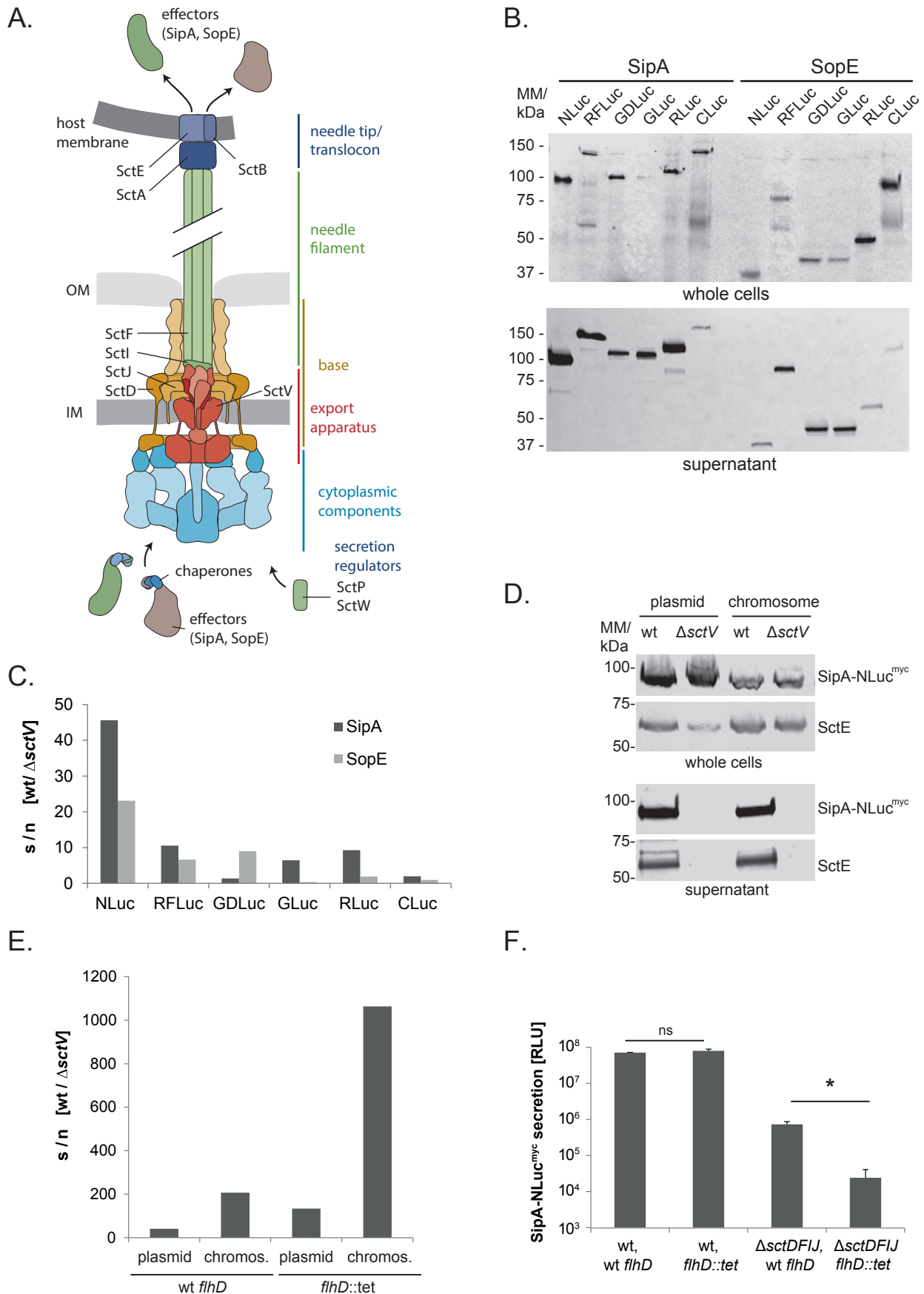
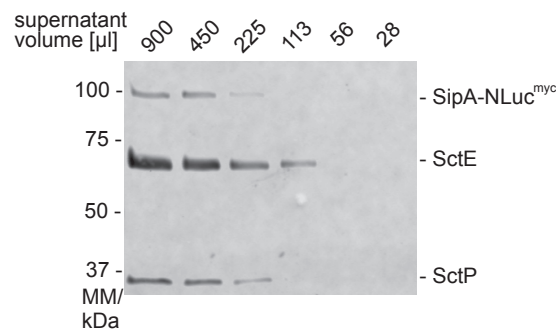
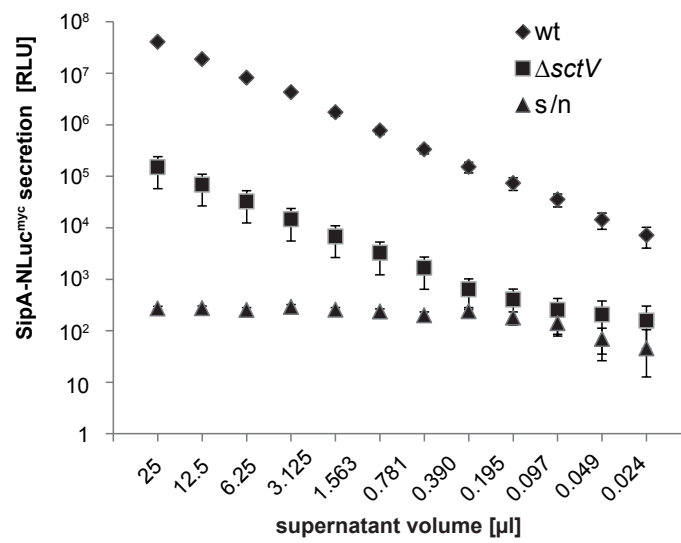


Figure 1

A.



B.



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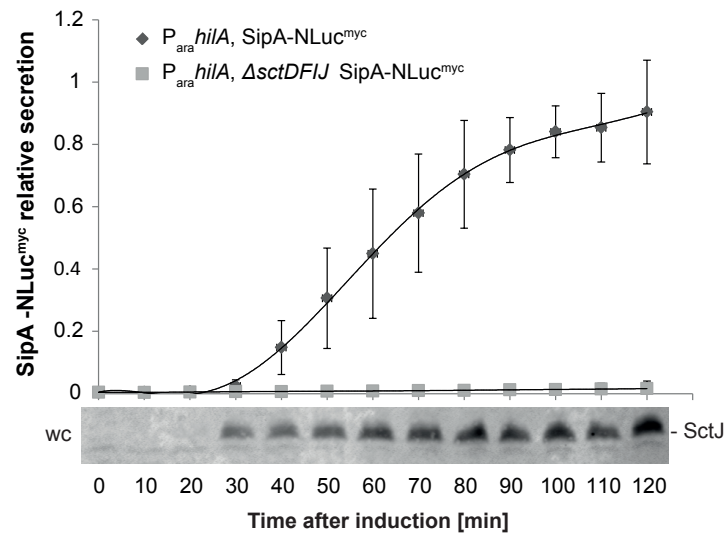


Figure 2

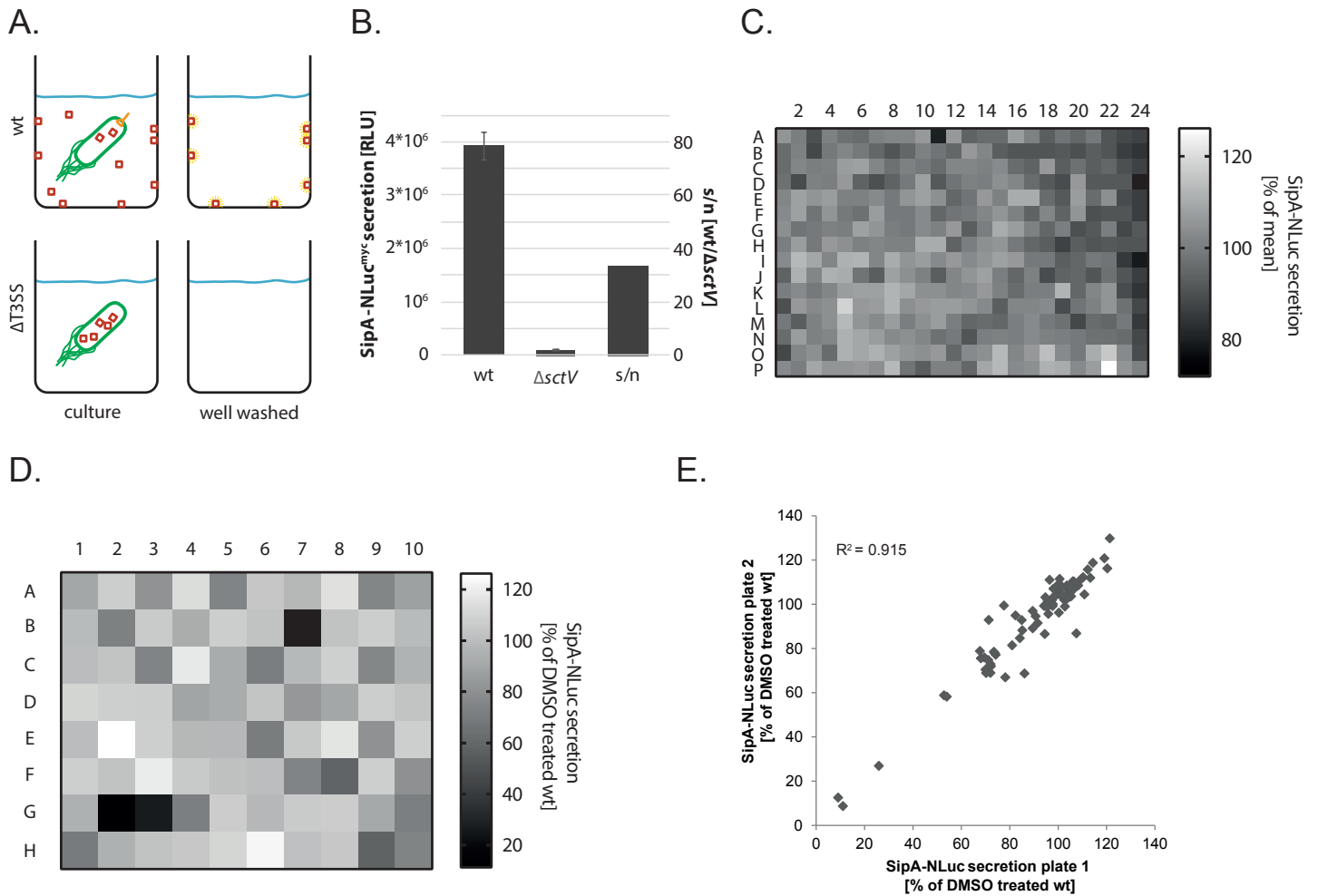


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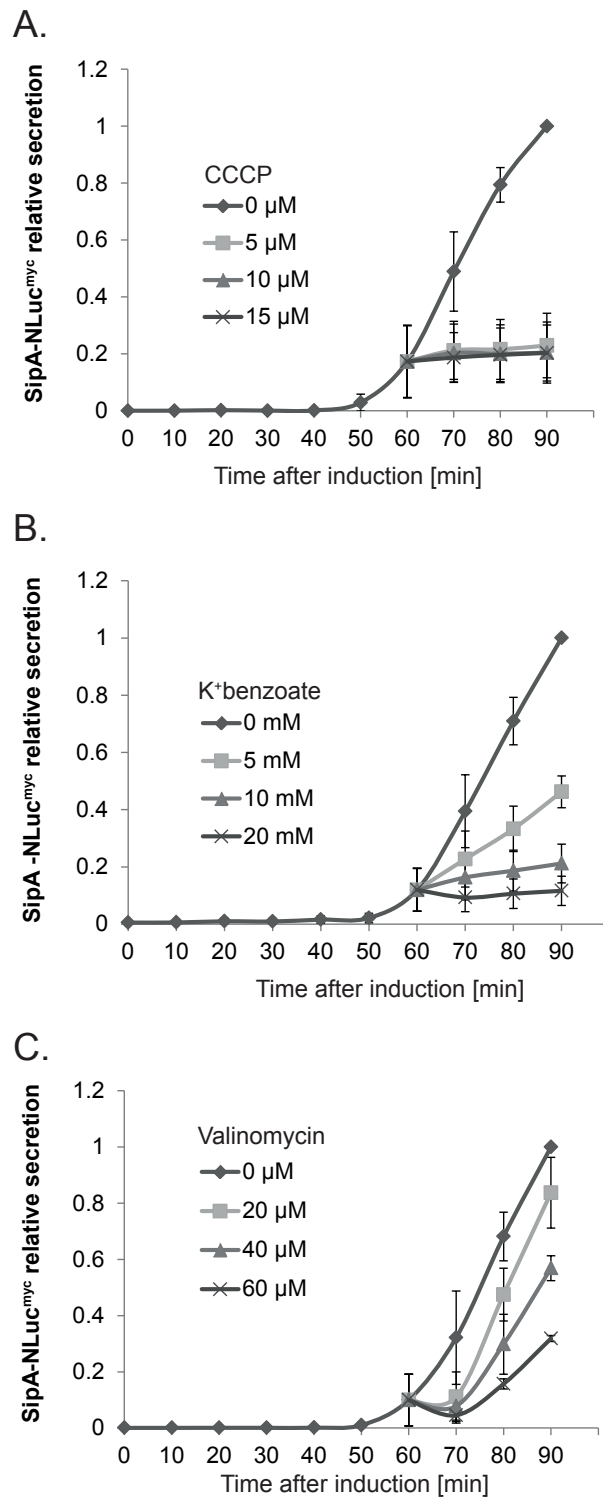


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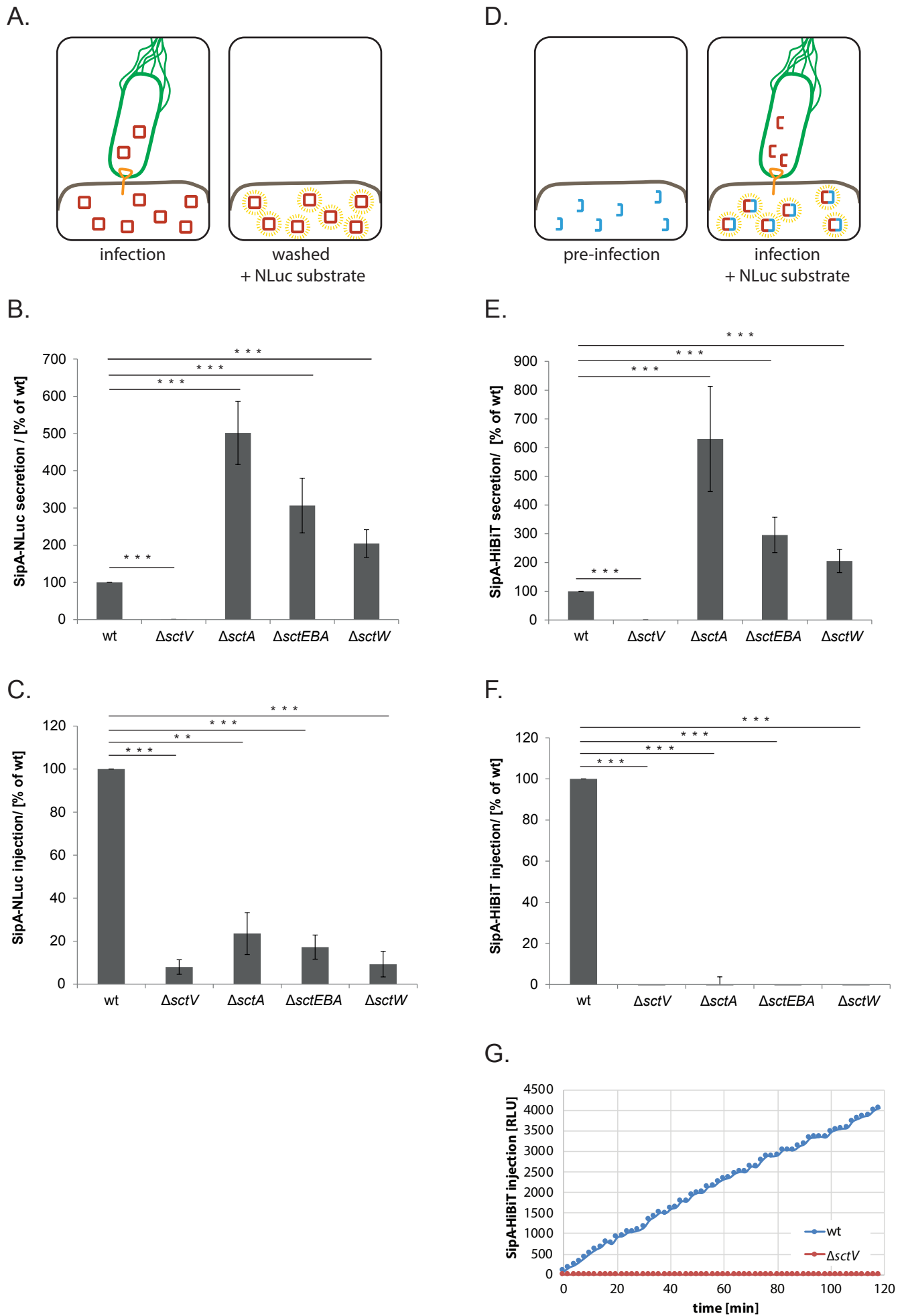


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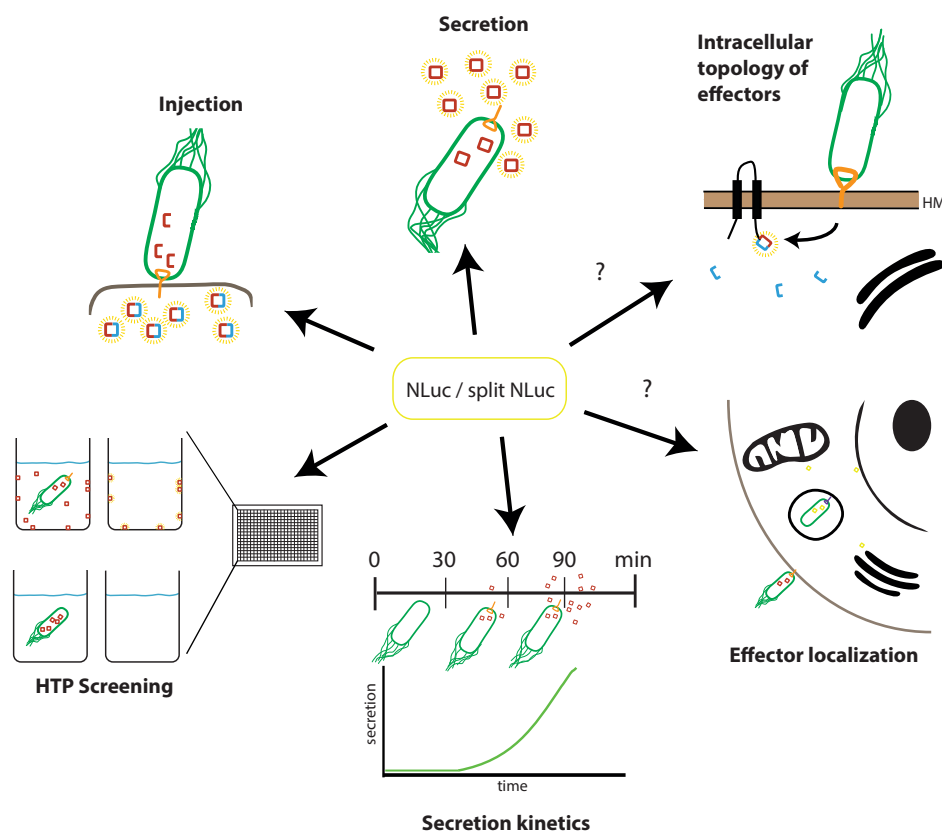


Figure 6

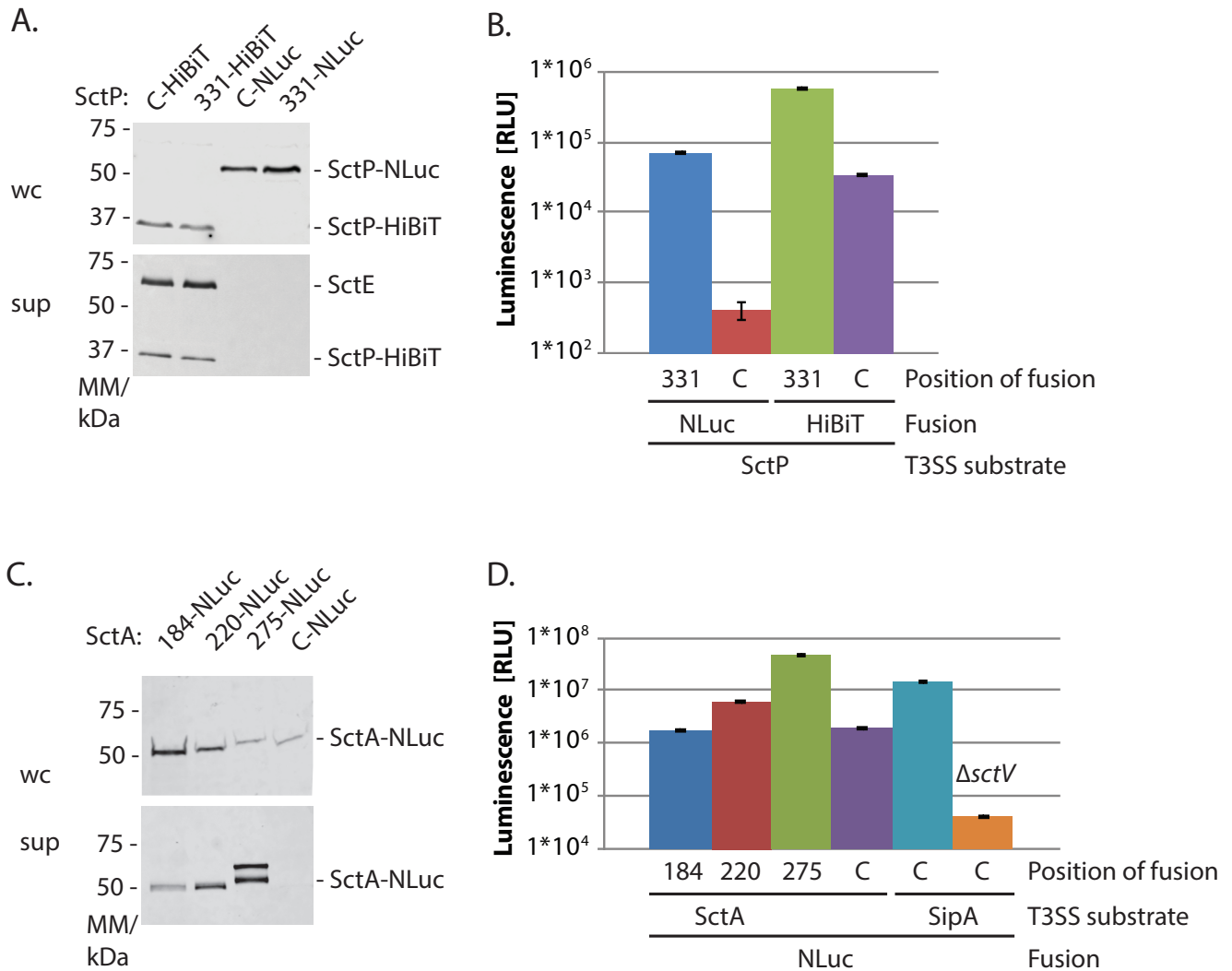


Fig S1 Expression and secretion of SctP-NLuc, SctP-HiBiT, and SctA-NLuc fusions.

(A) Immunodetection of the indicated SctP-NLuc and SctP-HiBiT fusions, and of SctE on Western blot of SDS PAGE-separated culture supernatants and whole cell lysates. 331 means that NLuc or HiBiT was inserted behind residue 331 of SctP, so that the Shine-Dalgarno sequence of *sctQ*, which is overlapping with the gene of SctP, was unaffected. Note that SctE is not secreted when expressing SctP-NLuc fusions, i.e. SctP-NLuc cannot complement the needle length regulating function of SctP, thus substrate specificity switching to the secretion of intermediate substrates is not induced.

(B) Luminescence of the indicated SctP-NLuc/HiBiT-fusions secreted into the culture supernatant. Data represent the mean (\pm standard deviation) of three technical replicates. Note that SctP₃₃₁-NLuc can be detected in the culture supernatant by luminometry but not by Western blotting. Also note that split-NLuc generally gives lower luminescence than regular NLuc.

(C) Immunodetection of the indicated SctA-NLuc fusions on Western blot of SDS PAGE-separated culture supernatants and whole cell lysates. The numbers (184, 220, 275) mean that NLuc was inserted behind these residues of SctA. The insertion positions were chosen based on the structure of *S. Typhimurium* SctA-1. Secreted SctA₂₇₅-NLuc reproducibly appeared as a double band for unknown reasons.

(D) Luminescence of the indicated SctA-NLuc and SipA-NLuc-fusions secreted into the culture supernatant. Data represent the mean (\pm standard deviation) of three technical replicates. Note that SctA_C-NLuc can be detected in the culture supernatant by luminometry but not by Western blotting. Also note that internal fusions of NLuc are accommodated well, with SctA₂₇₅-NLuc providing even stronger signal than SipA-NLuc.

Abbreviations: sup: culture supernatant, wc: whole cell lysates, C: C-terminus, RLU: relative luminescence units, NLuc: NanoLuc luciferase, T3SS: type III secretion system

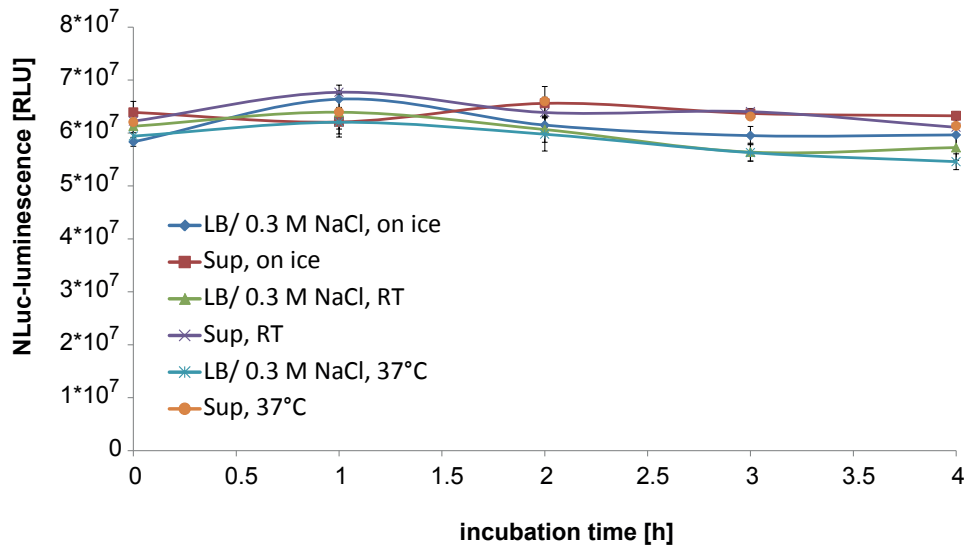


Fig S2 Stability of NLuc in LB/ 0.3 M NaCl and in culture supernatant. The enzymatic activity of purified NLuc was determined after incubation for 4 h at different conditions (on ice, room temperature (RT) and 37°C) in fresh LB/ 0.3 M NaCl and in filtered culture supernatant.