

## **A guide for membrane potential measurements in Gram-negative bacteria using voltage-sensitive dyes**

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1 **ABSTRACT**

2

3 Transmembrane potential is one of the main bioenergetic parameters of bacterial cells, and is  
4 directly involved in energising key cellular processes such as transport, ATP synthesis, and  
5 motility. The most common approach to measure membrane potential levels is through use of  
6 voltage-sensitive fluorescent dyes. Such dyes either accumulate or are excluded from the cell  
7 in a voltage-dependent manner, which can be followed by means of fluorescence microscopy,  
8 flow cytometry, or fluorometry. Since the cell's ability to maintain transmembrane potential  
9 relies upon low membrane ion conductivity, voltage-sensitive dyes are also highly sensitive  
10 reporters for the activity of membrane-targeting antibacterials. However, the presence of an  
11 additional membrane layer in Gram-negative (diderm) bacteria significantly complicates their  
12 use. In this manuscript, we provide guidance on how membrane potential and its changes can  
13 be reliably monitored in Gram-negatives using the voltage-sensitive dye DiSC<sub>3</sub>(5). We also  
14 discuss the confounding effects caused by the presence of the outer membrane, or by  
15 measurements performed in buffers rather than growth medium. We hope that the discussed  
16 methods and protocols provide an easily accessible basis for the use of voltage-sensitive dyes  
17 in Gram-negative organisms, and raise awareness of potential experimental pitfalls associated  
18 with their use.

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20

21 **Keywords:** membrane potential, voltage-sensitive dyes, depolarisation, *Escherichia coli*,  
22 *Salmonella enterica*

23

24 **Abbreviations:** DiSC<sub>3</sub>(5), 3,3'-Dipropylthiadicarbocyanine iodide; DiOC<sub>2</sub>(3), 3,3'-  
25 Diethyloxacarbocyanine Iodide; EDTA, Ethylenediaminetetraacetic acid; MIC, Minimal  
26 inhibitory concentration; ThT, Thioflavin T; PMB, Polymyxin B; PMBN, Polymyxin B  
27 nonapeptide. PBS, Phosphate-buffered saline; DMSO, Dimethyl sulfoxide

28 **INTRODUCTION**

29

30 Due to their misuse and overuse in both clinical and agricultural settings, antibiotic resistance  
31 is one of the biggest threats to global health today. This crisis is exacerbated by a deficit in  
32 antibiotic innovation, as demonstrated by the linear decline in discovery of new antibacterial  
33 molecules over the past 30 years [1]. There is, therefore, an urgent need for compounds with  
34 novel targets and modes of action. One emerging strategy is targeting bacterial membranes  
35 [2].

36 The bacterial cytoplasmic membrane is an essential macromolecular structure that  
37 harbours critical cellular processes such as nutrient and waste transport, respiration and ATP  
38 synthesis, protein secretion, motility, cell division, and cell wall synthesis [3]. Hence,  
39 maintaining the cytoplasmic membrane in an intact, biologically functional, and selectively  
40 permeable state is critical for the viability of bacterial cells. One of the essential features of the  
41 bacterial cytoplasmic membrane is its ability to maintain an electrical potential  
42 (transmembrane potential) which, alongside ATP, is a key cellular energy reserve used to  
43 drive important energy-demanding processes such as ion homeostasis, nutrient, protein and  
44 lipid transport, ATP synthesis, and motility.

45 The suitability of bacterial membranes as a vulnerable drug-target is perhaps best  
46 demonstrated by eukaryotic host defence peptides, which act by disrupting bacterial  
47 membranes and play a critical role in our innate immune system [4–6]. Despite a long history  
48 of co-evolution, bacteria have failed to evolve resistance mechanisms that fully protect  
49 themselves against membrane-targeting host defence peptides. More recently, the clinical  
50 efficacy of membrane-active antimicrobials has been demonstrated by the success of  
51 polymyxin B, daptomycin and colistin as last resort antibiotics used to treat life-threatening  
52 infections caused by multi-drug resistant pathogens [7–10]. Whilst the Gram-negative outer  
53 membrane and the associated lipopolysaccharide (LPS) layer are formidable barriers against  
54 many agents that target the cytoplasmic membrane, polymyxin B and colistin show selective  
55 activity against Gram-negative bacteria. Additionally, host defence peptides of the Cathelicidin

56 type are capable of disturbing the Gram-negative outer membrane as part of their antibacterial  
57 mode of action [4]. Hence, targeting the cytoplasmic membrane is a reasonable drug-  
58 development strategy, even against more challenging Gram-negative pathogens.

59 Membrane-targeting antibiotics commonly perturb membrane integrity by increasing  
60 permeability to ions or larger molecules, or by inducing more subtle changes such as forming  
61 or disturbing lipid domains, altering membrane fluidity, or delocalising membrane-associated  
62 proteins [11–15]. Large membrane-impermeable fluorescent dyes such as Propidium Iodide  
63 and Sytox Green are most frequently used to investigate antibiotic-induced changes in  
64 membrane permeability *in vivo*. These probes are DNA-intercalating and stain the nucleoid  
65 when large pores are formed in the cytoplasmic membrane, or when cell lysis is induced [4,  
66 16, 17]. However, these assays are unable to detect smaller-sized channels, increased ion  
67 permeability, or inhibition of respiration; all of which can still be lethal to the cell through  
68 dissipation of the transmembrane potential and associated cellular consequences [11, 18–21].  
69 Membrane depolarisation can be followed more directly using a number of voltage-sensitive  
70 fluorescent probes including 3,3'-Dipropylthiadicarbocyanine iodide DiSC<sub>3</sub>(5). Due to its  
71 hydrophobic and cationic nature, DiSC<sub>3</sub>(5) can penetrate the lipid bilayer and accumulate to  
72 high levels in polarized cells; a process which is associated with self-quenching of  
73 fluorescence. Upon membrane depolarisation, the dye is rapidly released from the cells  
74 resulting in a dequenching which can be followed fluorometrically, microscopically, and using  
75 flow cytometry [22, 23]. Whilst DiSC<sub>3</sub>(5), and a closely related voltage-sensitive dye 3,3'-  
76 Diethyloxacarbocyanine Iodide (DiOC<sub>2</sub>(3)), are frequently used in antibiotic mechanism-of-  
77 action studies in Gram-negative bacteria [24–26], the used protocols are relatively inconsistent  
78 and frequently include incubations in buffers of various compositions, presence of chelating  
79 agents such as EDTA, or in strains with hyperpermeable outer membranes. Accordingly,  
80 robust protocols for using these voltage-sensitive dyes to measure perturbations of the  
81 transmembrane potential in Gram-negative bacteria are missing.

82 Reliable and reproducible assays to follow changes in bacterial membrane potential  
83 are not only important in the context of antibiotic research. The use of DiSC<sub>3</sub>(5) as proxy for

84 the polarisation state of cells could be applied to study distinct cellular processes such as the  
85 energetic burden of flagellar formation and rotation. The assembly of the bacterial flagellum,  
86 as well as the flagellar-mediated swimming motility both rely on the proton motive force [27,  
87 28]. Recently, proton leakage via the stator units of the flagellar motor has been associated  
88 with a reduced growth rate in *Salmonella* [29]. Thus, voltage-sensitive dyes, such as DiSC<sub>3</sub>(5),  
89 could be used to monitor the effects of such energy-consuming processes on the polarisation  
90 state of individual cells and their current physiological condition.

91 In this manuscript, we will discuss and provide guidance on fluorescence-based  
92 techniques to measure membrane potential in the Gram-negative model organisms  
93 *Escherichia coli* and *Salmonella enterica*. We hope to raise awareness regarding the various  
94 confounding parameters and factors that can have a significant effect on the voltage-  
95 dependent behaviour of dyes in the context of Gram-negative bacteria. The discussed  
96 methods and protocols should provide a useful starting point for colleagues interested in  
97 identifying and characterising the antibacterial mode-of-action of membrane-targeting  
98 compounds against Gram-negative bacteria, or for analysing Gram-negative membrane  
99 potential levels in a more physiological context.

100

101

## 102 METHODS

103

### 104 Strains, media and growth conditions

105 Strains and genotypes are listed in Table 1. *E. coli* was grown in Lysogeny Broth (Miller) [10  
106 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl] and *S. enterica* in Lysogeny Broth (Lennox) [10  
107 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl]. For experiments performed in buffer, cells were  
108 collected by 3 min centrifugation at 6000×g, washed, and resuspended to an OD<sub>600</sub> of 0.3 in  
109 phosphate-buffered saline (PBS) [8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na<sub>2</sub>HPO<sub>4</sub>, 0.2 g/l KH<sub>2</sub>PO<sub>4</sub>,  
110 pH 7.3]. If indicated, PBS was supplemented with 0.2% glucose and 1 mM CaCl<sub>2</sub>.

111 **Minimal inhibitory concentration (MIC) determination**

112 *E. coli* overnight cultures were diluted 1:100 in appropriate growth medium and grown to mid-  
113 logarithmic phase. Cells were then diluted to give a final concentration of  $5 \times 10^5$  cells per well  
114 in a pre-warmed 96-well microtiter plate. This plate was prepared with an initial high  
115 concentration of the desired compound followed by a serial 2-fold dilution. After addition of the  
116 cells, the plate was incubated at 37°C for 16 hours with shaking at 700 rpm. MIC was defined  
117 as the lowest compound concentration able to inhibit visible bacteria growth.

118

119 **Fluorescence microscopy**

120 *E. coli* overnight cultures were diluted 1:100 in LB and incubated at 37°C upon shaking until  
121 an OD<sub>600</sub> of 0.3. 100-200 µl culture aliquots were transferred to 2 ml Eppendorf tubes with  
122 perforated lids followed by addition of 7 µM (10 µg/ml) polymyxin B (Sigma-Aldrich) or 31 µM  
123 (30 µg/ml) polymyxin B nonapeptide (Sigma-Aldrich) and incubation at 37°C upon shaking  
124 using a thermomixer. If applicable for the specific experiment, 200 nM of the membrane  
125 permeability indicator Sytox Green solved in H<sub>2</sub>O (ThermoFisher) was added alongside the  
126 antimicrobial compound, whilst 1 µM of the membrane potential-sensitive dye DiSC<sub>3</sub>(5)  
127 (Sigma-Aldrich) was added 5 min prior to imaging. The DMSO concentration of the final cell  
128 suspension was kept at 1-2%, which is critical for good DiSC<sub>3</sub>(5) solubility and staining while  
129 not affecting growth itself. Samples were immobilised on Teflon-coated multi-spot microscope  
130 slides (ThermoFisher) covered with a thin layer of H<sub>2</sub>O/1.2% agarose and imaged  
131 immediately. In case of data shown in Fig. 1, agarose was additionally supplemented with  
132 10% LB. For further details about this type of slide preparation, see te Winkel *et al.* [23].  
133 Microscopy was performed using a Nikon Eclipse Ti equipped with Nikon Plan Apo 100x/1.40  
134 Oil Ph3 objective, CoolLED pE-4000 light source, Photometrics BSI sCMOS camera, and  
135 Chroma 49002 (EX470/40, DM495lpxr, EM525/50) and Semrock Cy5-4040C (EX 628/40,  
136 DM660lp, EM 692/40) filter sets. Images were acquired with Metamorph 7.7  
137 (MolecularDevices) and analysed with Fiji [30].

138 **Time-lapse microscopy**

139 *E. coli* wild type cells were grown overnight at 30°C in M9 medium supplemented with 0.4%  
140 glucose, 0.2% casamino acids and 1mM thiamine, followed by a 1:10 dilution in M9 medium  
141 with diluted nutrients (0.02% glucose and 0.01% casamino acids) and incubation at 30°C. The  
142 time-lapse slide was prepared as described earlier [23, 31] with following modifications. For  
143 the preparation of the slide, a 2x stock of carbon source-diluted M9 medium (0.02% glucose,  
144 0.01% casamino acids) was preheated to 60°C and diluted 2-fold with 3% low-melting agarose  
145 kept at 60°C. DiSC<sub>3</sub>(5) solved in DMSO was added to the agarose to a final concentration of  
146 2.5 μM and a DMSO concentration of 1%, followed by pouring the time-lapse slide. The cells  
147 growing in nutrient arm M9 liquid medium were diluted to an OD<sub>600</sub> of 0.035, followed by  
148 transfer to the time lapse slide. Time-lapse microscopy was carried out at 30°C using an  
149 Applied Precision DeltaVision RT automated microscope equipped with a Zeiss Plan Neofluar  
150 63x/1.40 Oil Ph3 objective and Photometrics CoolSnap HQ camera, and a Cy5 filter set  
151 (EX632/22, DM645-700, EM 679/34). Phase contrast and fluorescence images were taken  
152 every 10 minutes over the duration of the microcolony growth (19h).

153

154 **Image analysis**

155 Quantification of DiSC<sub>3</sub>(5)-fluorescence for individual cells was performed in a semi-  
156 automated manner using Fiji [30]. To eliminate bias, the individual cells were identified and  
157 converted to regions of interest (ROIs) by thresholding of corresponding phase contrast  
158 images. If individual cells adhered to each other in a clearly identifiable manner, they were  
159 manually separated prior to automated cell detection. Larger cell aggregates were omitted  
160 from the analysis. The fluorescence intensity values for individual cells across the population  
161 were obtained from background-subtracted fluorescence images by using the phase contrast  
162 imaging-derived ROIs. To further eliminate bias, only multiples of full fields of view were  
163 analysed.

164 **Fluorometric determination of membrane potential levels**

165 *E. coli*:

166 Cultures were grown to logarithmic growth phase and, if needed, diluted to an OD<sub>600</sub> of 0.5 in  
167 growth medium supplemented with 0.5 mg/ml fatty acid free BSA (Sigma-Aldrich). Addition of  
168 BSA is critical to suppress DiSC<sub>3</sub>(5) binding to microtiter plate plastic surface. Immediately prior  
169 to measurement, the cells were transferred to black polystyrene 96-well plates (Porvair  
170 Sciences) and the autofluorescence of *E. coli* was measured for up to 5 min. DiSC<sub>3</sub>(5)  
171 dissolved in DMSO was then added to a final concentration of 0.5  $\mu$ M (1% DMSO) and the  
172 fluorescence quenching was monitored until a stable baseline was obtained, followed by  
173 addition of 7  $\mu$ M (10  $\mu$ g/ml) polymyxin B (Sigma-Aldrich) or 31  $\mu$ M (30  $\mu$ g/ml) polymyxin B  
174 nonapeptide (Sigma-Aldrich). Fluorometric measurements were taken every minute, with  
175 vigorous shaking in between readouts, using a BMG Clariostar multimode plate reader upon  
176 610 nm ( $\pm$  10) excitation, and 660 nm ( $\pm$  10) emission settings. All media, plates and  
177 instruments were warmed to 37°C prior to use. To investigate whether compounds of interest  
178 interfered with DiSC<sub>3</sub>(5) fluorescence at used concentrations, this assay was repeated, in the  
179 absence of cells, in PBS supplemented with BSA.

180

181 *S. enterica*:

182 For determination of membrane potential of stationary phase cells, the OD<sub>600</sub> of overnight  
183 cultures was determined and subsequently 2 ml were harvested by centrifugation for 3 min at  
184 21,000 $\times$ g to obtain cell free spent medium. To prevent re-energisation through resuspension  
185 in fresh medium, the cells were diluted to an OD<sub>600</sub> of 0.2 in spent medium, mixed with 0.5  
186 mg/ml fatty acid free BSA (Sigma-Aldrich) and incubated in a thermomixer for 15 min at 850  
187 rpm and 37°C with open lids. To pre-treat samples, polymyxin B nonapeptide (Merck) solved  
188 in H<sub>2</sub>O was added to a final concentration of 31  $\mu$ M (30  $\mu$ g/ml). After that, cell suspension was  
189 transferred into black 96-well polystyrene plates (Greiner Bio-One) and the autofluorescence  
190 of *S. enterica* was recorded at 610 nm ( $\pm$  9) excitation and 660 nm ( $\pm$  20) emission every  
191 minute for 3 min using a Tecan Infinite M200 plate reader with continuous shaking between

192 the measurements. Subsequently, DiSC<sub>3</sub>(5) (Eurogentech) was added to a final concentration  
193 of 1  $\mu$ M while maintaining 1% DMSO, and fluorescence measurement was continued for  
194 another 17 min in one minute intervals. For pre-treated samples, polymyxin B (Merck) was  
195 added to a final concentration of 14  $\mu$ M (20  $\mu$ g/ml), whilst water was added for untreated  
196 controls. For non-pre-treated samples, either polymyxin B nonapeptide or polymyxin B were  
197 added to final concentrations of 31  $\mu$ M (30  $\mu$ g/ml) or 14  $\mu$ M (20  $\mu$ g/ml), respectively. Membrane  
198 potential was followed for another 30 min in the plate reader with readings every minute. For  
199 measuring membrane potential of exponential growth phase growing cells, a fresh sub-culture  
200 was inoculated 1:100 from an overnight culture and incubated until an OD<sub>600</sub> between 0.3 and  
201 0.7 was reached. Subsequently, cells were diluted in fresh medium to an OD<sub>600</sub> of 0.2 and the  
202 protocol was performed as described above.

203

#### 204 **Statistical analyses**

205 The data presented here are representatives of at least two independent experiments. The  
206 minimal inhibitory concentration (MIC) and fluorometric assays were carried out as technical  
207 triplicates. The statistical significance was calculated as one-way, unpaired ANOVA with  
208 Tukey's post hoc test. Significance was depicted as \*\*\*\* for  $p < 0.0001$ , \*\*\* for  $p < 0.001$ , \*\* for  
209  $p < 0.01$ , \* for  $p < 0.05$ , ns for  $p > 0.05$ .

210

211

#### 212 **RESULTS AND DISCUSSION**

213

#### 214 **Inhibitory effects of used dyes and compounds**

215 A valid concern when using voltage-sensitive and other cell dyes is their potential to alter the  
216 cellular properties they are intended to monitor. Indeed, the amyloid [32], RNA [33, 34], and  
217 voltage-sensitive dye [35, 36] thioflavin T (ThT) was recently shown to dissipate the membrane  
218 potential itself if used in too high concentrations [37]. To test the growth inhibitory potential of  
219 DiSC<sub>3</sub>(5), and of the antimicrobial peptides used throughout this study, standard minimum

220 inhibitory concentration (MIC) assays were carried out with *E. coli* MG1655 and *S. enterica*  
221 LT2, used here as wild type strains. As shown in table 2, Polymyxin B (PMB) displayed strong  
222 antibacterial activity against both species. This, along with its well-documented ability to  
223 permeabilise both the outer and the cytoplasmic membranes [7, 38], confirmed its validity as  
224 a suitable positive control for this study. Crucially, PMB does not exhibit dye-interactions with  
225 DiSC<sub>3</sub>(5) (Fig. S1); a problematic phenomenon that frequently occurs between hydrophobic  
226 dyes and antimicrobials; which we recommend to always test and rule out [23]. As expected,  
227 a nonapeptide derivative of Polymyxin B (PMBN), which retains the ability to disrupt the Gram-  
228 negative outer membrane but is unable to form the depolarising cytoplasmic membrane pores  
229 [38], did not inhibit growth at any concentration tested (Table 2).

230 Previously, we have reported that DiSC<sub>3</sub>(5) is growth-inhibitory in the Gram-positive  
231 model organism *Bacillus subtilis* through a mechanism that is currently unknown but that does  
232 not involve membrane depolarisation [23]. However, it appeared that this inhibitory effect is a  
233 peculiarity of *B. subtilis* and does not occur in the close relative *Staphylococcus aureus*, or the  
234 Gram-negative species used in the present study (Table 2). When combined with the outer  
235 membrane permeabilising compound PMBN, an increased sensitivity of DiSC<sub>3</sub>(5) was  
236 observed with a MIC of 10 µM (Table 2), which is higher than the concentrations applied in  
237 subsequent experiments. We verified this by applying 1 µM of DiSC<sub>3</sub>(5) to *E. coli* cells in  
238 logarithmic phase and observed no effect on growth even in the presence of PMBN (Fig. S2).

239

240 **DiSC<sub>3</sub>(5) staining is influenced by both (inner) membrane potential and outer membrane  
241 permeabilisation**

242 As shown previously, single-cell DiSC<sub>3</sub>(5) fluorescence microscopy provides a simple and  
243 rapid method to measure membrane potential in the Gram-positive model organism *B. subtilis*  
244 [23]. To test whether this approach is also feasible in Gram-negative bacteria, we analysed  
245 the staining of wild type *E. coli* cells in the absence and presence of polymyxins (PMB and  
246 PMBN). For this aim, we included DiSC<sub>3</sub>(5) and the respective peptides in agarose pads,  
247 additionally supplemented with 10% LB, followed by addition of *E. coli* cells and rapid

248 microscopy. This was to observe the coarse kinetics of membrane depolarisation upon the  
249 imaging process. In untreated cells, DiSC<sub>3</sub>(5) fluorescence signals remained stable over 14  
250 min (Fig. 1). Surprisingly, an immediate increase in DiSC<sub>3</sub>(5) fluorescence intensity for approx.  
251 10 min was observed upon incubation with PMB, which was then followed by a loss of  
252 DiSC<sub>3</sub>(5) signal indicating membrane depolarisation. This increase was even more apparent  
253 upon PMBN-treatment where the cells remained highly stained for the duration of the  
254 experiment (approx. 15 min). This demonstrates that whilst DiSC<sub>3</sub>(5) staining is sensitive to  
255 (inner) membrane potential levels and exhibits the expected loss of staining upon  
256 depolarisation, the staining is also strongly influenced by outer membrane permeabilisation.  
257 In conclusion, DiSC<sub>3</sub>(5) is indeed well-suited for detection of cytoplasmic membrane potential  
258 levels in wild type *E. coli* cells directly in growth medium. However, care should be taken when  
259 interpreting the results if used under conditions that compromise the integrity of the outer  
260 membrane, or when comparing strains that have outer membranes of different composition or  
261 structure.

262

### 263 **Membrane potential measurements in buffer are possible but problematic**

264 Whilst DiSC<sub>3</sub>(5) has been previously used by our groups and others in the context of Gram-  
265 negative cells, the measurements are frequently carried out in cells washed with buffers of  
266 varying composition [25, 39, 40]. To investigate how washing and resuspending cells in buffer  
267 influences DiSC<sub>3</sub>(5) staining, we compared the DiSC<sub>3</sub>(5) signal levels between cells washed  
268 in phosphate-buffered saline (PBS) and cells stained directly in the growth medium. One  
269 important but frequently overlooked parameter when analysing membrane potential levels in  
270 buffer-suspended cells is the necessity to maintain a metabolisable carbon source. To test its  
271 effect on DiSC<sub>3</sub>(5) signal levels, we compared cells grown in LB supplemented with 0.2%  
272 glucose, and cells washed and resuspended in PBS with and without 0.2% glucose, followed  
273 by incubation for 15 min with shaking and staining. PMB-treatment for 15 min was used as a  
274 positive control for complete membrane depolarisation. The DiSC<sub>3</sub>(5) signal levels for cells  
275 washed in PBS with glucose were higher compared to cells in the growth medium and

276 extremely heterogeneous at the single-cell level (Fig. 2a, b). In the absence of a carbon  
277 source, the heterogeneity was still present. However, as shown in both the microscopy images  
278 and quantification of single-cell fluorescence levels, the DiSC<sub>3</sub>(5) staining was greatly reduced  
279 upon carbon source withdrawal indicating strongly reduced membrane potential (Fig. 2a, b).  
280 Whilst the higher initial DiSC<sub>3</sub>(5) staining levels in PBS may be due to differences in solubility  
281 of the dye between growth medium and buffer, we hypothesised that washing the cells could  
282 also remove divalent cations that are critical for outer membrane stability [38, 41, 42]. Hence,  
283 PBS may slightly permeabilise the outer membrane, thus explaining the increased DiSC<sub>3</sub>(5)  
284 staining. To test this, we repeated the experiment washing and resuspending the cells in PBS  
285 additionally supplemented with 1 mM CaCl<sub>2</sub>. Again, signals were diminished in the absence  
286 of a carbon source (Fig. 2a, b). However, supplementation of PBS with both glucose and CaCl<sub>2</sub>  
287 improved the consistency of signals and gave rise to DiSC<sub>3</sub>(5) fluorescence intensities more  
288 comparable to those measured in growth medium. This demonstrates that divalent cation  
289 removal, likely through destabilisation of the outer membrane, indeed affects DiSC<sub>3</sub>(5)  
290 staining.

291 At last, we performed a DiSC<sub>3</sub>(5) fluorescence microscopy time course experiment with  
292 cells washed and resuspended in PBS to investigate how long they remain energised. As  
293 demonstrated by the loss of DiSC<sub>3</sub>(5) fluorescence, *E. coli* cells gradually loose membrane  
294 potential in PBS buffer even when supplemented with a carbon source and CaCl<sub>2</sub> (Fig. 2c, d).

295 In conclusion, whilst DiSC<sub>3</sub>(5) can be used as a voltage-sensitive dye in buffers, assays  
296 carried out directly in the growth medium should be strongly favoured. If for experimental  
297 reasons measurements in buffer are essential, care must be taken to maintain both a carbon  
298 source to sustain central carbon metabolism and divalent cations to maintain outer membrane  
299 stability, and to carry out the assays rapidly after wash and resuspension into buffers.

300

301 **Compatibility of DiSC<sub>3</sub>(5) with time lapse microscopy and combination with other  
302 fluorophores**

303 As DiSC<sub>3</sub>(5) is not growth inhibitory in *E. coli*, we hypothesised that it could be compatible with

304 time-lapse experiments. To test this, we performed a time-lapse microscopy experiment with  
305 *E. coli* grown on DiSC<sub>3</sub>(5)-supplemented agarose pads using a method previously described  
306 in detail for other bacterial species [23, 31]. We chose to carry out this experiment in  
307 M9/glucose/casamino acids medium with both glucose and casamino acids concentrations  
308 reduced to 10% from normal. In this regime the growing microcolony exhausts the locally  
309 available carbon sources before exceeding the camera field of view. As shown in both Fig. 3  
310 and Movie S1, DiSC<sub>3</sub>(5) fluorescence can indeed be monitored in a time-lapse microscopy  
311 setting for an extended duration of time. Crucially, the cessation of growth coincides with a  
312 strong reduction of DiSC<sub>3</sub>(5) fluorescence, consistent with membrane depolarisation triggered  
313 by carbon source exhaustion.

314 Another useful property of DiSC<sub>3</sub>(5) is its far-red fluorescence emission spectrum  
315 (approx. 650-700 nm). While covered by commonly used Cy5-filters, it allows DiSC<sub>3</sub>(5) to be  
316 used in combination with even relatively weak green fluorophores such as GFP. This enables  
317 experiments that combine membrane potential readout with GFP-based protein localisation or  
318 expression reporter. One fluorophore combination that we have found to be very informative  
319 in the context of antibiotic research is co-staining with Sytox Green. Sytox Green is a  
320 membrane impermeable DNA intercalating dye that can stain the bacterial DNA but only when  
321 large pores are formed in the cytoplasmic membrane [16]. As shown in Fig. 4, *E. coli* can be  
322 simultaneously co-stained with both DiSC<sub>3</sub>(5) and Sytox Green. Upon treatment with the pore  
323 forming PMB, DiSC<sub>3</sub>(5) fluorescence is lost due to depolarisation whilst cells become strongly  
324 stained with Sytox Green indicating that the observed depolarisation is, as expected, caused  
325 by pore formation. This dual-dye technique thus enables the rapid fluorescence-based  
326 identification and differentiation between membrane depolarising and membrane pore-forming  
327 antimicrobial compounds or stresses *in vivo*, on a single-cell level.

328

### 329 **DiSC<sub>3</sub>(5)-based membrane potential measurements using fluorometry**

330 The approaches detailed above allow *E. coli* membrane potential levels to be monitored at the  
331 single-cell level using fluorescence microscopy and flow cytometry. Whilst sacrificing single-

332 cell resolution, DiSC<sub>3</sub>(5)-based membrane potential assays using fluorometry are perhaps  
333 more accessible, and provide better throughput and temporal resolution. This approach is  
334 based on self-quenching of DiSC<sub>3</sub>(5) fluorescence upon accumulation to high concentrations  
335 in polarised cells. When measured fluorometrically in a cell suspension, the accumulation of  
336 DiSC<sub>3</sub>(5) is observed as a gradual decline in fluorescence signal until a Nernstian equilibrium  
337 is achieved [43–45]. Upon loss of membrane potential, DiSC<sub>3</sub>(5) is released back into the  
338 medium, which leads to dequenching and an increase of overall measured fluorescence.  
339 Following DiSC<sub>3</sub>(5) fluorescence quenching behaviour, thus, enables live monitoring of mean  
340 membrane potential levels of a cell suspension. In the following, we will focus on such  
341 measurements for *S. enterica* cells exposed to the antimicrobials, PMB or PMBN.

342 The degree of DiSC<sub>3</sub>(5) fluorescence quenching is highly dependent on the used dye  
343 concentration and cell densities. Similar to the optimisation previously undertaken in Gram-  
344 positive bacteria [23], we first determined how the outer membrane barrier of Gram-negative  
345 bacteria affects the quenching of DiSC<sub>3</sub>(5) fluorescence upon accumulation in well energised  
346 cells. Upon addition of DiSC<sub>3</sub>(5) to exponentially grown but PMBN naïve cells, we observed  
347 only a slow and gradual quenching (Fig. 5a). When these cells were challenged with PMBN a  
348 further decrease in DiSC<sub>3</sub>(5) fluorescence was observed, indicating that outer membrane  
349 permeabilisation induced by PMBN stimulated further DiSC<sub>3</sub>(5) uptake, which is consistent  
350 with our microscopic observations in *E. coli* (Fig. 1). This effect was more pronounced when  
351 cells were exposed to PMB. Here, a sharp quenching followed by rapid dequenching was  
352 observed, indicating initial outer membrane permeabilisation followed by later inner membrane  
353 depolarisation. This is consistent with the two-step mode of action of PMB [46]. If the cells  
354 instead were pre-treated with PMBN, faster quenching was observed upon addition of  
355 DiSC<sub>3</sub>(5) (Fig. 5c). This was also accompanied by more extensive dequenching upon  
356 depolarisation induced by PMB addition.

357 It is well-established that bacterial membrane potential can differ according to the  
358 growth phase or growth conditions. Whilst logarithmic growth phase cells feature well-  
359 energised membranes, entry into stationary growth phase is associated with nutrient

360 limitations and other stresses that can lead to reduced membrane potential levels (also see  
361 movie S1) [47]. To establish that the fluorometric DiSC<sub>3</sub>(5) assay can also be applied for non-  
362 growing cultures, we repeated the experimental procedure with stationary phase cells  
363 obtained from an overnight culture. In the case of PMBN naïve cells, DiSC<sub>3</sub>(5) incorporation  
364 dynamics were similar for exponential and stationary phase cells (Fig. 5b), indicating that  
365 stationary phase *S. enterica* cells grown in rich medium maintain adequate membrane  
366 potential levels. Upon addition of PMB, a clear additional quenching step associated with outer  
367 membrane permeabilisation was observed. However, unlike in actively growing cells (Fig. 5a)  
368 this was not followed by dequenching associated with membrane depolarisation. Recently,  
369 Sabnis et al. demonstrated that inner membrane pore formation induced by Colistin  
370 (Polymyxin E) requires lipopolysaccharide (LPS), which is synthesised at the inner membrane  
371 prior to translocation to the outer membrane [7]. Very likely, the observed lack of depolarisation  
372 in non-growing cells is linked to this mechanism and caused by the absence of *de novo* LPS  
373 synthesis and, thus, inner membrane LPS. PMBN pre-treated stationary cells depicted a more  
374 rapid DiSC<sub>3</sub>(5) quenching behaviour compared to naïve cells, indicating that PMBN can  
375 permeabilise the outer membrane of stationary growth phase *S. enterica* to some degree (Fig.  
376 5d). However, the inability of full length PMB to trigger additional dequenching in these cells,  
377 and the lack of a significant response when PMBN is added to naïve stationary growth phase  
378 cells (Fig. 5c) does suggest that PMBN might be less active against non-growing cells. Finally,  
379 to confirm that the established fluorometric assays are robust, we repeated the measurements  
380 for actively growing *E. coli* cells in a different laboratory setting and using different  
381 instrumentations. Indeed, very comparable results were obtained for *E. coli* (Fig. 6).

382 Whilst the influence of both outer membrane permeabilisation and cytoplasmic  
383 membrane depolarisation on DiSC<sub>3</sub>(5) persists in the fluorometric assay, pre-incubation with  
384 PMBN allows this confounding factor to be largely eliminated at least in case of actively  
385 growing cells. Hence, with careful controls, DiSC<sub>3</sub>(5) can be used to reliably monitor  
386 membrane potential in a microtiter plate format using a fluorometric approach.

387 **SUMMARY**

388 Previously, we have published detailed methods and guidance on using carbocyanide-based  
389 voltage-sensitive dyes for the analysis of membrane potential in Gram-positive bacteria [23].  
390 Due to the additional outer membrane and its impact on dyes and membrane-active  
391 compounds, translating those methods to Gram-negative bacteria is not necessarily trivial. In  
392 this report, we summarise our experiences using DiSC<sub>3</sub>(5) as a voltage-sensitive dye in Gram-  
393 negative species including *E. coli* and *S. enterica*. Whilst the use of DiSC<sub>3</sub>(5) as a reporter for  
394 membrane potential in Gram-negative bacteria is not novel, the information about best  
395 practices and factors that can compromise the measurements are not well documented,  
396 making the use of such dyes without prior knowledge and experience challenging. The  
397 methods presented here should be easy to implement using commonly available equipment  
398 such as regular widefield fluorescence microscopes and fluorescence plate readers.  
399 Furthermore, these assays should in principle be directly transferrable to flow cytometry  
400 measurements, although not verified within this study. We hope that the included details and  
401 discussions, and information regarding the effects and problems associated with outer  
402 membrane permeabilisation and the use of buffers rather than growth media, will provide a  
403 valuable starting point for those interested in analysing bacterial membrane potential in a  
404 physiological context, or as an assay to study antimicrobial mode of action.

405

406

407 **Authors and contributors**

408 PFP and HS designed and coordinated research; JAB, MH, PFP performed the experiments;  
409 JAB, PFP, and HS analysed data; JAB, PFP and HS wrote the paper; ME and HS acquired  
410 the funding. All authors commented on the manuscript.

411

412 **Conflicts of interest**

413 The authors declare that there are no conflicts of interest.

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542 **FIGURES AND TABLES**

543

544 Table 1: Strains used in this study

Strain	Genotype	Reference
<i>E. coli</i> MG1655	$\lambda^+$ F- <i>ilvG</i> - <i>rfb</i> -50 <i>rph</i> -1	[48]
<i>S. enterica</i> TH437	<i>Salmonella enterica</i> serovar Typhimurium strain LT2	[49]

545

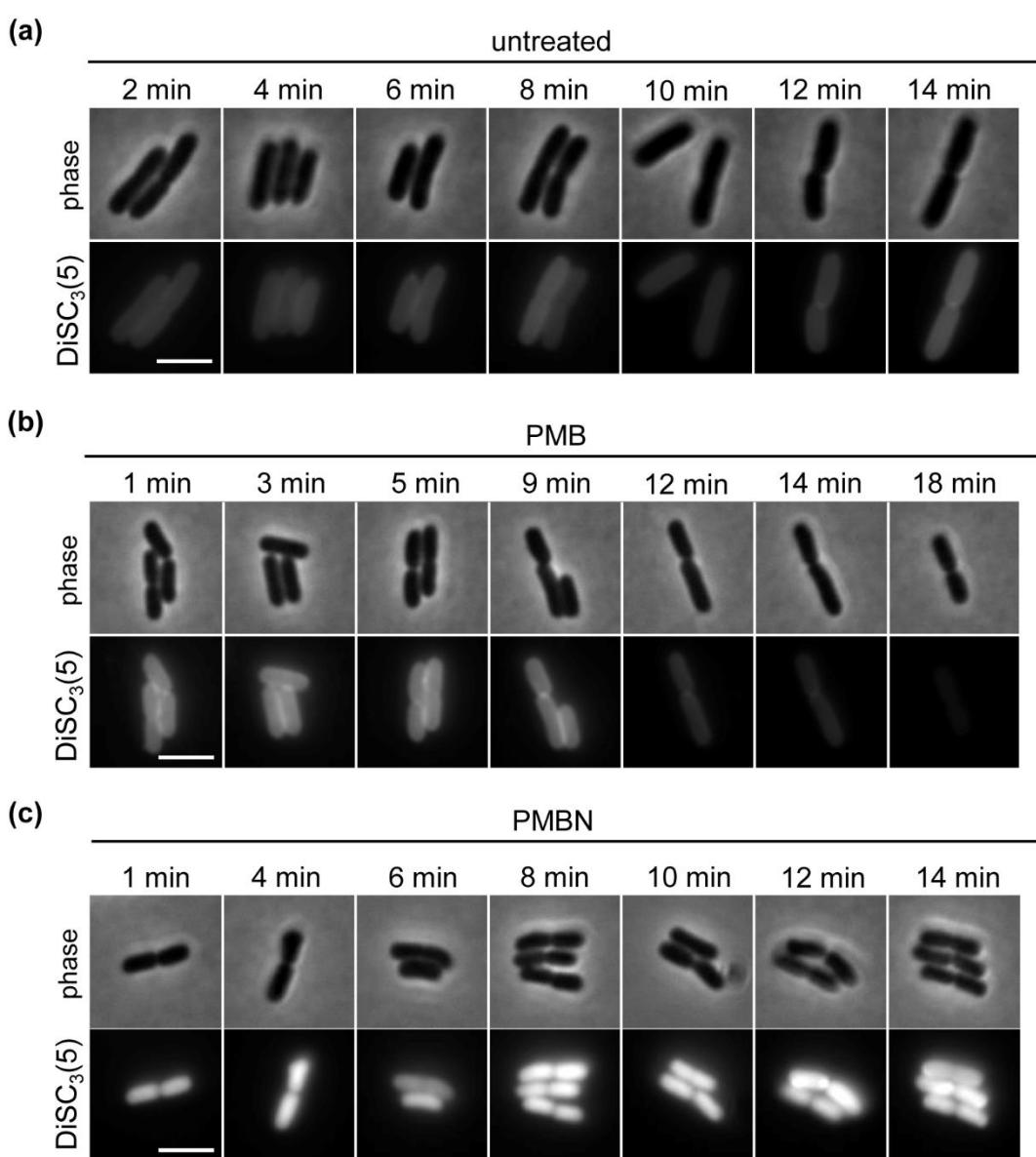
546

547 Table 2: Minimum inhibitory concentrations (MICs) of tested compounds and dyes for *E. coli*  
548 and *S. enterica* in the presence and absence of the outer membrane permeabilising agent  
549 Polymyxin B nonapeptide.

Compound	<i>E. coli</i> <sup>a</sup>		<i>S. enterica</i> <sup>a</sup>	
	MIC	MIC with PMBN	MIC	MIC with PMBN
PMB	0.25 $\mu$ M	NA	0.2 $\mu$ M	NA
PMBN	>100 $\mu$ M	NA	>100 $\mu$ M	NA
DiSC <sub>3</sub> (5)	>100 $\mu$ M	10 $\mu$ M	>100 $\mu$ M	10 $\mu$ M

550 <sup>a</sup>Values obtained from two biological replicates, each with technical triplicates. PMB:

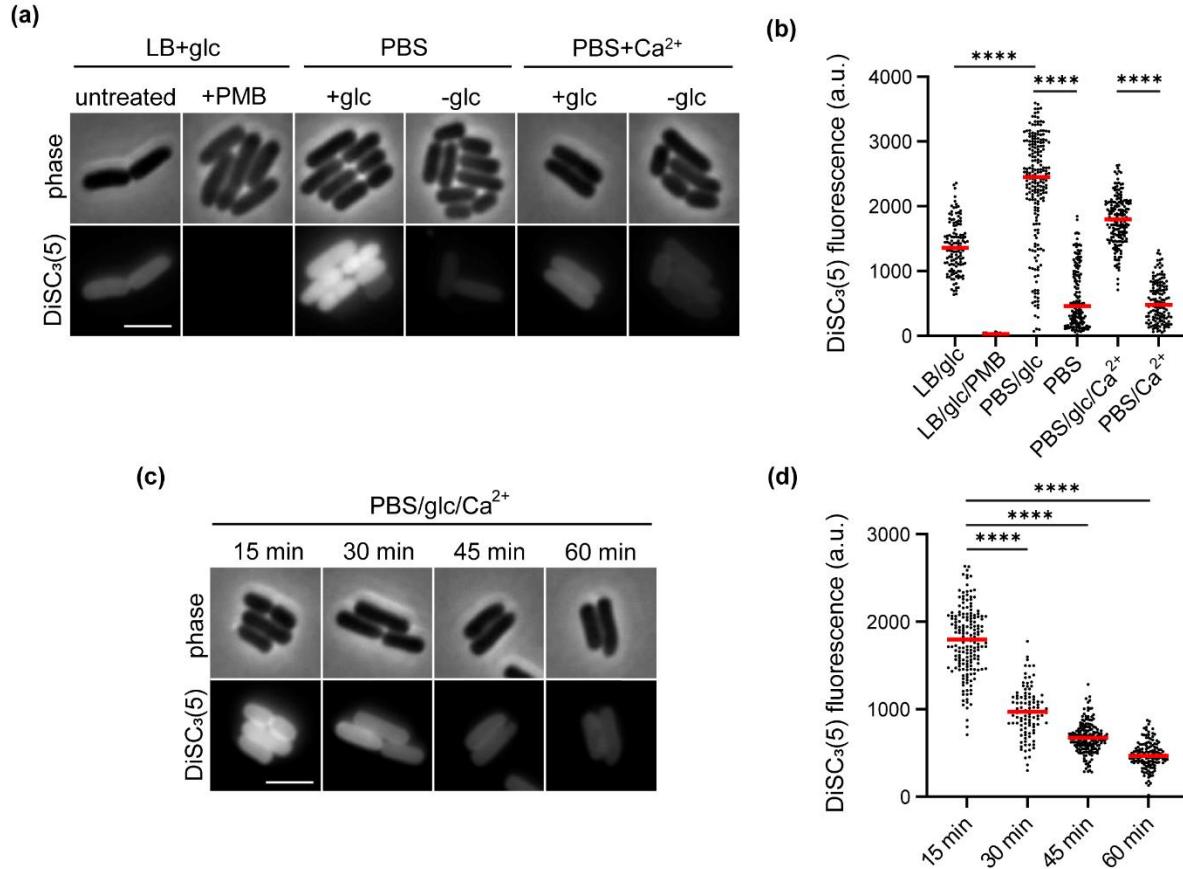
551 Polymyxin B, PMBN: Polymyxin B nonapeptide, NA: not applicable.



552

553 **Figure 1: *E. coli* DiSC<sub>3</sub>(5) staining is influenced by both outer membrane  
554 permeabilisation and (inner) membrane depolarisation.**

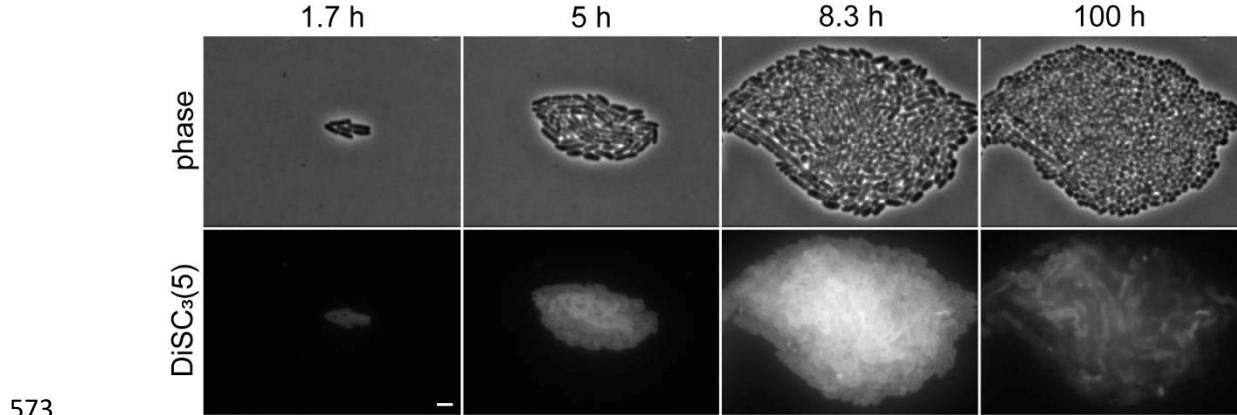
555 Phase contrast and fluorescence microscopy of *E. coli* stained with DiSC<sub>3</sub>(5) in the (a)  
556 absence and presence of (b) outer membrane permeabilising and inner membrane  
557 depolarising PMB (7  $\mu$ M) or (c) outer membrane permeabilising PMNBN (30  $\mu$ M) at different  
558 time points of incubation. Note that for this experiment the dye and antibiotics were added  
559 directly to the agarose pad supplemented with 10% LB. The time points indicate incubation  
560 after transfer of cells to the agarose pad. Scale bar: 3  $\mu$ m. Strain used: *E. coli* MG1655 (wild  
561 type).

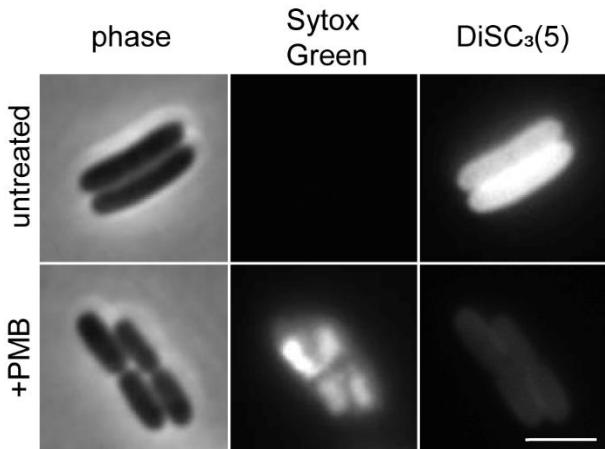


562

563 **Figure 2: Divalent cations, carbon source, and rapid imaging are critical for measuring**  
564 ***E. coli* membrane potential in buffer.**

565 (a) Phase contrast and fluorescence microscopy images of DiSC<sub>3</sub>(5)-stained *E. coli* in  
566 LB/0.2% glucose, in PBS with and without glucose (0.2%), and in PBS/glucose (0.2%) with 1  
567 mM CaCl<sub>2</sub>. As a positive control, the transmembrane potential was disrupted by the pore  
568 forming antibiotic PMB (7  $\mu$ M). (b) Quantification of DiSC<sub>3</sub>(5)-fluorescence for individual cells  
569 from the imaging dataset shown in panel a (n=128-211 cells). Median fluorescence intensity  
570 is indicated with a red line, together with P values of a one-way, unpaired ANOVA with Tukey's  
571 post hoc text. \*\*\*\* represents p < 0.0001. Scale bar: 3  $\mu$ m. Strain used: *E. coli* MG1655 (wild  
572 type).

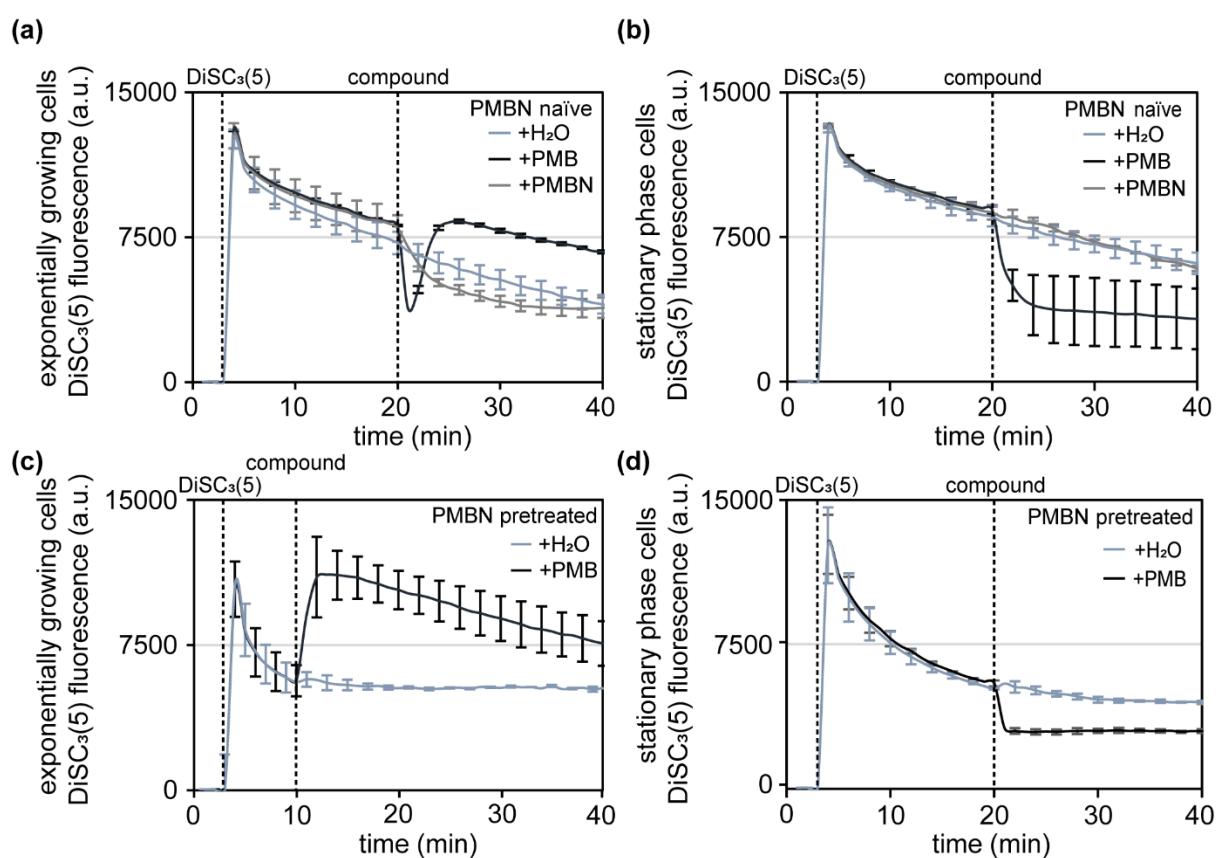




582

583 **Figure 4: Simultaneous detection of membrane potential and pore formation in *E. coli*.**

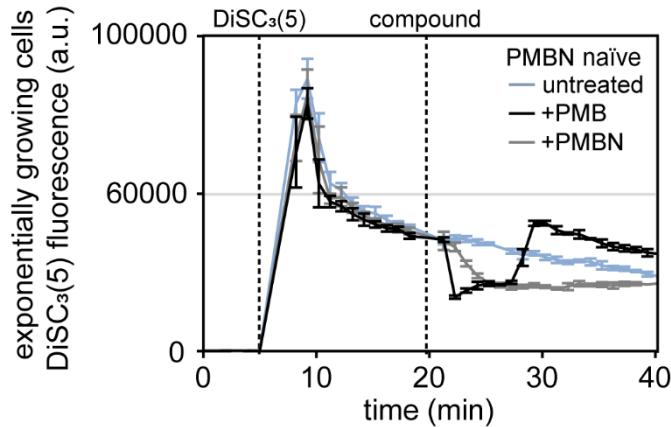
584 Phase contrast and fluorescence microscopy of *E. coli* cells co-stained with Sytox Green and  
585 DiSC<sub>3</sub>(5) in the absence or presence of PMB (7  $\mu$ M) for 15 min. Note the loss of membrane  
586 potential in PMB-treated cells that coincides with ability of Sytox Green to enter the cells  
587 indicating pore formation. Scale bar: 3  $\mu$ m. Strain used: *E. coli* MG1655 (wild type)



588

589 **Figure 5: DiSC<sub>3</sub>(5)-based fluorometric measurement of membrane potential in *S.*  
590 *enterica*.**

591 PMBN naïve exponential (a) and stationary growth phase (b) *S. enterica* cells were exposed  
592 to either PMBN (30 µg/ml), PMB (20 µg/ml), or solvent (H<sub>2</sub>O). Dashed vertical lines indicate  
593 the addition of DiSC<sub>3</sub>(5) and compounds, respectively. Note the quenching of the DiSC<sub>3</sub>(5)  
594 fluorescence upon accumulation in cells, and rapid further reduction upon OM  
595 permeabilisation by PMB and PMBN followed by dequenching upon membrane depolarisation  
596 by PMB. (c-d) Measurements were repeated in cells pre-treated with PMBN. Graphs depict  
597 the means of six replicates and standard deviation from two independent experiments. Strain  
598 used *S. enterica* TH437 (wild type).



599

600 **Figure 6: DiSC<sub>3</sub>(5)-based fluorometric measurement of membrane potential in *E. coli*.**

601 PMBN naïve exponential growth phase *E. coli* cells were exposed to either PMBN (30  $\mu$ M) or  
602 PMB (7  $\mu$ M). Dashed lines indicate addition of DiSC<sub>3</sub>(5) and compounds, respectively. Note  
603 the quenching of the DiSC<sub>3</sub>(5) fluorescence upon accumulation in cells, and further reduction  
604 upon OM permeabilisation triggered by PMB and PMBN, followed by dequenching upon  
605 membrane depolarisation triggered by PMB. The graph depicts mean and standard deviation  
606 from technical triplicates. Strain used *E. coli* MG1655 (wild type).