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1 **CLIQ-BID: A method to quantify bacteria-induced damage to eukaryotic**
2 **cells by automated live-imaging of bright nuclei**

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12

13 ABSTRACT

14 Pathogenic bacteria induce eukaryotic cell damage which range from discrete modifications
15 of signalling pathways, to morphological alterations and even to cell death. Accurate
16 quantitative detection of these events is necessary for studying host-pathogen interactions and
17 for developing strategies to protect host organisms from bacterial infections. Investigation of
18 morphological changes is cumbersome and not adapted to high-throughput and kinetics
19 measurements. Here, we describe a simple and cost-effective method based on automated
20 analysis of live cells with stained nuclei, which allows real-time quantification of bacteria-
21 induced eukaryotic cell damage at single-cell resolution. We demonstrate that this automated
22 high-throughput microscopy approach permits screening of libraries composed of
23 interference-RNA, bacterial strains, antibodies and chemical compounds in *ex vivo* infection
24 settings. The use of fluorescently-labelled bacteria enables the concomitant detection of
25 changes in bacterial growth. Using this method named CLIQ-BID (Cell Live Imaging
26 Quantification of Bacteria Induced Damage), we were able to distinguish the virulence
27 profiles of different pathogenic bacterial species and clinical strains.

28 INTRODUCTION

29 Bacterial toxins targeting eukaryotic cells can either directly affect plasma membrane
30 integrity or alternatively they may be internalized, translocated or injected inside the cells.
31 Independent of their route, toxins induce modifications of cell morphology and/or provoke
32 host-cell death. For example, the Anthrax Lethal Toxin (LT) is able to provoke pyroptosis or
33 apoptosis, depending on the cell type and the LT concentration. Furthermore, at sub-lethal
34 concentrations, it induces modification of the cytoskeleton and alters the distribution of
35 junction proteins in endothelial and epithelial cells¹. In Gram-negative bacteria, Type Three
36 Secretion System (T3SS) toxins hijack eukaryotic signalling pathways, leading to damage
37 ranging from modifications of the normal cytoskeleton function, to cell death, depending on
38 the cell type and the toxin².

39 Host-pathogen interaction studies therefore rely on detection and quantification of the
40 bacteria-induced eukaryotic cell injuries. Plasma membrane permeabilization leading to cell
41 death, the most dramatic outcome of the cell intoxication process, is usually monitored
42 through the enzymatic measurement of lactate dehydrogenase released after plasma
43 membrane rupture, or through the detection of nuclear stain incorporation by flow cytometry³⁻
44 ⁵. However, the analysis of early events such as the morphological changes induced by
45 cytoskeleton rearrangements are usually based on fixed and stained cells, rendering fine
46 kinetics studies laborious, or on expression of fluorescent chimeric markers, a time-
47 consuming procedure to which some cells are refractory⁶. These approaches are not easily
48 accessible to non-expert scientists.

49 Overall, there is a dearth of simple methods allowing real-time quantification of
50 morphological changes or cell death. Here, we present the CLIQ-BID method, based on
51 automated high-throughput monitoring of the fluorescence intensity of eukaryotic cell nuclei
52 stained with vital-Hoechst. This live-imaging method permits real-time quantification of

53 bacteria-induced cell damage at single-cell resolution. Starting from an observation in the
54 context of the *Pseudomonas aeruginosa* T3SS, it was extended to other Gram-positive and
55 Gram-negative bacteria equipped with diverse virulence factors. Towards identification of
56 new antibacterial therapeutic targets or research tools, this convenient approach could be
57 employed in functional high-throughput screening of interference-RNA, bacterial strains,
58 antibodies or small molecules. More generally, the CLIQ-BID method could also be used in
59 other cytotoxicity and cell-stress studies.

60

61 RESULTS

62 ***P. aeruginosa* induces a quantifiable nuclei size reduction**

63 The injection of the exotoxins ExoS, T, Y and ExoU by the T3SS machinery is one of the
64 main virulence determinants of *P. aeruginosa* clinical strains⁷. Those toxins have profound
65 effects on eukaryotic cell biology, provoking plasma membrane disruption or inhibition of
66 phagocytosis followed by a delayed apoptosis⁸. Visually, ExoS and ExoT action on host
67 cytoskeleton leads to a reduction of cell area and a “shrinkage” phenotype⁹. In the search for
68 robust descriptors of this phenomenon, we observed that the Hoechst-stained nuclei of Human
69 Umbilical Vascular Endothelial Cells (HUVECs) become gradually smaller and brighter
70 during incubation with the wild-type *P. aeruginosa* strain PAO1 harbouring ExoS and ExoT.
71 In addition this increased intensity of nuclear staining remarkably correlated with the decrease
72 of cell area (Fig. 1a, compare upper and lower images). The built-in Arrayscan image analysis
73 workflow was employed in order to obtain the nuclei mask (Fig. 1a insert – magenta outlines)
74 by intensity thresholding and the quantitative features corresponding to their areas and
75 fluorescence intensities. The graphical representation of these features extracted from 70
76 nuclei at different time points clearly shows a negative correlation between nuclei area and

77 intensity (Fig. 1b). Indeed, the condensation of the nuclei results in an increased concentration
78 of the fluorescent dye complexed to the DNA and thus in an enhanced fluorescence intensity.
79 Furthermore, a nuclear intensity threshold could readily be set to segregate cells with bright
80 nuclei (Fig. 1c). Therefore, a subpopulation of cells displaying bright nuclei, which
81 corresponds to the shrunk cells, could be automatically identified by monitoring the nuclear
82 staining intensity.

83 In order to determine whether the observed nuclei condensation was a phenomenon restricted
84 to HUVECs, the experiment was repeated on CHO, NIH 3T3, HeLa and A549 cells. Despite
85 slight differences in kinetics between the cell types, the nuclear staining intensity increased
86 during the time course of infection by *P. aeruginosa* (Supplementary Fig. S1 and
87 Supplementary Video S1). Of importance, increased brightness was strictly correlated with
88 cell shrinkage (compare upper and lower images of Supplementary Fig. S1).

89 *P. aeruginosa* induces a nuclear condensation that is responsible for the observed increase in
90 nuclear staining intensity. A statistical analysis was performed in order to determine which of
91 the nuclear features (i.e. area versus average intensity) better distinguishes between condensed
92 and non-altered populations. To that end, images from uninfected and 3h-infected cells were
93 obtained at 20x and 5x magnifications. Next, the nuclei images were analysed and three
94 features were compared: the mean nuclear area, the mean nuclear intensity and the percentage
95 of bright nuclei, which is the proportion of nuclei whose intensity is above a fixed threshold.
96 These three parameters were the most promising among the different features calculated by
97 the built-in software from the experiment described in Figure 1. The ability of these three
98 parameters to discriminate between the images obtained at the beginning and at the end of the
99 infection was assessed through the Z' -factor (abbreviated Z'). This statistical coefficient takes
100 into consideration the standard deviations as well as the difference between the means of the
101 positive and negative controls, and is often used for the optimization and validation of High

102 Throughput Screening assays¹⁰. The values reported in Supplementary Table S1 clearly
103 indicate that the percentage of bright nuclei is the most discriminant parameter with $Z'=0.64$
104 for 20x magnification, and that taking images at 5x magnification further increases the power
105 of the test ($Z'=0.75$).

106 The vital nuclear staining used here offers the possibility to monitor modifications of the
107 nuclei by live-imaging during infection. To obtain live kinetics, the nuclei of the five cell
108 types were labelled prior to infection with *P. aeruginosa* and images were taken at 5x
109 magnification every 15 minutes for 4 hours. Images of the same fields at one hour interval are
110 presented (Fig. 2a). Afterward, automated segmentation of the nuclei and measurement of
111 fluorescence intensities were performed as described above and the percentages of bright
112 nuclei were extracted from the built-in software. To visualize the cells that were considered to
113 be damaged upon bacterial infection, nuclei with intensities above a fixed threshold were
114 delineated in green while those below the threshold were delineated in magenta (Fig. 2a –
115 inserts). Nuclei segmentation and thresholding based on vital-Hoechst staining intensity
116 properly reflected the observed appearance of bright nuclei during the progression of
117 infection. Furthermore, images show that nuclei condensation of 3T3, HeLa and A549 cells
118 occurred earlier than for HUVEC and CHO cells. This was confirmed by the kinetic plots
119 (Fig. 2b) that successfully detected an increase of bright nuclei, occurring exponentially with
120 the duration of cell infection. These plots further depicted differences between cells in terms
121 of inflection time and curve steepness. It was therefore possible to identify kinetics signatures
122 for each of the five cell types. The method was named CLIQ-BID, standing for Cell Live
123 Imaging Quantification of Bacteria Induced Damage.

124

125 **Detection of bright nuclei is highly discriminant**

126 We then focused our analysis on HUVECs which are particularly relevant because they are
127 primary human cells, forming a polarized monolayer. The action of *P. aeruginosa* on
128 HUVECs have been extensively studied by cellular biology and the morphological changes
129 observed during infection have been described by microscopy approaches^{9,11-15}. Furthermore,
130 a “cell area” assay based on the quantification of fixed cells’ area by immunofluorescence
131 staining was previously reported^{9,14}. Therefore, we investigated how the CLIQ-BID method
132 compares to the previously published method which is based on immunofluorescence cell
133 staining. For this purpose, HUVECs were infected for different time periods, their nuclei were
134 observed with vital Hoechst staining and then immediately fixed, immunostained and
135 observed at the same position in the wells. A comparison of the results obtained with the
136 CLIQ-BID and “cell area” quantification methods is presented in Figure 3. Nuclei were
137 segmented and discriminated based on their fluorescence intensities (Fig. 3a, Hoechst and
138 bright nuclei images) while the cells’ area was quantified based on thresholding of the
139 vinculin staining (Fig. 3a, Vinculin and cell area images). Indeed, the area covered by the
140 cells decreased during infection with *P. aeruginosa*, in agreement with previous
141 observations¹⁴. Furthermore, the plots of the percentage of bright nuclei and the percentage of
142 the field area cleared by the cells after different infection durations exhibit a similar trend and
143 the correlation coefficient between the readouts of the image pairs was 0.96. However, the
144 standard deviations were much lower for the percentage of bright nuclei than for the cell area
145 (Figure 3b).

146 In order to confirm that the CLIQ-BID method reduces the variation between individual data
147 points, the comparison experiment was repeated with two modifications: i) the number of
148 replicate wells was set to 30 per condition and ii) the HUVECs were infected for 3 hours
149 either with a wild-type *P. aeruginosa* strain or the $\Delta pscF$ strain, which is unable to produce
150 the T3SS toxin injection needle and is therefore deficient for a functional T3SS. This

151 experiment substantiated that the CLIQ-BID method produces more robust and discriminant
152 results with a Z' -factor equal to 0.89 versus 0.14 for the cell area method (Supplementary
153 Table S2).

154 The high Z' -factor obtained with the CLIQ-BID method indicated that it is well-suited for
155 high throughput screening. To further examine this possibility, cell infections with wild-type
156 or $\Delta pscF$ *P. aeruginosa* strains were compared in 96- and 384-well plates. The live-imaging
157 monitoring of nuclei intensities allowed the obtaining of reproducible kinetics curves in the 48
158 replicates (Supplementary Fig. S2). Indeed, the Z' -factor values obtained by comparing the
159 wild-type and the T3SS deficient strains were close to or above 0.9 for both plate formats.
160 Importantly, an additional 384-well plate was inoculated with overnight *P. aeruginosa*
161 cultures, as opposed to exponential phase cultures, which is currently used to detect T3SS
162 activity (Supplementary Fig. S2). The removal of the subculture step significantly reduces the
163 handling procedure in the perspective of bacteria or molecule library screens. Despite higher
164 variation than with exponential cultures, the Z' -factor displayed values close to 0.8, higher
165 than the gold-standard of 0.7 above which a library could be screened in a single replicate
166 with an acceptable risk of false-positives and -negatives. Taken together, these results indicate
167 that the newly-developed CLIQ-BID method is adapted for HTS strategies.

168 In the search for a global descriptor of each kinetics plot, the Area Under the Curve (AUC)
169 was selected. As expected, large differences were observed between AUC obtained from
170 wells infected with wild-type or T3SS deficient strains (Supplementary Fig. S3). Indeed, the
171 statistical analysis showed that the AUC can robustly discriminate between infections by
172 these two strains, with Z' -factor values of 0.77 and 0.63 in 96- and 384-well plates,
173 respectively (Supplementary Table S3).

174

175 **Bright nuclei detection enables potent screening strategies**

176 Considering the encouraging results, the potential of the method to screen for inhibitors of
177 bacteria-induced cell damage was further investigated on a panel of molecules. In this proof-
178 of-concept experiment, compounds targeting the bacteria or the eukaryotic cells were tested,
179 along with siRNA. A major improvement was also made with the use of bacteria
180 constitutively expressing GFP in their cytosol. Measuring the global GFP fluorescence
181 increase in the wells thus enables the detection of possible bacteriostatic/bactericidal
182 properties of the tested compounds. These experiments were therefore analysed by plotting
183 the kinetics curves of bright nuclei (cell toxicity) and GFP fluorescence (bacterial growth), as
184 represented in Figure 4.

185 Cell damage were significantly delayed by known inhibitors of *P. aeruginosa* T3SS, like the
186 small molecule MBX2401 or polyclonal antibodies raised against the tip protein PcrV^{16,17}.
187 Conversely, neither DMSO nor antibodies targeting the translocator PopB had any effect on
188 the cell infection, as expected (Fig. 4a). Among molecules targeting the eukaryotic cells, the
189 prostaglandin PGE2 and forskolin, a cAMP inducer recently shown to inhibit *P. aeruginosa*
190 T3SS effects through Rap1 activation¹², significantly delayed the kinetics (Fig. 4c). On the
191 other hand staurosporine and chelerythrine, two potent cytotoxic inhibitors of protein
192 kinases^{18,19}, immediately induced the appearance of bright nuclei. Other compounds
193 moderately accelerated cell damage during infection, namely H-89 (protein kinase A
194 inhibitor), sphingosine-1-P (signalling phospholipid) and NSC23766 (Rac1 inhibitor), while
195 wortmannin (PI3-K inhibitor) had no effect. Of note, cells incubated with H-89, sphingosine-
196 1-P and Forskolin in the absence of bacteria exhibited a higher basal level of bright nuclei
197 (Supplementary Fig. S4). This moderate toxicity could explain the accelerated kinetics
198 observed when cell were infected in the presence of H-89 and sphingosine-1-P and the
199 relatively high baseline observed with foscokolin at the beginning of cell infection. Finally, cells

200 were grown after transfection with arbitrarily chosen siRNAs from a laboratory collection and
201 were subsequently infected. From the 18 tested siRNAs, some had no effect while some
202 exhibited promoting or inhibiting activities (Fig. 4e). The validation and the biological
203 investigation of the role of their targets are beyond the scope of this work.

204 Observing the effect of a particular treatment on bacteria growth (Fig. 4b, d, f) allowed the
205 determination as to whether any virulence inhibition was related to an antibiotic effect.
206 Indeed, none of the eukaryotic nor T3SS-specific targeting compounds exhibited a
207 bacteriostatic effect, while the antibiotic gentamicin prevented bacterial growth and,
208 consequently, host cell intoxication. Furthermore, the synthetic descriptors of the cell toxicity
209 and bacterial kinetics curves (respective AUCs) permitted the straightforward statistical
210 comparison of the kinetics by one-way ANOVA (Supplementary Fig. S5). In conclusion, the
211 developed method enables the identification of promotional or inhibitory effects from a
212 variety of molecular categories (small organic molecules, antibodies and siRNAs) and allows
213 one to simultaneously counter-screen for antibiotic effects.

214 Finally, the method was employed to compare the effects of different bacterial strains and
215 bacterial species. For this purpose, HUVECs were infected with 16 different bacteria and the
216 intensities of cell nuclei were monitored by live-imaging. Kinetics plots and the
217 corresponding AUCs (Fig. 5) show the diverse virulence potential of these bacteria. Among
218 the *P. aeruginosa* strains, the PP34 strain, injecting through its T3SS the powerful
219 phospholipase toxin ExoU^{3,7,20}, was the most active followed by IHMA87 and
220 PAO1 Δ *pscD::exlBA* (PAO1 *exlBA*) strains expressing the recently discovered Two Partner
221 Secreted toxin ExlA²¹⁻²³ and the CHA reference strain used throughout this study. Of interest,
222 the *Serratia marcescens* strain expressing the ShlA toxin²⁴ homolog to ExlA was as active as
223 the PP34 strain, while the isogenic mutant strain that has a transposon inserted into the *shlB*
224 gene²⁵, did not induce the appearance of bright nuclei. In *Staphylococcus aureus*, the 8325-4

225 strain exhibited lower effects than the closely related USA300 BEZIER and SF8300 strains,
226 both from the USA300 lineage known to be highly virulent²⁶. This workflow was also
227 successful in detecting cellular damage caused by *Yersinia enterocolitica*, which correlated
228 with its T3SS since the mutant depleted of T3SS toxins exhibited a significantly lower
229 activity. Finally, five bacteria species did not display measurable effects toward the
230 eukaryotic cells under the used experimental conditions, notably the relatively short time span
231 of infection and low MOI. Of interest, the statistical analysis of AUCs derived from the
232 kinetics plots of each replicate confirmed the depicted intra-species differences (Fig. 5b). The
233 sigmoid curves obtained with the virulent bacteria display different shapes, notably regarding
234 the lag phase and the slope. These parameters are not expected to be correlated because
235 different virulence mechanisms triggers effects with different delays and different degrees of
236 synchronicity in the target cell population. The AUCs calculation does not give access to
237 these variations and it is conceivable that they could compensate, resulting in some cases in
238 similar AUCs for different curve shapes. Therefore, the curves of each replicate of the same
239 experiment were fitted using a sigmoid equation and the calculated inflection points and curve
240 steepness were represented on a XY plot for each bacteria species (Fig. 5c). This analysis
241 clearly highlights inter- and intra-species differences while similar strains within *P.*
242 *aeruginosa* (PAO1 *exlBA* and IHMA87) and *S. aureus* (SF8300 and USA300) species
243 clustered together. Overall, real-time imaging allowed the observation of different cell-
244 damage kinetics, which are in agreement with what is expected for the corresponding bacteria
245 featuring diverse toxins and virulence mechanisms.

246

247 DISCUSSION

248 The new high-throughput image analysis strategy described in this work exhibits great
249 potential for monitoring cell damage induced by bacteria, as well as by other mechanisms.

250 Through nuclei monitoring, the method allows the observation of cell-reaction to Gram-
251 positive and Gram-negative bacteria secreting or injecting toxins. Furthermore, both cell lysis
252 induced by plasma-membrane targeting toxins ExoU and ExlA^{20,22}, as well as cell shrinkage
253 induced by ExoS^{9,27} were readily detected. Of note, this method can also be employed to
254 reveal cytotoxicity or cell stress from a variety of origins, as exemplified by the detection of
255 the effects of chelerythrine and staurosporine known to promote apoptosis. It is thus able to
256 detect cell shrinkage, necrosis and apoptosis.

257 The developed method allows cost-effective kinetic measurements of bacteria-induced action
258 on several cell lines, requiring a simple staining procedure, a microscope and an image-
259 analysis software. Detection of nuclei and quantification relies on the widely-used Hoechst
260 33342, an inexpensive vital stain of cell nuclei used for almost four decades²⁸. After image
261 acquisition on the microscope, images may be analysed with standard software like Cell
262 Profiler, ImageJ or Fiji²⁹⁻³¹, using basic algorithms to delineate nuclei and measure their
263 fluorescence intensities. In our work, we used an automated microscope along with its
264 proprietary analysis software to demonstrate the great potential of this method for High
265 Content Screening.

266 Indeed the Z'-factor values obtained in different assay configurations were often above 0.8.
267 This statistical descriptor reflects the quality of a screening method, i.e. its ability to identify
268 "hits"¹⁰. Screening of libraries are undertaken only if the Z'-factor is above 0.6 and a value
269 above 0.7 is considered to be fully satisfactory. The elevated Z'-factor value obtained in 384-
270 well microplates with fresh or overnight cultures of bacteria indicated that this assay could be
271 employed to screen large bacterial mutant or eukaryotic CRISPR/Cas9 libraries. Furthermore,
272 the screening approach was successfully applied to a set of antibodies and small molecules
273 targeting either bacteria or eukaryotic cells and to a panel of siRNAs. Indeed, it identified
274 three inhibitory activities among the tested small molecules and antibodies: the MBX2401

275 drug and anti-PcrV antibodies, both known to inhibit *P. aeruginosa* T3SS^{16,17}, and forskolin,
276 known to counteract the T3SS effect in eukaryotic cells¹². This represents a proof of concept
277 for a screening strategy. Moreover, the morphological readout of the method (brightness of
278 the nuclei) is a downstream event in the infection process, enabling the screening of
279 molecules that could target early or late events in the bacteria or the host.

280 In addition, the simultaneous quantification of GFP-expressing bacteria allows one to counter-
281 screen, in the same test, for bacteriostatic/bactericidal activities. Moreover, the same CLIQ-
282 BID method can be used in the absence of bacteria to detect the potential deleterious effects
283 of screened compounds. Therefore, this method can be the basis of a powerful 3-in-1
284 approach to rapidly identify treatments inhibiting bacteria virulence without affecting their
285 growth capacities or the eukaryotic cells' integrity. This is of particular interest since the
286 search for antivirulence treatments is receiving growing attention because they are thought to
287 reduce the risk of resistance emergence and microbiome destabilization^{32,33}.

288 The increasing accessibility to HCS/HCA equipment, with the help of the simple and cost-
289 effective CLIQ-BID method described here, should foster the understanding of bacterial
290 virulence as well as other scientific areas where early cell damage and cell stress are of
291 interest.

292 METHODS

293 **Bacteria strains**

294 *Pseudomonas aeruginosa* strains CHA, PAO1 Δ *pscD::exlBA*, IHMA87 and PP34; and
295 *Serratia marcescens* Db11 and Db11-tn-*shlB* were from our lab collection and previously
296 published^{14,21,22,25}. *Staphylococcus aureus* strains SF8300, USA300 BEZIER and 8325-4 were
297 a kind gift from Dr Karen Moreau. Other strains were *Yersinia enterocolitica* E40 and
298 Δ HOPEMN¹⁷, *Acinetobacter sp. genomospecies 13* ATCC 23220, *Burkholderia cepacia*

299 ATCC 17616, *Pseudomonas putida* KT2442, *Pseudomonas fluorescens* BG1 (environmental
300 isolate, gift from Dr John Willison) and *Stenotrophomonas maltophilia* (ATCC 13637).
301 Bacteria were grown in LB (Luria Bertani - Difco) except for *S. aureus* which were grown in
302 BHI (Brain Heart Infusion – Difco). *Y. enterocolitica* strains were grown at 28 °C and the
303 other strains at 37 °C. Unless otherwise stated, cultures were grown overnight under shaking
304 at 300 rpm and then diluted in fresh media to an optical density measured at 600 nm (OD₆₀₀)
305 of 0.1. When cultures reached OD₆₀₀ of 1, typically after 2.5 h of growth under shaking,
306 bacteria were mixed with eukaryotic cells at a multiplicity of infection (MOI) of 10.

307 **Chemicals and antibodies**

308 Hoechst 33342, Gentamicin, H-89, Sphingosine-1-P, Prostaglandin E2, Staurosporine,
309 Forskolin, NSC23766 and Wortmannin were from Sigma-Aldrich and chelerythrine from
310 Merck Millipore. MBX2401, an inhibitor of the Type Three Secretion System (T3SS) from *P.*
311 *aeruginosa*¹⁶ was synthesized as previously described³⁴. Antibodies raised against *P.*
312 *aeruginosa* PopB and PcrV (anti-PopB and anti-PcrV) were obtained in our laboratory and
313 previously characterized¹⁷. Antibodies to Vinculin were from Santa Cruz and the secondary
314 antibodies coupled to Alexa 488 were purchased from Molecular Probes.

315 **Cell culture**

316 Human umbilical vein endothelial cells (HUVECs) were isolated according to previously
317 described protocols⁹. The use of umbilical cords for scientific purposes is authorized by the
318 L1211-2 act from the French Public Health Code. Written informed consent was obtained
319 from each woman who donated an umbilical cord. The privacy of the donor's personal health
320 information was protected. Recovered cells were cultured in endothelial-basal medium 2
321 (EBM-2; Lonza) supplemented as recommended by the manufacturer. A549 (CCL-185) and
322 HeLa (CCL-2) cells were grown in RPMI supplemented with 10% foetal calf serum. 3T3

323 (CRL-2752) and CHO K1 (CCL-61) cells were grown in DMEM and F12 medium,
324 respectively, supplemented with foetal calf serum.

325 **siRNA**

326 Cells were seeded at 12,500 cells per/well in a 96-well plate and transfected with siRNAs
327 using Lipofectamine™ RNAiMax transfection reagent (Thermo Fisher Scientific), according
328 to the Reverse Transfection manufacturer's protocol. Briefly, 2.5 pmol of siRNA were diluted
329 in 10 µl of supplemented EBM-2 and mixed with 0.2 µl of transfection reagent, pre-diluted in
330 9.8 µl of supplemented EBM-2. After 15min at room temperature, the complexes were added
331 to the cells in a final volume of 100 µl of supplemented EBM-2. Cells were used 48 h later.

332 **Cell treatments, Hoechst staining and infection**

333 Black µclear 96-well plates (Greiner) were seeded at 12,500 cells per/well. Black µclear 384-
334 well plates (Greiner) were seeded at 3,000 cells per well. Cells were used 48 h later to obtain
335 highly confluent monolayers. Medium was replaced 3 h before infection with fresh medium
336 containing Hoechst 33342 (1 µg/ml). After incubation during 1 h, two washes with 80 µl of
337 fresh non-supplemented EBM-2 medium. All media were pre-heated at 37 °C.

338 For pharmacological and antibody treatments, medium was replaced 30 min before infection
339 with 80 µl of fresh medium supplemented with: gentamicin 200 µg/ml, H-89 10 µM,
340 sphingosine-1-P 2 µg/ml, prostaglandin E2 1 nM, staurosporine 1 µM, forskolin 10 µM,
341 NSC23766 50 µM, wortmannin 1 µM, chelerythrine 1 µM, MBX2401 and MBX2402 30 µM.
342 When applicable, the final DMSO concentration was 0.5%. Sera containing antibodies
343 directed against PopB and PcrV were diluted to a final concentration of 5%.

344 Cells were infected at a multiplicity of infection (MOI) of 10 by adding 20 µl of EBM-2
345 containing a 5x concentrated bacteria suspension. Plates were immediately observed by
346 Arrayscan microscopy.

347 **Automated High Content Imaging and High Content Analysis (HCA)**

348 The image acquisitions were performed on an automated microscope ArrayScanVTI (Thermo
349 Scientific) using a Zeiss 20x (NA 0.4) LD Plan-Neofluor or a Zeiss 5x (NA 0.25) Fluar air
350 objectives. In 96-well plates, four images per well were acquired with the 20x or 5x objectives
351 and one image per well was acquired with the 5x objective in 384-well plates. The dichroic
352 mirror used for Hoechst staining was BGRFR-386/23 nm and BGRFR-brightfield for
353 transmitted light imaging. Exposure times were set to reach 40% of intensity saturation in the
354 reference wells at the beginning of the experiment. The microplate was maintained at 37°C
355 and 5% CO₂ in the ArrayScan Live Cell Module and images were automatically acquired
356 every 15 min for up to five hours. Indicated times refer to the actual time of image acquisition
357 and are adjusted for delay between wells due to the plate displacement. Typically five minutes
358 are required to scan a complete 96-well plate.

359 Quantification of nuclei parameters was made using the Cell Health Profiling Bio-Application
360 of Thermo Scientific HCS Studio v6.5.0. Each nucleus was detected in the Hoechst channel
361 with the isodata thresholding method. Border-touch nuclei were rejected from each image.
362 Nuclei area and nuclei average intensity features, respectively named ObjectAreaCh1 and
363 ObjectAvgIntenCh1, were automatically calculated. When indicated in the text, a threshold
364 was applied on the ObjectAvgIntenCh1 feature to discriminate the population of cells with
365 bright nuclei. This threshold was set to 2,100 or 1,200 fluorescence arbitrary units (a.u.) for
366 20x or 5x magnification images respectively, and the proportion of cells with bright nuclei
367 were automatically calculated and named %HIGH_ObjectAvgIntenCh1. The arbitrary units
368 correspond to the raw fluorescence intensities obtained through the HCS Studio software.

369 **Immunofluorescence staining and quantification**

370 Cells were washed, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5%
371 Triton X-100 for 5 min and labelled with a mouse anti-Vinculin 7F9 primary antibody (Santa
372 Cruz) and donkey anti-mouse Cy3 secondary antibody (Jackson ImmunoResearch
373 Laboratories) for 1 h each. Nuclei were labelled with Hoechst 33258 (10 µg/ml, Sigma-
374 Aldrich). Under these conditions, total cellular vinculin is detected yielding a whole-cell
375 labelling. Images were captured using an automated microscope ArrayScanVTI with the 5x
376 magnification objective, in the BGRFR-549/15 nm channel, and treated with ImageJ software.
377 Briefly, images of vinculin staining were binarized and the total cell area was calculated for
378 each image. To facilitate interpretation, results were shown as the percentage of the field area
379 cleared by the cells.

380 **Statistics**

381 To evaluate the quality of the assay and its ability to identify “hits”, the Z' -factor was
382 calculated using the following equation, as described by Zhang et al ¹⁰:

$$383 \quad (1) Z' = 1 - 3 * (\sigma_p + \sigma_n) / |\mu_p - \mu_n|$$

384 where σ_p and σ_n are the standard deviations of the positive and negative conditions,
385 respectively, and μ_p and μ_n are the means of the positive and negative conditions,
386 respectively.

387 Statistical analyses were performed using SigmaPlot 12.5 (Systat software) for the
388 comparison of multiple groups by one-way ANOVA (two-tailed). When appropriate, *post hoc*
389 tests were Tukey or Dunnett for multiple comparisons or comparison to the control group,
390 respectively.

391 In figure legends, n represents the number of well replicates.

392 Curve fitting was done with SigmaPlot 12.5 using the following equation:

393
$$(2) y = y_0 + \frac{a}{1 + e^{-b(x-x_0)}}$$

394 where y_0 and a are the minimal and the range values of the bright nuclei percentage,
395 respectively, and x_0 and b respectively correspond to the inflection time and the curve
396 steepness.

397 **Data availability**

398 The datasets generated during and/or analysed during the current study are available from the
399 corresponding author on reasonable request.

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493

494 AUTHORS CONTRIBUTIONS

495 Y.W. and E.F. designed experiments, Y.W., S.B., P.H. and E.F. performed experiments, I.A.
496 and E.S. contributed reagents and methods, Y.W., E.S., P.H., I.A. and E.F. analyzed and
497 discussed the data and E.F. wrote the manuscript. All authors contributed to and edited the
498 manuscript.

499

500 ADDITIONAL INFORMATION

501 Competing Interests: The authors declare that they have no competing interests.

502

503 FIGURE LEGENDS

504 **Figure 1: Smaller and brighter cell nuclei reflect *P. aeruginosa* induced cell-damage.**

505 Human primary endothelial cells (HUVECs) were infected with *P. aeruginosa* and monitored
506 at different stages of infection by live-imaging microscopy with vital-Hoechst nuclear stain. a)
507 Cell surface and cell nuclei observed in transmitted light and by fluorescent labelling, upper
508 and lower images respectively, in the same field at one hour intervals. Nuclei from the same
509 set of images were automatically segmented (insert – magenta outlines). The scale bars shown
510 on the last nuclei image correspond to 50 μm . b) The area and fluorescence intensities of each
511 segmented nucleus were plotted. Data obtained from different time points are represented in
512 colors, from green (0 min) to dark red (240 min). a.u. = arbitrary units. c) Nuclear staining
513 intensities of cells at different time points of infection are represented in box plots. Whiskers
514 indicate the 10th and 90th percentiles; the top and bottom lines represent the 25th and 75th
515 percentiles; the middle line and dots respectively show median and outliers. Intensities from
516 the three first time points are statistically different from those of the two last time points (one
517 way ANOVA, $P < 0.05$). The horizontal dashed line represent a threshold that could
518 discriminate between normal and bright nuclei. In b) and c), $n = 70$ cells at each time point.
519

520 **Figure 2: Live-imaging quantification of *P. aeruginosa* cell infection by fluorescence**

521 **intensity measurement of Hoechst-labelled nuclei.** HUVEC, CHO, 3T3, HeLa and A549
522 cells were labelled with vital-Hoechst prior to infection with *P. aeruginosa* and monitored by
523 microscopy at a 5x magnification. a) Cell nuclei observed by fluorescent labelling and
524 automatically segmented (insert). Nuclei with intensities below a fixed threshold were
525 delineated in magenta while those above the threshold were delineated in green. The scale bar
526 shown on the first image of the HUVECs cells corresponds to 200 μm . b) Kinetic plots

527 representing the percentage of nuclei with intensities above the thresholds in the images taken
528 every 15 min.

529

530 **Figure 3: Comparison of the CLIQ-BID and “cell area” methods.** HUVECs were infected
531 with *P. aeruginosa* for different durations; images of the nuclei were acquired before cell
532 fixation, immunostaining and acquisition of cell area images. a) Image sets at different time
533 points for i) Hoechst: cell nuclei; ii) Bright nuclei: nuclei automatically segmented and sorted
534 for intensities below (red) or above (green) a fixed threshold; iii) Vinculin: cell area detected
535 with a cytoplasmic vinculin staining; iv) Cell area: automated thresholding of the
536 immunostaining allowing the calculation of the field area covered by the cells. The scale bar
537 shown on the last nuclei image corresponds to 50 μm . b) Plots of the percentage of nuclei
538 with intensities above the thresholds and of the percentage of area cleared by the cells after
539 different infection durations. Error bars represent the standard deviation (n=8).

540

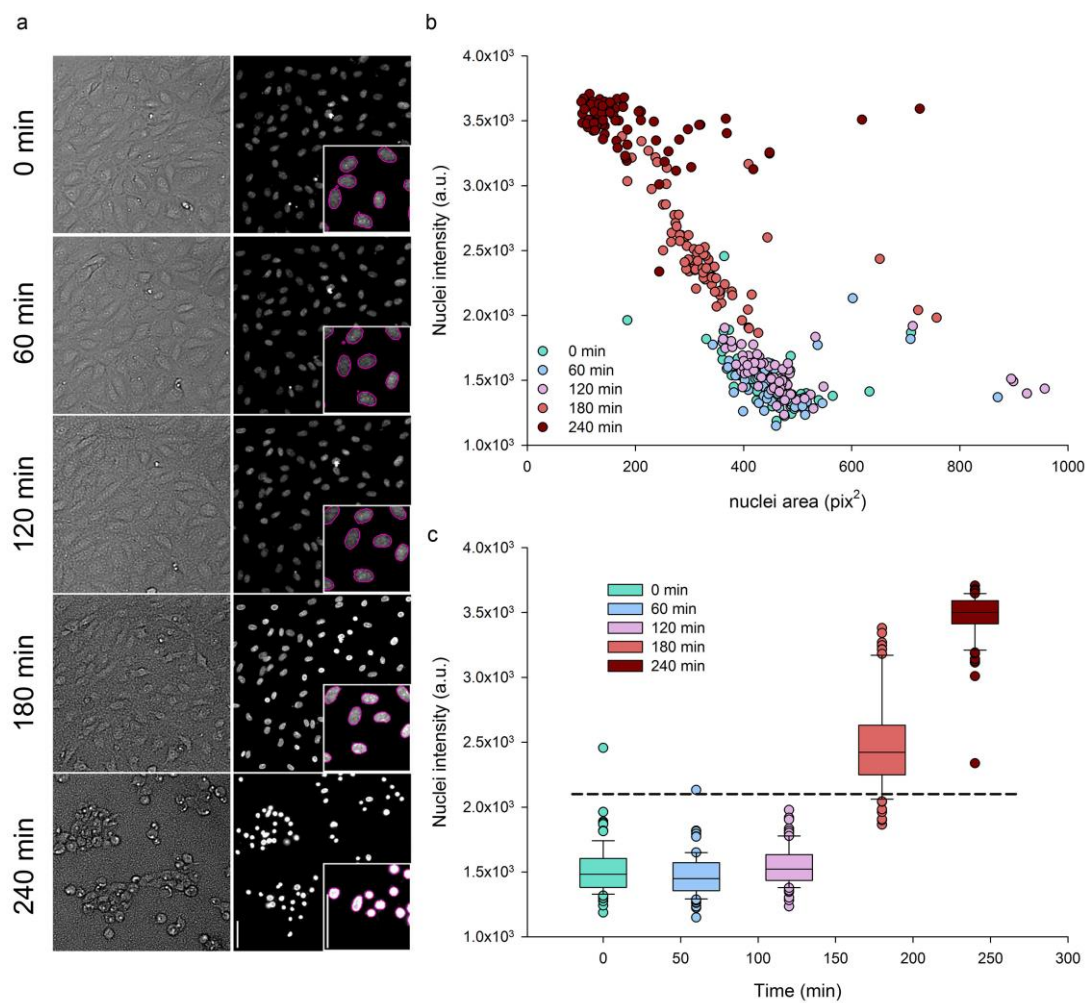
541 **Figure 4: Screening of a panel of molecules.** HUVECs were infected with *P. aeruginosa*
542 wild-type strain in the presence of molecules targeting the bacteria (a, b) or targeting the
543 eukaryotic cells (c, d) and their respective controls. To assess the effect of siRNA transfection
544 in the cells, HUVECs were transfected two days before infection (e, f). A strain deficient for
545 the production of the T3SS needle subunit ($\Delta pscF$) was used as control. The kinetics of bright
546 nuclei appearance (a, c, e) and bacteria growth (b, d, f) were simultaneously recorded by live-
547 imaging and analysed. a.u. = arbitrary units.

548

549 **Figure 5: Comparison of cell damage kinetics with different bacteria.** HUVECs were
550 infected with 16 different bacteria and monitored by live-imaging. The percentages of bright
551 nuclei were used to derive kinetics plots (a) and the corresponding Area Under the Curves

552 (AUC) (b). Each point in the kinetics plots correspond to the means of triplicates and AUC
553 histograms represent the means of the AUCs obtained for the kinetics of each replicate. Error
554 bars represent the standard deviation (n=3). Stars indicate statistically significant differences
555 between strains of the same species and NS a non-significant difference (one-way ANOVA, P
556 < 0.05). Each of Tthe kinetics plot replicatess obtained with different bacterial species
557 inducing “bright nuclei” were fitted with sigmoid model curves and the inflection point and
558 curve steepness were calculated and represented as XY pairs (c). Means and standard
559 deviations are represented.
560

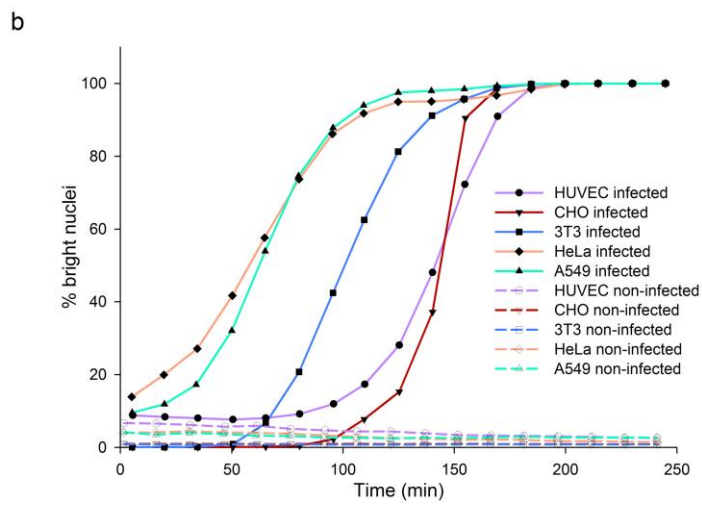
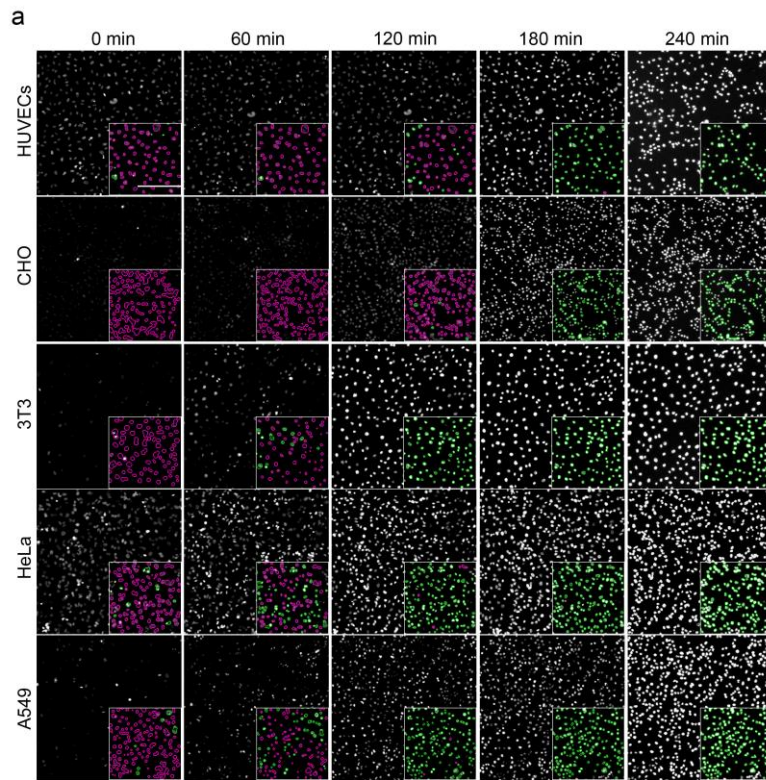
561 Figure 1



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563

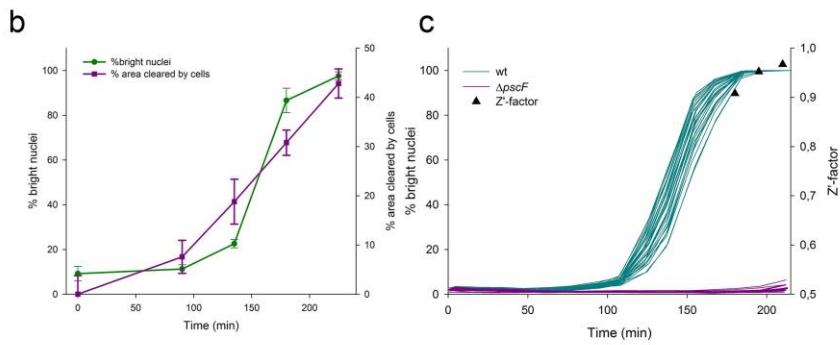
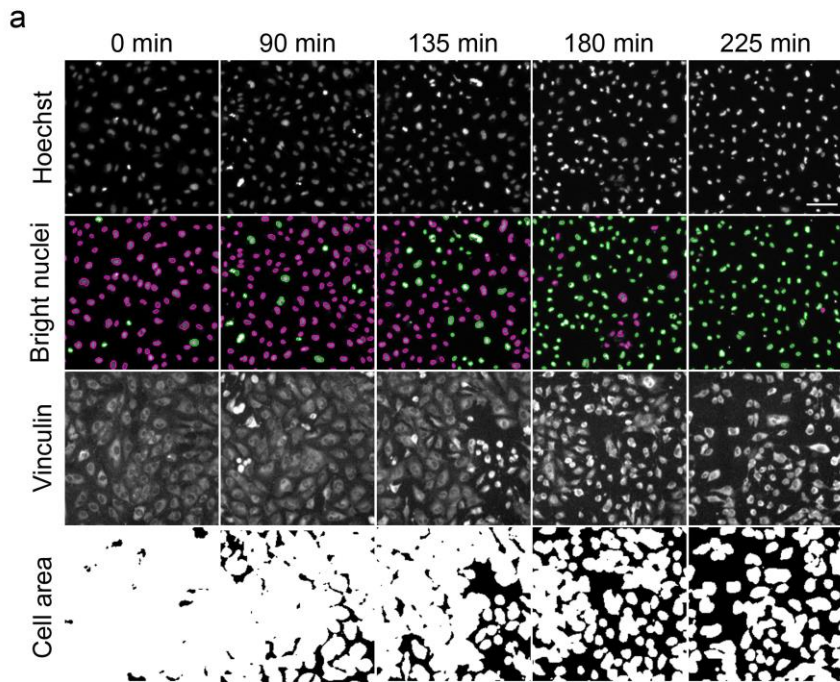
564 Figure 2



565

566

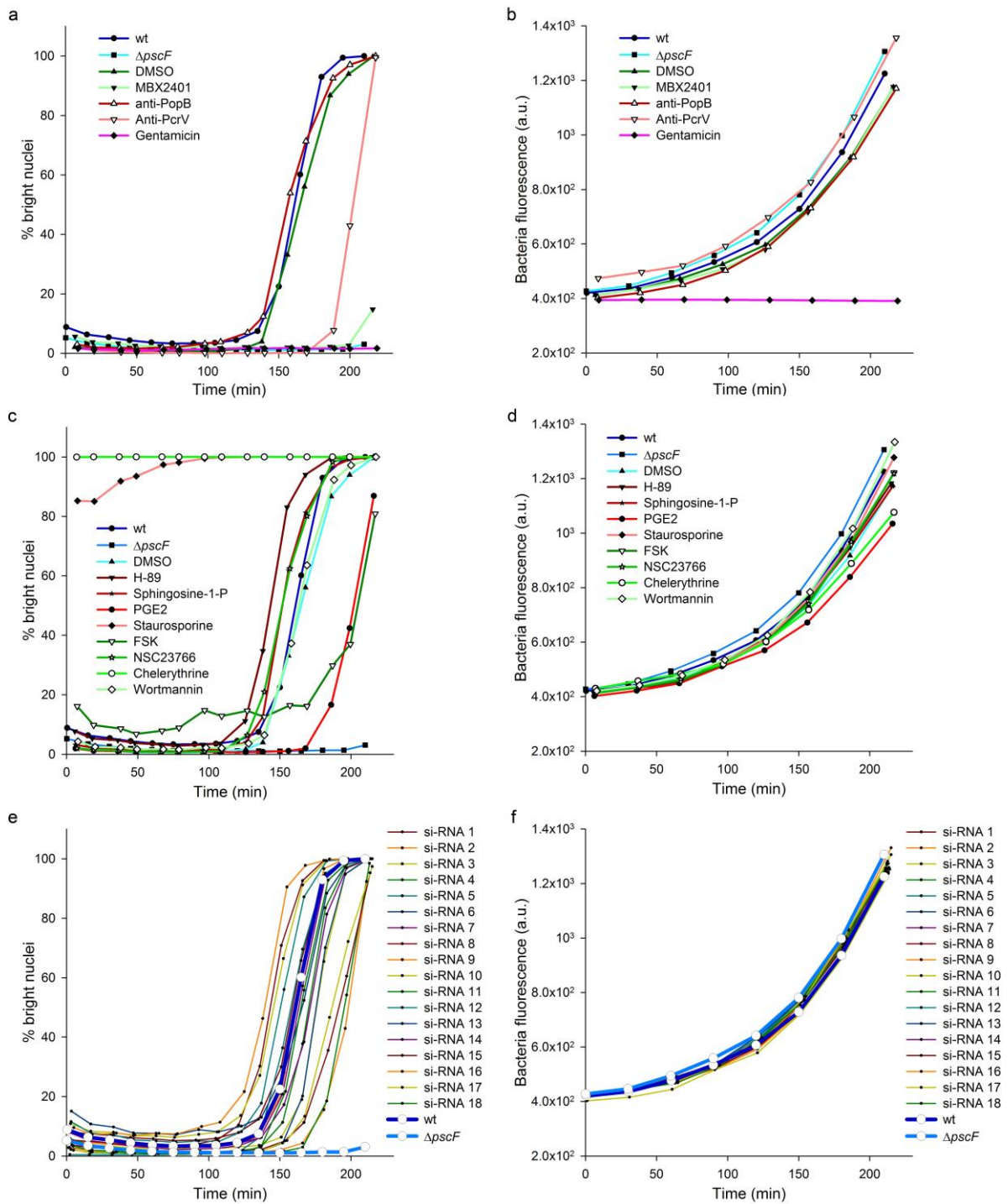
567 Figure 3



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569

570 Figure 4



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572

