High-efficiency transfection of *Acanthamoeba castellanii* using a cationic polymer

Anaísa B. Moreno*[1], Viktor Ek, Jens Eriksson, Mikael E. Sellin, and Lionel Guy*

Department of Medical Biochemistry and Microbiology, Science for Life Laboratory, Uppsala University, Uppsala, Sweden

*Corresponding authors: amoreno@imm.uzh.ch, lionel.guy@imbim.uu.se

[1] Current address: Institute of Medical Microbiology, University of Zürich, Zürich, Switzerland

Abstract

The free-living amoeba *Acanthamoeba castellanii* is an ecologically, clinically, and evolutionarily important microorganism. *A. castellanii* amoebae are directly pathogenic to humans and serve as reservoirs for bacterial pathogens (e.g., *Legionella pneumophila*) but also regulate the proliferation of other microorganisms in the soil. Despite their importance, no reliable genetic system has been developed, hampering the use of *A. castellanii* and related species as model organisms. Transfecting *A. castellanii* with plasmids is possible with commercial kits, but it is expensive, inefficient, and vulnerable to product discontinuation. In this contribution, we present a method for efficient transfection of *A. castellanii* with readily available and inexpensive polyethylenimines. We systematically explore the method's parameters, obtaining up to 100-fold higher efficiency than currently used protocols. The method presented here provides a robust step towards a complete genetic toolbox for *A. castellanii*, hence expanding its use as a model organism.

Keywords *Acanthamoeba*, transfection, cationic polymers, polyethylenimine, PEI

Introduction

28 *Acanthamoeba castellanii* is a free-living amoeba which is ubiquitous in various environments. It is a
29 human pathogen responsible for two major infections – *Acanthamoeba* keratitis and granulomatous
30 amoebic encephalitis (GAE) [1–3]. It also serves as a reservoir for other microorganisms, such as bacteria,
31 viruses and other protists, in either a parasitic or mutualistic relationship [1]. *A. castellanii* has been used
32 as a model organism to study cytoskeleton motility, host-pathogen interactions, gene expression, and
33 the evolution of pathogenicity [3,4]. However, this species is still underutilised relative to other amoebae,
34 such as *Dictyostelium discoideum* [3]. One of the reasons for this is its genomic complexity [5,6], which has
35 hampered the development of molecular biology tools [5,7]. The ability to effectively introduce and
36 express exogenous genes in these amoebae is, thus, of particular interest and significance [8].
37 Transfection of *Acanthamoeba* with plasmid vectors has been successfully reported using physical
38 methods (electroporation) [9], but more frequently with chemical methods, using the commercially
39 available reagents SuperFect® (Qiagen) [10,11], and Viafect™ (Promega) [12]. However, these methods'
40 reported transfection efficiency is low, typically 5% or less [2,7]. Commercial kits offer a quick and
41 straightforward technique for transfection but are costly, which restricts the freedom to optimise the
42 method for specific applications. Moreover, kits may be suddenly discontinued, as the example of
43 SuperFect® shows. Since the components for such reagents are patented, finding a replacement is
44 difficult.
45 Cationic polymers such as polyethylenimines (PEIs), poly-L-lysines (PLLs), polyamidoamines
46 (PAMAMs), and chitosans have been employed as gene delivery systems in eukaryotic cells [13–15]. With
47 their high number of positively charged (e.g., amines) residues, they form polyplexes with the
48 negatively charged phosphate groups in DNA molecules via electrostatic interactions [15]. This
49 complexation condenses the DNA into small, compact particles, which cells internalise, and make their
50 way to the nucleus where genes can be expressed [14,15]. PEIs are among the best-studied cationic
51 polymers due to their high cation charge density, wide availability, and low-cost [14,16,17]. In addition,
52 their amine groups can be polymerised in two ways to create linear or branched isomers. The degree of
53 polymerisation, and branching of PEIs, along with other physio-chemical properties, affect their
54 molecular weight, polydispersity, and buffering capacity, reflected in how well they interact with DNA.
55 Although PEIs have been established as DNA carrier vectors for a variety of cell types [15,17], transfection

56 efficiency may be affected by the nucleic acid quantity and purity, the preparation and composition of
57 the complexes and the transfection conditions employed [14,17,18].
58 While PEIs have been used to transfect another free-living amoeba – *Naegleria* [19], they remain
59 unexplored for *Acanthamoebae*. In this contribution, we describe the use of PEIs for high-efficiency
60 transfection of *A. castellanii*. We systematically explore three types of PEI and assess their effect on
61 cell toxicity and transfection efficiency. Importantly, we show that the here presented PEI-based
62 protocol is vastly superior to current commercial standards for the transfection of *A. castellanii*. This
63 provides a foundation for developing a full genetic system for this ecologically important amoeba.
64
65 **Results**
66 ***DNA condensation by PEI is affected by the complexation media***
67 We first investigated how different complexation media and PEI amounts influenced the condensation
68 of DNA. The media tested were selected for supporting *A. castellanii* growth – PYG, Ac medium and
69 LoFlo, or for having previously been described in the literature as buffers used for PEI transfections –
70 HBS, NaCl, and ultrapure water. Agarose-gel retardation assays were performed to evaluate the DNA
71 condensation ability of the PEIs. After gel electrophoresis, the migration of the polyplexes was
72 compared to that of naked plasmid DNA (pDNA) (**Figure 1A and S1**). In all conditions, increasing
73 concentrations of PEI led to better complexation of DNA. However, the media used affected the
74 complexation, most noticeably comparing the linear PEIs (PEI-25 and PEI-40) to the branched PEI
75 (PEI-Br) (**Figure S1**). Nevertheless, a high stoichiometry of PEI to pDNA resulted in successful
76 complexation across all buffer conditions. Retardation of the polyplexes can be due to the neutralisation
77 of the negatively charged DNA (which would then not move through the gel), polyplex size change, or
78 a combination of both. Overall, PYG appeared to work best with all PEIs (**Figure 1A**, compared to
79 **Figure S1**), and since this is also the preferred medium for *A. castellanii*, PYG was selected for the
80 remaining studies.
81
82 ***Evaluation of PEI toxicity on* A. castellanii *cells***

83 One factor often considered in transfection studies involving mammalian cells is how the transfection
84 reagent affects the cells, as high concentrations can be toxic. Therefore, a balance between a high
85 transfection rate and cytotoxicity should be struck. Here, a resazurin assay was performed to estimate
86 the effect of PEI concentration on the amoebae. The use of this assay to evaluate *A. castellanii* viability
87 has been described [20], but the assay was further optimised here to ascertain its sensitivity for the
88 conditions used (**Figure S2**). Cellular toxicity varied between the PEIs, with the most prominent
89 contrast between the linear and the branched PEIs (**Figure 1B**). PEI-40 had the least toxic effect on the
90 *A. castellanii* cells, with only a 20% decrease in cell viability at the highest quantity tested (2.5 µg),
91 followed by PEI-25 and PEI-Br with a 40% and 80% decrease, respectively. Altogether, the results
92 suggest a threshold at a maximum of ~1.5 µg of PEI, whereafter the most substantial decrease in
93 viability is seen.

94

95 ***Optimisation of transfection conditions***

96 Transfection efficiency is highly dependent on the effective formation of DNA-PEI polyplexes, i.e., all
97 the DNA should be condensed by the PEI so it can be delivered to the cell. For this to happen, there
98 should be enough available nitrogen (N) in the PEI to cationise the phosphate (P) in the DNA, this is
99 referred to as the N/P ratio, and it is used to find the optimal conditions for transfection. Since no
100 previous work has described PEI-based transfection of *Acanthamoebae*, a systemic approach was
101 employed to evaluate the performance of each PEI used in this study. A combinatorial matrix using a
102 range of pDNA and PEI amounts resulted in an encompassing assay of 100 different combinations, with
103 N/P ratios ranging from ~ 0.7 to 70 (**Figure 1C**).

104

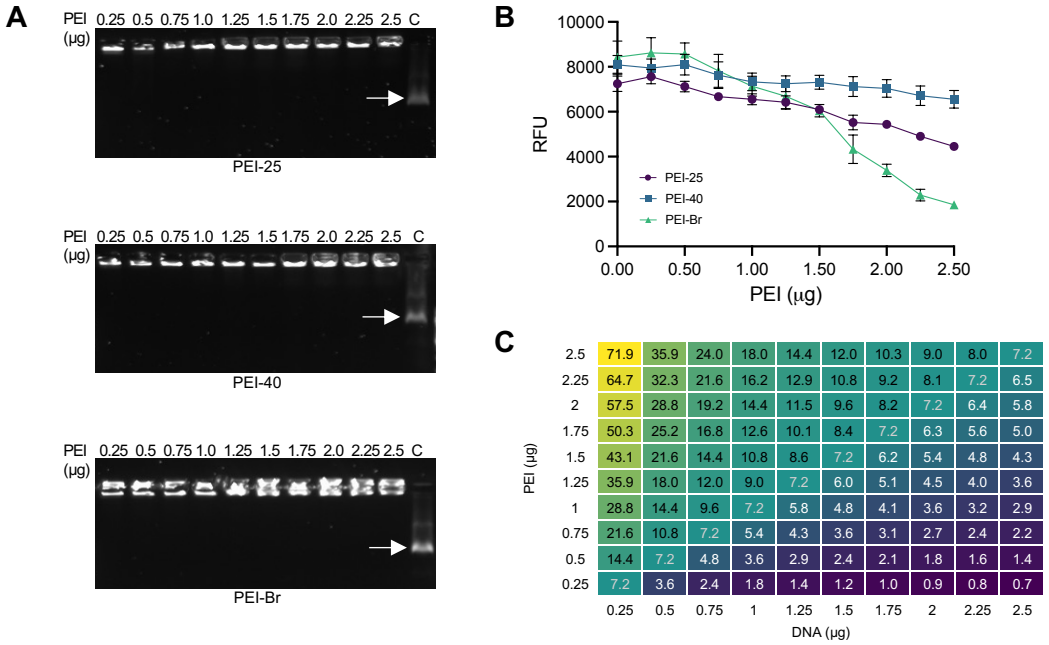

Figure 1. Condensation of PEI in PYG, cytotoxicity, and optimisation of transfection conditions. **(A)** DNA condensation ability of PEI in PYG. Increasing amounts of PEI-25, PEI-40 and PEI-Br were used to form complexes with 0.25 µg of pDNA. The migration of the pDNA-PEI polyplexes was compared to that of non-complexed pDNA, indicated by the white arrows (C, untreated control). **(B)** Cytotoxicity of PEI on *A. castellanii*. Viability of *A. castellanii* with increasing amounts of PEI in the medium was evaluated by resazurin assay. Resorufin fluorescence was measured (ex: 530 nm, em: 590 nm) at 6 h post-addition of the solution to the cells. Results are from one of three independent experiments, data points in the graph are the mean of 6 technical replicates, error bars are standard deviation. **(C)** Table of N/P ratios at different combinations of pDNA and PEI quantities.

Transfection efficiency is dependent on minimum quantities of pDNA and PEI

The results of the combinatorial transfection screen for the two constitutive fluorescence plasmids pGAPDH-EGFP and pGAPDH-mScarlet (**Figure S3**) illustrate a significant impact of PEI choice for transfection of *A. castellanii*. Overall, there is a minimum threshold for efficient transfection, contingent on the concentration of PEI. The results for PEI-Br concur with the toxicity data, as there were few transfectants at PEI quantities above 1.5 µg, with most fluorescent cells observed at N/P ratios ranging from ~ 2.9 - 4.5 (**Figure 1C and Figure 2**).

The linear PEIs (PEI-25 and PEI-40) had the highest transfection efficiencies, but the results were not directly correlated with higher N/P ratios. Although the results varied considerably between the two plasmids, the overall distribution trends were similar. For PEI-25 and PEI-40, the minimum threshold of PEI per reaction appeared to be above 0.5 µg and 0.75 µg PEI per reaction, respectively. Over this threshold, there were transfectants even with the lowest amounts of pDNA. The highest transfection efficiencies were observed at low to modest N/P ratios, i.e., ~5 – 10 (**Figure 2**). Interestingly, the highest fluorescent cell count with PEI-40 for both plasmids was at the same N/P ratio of 8.6, and with PEI-25, there was a high number of fluorescent cells even at higher amounts of PEI.

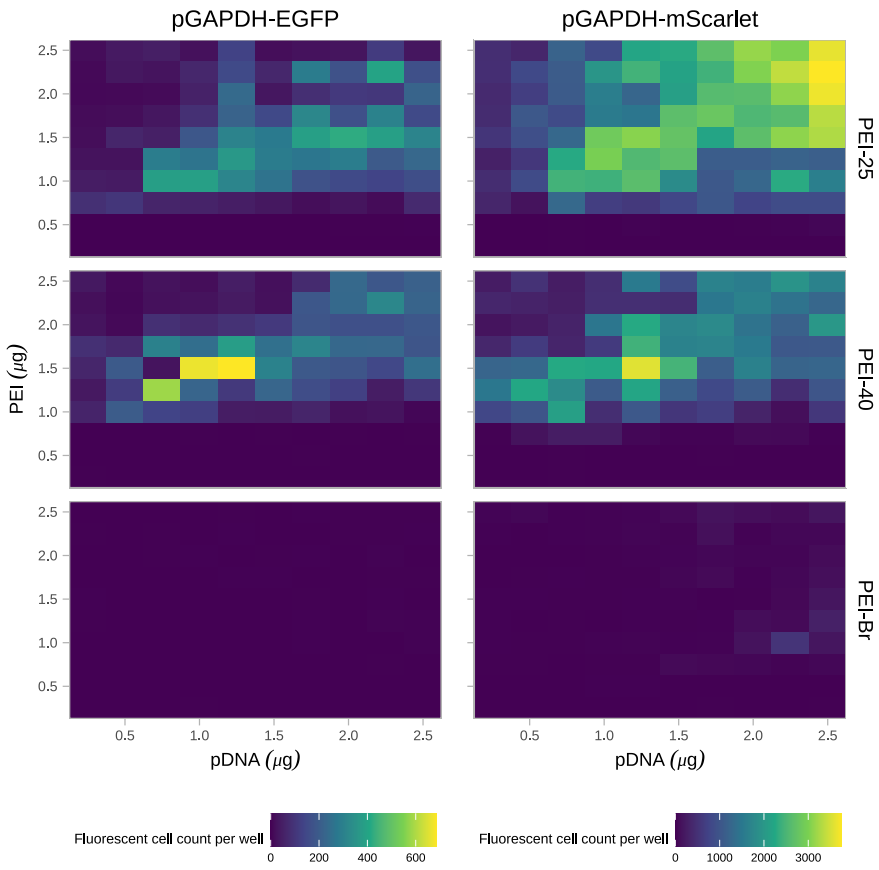

Figure 2. Effect of DNA and PEI quantities on transfection efficiency. The left and right panels show the results for cells transfected pGAPDH-EGFP and pGAPDH-mScarlet, respectively. The top, middle and bottom panels correspond to transfections with PEI-25, PEI-40 and PEI-Br, respectively.

136 The x- and y-axis represent the amount of DNA and PEI, respectively. The colour of the tiles represents
137 the sum of fluorescent cells detected per well. Note that scales are different for the left and right panels.

138

139 ***Fluorescence intensity varies between* A. castellanii *transfectants***

140 Through microscopy, we observed that the level of fluorescence varied considerably between
141 transfectants (**Figure 3** and **S4**). A notable variation in individual cell fluorescence appeared throughout
142 all transfected cells (Figures **3A** and **3B**), but this was most pronounced on pGAPDH-mScarlet
143 transfectants and particularly at high amounts of pDNA and PEI (**Figures 3C** and **3D**).

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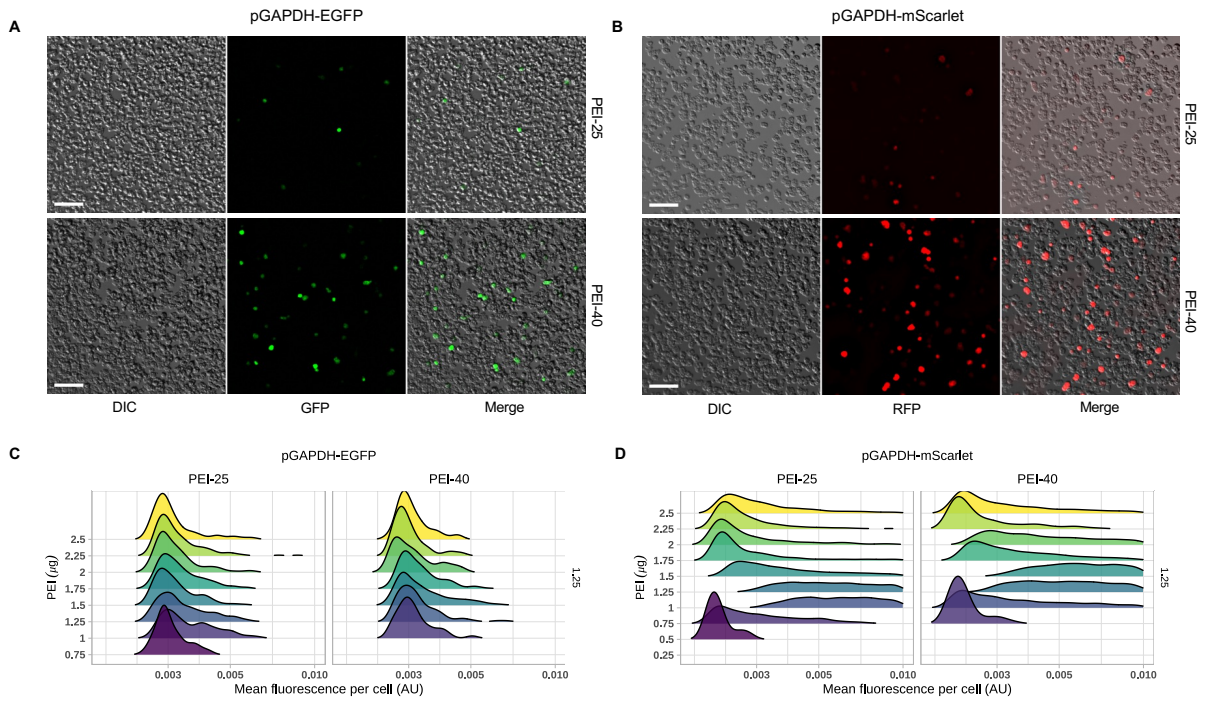

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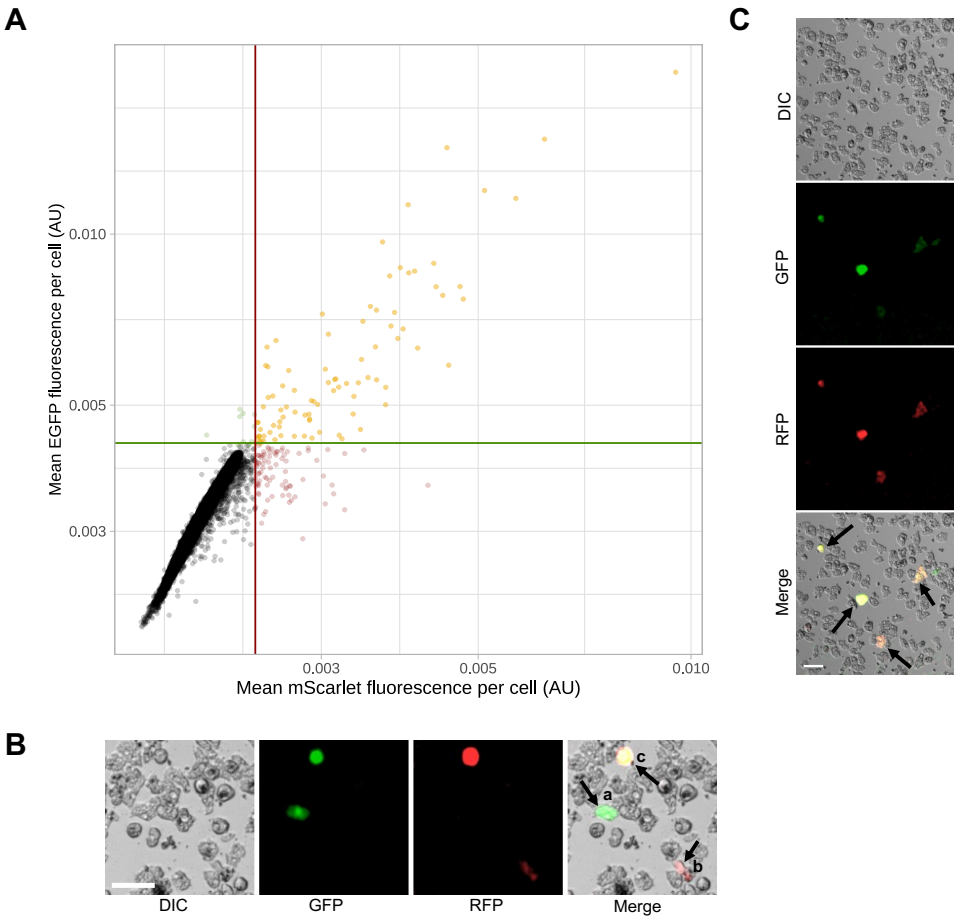
146 **Figure 3. Individual fluorescence variation of *A. castellanii* transfectants.** Representative
147 micrographs of *A. castellanii* transfected with pGAPDH-EGFP (**A**) or -mScarlet (**B**) using PEI-25 or
148 PEI-40. Scale bars, 100 µm. Mean fluorescence distribution per cell upon transfection with PEI-25 or
149 PEI-40 and 1.25 µg of pGAPDG-EGFP (**C**) or pGAPDH-mScarlet (**D**). Each ridge corresponds to rising
150 concentrations of PEI (bottom to top) within each panel, marked with different colours, showing the
151 distribution of the cell fluorescence per condition (x-axis, logarithmic scale).

152

153 To discern whether the fluorescence variability was due to the number of plasmids per cell or to
154 differential gene expression the amoebae were then transfected with both plasmids simultaneously.
155 From the transfection screen results, it was clear that pGAPDH-mScarlet yields more transfectants than
156 pGAPDH-EGFP, so it was imperative to optimise the ratio of pGAPDH-EGFP:pGAPDH-mScarlet
157 plasmids per transfection reaction. For this analysis, the *A. castellanii* cells were co-transfected with
158 PEI-40 at the optimal pDNA:PEI ratios (1.25:1.5 µg, as experimentally observed; **Figure 2**); and using
159 a range of pGAPDH-EGFP:pGAPDH-mScarlet plasmid ratios from 10:0 to 0:10 (**Figure S5**).
160 As expected, pGAPDH-mScarlet yielded more transfectants also in this setup. Most importantly, at a
161 9:1 pGAPDH-EGFP:pGAPDH-mScarlet plasmid ratio, we observed a substantial fraction of EGFP+
162 and mScarlet+ co-transfectants (**Figure 4** and **S5**). This experiment illustrates that *A. castellanii* can be
163 readily co-transfected with more than one plasmid per cell with the current protocol and that titration
164 of protein expression levels can also be accomplished by altering pDNA amounts.
165



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167

168 **Figure 4. Distribution of transfectants according to their fluorescence.** Co-transfection of *A.*
169 *castellanii* at a 9:1 pGAPDH-EGFP:pGAPDH-mScarlet plasmid ratio. (**A**) Black dots are non-
170 transfected cells, red and green are pGAPDH-EGFP and -mScarlet single-plasmid transfectants,
171 respectively, and yellow are transfectants harbouring both plasmids. The x- (red/mScarlet) and y-
172 intercept (green/EGFP) lines represent the minimum fluorescence threshold for a cell to be considered
173 a positive transfectant. (**B**) Representative micrographs of transfected *A. castellanii*, arrows point to
174 cells with plasmids expressing (a) EGFP, (b) mScarlet, and (c) both. (**C**) Representative micrographs
175 of *A. castellanii* co-transfectants with varying EGFP and mScarlet-fluorescence intensities. Scale bars,
176 50 µm.

177

178 ***The use of PEI greatly improves transfection efficiency over currently used reagents***

179 The efficiency of PEI as a transfection reagent for *A. castellanii* was next compared to that of the
180 currently used commercial reagents, namely SuperFect® and ViaFect™. A common transfection
181 protocol for each reagent was derived from published studies on *A. castellanii* [11,12]. The two resulting
182 protocols differed in that transfection was done with 1 µg of pDNA for SuperFect®, and 0.2 µg for
183 Viafect™, and incubation times of the complexes with cells were 3 h for SuperFect® and 16 h for
184 Viafect™. The two protocols were then adapted for PEI-25 and -40 (i.e., using the same quantity of
185 pDNA and incubation period) in combination with the optimal quantity of each PEI for these pDNA
186 concentrations (informed by results in **Figure 2**). The outputs of each protocol using the original reagent
187 were then compared to PEI-25 and -40 and quantified using CellProfiler, as before (**Figure 5**).
188 In both comparisons, the PEIs were vastly more efficient at transfecting the amoebae than either
189 commercial reagent (**Figure 5**). Compared to SuperFect®, both PEIs were at least 5x to up to ~100x
190 more efficient when using PEI-25 and pGAPDH-mScarlet (**Figure 5A**). In the PEIs *vs* Viafect™
191 comparisons, although the relative data was similar to SuperFect®, the absolute number of transfectants
192 was lower on average, which is congruent with this study's previous results from transfections with low

193 pDNA amounts (**Figure 2**). Also here, both PEIs were more efficient, at 8 to over 100-fold the
194 transfection efficiency of Viafect™ (**Figure 5B**).
195 As the transfections using PEIs had not been tested with shorter incubation periods, replica transfections
196 were performed in which the *A. castellanii* were incubated with complexes for 3 h (**Figure 5A,**
197 **triangles**). These results indicate that pDNA:PEI complexes can be transfected with broadly similar
198 efficiency even using significantly shorter incubation periods. In summary, PEI-25 and -40 vastly
199 outperform SuperFect® and ViaFect™ even under (for PEI) sub-optimal conditions.
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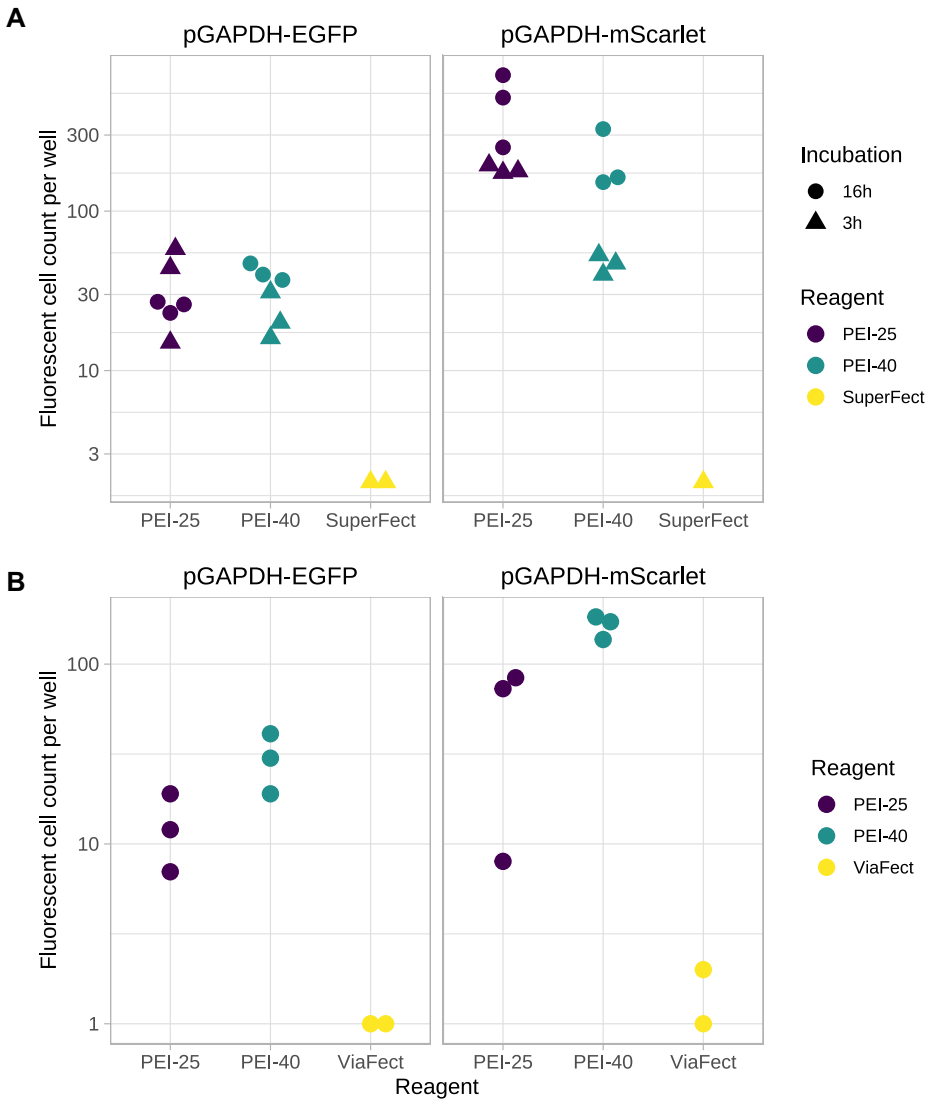

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202

203 **Figure 5. Comparison of the transfection efficiency of PEI-25 and 40 *vs* SuperFect® or Viafect™**
204 **during 3 h or 16 h incubation.** (**A**) Fluorescent cell count per well after transfection of *A. castellanii*
205 with pGAPDH-EGFP or -mScarlet using PEI-25, PEI-40 or SuperFect®. Per manufacturer instructions
206 pDNA:SuperFect® complexes were incubated with the cells for 3 h, while pDNA:PEI complexes were
207 incubated with the cells for 3 h or 16 h. The symbol colours correspond to the different reagents and
208 the y-axis to the total transfectant count. Each symbol is a technical replicate. Experiments were
209 conducted in triplicates. Some experiments with SuperFect® or Viafect™ did not yield any transfectant
210 and are thus not displayed. The triangle and dot shapes represent the incubation period of the complexes
211 with the cells (3 and 16 h, respectively). (**B**) shows the same comparison between PEIs and Viafect™.

212

213 **Discussion**

214 *A. castellanii* is a free-living protist, ubiquitous and abundant in freshwater and soil habitats. It is one
215 of the most often isolated amoebal species worldwide [21,22]. Its status as an emerging human pathogen is
216 due not only to the infections it causes as an opportunistic human pathogen but also because of its role
217 as a reservoir for bacteria, viruses, and fungi [21,22,23]. Furthermore, due to the cellular and functional
218 similarities between *A. castellanii* and mammalian phagocytic cells, the former can be considered
219 training grounds for these pathogens to cause human infection [21,22,24]. These factors make *A. castellanii*
220 an important and exciting model organism. Still, due to the shortage of molecular biology techniques
221 (among others), it has not been as widely used as other amoebae [21–24].

222 Here, we demonstrated that the readily available and inexpensive cationic polymer PEI can be used to
223 efficiently transfect *A. castellanii* with plasmid DNA. We systematically explored the use of three PEIs,
224 PEI and DNA quantities, on transfection efficiency and their effects on cell viability. Our combinatorial
225 approach allowed us to test a broad range of PEI and pDNA amounts and ratios in a high-throughput
226 manner. The N/P ratio range tested was sufficient to highlight how there are none or very few
227 transfectants at the extremes of the N/P ratio distribution. It also highlights how the actual quantity of
228 the reagents in the solution, and not just the N/P ratio, plays an important role in efficient transfection.

229 These observations provide a comprehensive guide to choosing optimal transfection parameters for *A.*
230 *castellanii*.
231 We also demonstrate that co-transfection of more than one plasmid per cell is possible and prevalent.
232 Furthermore, the quantity of PEI used affected not only the absolute transfection efficiency, but also
233 the distribution of the different transfected plasmids during co-transfection, making it possible to
234 modulate the level of co-transfection. This could be used to normalise or titrate the number of each
235 plasmid transfected per cell, should it be desirable to have a homogenous or a graded protein expression
236 within the population. However, the differing transfection efficiency between the two fluorescence
237 plasmids remains unclear. Two hypotheses may explain this phenomenon: (i) mScarlet is a brighter
238 fluorescent molecule, compared to EGFP (brightness of 70 vs 33.54, respectively, according to
239 www.fpbase.org), and so the plasmids may be similarly transfected, but mScarlet fluorescence may be
240 more easily detected; alternatively, (ii) while EGFP was codon-optimised for expression in humans, the
241 mScarlet sequence was codon optimised for *A. castellanii*, which should improve the expression of the
242 latter. This effect has been described before, e.g. Bateman observed that: "*(...) high levels of monomeric*
243 *red fluorescent protein (mRFP) expression could be serendipitously obtained.*" when using pTPBF-
244 mRFP *versus* pTPBF-EGFP [25]. Another report mentions using a 1.7 ratio of mVenus- over tdTomato-
245 expressing plasmid when co-transfecting to account for differences in brightness and quantum yield of
246 the fluorescent proteins [26,27]. Temperature, an element to consider when choosing fluorescent proteins
247 [27], might also provide some insight into this phenomenon; since EGFP has been optimised for
248 expression in human cells, it stands to reason that this protein would also be best expressed at 37°C and
249 not at 30°C used here. Ultimately, no generalisable conclusions regarding if one plasmid is always
250 *better* than another should be drawn from the present work. Nevertheless, we demonstrate that PEI
251 transfection provides a vastly improved protocol over current commercial standards, both for an EGFP
252 and a mScarlet fluorescence plasmid.
253 The cytotoxicity results show that even high concentrations of PEI-25 and PEI-40 are non-toxic. Yet, it
254 is important to be wary that high concentrations of PEI could affect the cells in other ways not assessed
255 here. Therefore, other cell characteristics, such as morphology, should be considered in future
256 experiments. Also not investigated here, but of interest, is whether PEI is also effective for transfection

257 of siRNA in *A. castellanii*. Many studies demonstrate PEI-mediated gene knockdown with siRNA in
258 other cell types. In studies where *A. castellanii* is transfected with siRNA the reagents used are the same
259 as those used for transfection with plasmid DNA [12], so it can be surmised that PEI would also work to
260 achieve this in *A. castellanii*.
261 In summary, we here provide evidence for a high-efficiency transfection protocol in *A. castellanii* using
262 the cationic polymer PEI and comprehensively optimise the relevant transfection conditions.
263

264 **Materials and Methods**

265 **Acanthamoeba *culture***

266 *Acanthamoeba castellanii* (ATCC 30010) was cultured in PYG medium with additives (ATCC medium
267 712) at 30°C, and cultures were split three times per week.
268

269 ***Plasmid DNA***

270 The pGAPDH-EGFP plasmid used in this study was first described by Bateman [25], and it was kindly
271 sent to us by Prof. Yeongchul Hong (Kyungpook National University, Korea) (**Figure S3**). To make
272 pGAPDH-mScarlet (**Figure S3**), the EGFP fluorescence reporter gene was removed by enzymatic
273 digestion of the plasmid with *Nde*I and *Xba*I restriction enzymes. The plasmid backbone was gel
274 purified and used for ligation with mScarlet cut with the same restriction enzymes as above. The
275 sequence of the mScarlet gene [28] was codon optimised according to the codon profile of *A. castellanii*,
276 using a codon optimisation tool (https://dnahive.fda.gov/dna.cgi?cmd=codon_usage&id=537&mode=
277 cocoputs) [29]. The restriction sites – *Nde*I and *Xba*I – were added at the 5' and 3' ends, and Integrated
278 DNA Technologies® synthesised the sequence.
279

280 ***Transfection reagents***

281 The 25kDa PEI 25™ (PEI-25) and 40 kDa PEI Max® (PEI-40) polymers were acquired from
282 Polysciences and were prepared according to manufacturer instructions to get a stock solution of 1
283 mg/ml. Branched PEI (PEI-Br) was purchased from Sigma-Aldrich (408727) and diluted in ultrapure
284 water to 1 mg/ml concentration.

285

286 ***Plasmid DNA-PEI affinity in different media***

287 To test the effect of different media and buffers on the ability of the PEI to form complexes with the
288 plasmid DNA at different N/P ratios, increasing amounts of PEI (0.25 – 2.5 µg) and 0.25 µg of pDNA
289 were each diluted in 10 µl of the indicated complexation buffers, mixed by pipetting and incubated at
290 RT for 20 minutes. After incubation, 30 µl of the respective buffer was added to the pDNA-PEI complex
291 mix. An aliquot of this was used to load a 0.7% agarose gel (with GelRed), and gel electrophoresis was
292 carried out for 1h at 100V. The different media used were Peptone Yeast Glucose (PYG) (ATCC 712),
293 Ac medium (PYG medium without protease peptone, yeast extract, and glucose), and LoFlo
294 (ForMedium); and HEPES buffered solution (HBS), NaCl (150 mM) solution, and ultrapure water
295 (ddH_2O).

296

297 ***Viability Assay standardisation***

298 To assess the toxicity of the transfection reagents, viability assays were performed using a resazurin
299 assay [20]. This assay reduces the non-toxic and non-fluorescent dye resazurin to fluorescent resorufin via
300 mitochondrial reductase. Changes in fluorescence indicate mitochondrial activity, a proxy for cell
301 viability. Resazurin sodium salt (Sigma) was diluted in PBS to 2 mM, filter-sterilised, and kept at 4°C,
302 protected from light.

303 To determine the optimal cell number and incubation period with the resazurin salt solution for the
304 assay, $2.5x10^3$-$8.0x10^4$ *A. castellanii* cells were seeded per well, in triplicates, in a black 96-well plate,
305 in a final volume of 100 µl of PYG. The plate was incubated at 30°C for 16 h. Then, 10 µl of the
306 resazurin solution was added to each well, the plates were further incubated, and fluorescence
307 measurements were taken at 2, 4 and 6 h. The resorufin fluorescence was measured at 530 nm
308 (excitation) and 590 nm (emission) at 5 locations in the well using a Tecan Spark fluorometer®.
309 Measuring the fluorescence produced by the amoebae with a 2 mM resazurin solution and 6 h of
310 incubation proved sensitive enough to discriminate cell viability even at low cell concentrations.

311

312 ***PEI toxicity assays***

313 *A. castellanii* tolerance to each PEI reagent was assessed using the resazurin assay. Briefly, $2x10^4$ cells
314 were seeded per well in a black 96-well plate and incubated overnight at 30°C. The following day the
315 medium was replaced by 50 µl of PYG containing increasing concentrations of PEI (0.25-2.5 µg), and
316 the plates were incubated for 24 h. The medium was removed, and 100 µl PYG was added before
317 initiating the resazurin assay (described above).
318
319 ***Nitrogen/Phosphate ratio theoretical approximation***
320 The ratio of the amount of nitrogen in PEI per phosphate in DNA in a reaction mixture (N/P ratio) was
321 estimated, as it plays an essential role in polyplex formation and transfection efficiency. The theoretical
322 amine (nitrogen) groups in PEI are derived from the polymer's chemical formula for a single repeat unit
323 (monomer). Using the 25kDa lPEI ($-NHCH_2CH_2-$) as an example, a monomer's molecular weight (MW)
324 would be 43 g/mol. Therefore, 1 µg would contain 23 nmol of nitrogen. The number of phosphate
325 groups in the DNA is calculated using the equations (1) and (2)[13,14,18]:
326 $$(1)\ P\ moles\ =\ dsDNA\ moles\ \times dsDNA\ length\ (bp)\ \times 2$$
327 where
328 $$(2)\ dsDNA\ moles\ =\ \frac{dsDNA\ moles\ mass\ (g)}{dsDNA\ length\ (bp)\ \times\ MWbp}$$
329
330 Hence, 1 µg pGAPDH-EGFP (5831 bp) would have 3.2 nmol of phosphate. The N/P ratio of this
331 reaction is then calculated with the following equation (3):
332 $$(3)\ N/P\ =\frac{23\ nmol}{3.2\ nmol}\ \approx 7.2$$
333
334 **Acanthamoeba *transfection***
335 A day before transfection, $1x10^4$ cells were seeded per well in a 96 black well plate, in 100 µl of PYG
336 with 1% Penicillin-Streptomycin (PS). On the day of transfection, pDNA and PEIs were each first
337 diluted in 10 µl of PYG, then mixed by pipetting, and incubated at room temperature for 20 mins.
338 During this incubation, the amoebae plates were prepared by aspirating the PYG, and washing once.

339 Lastly, 30 µl of PYG was added to the pDNA-PEI mix, the total volume (50 µl) was to each well, drop-
340 wise, and the plates were then incubated at 30°C for 16 h.
341
342 ***Transfection with SuperFect® and Viafect™***
343 To compare PEI-25 and PEI-40 and the commercial reagents – Viafect™ and SuperFect®, *A. castellanii*
344 cells were prepared and seeded as described above. The ratios of pDNA to transfection reagent were
345 based on published methods [11,12] and the manufacturer's protocols. For transfections with SuperFect, 1
346 µg pDNA was mixed in 30 µl of PYG, before adding 5 µl of SuperFect®. This reaction was incubated
347 at room temperature for 20 mins, then 150 µl of PYG was mixed in and added to the cells. The cells
348 were then incubated for 3 h at 30°C, after which the DNA-SuperFect® solution was carefully aspirated,
349 the cells were washed once, and fresh PYG with PS was added, followed by overnight incubation. For
350 the paired comparison assays, 1 µg pDNA was transfected with 1.5 µg of PEI-25/PEI-40, as described,
351 cells were incubated with the DNA: PEI complexes, for 3 h or 16 h, before replacing the medium with
352 PYG with PS. Viafect™ transfections were performed similarly: 0.2 µg pDNA and 1 µl reagent were
353 mixed in 50 µl PYG; this solution was incubated with the cells for 3 h, before adding 100 µl of PYG
354 and further incubating for a total of 16 h. To compare the PEIs with Viafect™, 0.2 µg pDNA was mixed
355 with 1 µg PEI-25/PEI-40 and incubated with the cells for 16 h.
356
357 ***Detection of transgene expression***
358 To assess the efficiency of transfection, a microscopy-based quantification approach was taken. Using
359 a Nikon T*i*-2 Eclipse microscope equipped with a Spectra-X light engine (Lumencor), 4 pictures were
360 taken at different locations per well, with a 10x /0.45 NA Plan Apochomat objective and a Photometrics
361 Prime 95B camera (1.12 µm pixel size), covering a total area of approximately 38% of the well.
362 Micrographs were taken using DIC contrast and in the GFP (excitation: 475/34, emission filter: Chroma
363 ET510/20) or RFP (excitation: 575/35, emission Chroma 89403) channels. The images were segmented
364 using the Cellpose 2.0 segmentation algorithm [30], processed and analysed in CellProfiler v4.2.1[31]. Data
365 were further analysed with R [32], and figures were produced with the R package ggplot2 [33] and GraphPad
366 Prism (version 9.4.0 for Mac).

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Author contributions

A.B.M.: Conceptualisation, Methodology, Investigation, Formal Analysis, Visualisation, Writing - Original draft. V.E.: Visualisation, Software. J.E.: Methodology, Software. M.E.S.: Methodology, Resources. L.G.: Formal Analysis, Visualisation, Resources. All authors reviewed and edited the manuscript.

Declaration of interests

The authors declare no competing interests.

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Supplementary Figures

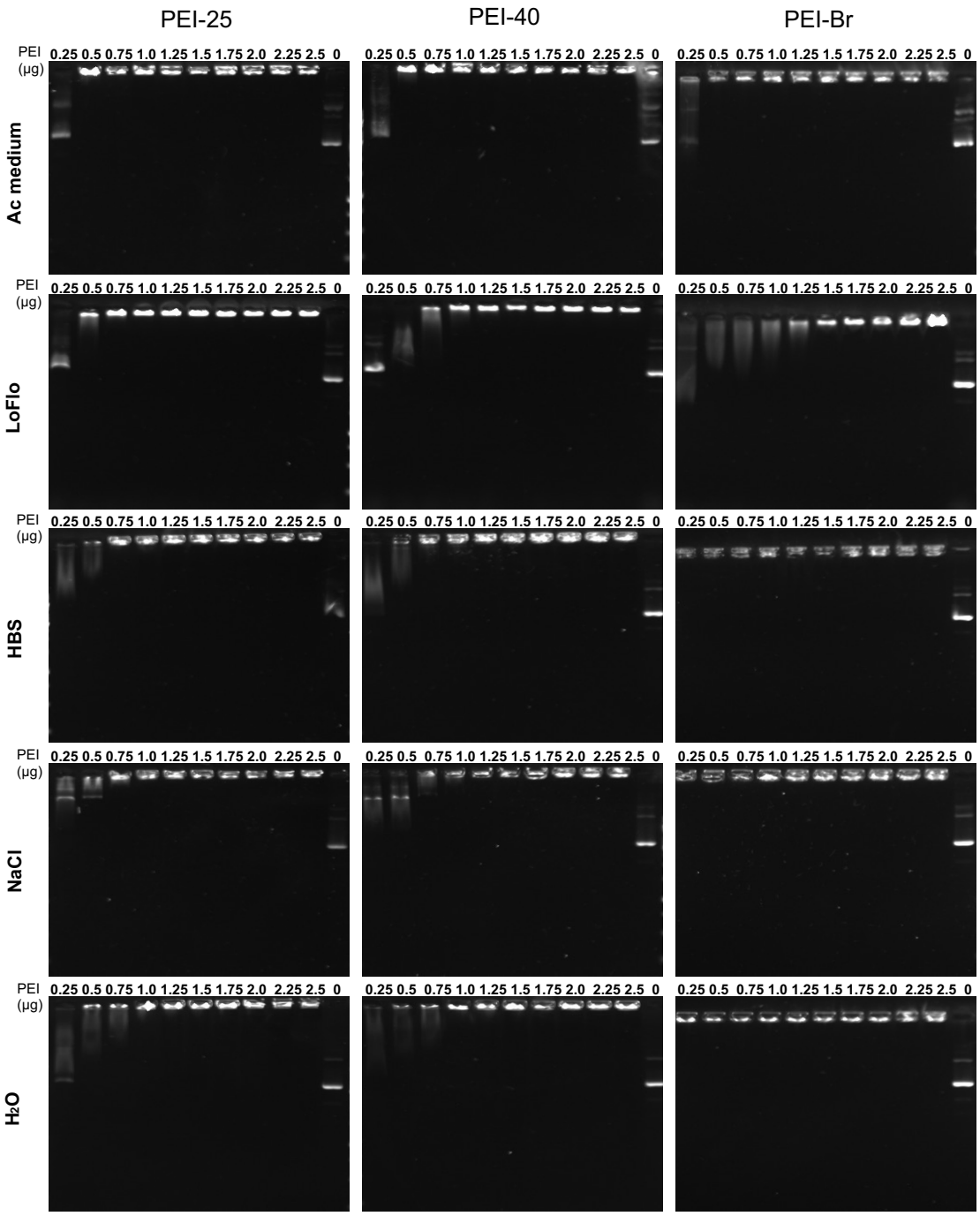


Figure S1. Condensation of pDNA with PEIs in different media. Increasing concentrations of PEI were used to form complexes with 0.25 µg of pDNA. The migration of the DNA-PEI polyplexes was compared to that of non-complexed/naked pDNA (C, untreated control).

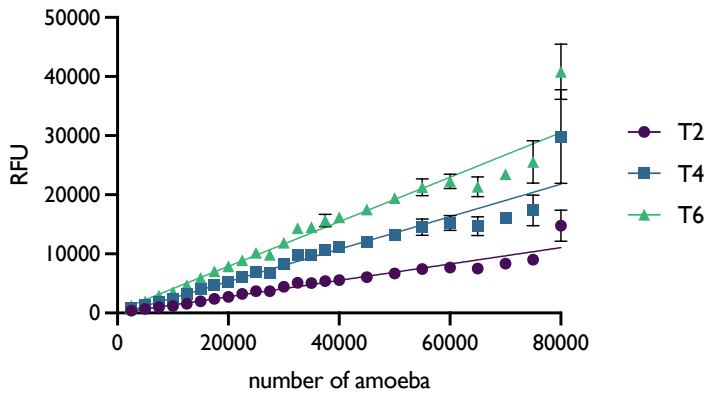


Figure S2. Resazurin viability assay optimisation. Fluorescence signal produced by *A. castellanii* using 2 mM resazurin sodium salt solution at different incubation periods (2, 4 and 6 hours). Results are the mean of 6 technical replicates and 3 independent experiments; error bars are standard deviation.

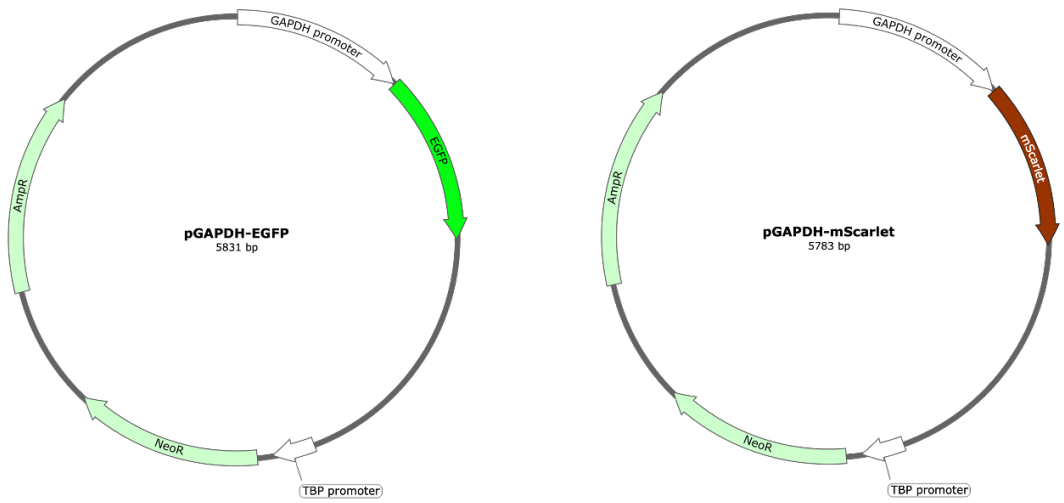


Figure S3. Plasmids used in this study. Plasmid pGAPDH-EGFP was first described in Bateman (2010) [25], and plasmid pGAPDH-mScarlet was constructed as part of this study. Both plasmids contain the *Acanthamoeba* GAPDH gene promoter upstream of the fluorescence marker (EGFP or mScarlet) for constitutive expression in *A. castellanii*; an ampicillin resistance gene (AmpR) for growth and selection in *E. coli*; a neomycin resistance gene (NeoR) downstream of the *Acanthamoeba* TBP gene promoter for selection in *A. castellanii.*

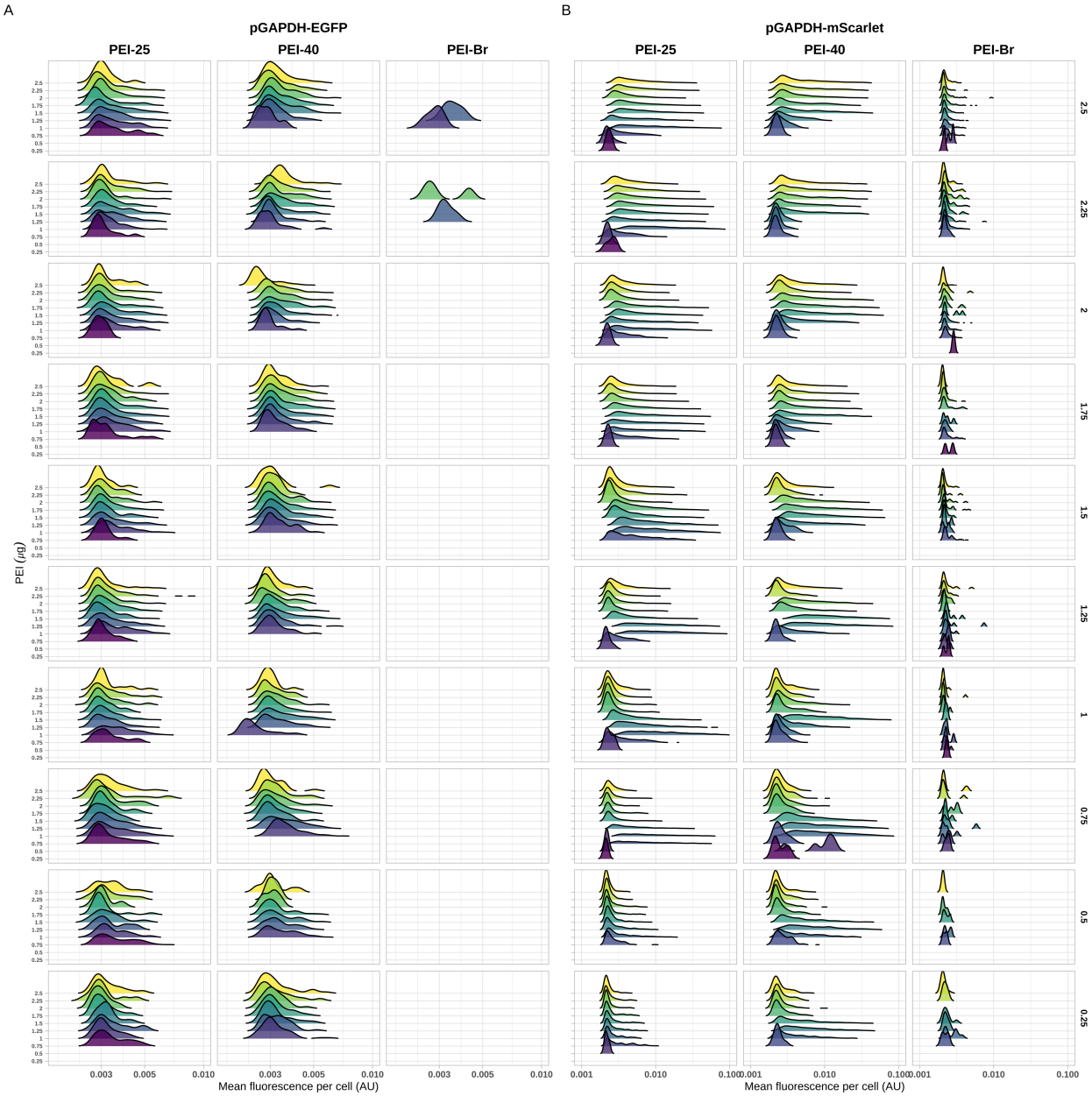
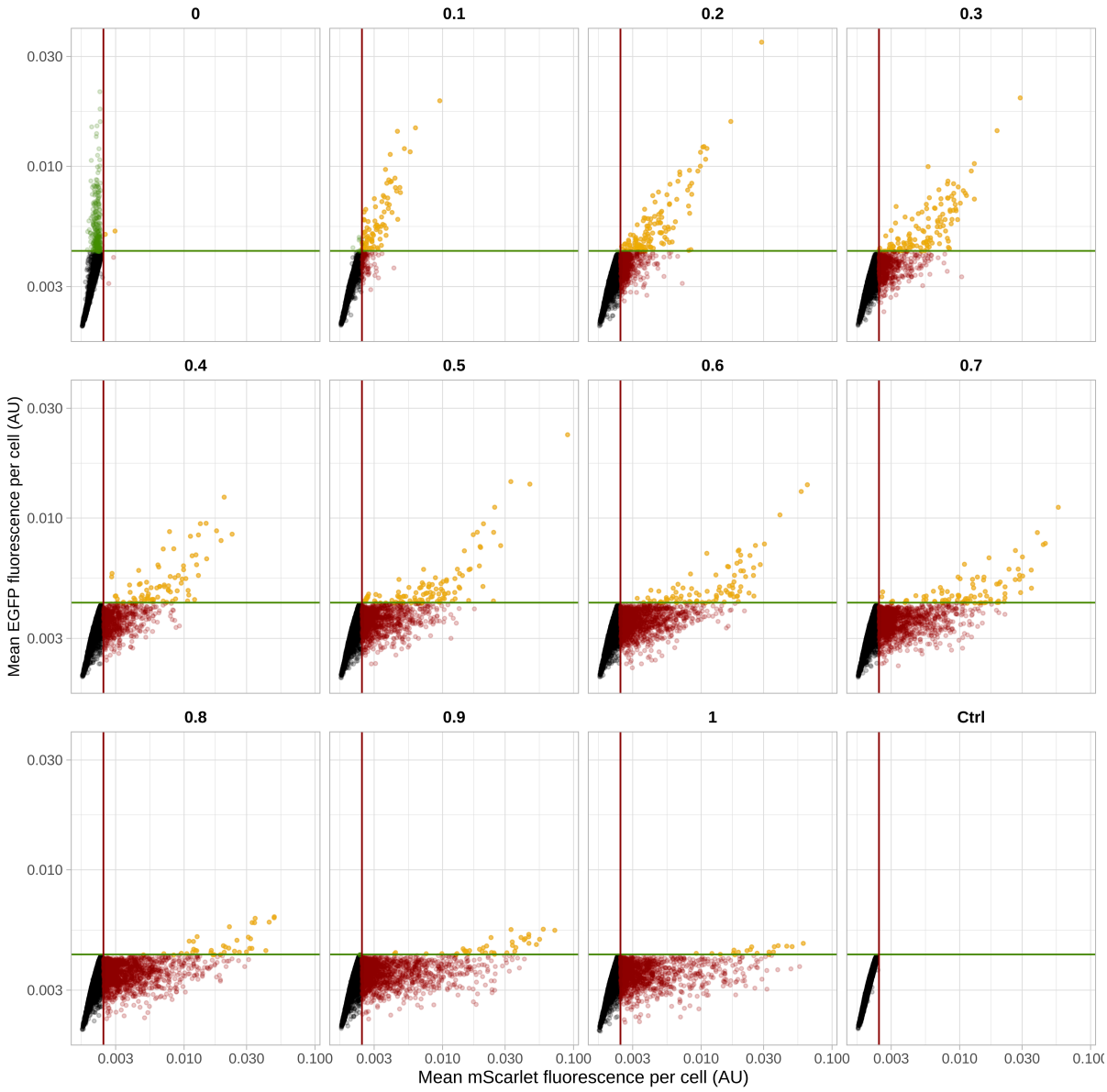


Figure S4. Fluorescence distribution per cell transfected with different pDNA and PEI quantities. Transfection of *A. castellanii* with pGAPDH-EGFP (**A**) or pGAPDH-mScarlet (**B**) plasmids using PEI-25, -40 and -Br. The rows of panels correspond to, from top to bottom, decreasing concentrations of pDNA. In each panel, the ridges illustrate the fluorescence distribution per cell with increasing quantities of PEI.



485

486 **Figure S5. Distribution of *A. castellanii* co-transfectants according to their fluorescence.**
487 Transfection of *A. castellanii* with different ratios of pGAPDH-EGFP and pGAPDH-mScarlet plasmids
488 (indicated above each plot). In each plot: black dots are non-transfected cells; red and green dots are
489 pGAPDH-EGFP and -mScarlet-single plasmid transfectants, respectively; and yellow dots are co-
490 transfectants with both pGAPDH-EGFP and -mScarlet plasmids. The X- (red/mScarlet) and Y-intercept
491 (green/EGFP) lines represent the threshold fluorescence for a cell to be considered a positive
492 transformant. The 'Ctrl' plot is non-transfected cell control.