

1       **Cultured pluripotent planarian stem cells retain potency and**  
2       **express proteins from exogenously introduced mRNAs**

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21       **Key words:** cell culture, planaria, neoblast, transgenesis, SiR-DNA

22

23 **Abstract:**

24 Planarians possess naturally occurring pluripotent adult somatic stem cells  
25 (neoblasts) required for homeostasis and whole-body regeneration. However,  
26 methods for culturing neoblasts are currently unavailable, hindering both  
27 mechanistic studies of potency and the development of transgenic tools. We report  
28 the first robust methodologies for culturing and delivering exogenous mRNA into  
29 neoblasts. We identified culture media for maintaining neoblasts in vitro, and  
30 showed via transplantation that the cultured stem cells retained pluripotency. By  
31 modifying standard flow cytometry methods, we developed a new procedure that  
32 significantly improved yield and purity of neoblasts. These methods facilitated the  
33 successful introduction and expression of exogenous mRNAs in neoblasts,  
34 overcoming a key hurdle impeding the application of transgenics in planarians. The  
35 tissue culture advances reported here create new opportunities to advance  
36 detailed mechanistic studies of adult stem cell pluripotency in planarians, and  
37 provide a systematic methodological framework to develop cell culture techniques  
38 for other emerging research organisms.

39

## 40 **Introduction**

41 Stem cell pluripotency remains an important and still unresolved problem in  
42 biology. Several systems have been established to study pluripotency regulation  
43 in germlines, embryonic, and induced pluripotent stem cells <sup>1-4</sup>. However, no  
44 naturally occurring adult pluripotent stem cells have been identified in traditional  
45 model systems, including round worms, flies, fishes, and mice. Unlike traditional  
46 research organisms, planarians harbor an abundant population of adult stem cells  
47 collectively known as neoblasts. These cells are characteristic of flatworms and  
48 acoels <sup>5</sup>, and in planarians include a subpopulation of pluripotent stem cells termed  
49 clonogenic neoblasts <sup>6-8</sup>. Neoblasts confer planarians with remarkable  
50 regenerative abilities and a seemingly limitless capacity for tissue homeostasis. Of  
51 the many freshwater planarian species known to exist, *Schmidtea mediterranea*  
52 has become one of the most widely studied <sup>9</sup>. Planarians thus provide a unique  
53 context in which to explore how nature has solved the complex problem of  
54 maintaining stem cell pluripotency in a long-lived adult animal.

55 Expression of conserved genes regulating pluripotency have been identified in  
56 planarian neoblasts and functionally studied using RNA interference <sup>10-13</sup>. However,  
57 due in part to the lack of methodologies for cell culture, exogenous gene  
58 expression, and transgenesis in planarians, the mechanisms regulating the  
59 pluripotency of these adult stem cells *in vivo* are poorly understood. Therefore,  
60 developing planarian transgenesis is of great significance <sup>14</sup>. A review of the history  
61 of cell culture methodologies and attempts to develop transgenics in planarians

62 indicated that successful neoblast culture may be a critical first step to develop  
63 transgenic methodologies in planarians.

64 Transgenic approaches typically take advantage of either early stage embryos  
65 or cultured stem cells. Invertebrates, such as *Caenorhabditis elegans*, *Drosophila*  
66 *melanogaster*, Hydra, *Nematostella vectensis*, and the flatworm *Macrostomum*  
67 *lignano*, have large syncytial germ cells or embryos, respectively, that are highly  
68 amenable to genetic manipulation <sup>14-18</sup>. In vertebrates, such as mice, both zygotes  
69 and cultured embryonic stem cells are used to deliver exogenous genetic material  
70 <sup>19</sup>. Unlike these research organisms, planarians do not possess large, easily  
71 accessible germ cells or early-stage blastomeres amenable to manipulation or  
72 transplantation. Instead, in asexually reproducing planarians, neoblasts are the  
73 only known proliferating cells in the animal <sup>20</sup>. Neoblasts from one animal can be  
74 readily transplanted into a host devoid of its own endogenous neoblasts after lethal  
75 irradiation, resulting in neoblast repopulation and host rescue within 1 month <sup>6,8</sup>.  
76 Thus, introduction of exogenous DNA into cultured neoblasts prior to  
77 transplantation is a potential strategy to produce transgenic planarians. Cultured  
78 neoblasts would also be ideal for rapidly screening conditions for delivering and  
79 expressing exogenous mRNA or DNA. Therefore, we aimed to establish a robust  
80 method for culturing pluripotent neoblasts that may allow rapid screening of  
81 conditions for delivering and expressing exogenous mRNA or DNA.

82 Previous efforts to culture planarian cells were conducted at a time when our  
83 mechanistic understanding of neoblast self-renewal and heterogeneity were  
84 limited <sup>21-24</sup>. In some of these studies, cells with gross morphology similar to

85 neoblasts survived in an isotonic medium for a couple of weeks, yet neither  
86 functional nor molecular tests on the cultured cells were performed, leaving an  
87 open question as to their identity and potency<sup>23</sup>. Since then, the pan-neoblast  
88 marker *smedwi-1* (a homolog of the Argonaute family of proteins) was identified<sup>25</sup>,  
89 allowing us to molecularly define and visualize neoblasts using gene expression  
90 profiling or whole mount in situ hybridization (ISH). Techniques that enrich  
91 neoblasts using flow sorting have also been developed. A cell cycle-based flow  
92 sorting method using Hoechst 33342 staining has been used to isolate S and G2/M  
93 cell cycle phase neoblasts (X1 cells; nearly 90% of X1 cells are *smedwi-1*+  
94 neoblasts)<sup>25,26</sup>. However, Hoechst 33342 is cytotoxic, and X1 neoblasts cannot  
95 proliferate *in vivo* after transplantation into lethally irradiated planarians lacking  
96 stem cells. To solve this technical limitation, a DNA dye free back-gating strategy  
97 using forward scatter (size) and side scatter (complexity) was shown to enrich for  
98 a heterogeneous cell population containing neoblasts (X1(FS))<sup>8</sup>. Unlike X1  
99 neoblasts, X1(FS) neoblasts proliferate and successfully rescue lethally irradiated  
100 planarians upon transplantation making the X1(FS) population suitable for the  
101 development of an *in vitro* neoblast culture protocol<sup>8</sup>. When considered alongside  
102 the formulation of new types of cell culture media<sup>23,27</sup>, these advances provide a  
103 groundwork to attempt establishing new, robust methods for *in vitro* culture of  
104 pluripotent and transplantation-competent neoblasts.

105 In this study, we performed an unbiased screen of 23 different formulations of  
106 cell culture media to identify the best nutrient conditions for flow cytometrically  
107 isolated neoblasts. Cell morphology, viability, percentage of *smedwi-1*+ cells,

108 clonogenic capacity after transplantation, and rescue efficiency were assayed to  
109 identify the optimal conditions for culturing pluripotent neoblasts. Importantly, time-  
110 lapse imaging captured neoblast division for the first time in culture in real-time.  
111 Moreover, a novel neoblast isolation method using the vital dye SiR-DNA was  
112 developed, improving the purification yields for neoblasts relative to X1(FS), while  
113 preserving the clonogenic and rescue capacity of neoblasts following  
114 transplantation. Finally, we developed electroporation conditions that can deliver  
115 exogenous mRNA into cultured neoblasts providing unambiguous evidence that  
116 exogenous mRNAs can be expressed, albeit with low efficiency, in cultured  
117 neoblasts. Cumulatively, our work provides a foundation for developing long-term  
118 neoblast culture methods and, ultimately, transgenic planarians. It also provides a  
119 systematic methodological framework that may be applied to the development of  
120 cell culture techniques in other invertebrate research organisms.

121

## 122 **Results**

### 123 **Identification of seven culture conditions that maintain viable dividing** 124 **neoblasts**

125 To test different culture conditions for neoblasts, we first used an established  
126 back-gating method to sort X1(FS) cells, which contain approximately  $23.4\% \pm 2.5\%$   
127 neoblasts (*smedwi-1+*) (Fig. 1a-c). We then systematically screened 23 different  
128 types of media, representing most commercially available or previously reported  
129 formulations in the presence (+) or absence (-) of 5% CO<sub>2</sub> (Supplementary Table  
130 1)<sup>23,24</sup>. To assess each culture condition, five criteria were assayed: 1) cell

131 morphology and viability (viability); 2) percentage of *smedwi-1+* cells maintained  
132 in culture (%neoblasts); 3) cell division; 4) clonogenic capacity after transplantation  
133 (colony expansion); and 5) rescue efficiency in lethally irradiated planarians  
134 (pluripotency) (Fig. 1a).

135 After 1 day of culture, cell morphologies were observed using transmitted light  
136 microscopy. Cells cultured in CMFB +/- 5% CO<sub>2</sub> displayed abnormally roughened  
137 cell morphologies accompanied by abundant cellular debris in the plate,  
138 suggesting poor viability (Fig. 1d). In contrast, cells in all other conditions, such as  
139 IPM +/- 5% CO<sub>2</sub>, had normal morphology, suggesting high viability (Fig. 1d). Cells  
140 in Leibovitz's L-15 medium (L15) without 5% CO<sub>2</sub> extended long processes that  
141 were visible even after 6 days of culture (Supplementary Fig. 1), suggesting  
142 neuronal differentiation as previously observed in cultured *Caenorhabditis elegans*  
143 embryonic cells<sup>28</sup>.

144 To measure viability, cells cultured for 1 day were stained with propidium iodide  
145 (PI), which labels the DNA of dying cells, and the percentage of PI negative cells  
146 was determined using flow cytometry. Consistent with the microscopic evaluation,  
147 cells cultured in CMFB displayed poor survival +/- 5% CO<sub>2</sub> (>60% dead cells) (Fig.  
148 1e). In fact, of all media conditions tested, seventeen yielded a viability of at least  
149 60% (Fig. 1e), with only seven of the media performing significantly different in the  
150 presence and absence of 5% CO<sub>2</sub> (Fig. 1e).

151 To determine what proportion of viable cells were neoblasts after 24 hours of  
152 culture, we quantified the number of *smedwi-1+* X1(FS) cells by fluorescent *in situ*  
153 hybridization (FISH). Importantly, all cultures without 5% CO<sub>2</sub> maintained fewer

154 *smedwi-1+* neoblasts compared to those cultured in the presence of 5% CO<sub>2</sub>,  
155 except for diluted (d) SFX (Fig. 1f). Furthermore, of the 5% CO<sub>2</sub> cultures, seven  
156 media maintained significantly more *smedwi-1+* neoblasts than all other culture  
157 conditions, including dGrace's medium, IPM, KnockOut DMEM, dL15 medium,  
158 dKnockOut DMEM, dSchneider's medium, and dDMEM (Fig. 1f). Because dSFX  
159 without 5% CO<sub>2</sub> failed to support neoblast culture as well as the seven media with  
160 5% CO<sub>2</sub> we identified above (Fig. 1e), we did not explore it further in this study.  
161 This result was supported by co-staining cells cultured in IPM + 5% CO<sub>2</sub> with  
162 *smedwi-1* and the apoptotic/dead-cell marker, Annexin V (Supplementary Fig. 2);  
163 no co-labeling was observed, indicating that neoblasts were viable  
164 (Supplementary Fig. 2). Consistently, cell viability in these seven media + 5% CO<sub>2</sub>  
165 was consistently greater than 60% (Fig. 1e). We next examined whether *smedwi-*  
166 *1+* neoblasts were maintained after 3 days in culture using these seven media +  
167 5% CO<sub>2</sub>, and observed persistent *smedwi-1+* cells in all culture conditions tested  
168 (Fig. 1g). Thus, neoblasts can be maintained for at least 3 days *in vitro*. Therefore,  
169 we focused on testing dGrace's, IPM, KnockOut DMEM, dL15, dKnockOut DMEM,  
170 dSchneider's, and dDMEM media in subsequent optimization experiments.

171 Next, we assessed whether cultured neoblasts were capable of dividing *in vitro*.  
172 Although an obvious increase in cell number was not noticed, low levels of both  
173 symmetric and asymmetric neoblast divisions were observed in 1 day cultured  
174 cells, as judged by cell pair size and distribution of *smedwi-1* transcripts (Fig. 1h)  
175 <sup>13</sup>. Confirmation that neoblasts can divide *in vitro* was obtained using time-lapse  
176 imaging microscopy to record the behavior of X1(FS) cells in culture. Both



177 symmetric and asymmetric cell divisions were observed within the first 24 hours  
178 after culture (Fig. 1i and Supplementary Movies 1 and 2) in IPM, KnockOut DMEM,  
179 and dL15 medium, but not in the other four media tested (Fig. 1i). Consistently, the  
180 percentage of *PCNA*<sup>+</sup> cells in the cultures of IPM, KnockOut DMEM, and dL15  
181 medium were significantly higher than those in CMFB, Schneider's, and DMEM  
182 medium (Supplementary Fig. 3). Even though we cannot exclude the possibility  
183 that these conditions only allow neoblasts in M phase to complete the cell cycle,  
184 to our knowledge, this is the first time that neoblast divisions have been observed  
185 and recorded *in vitro*. These results suggest that a fraction of X1(FS), *smedwi-1*<sup>+</sup>  
186 cells can execute cell division within 24 hours after isolation in culture.

### 187 **Cultured neoblasts maintain clonogenic capacity**

188 To determine if X1(FS) neoblasts could divide *in vivo* following *in vitro* culture,  
189 we next examined their clonogenic capacity following transplantation into lethally  
190 irradiated planarians, an experimental manipulation that normally leads to robust  
191 neoblast expansion (Fig. 2a). Serial cell dilution experiments indicated that 1,000  
192 freshly collected X1(FS) cells undergo consistent colony expansion in  $\geq 80\%$  hosts  
193 upon bulk cell transplantation (Supplementary Fig. 4). Considering the rate of cell  
194 death in culture, 5,000 X1(FS) cells were cultured for each test condition to ensure  
195 that enough cells were viable at the time of transplant. We transplanted X1(FS)  
196 cells cultured in the seven different media + 5% CO<sub>2</sub> that showed higher than 15%  
197 *smedwi-1*<sup>+</sup> cells (Figure 1f) for 1, 2, or 3 days. At 8 days post-transplantation (dpt),  
198 the presence or absence of *smedwi-1*<sup>+</sup> neoblast colonies and the number of  
199 *smedwi-1*<sup>+</sup> neoblasts in each colony were determined. All X1(FS) neoblasts

200 cultured for 1 or 2 days efficiently proliferated *in vivo*, except for those cultured in  
201 dGrace's medium + 5% CO<sub>2</sub> (Fig. 2b–d). By comparing the number of *smedwi-1+*  
202 neoblasts in each transplant, X1(FS) cells cultured for 1 day in either IPM or  
203 KnockOut DMEM formed the largest colonies *in vivo* (Fig. 2b, d). X1(FS) cells  
204 cultured for 2 days displayed decreased expansion potential in all conditions, but  
205 all were still capable of forming colonies *in vivo* with the exception of those cultured  
206 in dGrace's medium + 5% CO<sub>2</sub>. X1(FS) cells cultured for 3 days were largely  
207 incapable of forming colonies following transplantation, though small colonies  
208 formed from cells cultured in dSchneider and dL15 media (Fig. 2c, d). In summary,  
209 IPM and KnockOut DMEM performed best following the first day in culture, but  
210 performed similarly to dKnockOut DMEM, dSchneider's, dL15, and dDMEM after  
211 two days of culture. In addition, we observed that clonogenic capacity of X1(FS)  
212 neoblasts diminished greatly following three days in culture, regardless of the  
213 media used. These results suggest that IPM, KnockOut DMEM, dL15, dKnockOut  
214 DMEM, dSchneider's, and dDMEM are capable of maintaining the proliferation  
215 potential of neoblasts for up to two days in culture in the presence of 5% CO<sub>2</sub>.

## 216 **Cultured neoblasts can rescue stem cell-depleted planarian hosts**

217 To evaluate the functional pluripotency of neoblasts cultured in these six media  
218 (IPM, KnockOut DMEM, dKnockOut DMEM, dL15, dSchneider's, and dDMEM),  
219 rescue was assessed following bulk-cell transplantation. Genotyping PCR and  
220 restriction fragment length polymorphism (RFLP) assays were performed to test  
221 whether sexual hosts had been transformed into the asexual biotype following  
222 transplantation of the asexual neoblasts (Supplementary Fig. 5a)<sup>8</sup>. For non-

223 cultured, freshly collected X1(FS) cells, 30–50% of the lethally irradiated (6,000  
224 rads) sexual *S. mediterranea* hosts were rescued (Fig. 3b, c, and Supplementary  
225 Fig. 5b, e). Next, X1(FS) cells cultured in the indicated media for 1, 2, or 3 days  
226 were transplanted into lethally irradiated hosts using the same method. X1(FS)  
227 cells cultured in IPM, dL15, and KnockOut DMEM for 1 and 2 days were capable  
228 of rescuing hosts devoid of stem cells (Fig. 3c and Supplementary Fig. 5c-e), of  
229 which X1(FS) cells cultured in KnockOut DMEM displayed the highest and most  
230 robust rescue efficiency (Fig. 3c and Supplementary Fig. 5e). Consistent with the  
231 clonogenic assay results, none of the X1(FS) neoblasts cultured for 3 days rescued  
232 lethally irradiated hosts. These data indicate that of all conditions tested, KnockOut  
233 DMEM +5% CO<sub>2</sub> is the best one for maintaining pluripotent neoblasts in culture for  
234 up to 2 days. IPM and dL15 medium were also capable of maintaining pluripotent  
235 neoblasts in culture for up to 2 days albeit with reduced rates of irradiate animal  
236 rescue after transplantation (Fig. 3c and Supplementary Fig. 5e).

237 In summary, we found that after screening 23 media followed by assaying 5  
238 criteria (*i.e.*, viability, *smedwi-1* expression, cell division, clonogenic capacity and  
239 rescue efficiency of irradiated animals), three types of media (KnockOut DMEM,  
240 IPM, and dL15) were capable of maintaining pluripotent neoblasts *in vitro*. Of these  
241 three different media, KnockOut DMEM produced cultured neoblasts with the  
242 strongest performance across the multiple measured criteria, with IPM and dL15  
243 medium performing slightly less well (Fig. 3d).

244 **Electroporation delivers fluorescent dextran into neoblasts**

245        Following the successful optimization of *in vitro* culture conditions for the  
246 maintenance of pluripotent neoblasts, we next attempted to test conditions for the  
247 delivery of exogenous molecules into neoblasts, the next step required for  
248 developing transgenic methods for planarians. We first used dextran-FITC as a  
249 fluorescent indicator to screen suitable electroporation conditions for neoblasts  
250 labeled by Hoechst 33342 staining (Fig. 4a). We tested 52 electroporation  
251 programs and 10 different buffers using X1 cells<sup>25,26</sup>, and found that dextran-FITC  
252 was delivered into neoblasts most efficiently in IPM buffer with electroporation at  
253 100-120V (Supplementary Table 2 and Fig. 4b-d). When similarly applying the  
254 electroporation method to X1(FS) cells, rather than Hoescht 33342 sorted X1 cells,  
255 dextran-FITC+ populations could only be detected with electroporation values of  
256 110V and 120V. However, less than 6% of dextran-FITC+ X1(FS) cells were  
257 *smedwi-1+* neoblasts and virtually no *smedwi-1+* cells could be detected after 1  
258 day culture in KnockOut DMEM +5% CO<sub>2</sub> (Fig. 4e). Consistent with the drastic  
259 reduction in *smedwi-1+* cell viability post-electroporation, none of the donor X1(FS)  
260 cell populations subjected to more than 100V formed colonies following  
261 transplantation into lethally irradiated donors (Fig. 4f). We reasoned that the failure  
262 was likely due to the low purity of *smedwi-1+* neoblasts in X1(FS), which was even  
263 further reduced after electroporation. Therefore, it was necessary to develop a new  
264 strategy for neoblast isolation that would result in both higher clonogenic and  
265 pluripotent *smedwi-1+* cell enrichment than the X1(FS) sorting protocol.

266        We also tested whether X1(FS) can express exogenously delivered mRNA in  
267 current culture conditions. We cloned a planarian endogenous gene, *Smed-*

268 *histone3.3* and fused with two copies of flag tag (2×flag). After electroporation and  
269 one day of culture, cells electroporated with *Smed-histone3.3-2×flag* mRNA had  
270 more anti-FLAG staining positive cells ( $9.7 \pm 1.4\%$ ) than electroporated cells  
271 without mRNA ( $1.2 \pm 0.7\%$ ) (Fig. 4g). Even though the anti-FLAG antibody stained  
272 enucleated cells, nuclear localization signal in nucleated cells suggested  
273 successful expression of *Smed-histone3.3-2×flag* mRNA (Fig. 4g and 4h). Even  
274 though we did not detect the signal of Smed-HISTONE3.3-2×FLAG in *smedwi-1+*  
275 cells (Supplementary Fig. 6), these data encouraged us to further enrich for  
276 neoblasts to optimize cell culture conditions.

### 277 **A new flow cytometry protocol using SiR-DNA and Cell Tracker improves** 278 **yield of clonogenic, pluripotent, transplantable neoblasts**

279 To enrich for neoblasts, we tested three major types of cell-permeable DNA  
280 stains to enrich cycling neoblasts at G2/M cell cycle phases (DRAQ5, Vybrant  
281 DyeCycle, and SiR-DNA). DRAQ5 staining remained cytotoxic similarly to Hoechst  
282 33342. Vybrant DyeCycle staining failed to unambiguously discriminate among  
283 distinct neoblast cell cycle phases by flow cytometry. However, the recently  
284 developed DNA stain, SiR-DNA<sup>29</sup> proved to have low toxicity and enriched  
285 *smedwi-1+* neoblasts to a ratio ~60% (Fig. 5a, b, f and Supplementary Fig. 7).  
286 Comparison of *smedwi-1+* and *smedwi-1-* cell morphology in the isolated  
287 populations showed that *smedwi-1-* cells were generally smaller than *smedwi-1+*  
288 cells (Fig. 5b). To discriminate between small and large cells in the SiR-DNA+  
289 population, the cytoplasmic dyes Cell Tracker Green (CT) and Calcein AM (CAM)  
290 were tested in combination with SiR-DNA in neoblast isolation (Fig. 5c, d). Using

291 a dual dye staining strategy resulted in a significant increase in neoblast  
292 enrichment, as judged by *smedwi-1+* ISH (Fig. 5e, f); SiR-DNA/Cell Tracker Green  
293 costaining performed comparably to Hoechst 33342 staining for enriching *smedwi-*  
294 *1+* neoblasts (Fig. 5e, f). We termed this new sorted cell population SirNeoblasts.  
295 Unlike neoblasts derived from Hoechst 33342 sorts, SirNeoblasts proliferated *in*  
296 *vitro* and underwent colony expansion *in vivo* after transplantation into lethally  
297 irradiated planarians (Fig. 5g). Facilitated by SiR-DNA staining of DNA, the  
298 separation dynamics of chromosomes in dividing SirNeoblasts were observed *in*  
299 *vitro* (Supplementary Movies 3-5), confirming the occurrence of *bona fide* cell  
300 division in the tested culture condition. Importantly, no noticeable difference in  
301 colony sizes was observed at 7 dpt between X1(FS), single (SiR-DNA), and double  
302 dye (SiR-DNA/CT) stained populations (Fig. 5g). Finally, both freshly isolated  
303 SirNeoblasts and those cultured in KnockOut DMEM +5% CO<sub>2</sub> for one day were  
304 capable of rescuing lethally irradiated planarians at frequencies comparable to  
305 those seen with X1(FS) cells (Fig. 3c and Fig. 5h). Based on these results, we  
306 conclude that SiR-DNA/CT dual labeling-based cell sorting can be used to isolate  
307 clonogenic, pluripotent neoblasts that can be maintained in primary culture and  
308 serve as donor cells in transplantation assays. To further characterize the  
309 SirNeoblasts, we stained SirNeoblasts with Hoechst 33342 to analyze their cell  
310 cycle. However, co-staining of SiR-DNA and Hoeschst 33342 resulted in a failure  
311 to detect SiR-DNA staining. We then tested whether Hoeschst 33342 can stain  
312 SiR-DNA stained cells, and found that Hoechst 33342 can replace the staining of  
313 SiR-DNA, which showed the cell cycle distribution of SirNeoblasts consisted of

314 ~17.89% at G1, 13.02% at S, and ~69.09% at G2/M cell cycle phases  
315 (Supplementary Fig. 7). This reversible staining of SiR-DNA may also explain the  
316 reason why SirNeoblasts can proliferate after staining unlike Hoechst 33342  
317 stained X1 cells.

318 Next, we determined conditions to optimize electroporation efficiency and  
319 viability for SirNeoblasts (Fig. 6a). Consistent with previous studies,  
320 electroporation at 110V-120V was required for dextran-TMR entry into SirNeoblasts  
321 (Fig. 6b, c). As expected, *smedwi-1+* cells were more abundant in the 110 V and  
322 120V electroporated SirNeoblasts compared to X1(FS) cells, and some  
323 electroporated SirNeoblasts persisted for one day in culture (Fig. 6d). Importantly,  
324 110V – 120V electroporated SirNeoblasts were capable of forming colonies and  
325 rescuing lethally irradiated hosts upon transplantation (Fig. 6e, f). However, 120V  
326 electroporations resulted in comparably fewer irradiated hosts being rescued after  
327 SirNeoblast transplantations (Fig. 6e, f), indicating that high voltages may have a  
328 negative impact on pluripotency.

### 329 **Exogenous mRNA delivered by electroporation can be successfully** 330 **expressed in SirNeoblasts**

331 To assess whether exogenous mRNA could be delivered into SirNeoblasts  
332 using the described electroporation conditions, *tdTomato* mRNA was added to the  
333 electroporation reaction along with Dextran. Dextran positive SirNeoblasts were  
334 sorted and cultured in KnockOut DMEM + 5% CO<sub>2</sub>. To determine whether mRNA  
335 was successfully delivered, we probed cells via FISH 20 hours after  
336 electroporation. *tdTomato* mRNA signal was detected in both 110V and 120V

337 electroporated cells, suggesting a successful delivery of exogenous mRNA into  
338 SirNeoblasts (Fig. 6g, h). However, costaining with *smedwi-1+* revealed that not  
339 all *tdTomato* mRNA<sup>+</sup> cells retained neoblast identity in culture. The number of  
340 sorted SirNeoblasts responded similarly to X1 and X1(FS) cells with respect to  
341 electroporation in that the number of cells positive for both *tdTomato* mRNA and  
342 *smedwi-1* expression was significantly higher after 110V electroporation than after  
343 120V (Fig. 6h). This result indicates that under the conditions tested, 110V  
344 electroporation may be the most suitable to both introduce exogenous, charged  
345 molecules such as RNA into neoblasts, while maintaining their viability and  
346 potency.

347 Unfortunately, expression of *tdTomato* was not detected by either microscopy  
348 or antibody staining. Two possibilities were suspected: 1) The culture condition is  
349 not good enough to support the translation of the delivered mRNA; 2) There is an  
350 unknown mechanism that prevents the translation of the delivered mRNA. A recent  
351 discovery in *C. elegans* indicated that endogenous piRNAs can target on the  
352 exogenous transgene sequences and prevent their translation<sup>30</sup>. Similarly,  
353 planarian neoblasts contain abundant PIWI and piRNAs. We thus hypothesize that  
354 a similar piRNA targeting mechanism may exist in planarian neoblasts, which may  
355 prevent the translation of the delivered mRNAs. We tested this hypothesis by  
356 synthesizing multiple mRNAs encoding the fluorescent protein mCherry in which  
357 conservative nucleotide substitutions were introduced in order to minimize  
358 potential pairing of the exogenous mRNA with endogenous piRNAs, as was  
359 recently described in *C. elegans*<sup>30</sup>. The synthetic mCherry mRNAs were tested



360 via electroporation into SirNeoblasts (Fig. 7a). Significantly, we found one mCherry  
361 mRNA construct that resulted in robust mCherry+ cultured SirNeoblasts (Fig. 7b-  
362 e, twice with high expression, five times with medium/low expression, ten times  
363 without expression). Even though we have yet to fully comprehend the  
364 mechanisms that may be underpinning piRNA targeting in neoblasts, the  
365 successful expression results indicated that the culture and electroporation  
366 conditions defined in our study are capable of maintaining neoblasts in culture  
367 capable of retaining both pluripotency (Figures 5g, h and 6e, f) and translational  
368 activity (Figure 7b). Although the efficiency by electroporation is low for mRNA  
369 delivery, our current study is focused on developing a reliable method for culturing  
370 neoblasts. Increasing the efficiency of delivery for mRNA and testing Cas9 and  
371 guide RNAs is clearly necessary and will require further studies.

372 In summary, we defined a novel FACS isolation strategy and primary cell  
373 culture conditions capable of maintaining clonogenic, pluripotent neoblasts *in vitro*  
374 that are compatible with transplantation, repopulation and rescue of lethally  
375 irradiated hosts. In addition, we optimized electroporation conditions that  
376 successfully introduced fluorescent dextran and exogenous mRNA into  
377 clonogenic, pluripotent neoblasts. These technical milestones are prerequisites for  
378 the successful generation of transgenic planarians.

## 379 **Discussion**

380 Past efforts to culture planarian cells have been unable to convincingly  
381 demonstrate that pluripotent neoblasts could be maintained in culture <sup>23,24,31,32</sup>.  
382 Here, we provide definitive molecular and functional evidence that pluripotent

383 neoblasts can be maintained *in vitro*. This technical advance facilitated the first  
384 real-time observation of neoblast cell division within the first 24 hours after cell  
385 culture *in vitro* (Supplementary Movies 1 and 2) and the first demonstration that  
386 exogenous molecules, including fluorescent conjugated dextrans and mRNA, can  
387 be delivered into planarian cells. This method establishes the required foundation  
388 for future transgenic and genome editing technique development in planarians,  
389 and opens exciting new avenues for a systematic investigation of the biology of a  
390 naturally occurring population of pluripotent adult stem cells.

### 391 **The vital fluorescent dye SiR-DNA improves purification of pluripotent** 392 **neoblasts**

393 Prior to this study, The use of Hoechst 33342 staining has been broadly  
394 adopted for isolating cycling neoblasts (X1 cells) by FACS. However, X1 cells  
395 labeled with this nuclear dye cannot proliferate *in vivo* following their  
396 transplantation into irradiated hosts. We sought to overcome this limitation by  
397 testing alternative DNA dyes, such as DRAQ5 and Vybrant Dye Cycle, yet they  
398 resulted in cytotoxicity and failed to unambiguously discriminate between distinct  
399 neoblast cell cycle phases by flow cytometry. However, we found that unlike other  
400 vital DNA dyes tested, the recently developed SiR-DNA dye <sup>29</sup> was not cytotoxic,  
401 and when combined with Cell Tracker Green staining, significantly improved  
402 pluripotent neoblast yields by flow cytometry. Together with cell subtype-specific  
403 antibodies, SiR-DNA may allow for more specific dissection of the pluripotent  
404 neoblast population by facilitating the isolation and functional characterization of

405 different neoblast subpopulations. Furthermore, this reagent may prove useful for  
406 the isolation of viable proliferating cells in other organisms.

#### 407 **Neoblast cell culture paves the way for transgenesis in planarians**

408 Transgenesis in planarians has been lacking for decades. Without a planarian-  
409 specific positive control, it has been difficult to determine why exogenous nucleic  
410 acids fail to be translated when introduced into neoblasts. Isolated neoblasts  
411 provide an obvious proving ground for delivery of exogenous materials. While  
412 neoblast transplantation can be performed immediately after delivering exogenous  
413 molecules, the uncertain viability of neoblasts during and after transplantation  
414 made this strategy ineffective. The cell culture system we have developed makes  
415 it possible to trace and study each cell following delivery of exogenous materials  
416 *in vitro*. First, it allows for ease of screening of constructs using a small number of  
417 cells under conditions where neoblast purity and viability are well-established.  
418 Second, when introducing transformed cells into lethally irradiated hosts to monitor  
419 behavior *in vivo*, we can enrich for positive cells via FACS prior to transplantation,  
420 minimizing the effects of cell-cell competition in a heterogeneous donor cell  
421 population. Hence, neoblasts cultured using the methods described here lend  
422 themselves accessible for testing a diversity of delivery methods. For instance,  
423 custom-engineered liposomes were shown to facilitate the transfection of double-  
424 stranded RNA and anti-miRNAs into planarian cells *in vivo*<sup>33</sup>. As such, it should  
425 be possible to use liposomes to deliver larger molecules and genome-editing tools  
426 in an effort to obtain higher neoblast transfection efficiency and further improve the  
427 likelihood of producing transgenic animals. Thus, our methodology not only stands

428 to facilitate cell transformation, but may also play a key role in efforts to establish  
429 long-term culture systems and/or cell lines.

430 **piRNA silencing mechanism for transgenes may be of broad occurrence**  
431 **across metazoans**

432 Efforts to generate transgenic planarians span several decades with little to no  
433 success reported thus far. The reasons for this state of affairs have been generally  
434 associated with technical limitations of both culture conditions and delivery  
435 methods of exogenous nucleic acids into neoblasts. Little consideration, however,  
436 has been given to the possibility that such prolonged failure may be underscored  
437 by unknown aspects of neoblast biology. Given that neoblasts are the *de facto*  
438 units of selection in planarians and that the viability of these animals heavily  
439 depends on the proper function and health of neoblasts, strong positive selection  
440 for evolving robust mechanisms to protect the genome of these cells should be  
441 expected.

442 piRNAs are small non-coding RNAs that have been shown to be essential for  
443 safeguarding genome integrity by silencing transposable elements<sup>34</sup>. However, it  
444 is also known that many piRNAs do not map to transposable element sequences  
445 in various animals, including mice, *C. elegans* and planarians<sup>35-37</sup>. In fact, the  
446 function of these piRNAs remains largely unknown. Additionally, it has also been  
447 known for decades that transgenes with foreign sequences can be frequently  
448 silenced in the germline of *C. elegans*<sup>38</sup>. Recent studies have begun to shed light  
449 on piRNA function in both *Drosophila*<sup>39</sup> and *C. elegans*<sup>40</sup>. It was recently reported  
450 that the repression of transgenes in the germline of fruitflies could be lifted by using

451 a UAS-promoter free of interference by Hsp70 piRNAs as silenced<sup>39</sup>. Also, it is known  
452 that the PIWI protein PRG-1 is required for the silencing phenomenon observed in  
453 the germline of *C. elegans*, suggesting a function of piRNAs in this process<sup>40</sup>.  
454 More recently, it was discovered that a mechanism targeting transgene sequences  
455 introduced into the syncytial ovary of *C. elegans* involves piRNAs, and that a  
456 sequence-based strategy to bypass transgene silencing by these small non-coding  
457 RNAs allowed expression of exogenously added genes in the germline of this  
458 animal<sup>30</sup>.

459 Given the ancestral origin of PIWI proteins and piRNAs, we hypothesized that  
460 similar mechanisms may be operating in planarian neoblasts. Our current study  
461 showed that exogenous mRNAs in which predicted piRNA targeting sequences  
462 were changed overcame silencing and allowed the translation of the reporter  
463 protein (Figure 7b). However, we do not yet fully understand the piRNA recognition  
464 rules in *S. mediterranea*. The size of planarian piRNAs are ~32nt, in contrast to  
465 ~22 nt in *C. elegans*<sup>37,41</sup>, so the models predicting targeting of piRNA in  
466 nematodes do not fully transpose to planarians. Additionally, planarians have at  
467 least three PIWI proteins<sup>25,37</sup>, raising another question as to which PIWI proteins  
468 may or may not be required for producing piRNAs that may potentially target  
469 exogenous nucleic acids. Definitive experiments to test this hypothesis are  
470 necessary and future and ongoing research will help in resolving these issues and  
471 testing and refining our predictive piRNA targeting models in the hopes of  
472 producing the most stable exogenous nucleic acid molecules for introduction into  
473 neoblasts.

474 **A method for mechanistic studies of neoblast proliferation and**  
475 **differentiation *in vitro***

476 The paucity of cell culture conditions for invertebrates in general, and  
477 planarians in particular, has hampered our ability to test and identify factors directly  
478 regulating the functions of neoblasts, a remarkably abundant and pluripotent adult  
479 stem cell population. For example, our understanding of how extracellular growth  
480 factors modulate neoblast proliferation is still in its infancy. In planarians, several  
481 of these factors have been shown to have important functions in neoblast  
482 proliferation or homeostasis. For instance, knockdown of *smed-neuregulin (nrg)-7*  
483 or *smed-insulin-like peptide-1* impairs neoblast proliferation *in vivo*<sup>13,42</sup>. We  
484 hypothesize that addition of these factors, or potentially other purified extracellular  
485 growth factors, may boost neoblast proliferation *in vitro*. However, no *in vitro*  
486 culture system had been developed to test this hypothesis. With the methods and  
487 results presented here open the door to test the effects of planarian extracellular  
488 extracts or purified extracellular growth factors from planarian species on the  
489 proliferation and maintenance of neoblasts. Additionally, our protocols lend  
490 themselves to initiate a systematic comparison of the metabolomics of cultured  
491 neoblasts with those found *in vivo*. Such studies will aid in further optimization of  
492 culture conditions and may ultimately lead to the controlled manipulation of cell  
493 metabolism to predictably regulate neoblast proliferation and differentiation *in vitro*.

494 **Defining the neoblast niche in planarians**

495 The existence of a niche that supports the proliferation and differentiation of  
496 neoblasts has been previously proposed<sup>43</sup>. This hypothesis has been supported

497 by indirect evidence<sup>13,44,45</sup>. However, the molecular and cellular nature of the niche  
498 is largely unknown. Transplant experiments carried out in this study showed that  
499 a limited number of neoblasts can be maintained in the transplanted location and  
500 may continue their proliferation and differentiation. Because of the limited number  
501 of cells surviving after transplantation, dissecting the cellular microenvironment of  
502 transplanted neoblasts is likely to be a promising context for a mechanistic  
503 characterization of the proposed neoblast niche. Together with sublethal irradiation  
504 assays, the cell culture tools reported here should afford us the opportunity to  
505 understand how pluripotency and cell fate may be cell- and non-cell autonomously  
506 regulated in a highly regenerative context.

#### 507 **A framework for establishing cell culture in new research organisms**

508 Since the development of cell lines in the 1950s<sup>46</sup>, cell culture has enabled  
509 scientists to study fundamental aspects of cell biology. In recent years, the number  
510 of research organisms being employed to address and discover new biology has  
511 steadily increased. However, a comparatively small number of cell types have  
512 been successfully cultured *in vitro*, particularly for invertebrates. The current study  
513 systematically screened the majority of published cell culture media and optimized  
514 culture conditions for planarian neoblasts. Thus, the systematic development of  
515 cell culture methods reported here not only advances the study of cell biology in  
516 the highly regenerative planarian *S. mediterranea*, but should also facilitate the  
517 establishment of culture methods for other species, particularly underrepresented  
518 invertebrate research organisms.

519

## 520 **Experimental Procedures**

### 521 **Planarian care and irradiation treatment**

522 Asexual (Clone CIW4) and sexual (Clone S2F1L3F2) strains of *Schmidtea*  
523 *mediterranea* were maintained in Montjuïc water at 20°C as previously described  
524 <sup>8,20</sup>. Animals were starved for 7–14 days before each experiment. Animals exposed  
525 to 6,000 rads of  $\gamma$  rays were used as transplant hosts <sup>8</sup>. After transplantation, hosts  
526 were maintained in Montjuïc water with 50  $\mu$ g/ml Gentamicin (GEMINI, 400-100P).  
527 For transplant rescue experiments, host animals were maintained in 3.5 cm Petri  
528 dishes (1 worm/dish), and Montjuïc water was changed every 2–3 days.

### 529 **Cell collection and culture**

530 X1(FS) cells were collected as previously described with minor modifications  
531 <sup>8,25</sup>. Tails from planarians (>8 mm in length) were macerated in Calcium  
532 Magnesium free buffer with 1% Bovine Serum Albumin (CMFB) (see Recipe in  
533 Table S1) for 20–30 min with vigorous pipetting every 3–5 min. After maceration,  
534 dissociated cells were centrifuged at 290g for 10min. Cells were then resuspended  
535 in IPM with 10% Fetal Bovine Serum for either Hoechst 33342 or SiR-DNA  
536 staining. To gate the X1(FS) cells, the X1 population from a control sample stained  
537 with 0.4 mg/ml Hoechst 33342 (ThermoFisher Technologies, H3570) was used to  
538 define the forward scatter/side scatter gate. To obtain SirNeoblasts, dissociated  
539 cells were stained with SiR-DNA (1 $\mu$ M, Cytoskeleton Inc., CY-SC007) and  
540 CellTracker Green CMFDA Dye (2.5 $\mu$ g/ml, Thermo Fisher Technologies, C7025)  
541 for 1 hour and 10 min sequentially. Cells were sorted with an Influx sorter using a  
542 100- $\mu$ m tip. For time-lapse imaging experiments, X1(FS) cells were incubated in



543 either 5 mL of the indicated culture medium per well in 6-cm dishes (MatTek,  
544 P35G-1.5-14-C) or in 1 mL of the indicated culture medium per well in a 24-well  
545 plate (MatTek, P24G-1.5-13-F). For other experiments, X1(FS) were incubated in  
546 50  $\mu$ L of the indicated culture medium per well in 384-well plates (Greiner bio-one,  
547 781090). Cells were cultured in indicated media containing 5% Fetal Bovine Serum  
548 (Sigma-Aldrich, F4135) at 22°C, +/- 5% CO<sub>2</sub>. Dishes and multi-well plates were  
549 pre-coated with poly-D-lysine (50 $\mu$ g/ml, BD Biosciences).

### 550 ***In situ* hybridization and antibody staining**

551 Whole-mount *in situ* hybridization was carried out as previously described<sup>13,47-</sup>  
552 <sup>49</sup>. For ISH on cultured cells, cell culture plates were centrifuged in an Eppendorf  
553 horizontal centrifuge (Centrifuge 5810 R) at 300 *g* x 3 min. Cells were fixed with  
554 3.7% formaldehyde (Sigma-Aldrich, F8775) or 4% paraformaldehyde (Electron  
555 Microscopy Sciences, 15710) for 20 min. After washing with 1 $\times$  PBS, cells were  
556 hybridized with riboprobes at 56°C for at least 15 h. After washing with 2 $\times$  SSC  
557 and 0.2 $\times$  SSC, cells were incubated with anti-digoxigenin-POD (Roche  
558 Diagnostics, 11207733910) or anti-fluorescein-POD (Roche Diagnostics,  
559 11426346910) at room temperature for 2 h. After washing with 1 $\times$  PBS/0.3%  
560 TritonX-100, the signal was developed using tyramide-conjugated Cy3 (Sigma-  
561 Aldrich, PA13101) or Cy5 (Sigma-Aldrich, PA15101).

562 Anti-phospho-Histone H3 (Ser10) (H3P) antibody (1:1,000, Abcam, ab32107)  
563 and Alexa 555-conjugated goat anti-rabbit secondary antibodies (1:1,000, Abcam,  
564 ab150086) were used to stain proliferating cells at the G2/M phase of the cell cycle.

### 565 **Annexin V staining**

566 Fifty microliters of cultured cells were re-suspended and stained with 2.5  $\mu$ l of  
567 Annexin V FITC Conjugate (BioLegend, 640905) at room temperature for 15 min.  
568 After washing twice with IPM + 10%FBS, cells were subjected to *smedwi-1* ISH.  
569 Thereafter, anti-fluorescein-POD (Roche Diagnostics, 11426346910) was used to  
570 stain Annexin V for apoptotic and dead cells detection.

### 571 **Cell transplantation**

572 X1(FS) cells collected by flow cytometry were transplanted into irradiated hosts  
573 (6,000 rads) as previously described with minor modifications<sup>8</sup>. Approximately 1  
574  $\mu$ L of an X1(FS) cell suspension (5,000 cells/ $\mu$ L) was injected into either the post-  
575 pharyngeal midline (of asexual CIW4 hosts) or the post-gonopore midline (of  
576 sexual S2F1L3F2 hosts) at 0.75–1.0 psi (Eppendorf FemtoJet) using a borasilicate  
577 glass microcapillary (Sutter Instrument Co., B100-75-15).

### 578 **mRNA synthesis and electroporation**

579 mRNAs used for electroporation were prepared following the standard  
580 protocols in the mMESSAGE mMACHINE T7 ultra Transcription Kit (ThermoFisher  
581 Technologies, AM1345) and the Ambion RNA Purification Kit (ThermoFisher  
582 Technologies, AM1908). tdTomato mRNA was transcribed from the linearized  
583 plasmid pcDNA3.1(+)-tdTomato. mCherry and T7 promoter sequences were  
584 cloned into the pIDT vector and synthesized by IDT Inc. Primers used to amplify  
585 the template were 5'-CAGATTAATACGACTCACTATAGG-3' and 5'-  
586 ACTGATAATTAACCCTCACTAAAG-3'.

587 To screen electroporation conditions, cells from four tail fragments were  
588 suspended in 20  $\mu$ L electroporation buffers following Hoechst 33342 staining. 20

589  $\mu\text{g}$  Dextran-FITC (ThermoFisher Technologies, D3306) were mixed with cells and  
590 loaded into a 1mm electroporation cuvette for BTX ECM830 electroporator or a  
591 12-well electroporation strip for Lonza 4D electroporator. The buffer SE, SG, SF,  
592 P1-5 were electroporation buffers in Lonza Cell Line and Primary Cell 4D-  
593 Nucleofector Optimization kits (V4XC-9064 and V4XP-9096). Cell viability and  
594 electroporation efficiency were assessed using an Influx sorter.

595 For exogenous mRNA electroporation,  $\sim 1 \times 10^8$  cells were suspended in 50  $\mu\text{L}$   
596 IPM following SiR-DNA staining. 50  $\mu\text{g}$  Dextran-FITC and  $\sim 5$   $\mu\text{g}$  mRNA were  
597 mixed with cells and loaded into a 1mm electroporation cuvette. BTX ECM830  
598 electroporator was used to apply a 110 V and 1 millisecond square wave pulse to  
599 deliver dextran-FITC and mRNA into planarian cells. Dextran-FITC+ SirNeoblasts  
600 were purified using an Influx sorter and cultured in KnockOut DMEM + 5%FBS.

### 601 **Microscopy and time-lapse imaging**

602 The Celigo imaging cell cytometer (Celigo, Inc.) and the Falcon 700 confocal  
603 microscope were used to take pictures of X1(FS) and SirNeoblasts following ISH.  
604 Celigo or ImageJ software was used for quantitative analyses. Time-lapse imaging  
605 of cultured cells was performed using a Nikon Eclipse TE2000-E equipped with  
606 Perfect Focus and a Plan Fluor ELWD 20X/0.45 NA Ph1 objective. Micro-manager  
607 was used to control the microscope and Hamamatsu Orca R2 CCD<sup>50</sup>. Multiple  
608 positions were acquired at 5-min intervals for 24–48 h. *In situ* hybridization  
609 samples were imaged with a Nikon Eclipse Ti equipped with a Yokogawa W1  
610 spinning disk head and a Prior PLW20 Well Plate loader. Several slides were  
611 prepared at once and then loaded and processed automatically using a

612 combination of Nikon Elements Jobs for all robot and microscope control and Fiji  
613 for object-finding and segmentation. Slides were imaged at low magnification and  
614 objects identified before re-imaging tiled z-stacks using a Plan Apo 10X 0.5NA air  
615 objective. Tiled images were stitched, projected, and *smedwi-1+* puncta were  
616 counted using custom macros and plugins in Fiji.

### 617 **Generation of optimized mCherry sequence**

618 mCherry candidate sequences were generated by means of a custom python  
619 script. Amino acid sequences were back translated to 21 nucleotide sequences  
620 from 7 amino acid words at a time. Each potential nucleotide sequence was  
621 screened against a list of known piRNAs to generate the sequence with the fewest  
622 piRNA matches. A piRNA match consists of no more than a single G/T mismatch  
623 in the 6 nucleotide seed region (positions 2-7 of a piRNA) <sup>30</sup>. Additional G/T  
624 mismatches were scored as .5 and other mismatches as 1. Only the first 21  
625 basepairs of the piRNAs were aligned. The highest scoring piRNA determined the  
626 score for that potential nucleotide sequence. The 21 nucleotide sequence with the  
627 lowest score was retained. The script was run with four alternate coding  
628 tables. The “all” coding table contained all possible codons for each amino acid.  
629 The “smed” coding table contained only those codons known to be most used in  
630 *S. mediterraea* <sup>51</sup>. “lowgc” contained only those codons with the fewest G or C  
631 nucleotides. “highgc” contained only those codons with the most G or C  
632 nucleotides. The “highgc” sequence is shown in Figure 7. The other three  
633 sequences as well as 5 additional sequences generated by shuffling the four

634 generated sequences and one sequence generated by backtranslating the amino  
635 acid sequence with sms failed to show fluorescence<sup>52</sup>.

### 636 **Data availability**

637 All codes used for plugins in Fiji are available at:<https://github.com/jouyun>. All  
638 original data underlying this manuscript can be accessed from the Stowers Original  
639 Data Repository at: <http://www.stowers.org/research/publications/libpb-1281>. All  
640 reagents are available from the corresponding author upon reasonable request.

### 641 **Statistical analyses**

642 Microsoft Excel and Prism 6 were used for statistical analysis. Mean  $\pm$  s.e.m.  
643 is shown in all graphs. Unpaired two-tailed Student's *t*-test was used to determine  
644 the significant differences between two conditions.  $p < 0.05$  was considered a  
645 significant difference.

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656 **Author contributions**

657 K.L. and A.S.A. conceived the project, designed experiments, analyzed data,  
658 and wrote the manuscript. K.L. performed all experiments and data acquisition.  
659 S.A.M. performed the time-lapse imaging experiments and analyzed raw spinning-  
660 disk imaging data. E.J.R. and H.-C.L. designed the variant sequences for mCherry.

661 **Competing interests**

662 The authors declare no conflicts of interest.

663

1 **Figure Legends**

2  
3 **Figure 1. Systematic screen identifies cell culture conditions for maintaining**

4 **X1(FS) neoblasts *in vitro*.** (a) Flowchart illustrating steps of X1(FS) cell culture and  
5 criteria used to identify best culture condition for neoblasts: cell viability, percentage of  
6 *smcdwi-1+* neoblasts (%*smcdwi-1+*), cell division *in vitro*, colony expansion after  
7 transplantation, and rescue efficiency of irradiated hosts after transplantation  
8 (pluripotency). (b) Plots showing the FACS gating to sort X1(FS) cells. (c) Representative  
9 images showing *smcdwi-1+* neoblasts among the sorted X1(FS) cells. Scale bar, 20  $\mu$ m.  
10 X1(FS) cells consistently contains 23.4% $\pm$ 2.5% neoblasts in total DAPI+ cells scored.  
11 Three replicates were assayed, n=100 to 150. (d) Representative images of cell  
12 morphologies observed after 1 day of culture +5% CO<sub>2</sub>, including poor cell morphology in  
13 CMFB and healthy cell morphology in IPM (arrowheads). Scale bar, 20  $\mu$ m. (e)  
14 Percentages of live cells (Propidium Iodide-negative) among 23 media, +/- 5% CO<sub>2</sub>, after  
15 1 day of culture. Knockout DMEM + 5% CO<sub>2</sub> yielded best overall cell viability. Three  
16 replicates were assayed, n=500 to 1200. (f) Percentage of *smcdwi-1+* neoblasts after 1  
17 day of culture under indicated conditions. Significantly more *smcdwi-1+* neoblasts were  
18 maintained in seven media + 5% CO<sub>2</sub> compared to all other conditions. Three replicates  
19 were assayed, n>500. (g) Percentage of *smcdwi-1+* neoblasts after 3 days of culture in  
20 indicated media + 5% CO<sub>2</sub>. (h) Representative images of dividing cells undergoing either  
21 symmetric cell division (SCD) or asymmetric cell division (ASCD). Scale bar, 10  $\mu$ m. (i)  
22 Time-lapse images of dividing cells undergoing either SCD or ASCD in IPM + 5% CO<sub>2</sub>.  
23 Scale bar, 10  $\mu$ m. Both SCD and ASCD can be observed in ~300 X1(FS) cells cultured  
24 in IPM, KnockOut DMEM, and dL15 + 5% CO<sub>2</sub>.

1  
2 **Figure 2. Cultured X1(FS) neoblasts expand after transplantation.** (a) Flowchart  
3 showing steps of X1(FS) cell transplantation following *in vitro* culture. (b) Representative  
4 images showing colonies of *smedwi-1+* neoblasts at 8 days post-transplantation (dpt)  
5 cultured in the indicated conditions + 5% CO<sub>2</sub>. Only X1(FS) cells cultured in dGrace's  
6 medium + 5% CO<sub>2</sub> did not efficiently form colonies *in vivo*. Scale bar, 200 μm. (c)  
7 Percentage of hosts receiving X1(FS) cells cultured in indicated media + 5% CO<sub>2</sub> for 1, 2,  
8 or 3 days that possessed *smedwi-1+* colonies (green bars) or H3P+ colonies (red bars)  
9 at 8 dpt. (d) Number of *smedwi-1+* neoblasts in colonies formed by X1(FS) cells at 8 dpt  
10 following culturing in indicated media + 5% CO<sub>2</sub> for 1, 2, or 3 days. Ten to twelve animals  
11 assayed per condition.

12  
13 **Figure 3. Cultured X1(FS) cells rescue neoblast-depleted planarians.** (a) Flowchart  
14 illustrates steps of rescue assay using cultured X1(FS) cells. (b) Representative images  
15 showing rescue of lethally irradiated hosts following transplantation of freshly isolated  
16 X1(FS) cells, culminating in fission at 95 dpt. Scale bar, 200 μm. (c) Rescue rates for  
17 lethally irradiated hosts following the transplantation of X1(FS) cells cultured in the  
18 indicated media + 5% CO<sub>2</sub> for 1, 2, or 3 days. Histogram indicates averaged percent from  
19 replicate experiments. Ten to twelve animals assayed per condition in each replicate  
20 experiment. (d) Summary of 23 cell culture media screen using the following criteria: cell  
21 morphology, cell viability, %*smedwi-1+* neoblasts, ability of transplanted cells to form  
22 colonies and expand *in vivo* (clonogenesis), and ability to rescue lethally irradiated



1 animals (pluripotency). Overall, KnockOut DMEM was the most effective medium for  
2 maintaining pluripotent neoblasts in culture for 2 days.

3

4 **Figure 4. Electroporation can deliver Dextran-FITC into neoblasts. (a)** Flowchart  
5 describing electroporation assay steps to screen for best conditions for cell viability and  
6 Dextran-FITC delivery efficiency. **(b)** Plots of X1 viability (upper) and electroporation  
7 efficiency (lower) by using IPM as the electroporation buffer to deliver Dextran-FITC at  
8 120V compared to 0 V controls. **(c)** Representative images of sorted Dextran-FITC<sup>low</sup> and  
9 Dextran\_FITC<sup>high</sup> cells indicate successful delivery of Dextran-FITC at 120V. **(d)** Viability  
10 (blue) and electroporation efficiency (red) on X1 cells after electroporation using IPM as  
11 electroporation buffer. **(e)** %*smedwi-1+* neoblasts in X1(FS) cells after 100V, 110V, and  
12 120V electroporation immediately (black column) and after 1 day of culture in KnockOut  
13 DMEM + 5% CO<sub>2</sub> (white column). Four random fields assayed per condition. p<0.05 for  
14 120 V. N>40. **(f)** Electroporated X1(FS) cells receiving greater than 100 V failed to form  
15 colonies following transplantation. Ten animals assayed per condition. **(g)** Representative  
16 images of electroporated X1(FS) with (upper panel) or without (lower panel) *Smed-*  
17 *histone3.3-2xflag* mRNA. Arrowheads: anti-FLAG+ nucleated cells. Stars: anti-FLAG+  
18 enucleated cells. Scale bar, 20 μm. **(h)** Z-stack images of an nucleated anti-FLAG+ cell.  
19 Scale bar, 10 μm.

20

21 **Figure 5. SiR-DNA plus Cell Tracker staining and cell sorting protocol enriches for**  
22 **clonogenic, pluripotent *smedwi-1+* neoblasts. (a)** Plots showing the gate used to  
23 isolate SiR-DNA+ cells for *smedwi-1* ISH. **(b)** *smedwi-1* ISH on isolated cells from the

1 SiR-DNA+ gate shown in Supplementary Fig. 7a. *smedwi-1*- cells (arrows) were generally  
2 smaller than *smedwi-1*+ cells (stars). Scale bar, 20  $\mu$ m. **(c-d)** Plots showing the gates  
3 used to isolate SiR-DNA+, calcein-AM+ cells **(c)** and SiR-DNA+, Cell Tracker Green+  
4 cells **(d)** for *smedwi-1* ISH. **(e)** *smedwi-1* ISH for SIR-DNA+ neoblasts populations  
5 indicated in **(c)**. Scale bar, 20  $\mu$ m. **(f)** %*smedwi-1*+ neoblasts in indicated FACS isolated  
6 populations. SiR-DNA and Cell Tracker Green dual staining enriches for *smedwi-1*+  
7 neoblasts (SirNeoblasts) comparably to the Hoechst 33342 stained X1 population. \*,  
8  $p < 0.05$ . \*\*,  $p < 0.01$ . n.s., no significance. Four random fields assayed per condition.  $N > 70$ .  
9 **(g)** Representative images showing the clonogenic capacity of transplanted neoblasts  
10 obtained using different FACS isolation protocols. No noticeable difference in the colony  
11 expansion was observed among single and double dye staining populations at 7dpt.  
12 Scale bar, 200  $\mu$ m. Ten animals assayed per condition. **(h)** Rescue efficiency of fresh and  
13 1-day cultured SirNeoblasts. CT: cell tracker green.

14  
15 **Figure 6. SirNeoblasts can be used for exogenous mRNA electroporation. (a)**  
16 Flowchart presenting the steps of neoblast electroporation using SirNeoblasts. **(b)** Plots  
17 showing electroporation efficiency of SirNeoblasts at 100V, 110V and 120V compared to  
18 0V. **(c)** Neoblasts after electroporation of Dextran-FITC showing 100% isolation of  
19 positive cells after electroporation at 110V and 120V. All SirNeoblasts were free of  
20 Dextran-FITC without electroporation treatment. Scale bar, 20  $\mu$ m. **(d)** Percentage of  
21 *smedwi-1*+ cells after electroporation, suggesting a relative high ratio of neoblasts after  
22 electroporation by using SirNeoblasts compared to X1(FS) in **Fig. 4e**. Four random fields  
23 assayed per condition. \*,  $p < 0.05$  (120V SirNeoblasts vs. 120V X1(FS) at 1 day) . \*\*,

1 p<0.005 (110V SirNeoblasts vs. 120V SirNeoblasts at 1 day, 110V SirNeoblasts vs. 110V  
2 X1(FS) at 0 day, and 120V SirNeoblasts vs. 120V X1(FS) at 0 day). \*\*\*, p<0.001 (110V  
3 SirNeoblasts vs. 110V X1(FS) at 1 day). **(e)** Representative images showing the colony  
4 expansion of electroporated SirNeoblasts after transplanation Scale bar, 200  $\mu$ m. N=14  
5 for 110V and =10 for 120 V. **(f)** Rescue efficiency of electroporated SirNeoblasts. Scale  
6 bar, 200  $\mu$ m. **(g)** Representative images showing the mRNA signals (white dots) in cells  
7 1 day after 110V and 120V electroporation. Scale bar, 20  $\mu$ m. **(h)** Percentage of total cells  
8 and *smedwi-1+* cells containing mRNA 1 day after 110V and 120V electroporation. n.s.:  
9 not significant. \*\* < 0.01.s

10

11 **Figure 7. mCherry mRNA is expressed in SirNeoblasts.** **(a)** A flowchart describes  
12 steps of SirNeoblast electroporation using mCherry mRNA. **(b)** Representative images of  
13 mCherry mRNA electroporated SirNeoblasts cultured in KnockOut DMEM for 1 day.  
14 Upper: electroporated SirNeoblast without mRNA in culture. Lower: mCherry mRNA  
15 electroporated SirNeoblasts in culture. Scale bar, 20  $\mu$ m. **(c)** Plot showing no mCherry  
16 expression after 110V electroporation without mCherry mRNA. **(d)** Plot showing ~5%  
17 mCherry+ cells after 110V electroporation with mCherry mRNA. **(e)** Representative  
18 images of cells from mCherry- population in (upper row) and mCherry+ population in  
19 (lower row). Cells from mCherry+ population showed obvious mCherry localization in  
20 cytoplasm. Scale bar, 20  $\mu$ m.

21

1 **Supplementary Figure 1. X1(FS) cells cultured in L15 extend long cellular**  
2 **processes. (a–d)** Four representative images showing long cellular processes from cells  
3 after 6 days of culture in L15 without 5% CO<sub>2</sub>. Scale bar, 20 μm.

4  
5 **Supplementary Figure 2. *smcdwi-1+* X1(FS) neoblasts are viable.** X1(FS) cells were  
6 cultured in IPM + 5% CO<sub>2</sub> for 2 days. Representative images of apoptotic cells (Annexin  
7 V, green, arrowheads) co-stained with the pan-neoblast marker *smcdwi-1* (magenta),  
8 n=37. Two independent replicate experiments were performed. No co-labeling was  
9 observed, suggesting neoblasts examined in study were viable. Scale bar, 20 μm.

10  
11 **Supplementary Figure 3. IPM, Knockout DMEM, and dL15 maintain more *PCNA+***  
12 **cells.** Percentage of *smcdwi-1+* neoblasts after 1 day of culture in indicated media + 5%  
13 CO<sub>2</sub>.

14  
15 **Supplementary Figure 4. Determining the number of X1(FS) cells needed for**  
16 **efficient colony expansion. (a)** Percentage of lethally irradiated hosts displaying robust  
17 neoblast colony expansion following transplantation with the indicated numbers of sorted  
18 X1(FS) cells. At 7 days post-transplantation (dpt), > 80% of all hosts displayed colony  
19 expansion when 1,000 X1(FS) were transplanted. **(b)** Representative images of hosts  
20 transplanted with X1(FS) cells at 7 dpt. *smcdwi-1+* neoblasts: green. DAPI: blue. Scale  
21 bar, 200 μm. Ten animals assayed per condition.

22

1 **Supplementary Figure 5. Sexual hosts are rescued and reconstituted by**  
2 **transplantation of cultured asexual X1(FS) cells. (a)** Sequence showing the HpaI  
3 enzyme restriction site, which was used to distinguish between the asexual (donor) and  
4 sexual (host) biotypes by RFLP analyses. **(b)** RFLP data showing rescue of lethally  
5 irradiated sexual worms transplanted with freshly collected, non-cultured X1(FS) cells. **(c-**  
6 **d)** RFLP data showing rescue of lethally irradiated sexual worms transplanted with 1 and  
7 2 day cultured X1(FS) cells. Data from two independent experiments shown replicate 1  
8 (panel c); replicate 2 (panel d). **(e)** Rescue rates for lethally irradiated hosts following  
9 transplantation of X1(FS) cells cultured in the indicated media + 5% CO<sub>2</sub> for 1 or 2 days.  
10 None of the conditions rescued lethally irradiated hosts after 3 days. Blue and orange  
11 dots show value of rescue rate from replicate experiments, respectively.

12  
13 **Supplementary Figure 6. No expression of exogenously delivered *Smed-***  
14 ***histone3.3-2xflag* mRNA in *smedwi-1+* cells.** Representative images of electroporated  
15 X1(FS) without (upper panel) or with (lower panel) *Smed-histone3.3-2xflag* mRNA. Cells  
16 cultured in Knockout DMEM + 5% CO<sub>2</sub> for 1 day were stained with *smedwi-1* riboprobe  
17 and anti-FLAG antibody. Arrowhead: anti-FLAG+ nucleated cells. Scale bar, 20 μm.

18  
19 **Supplementary Figure 7. Compare SiR-DNA sorted cells. (a)** A plot showing how SiR-  
20 DNA-stained cells are displayed without gates in the flow cytometry analysis using SiR-  
21 DNA versus side scatter. **(b)** A plot showing how gates were defined to isolate two SiR-  
22 DNA staining cell populations based on DNA content (SiR-DNA 4n and 2n). **(c)** *smedwi-*  
23 *1* in situ staining for neoblasts in two isolated cell populations based on DNA content as

1 indicated in (b). SiR-DNA 4n population contains 56.4%±2.6% *smedwi-1*+ neoblasts (also  
2 see Fig. 4f) compared to 26.8%±3.2% in SiR-DNA 2n population, p value = 0.0017. Scale  
3 bar, 20 µm. **(d-g)** Plots showing the cell cycle distribution of SirNeoblasts (SiR-DNA 4n +  
4 CT) (d), cells between SiR-DNA 4n and 2n (e), SiR-DNA 2n (f), and all SiR-DNA+ cells  
5 (g). Sorted cells were stained with Hoechst 33342. Hoechst 33342+ (square gate) cells  
6 were analyzed for cell cycle distribution.

7

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9

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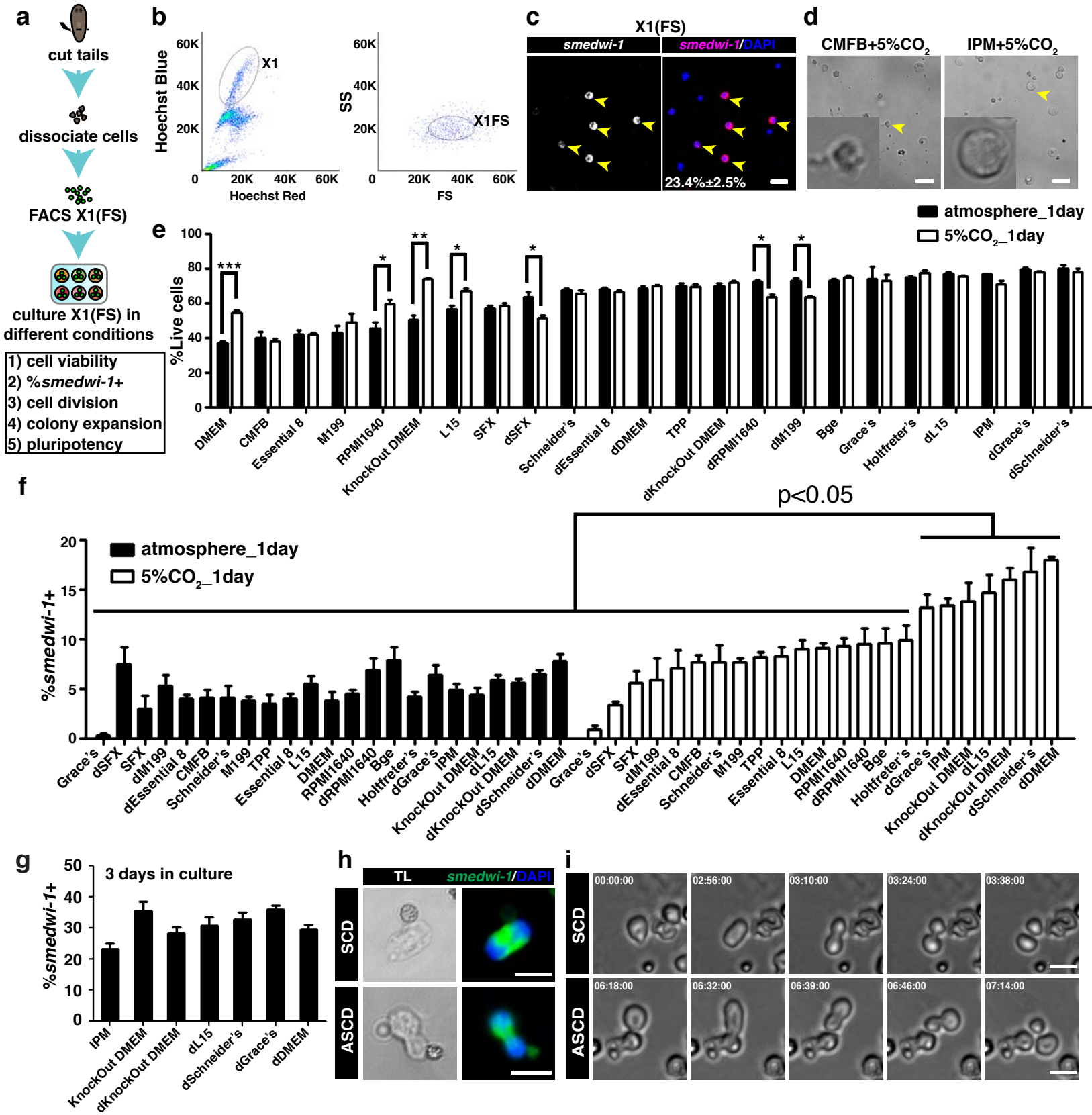
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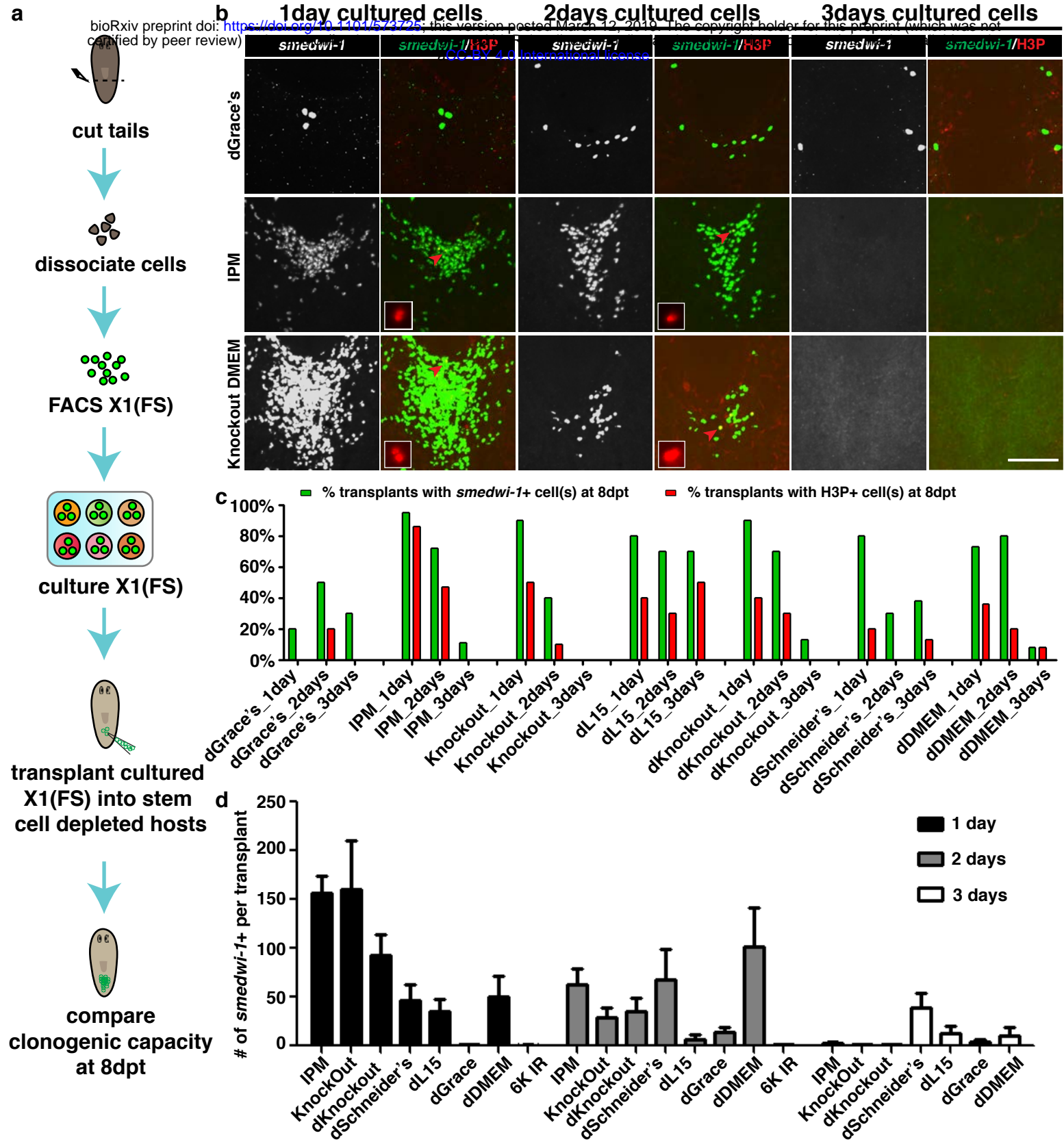
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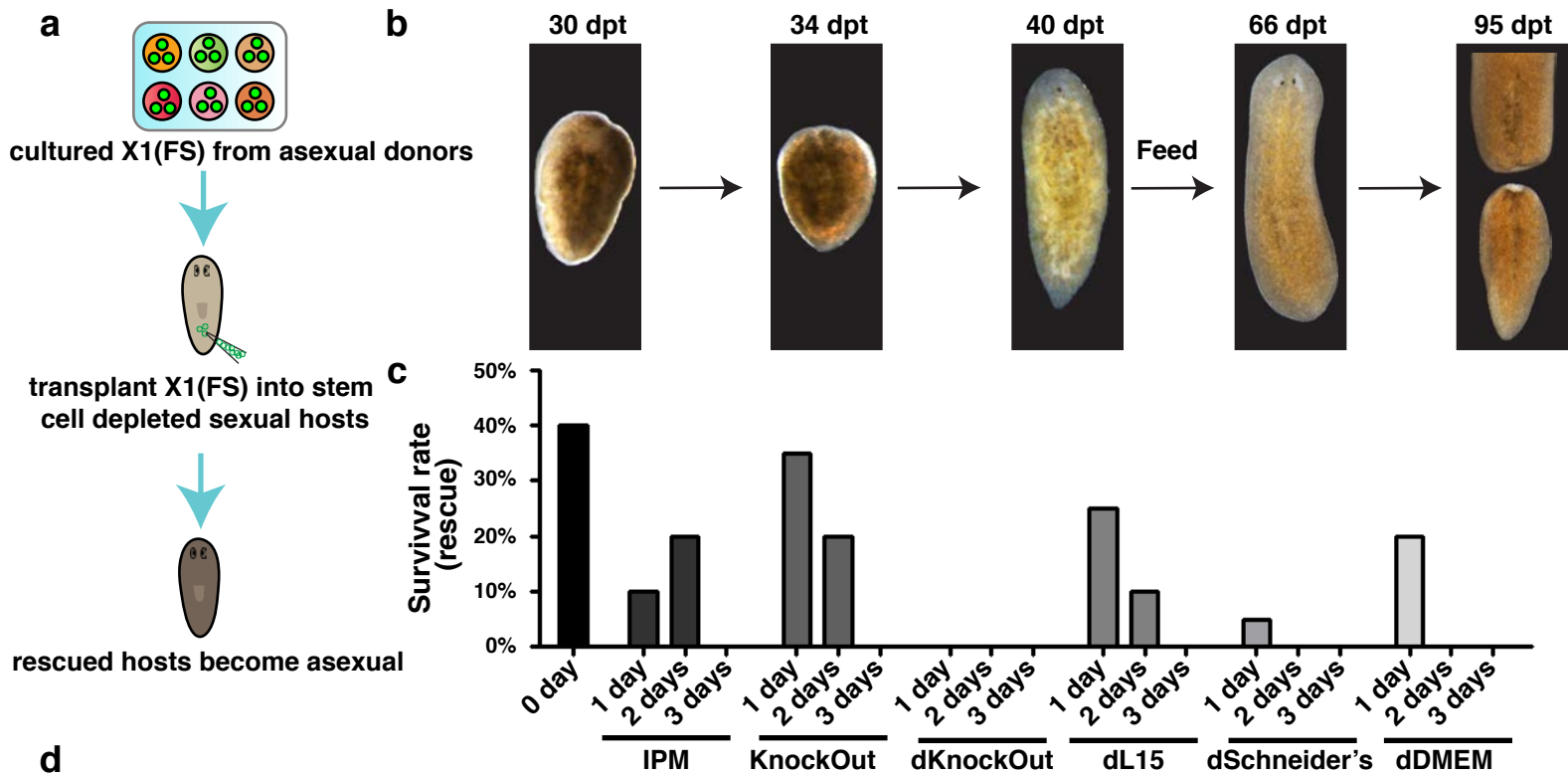
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# Lei et al. Figure 2

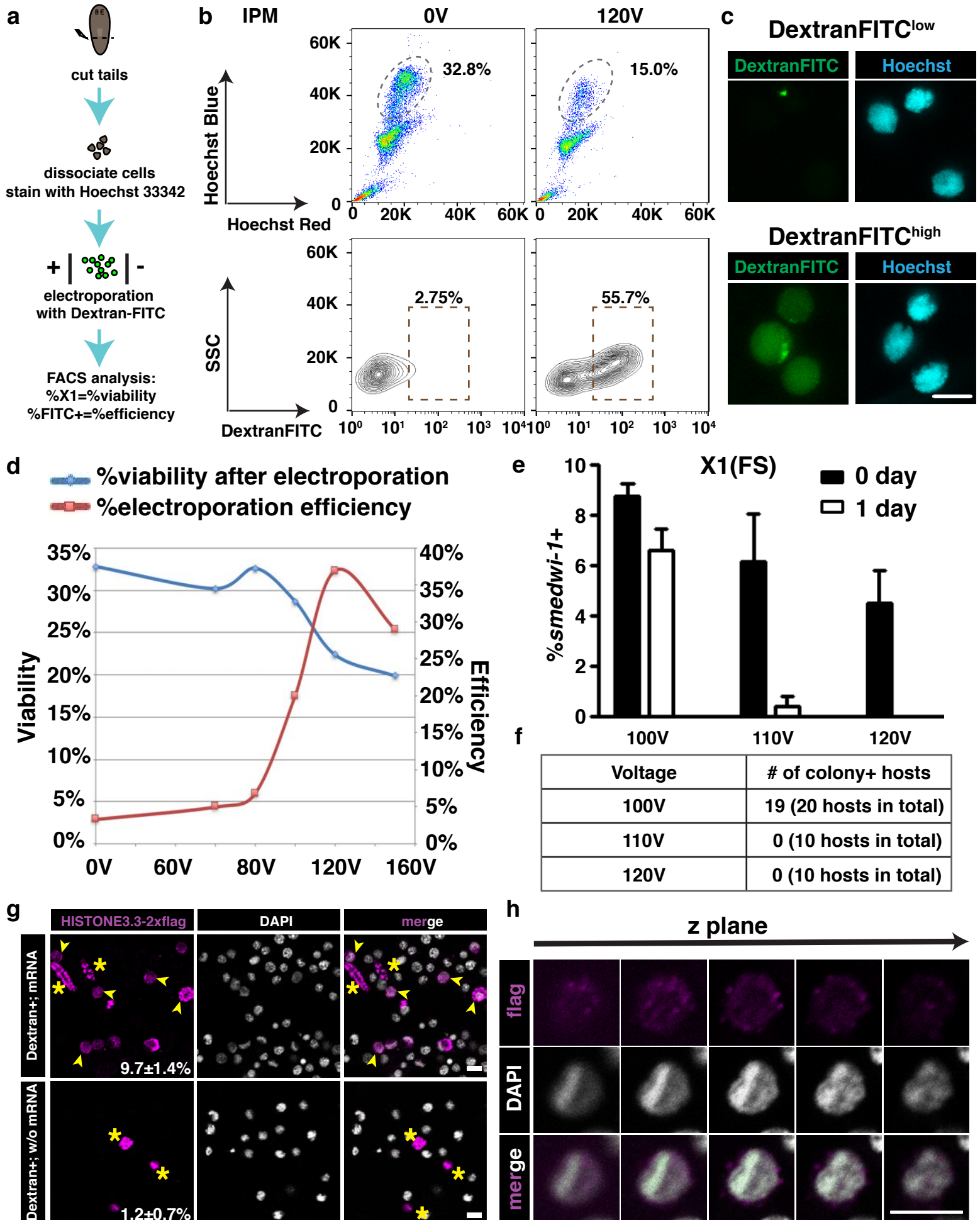


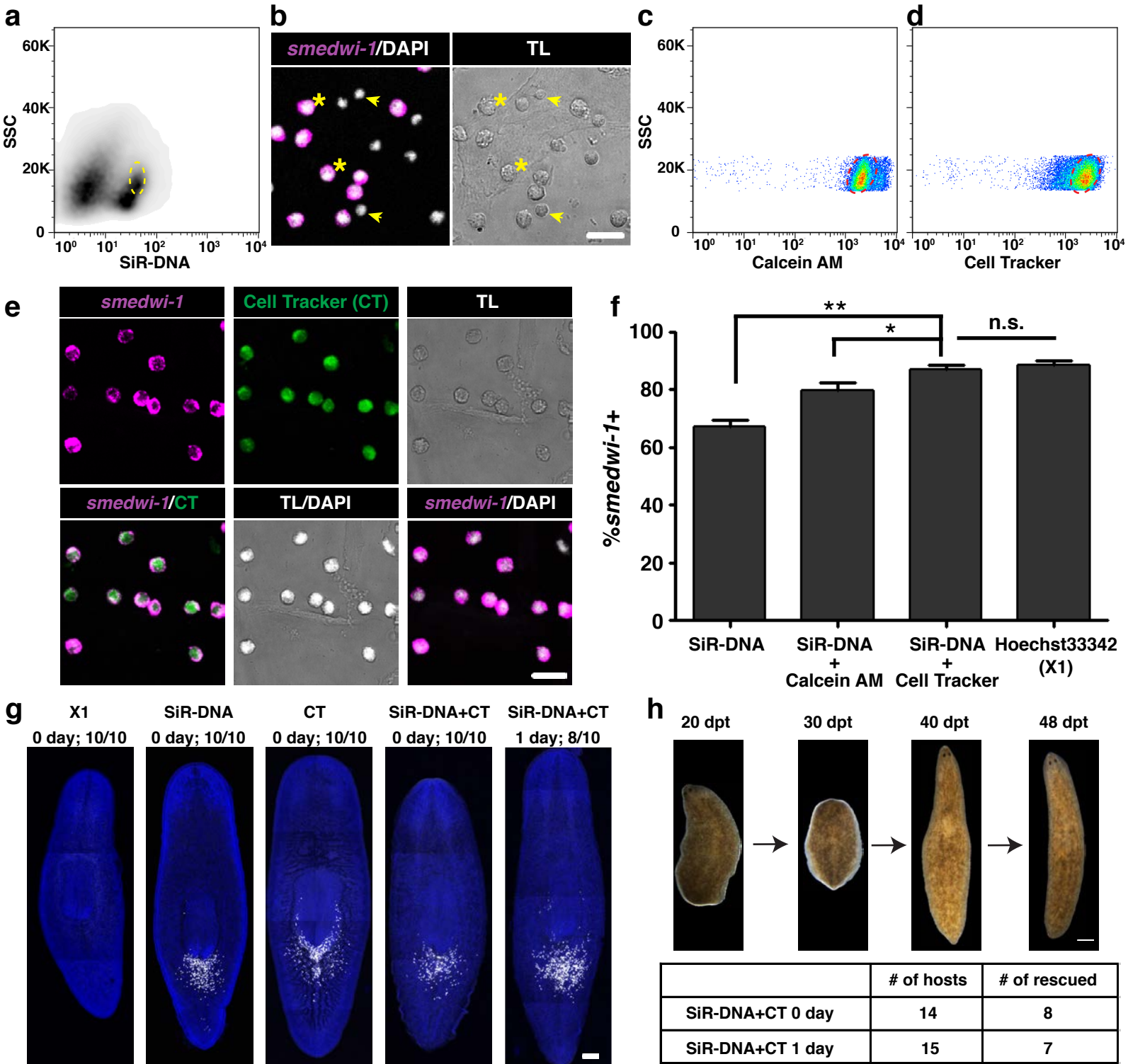


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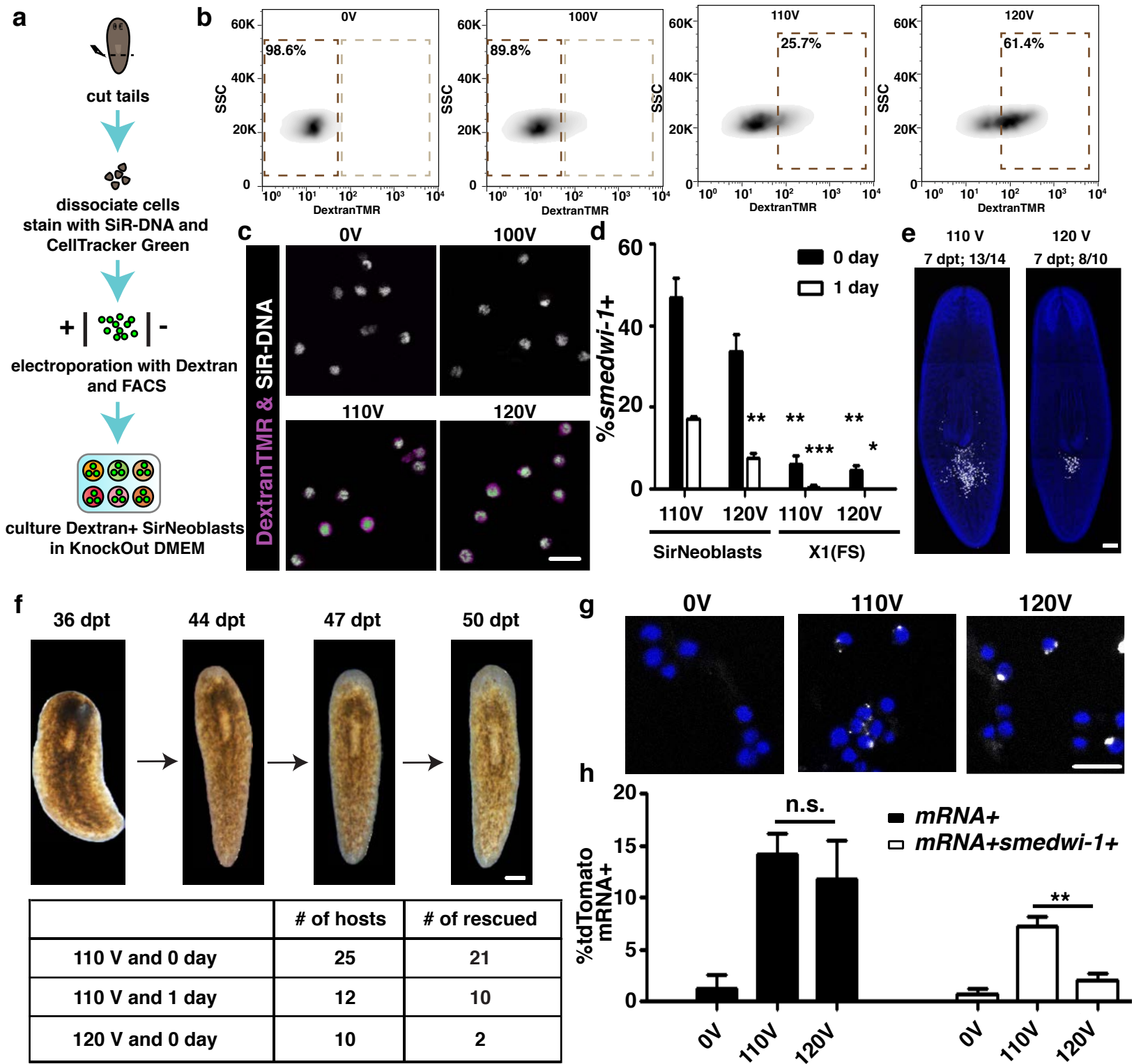
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1	IPM	YES	YES	YES	YES	YES
2	KnockOut DMEM	YES	YES	YES	YES	YES and Best
3	dKnockOut DMEM	YES	YES	YES	YES	NO
4	dL15	YES	YES	YES	YES	YES
5	dSchneider's	YES	YES	YES	YES	NO
6	dDMEM	YES	YES	YES	YES	NO
7	dGrace's	YES	YES	YES	NO	Not tested
8	Holtfreter's	YES	YES	NO	Not tested	Not tested
9	Bge	YES	YES	NO	Not tested	Not tested
10	dRPMI1640	YES	YES	NO	Not tested	Not tested
11	RPMI1640	YES	YES	NO	Not tested	Not tested
12	DMEM	YES	YES	NO	Not tested	Not tested
13	L15	YES	YES	NO	Not tested	Not tested
14	Essential 8	YES	NO	NO	Not tested	Not tested
15	TPP	YES	YES	NO	Not tested	Not tested
16	M199	YES	NO	NO	Not tested	Not tested
17	Schneider's	YES	YES	NO	Not tested	Not tested
18	CMFB	NO	NO	NO	Not tested	Not tested
19	dEssential 8	YES	YES	NO	Not tested	Not tested
20	dM199	YES	YES	NO	Not tested	Not tested
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23	Grace's	YES	YES	NO	Not tested	Not tested



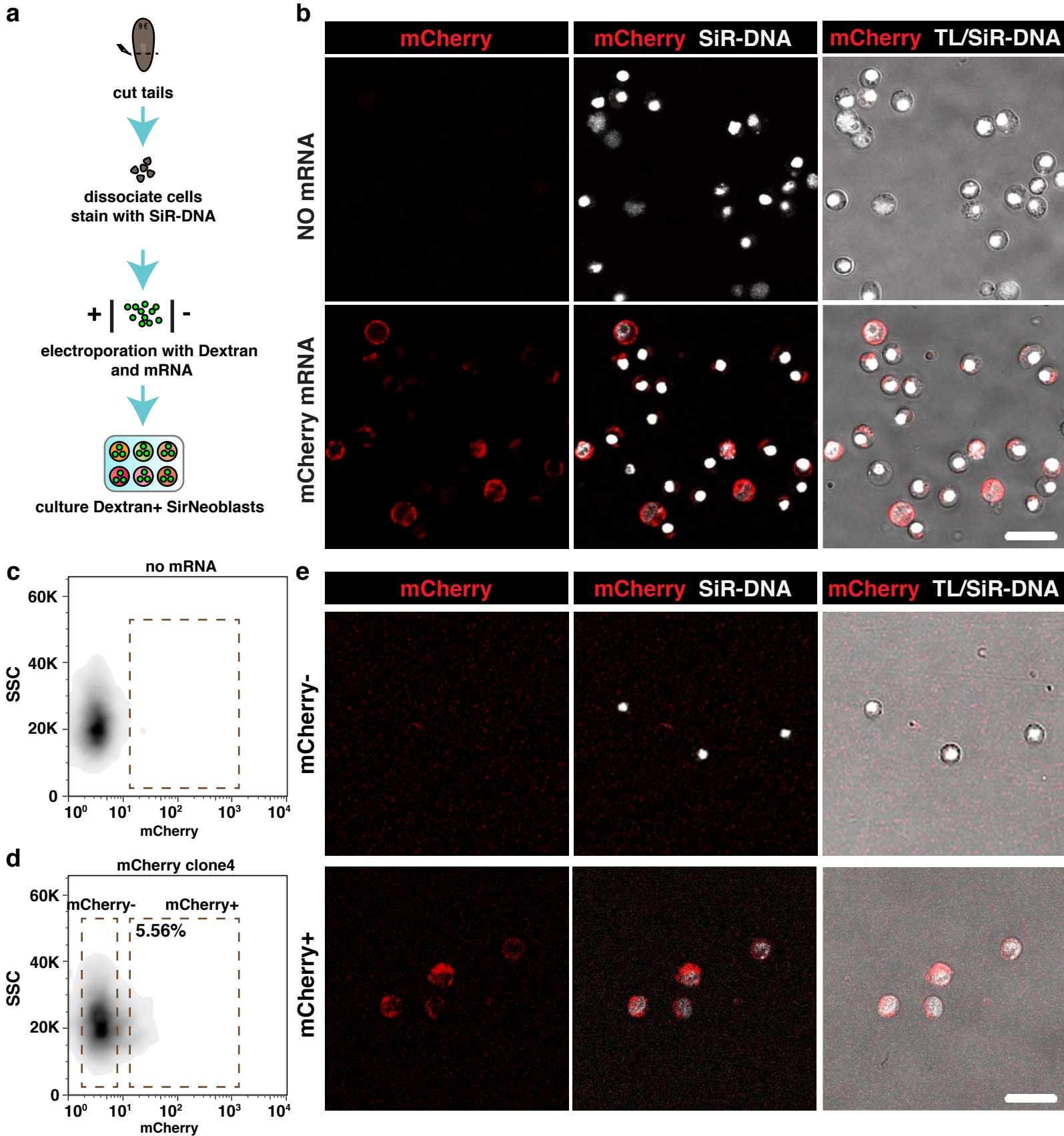








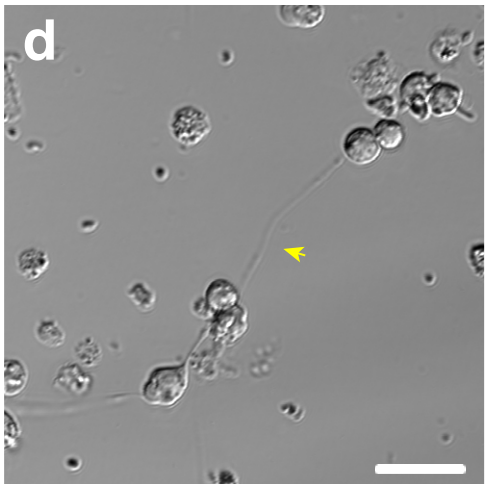
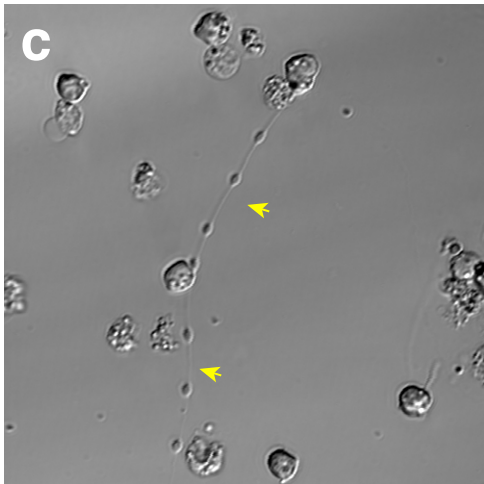
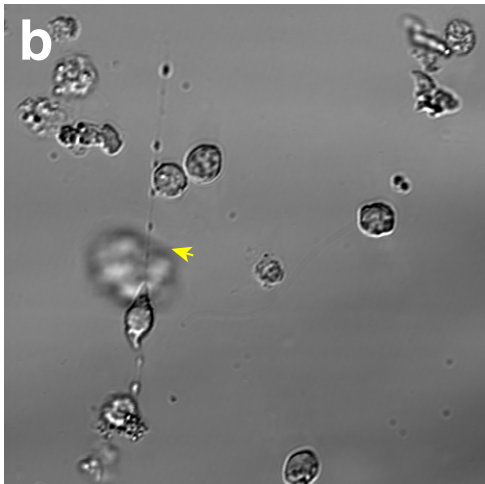
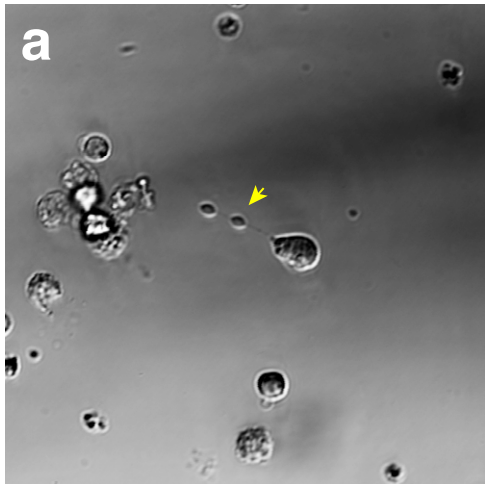
# Lei et al. Figure 7



# Lei et al. Supplementary Figure 1

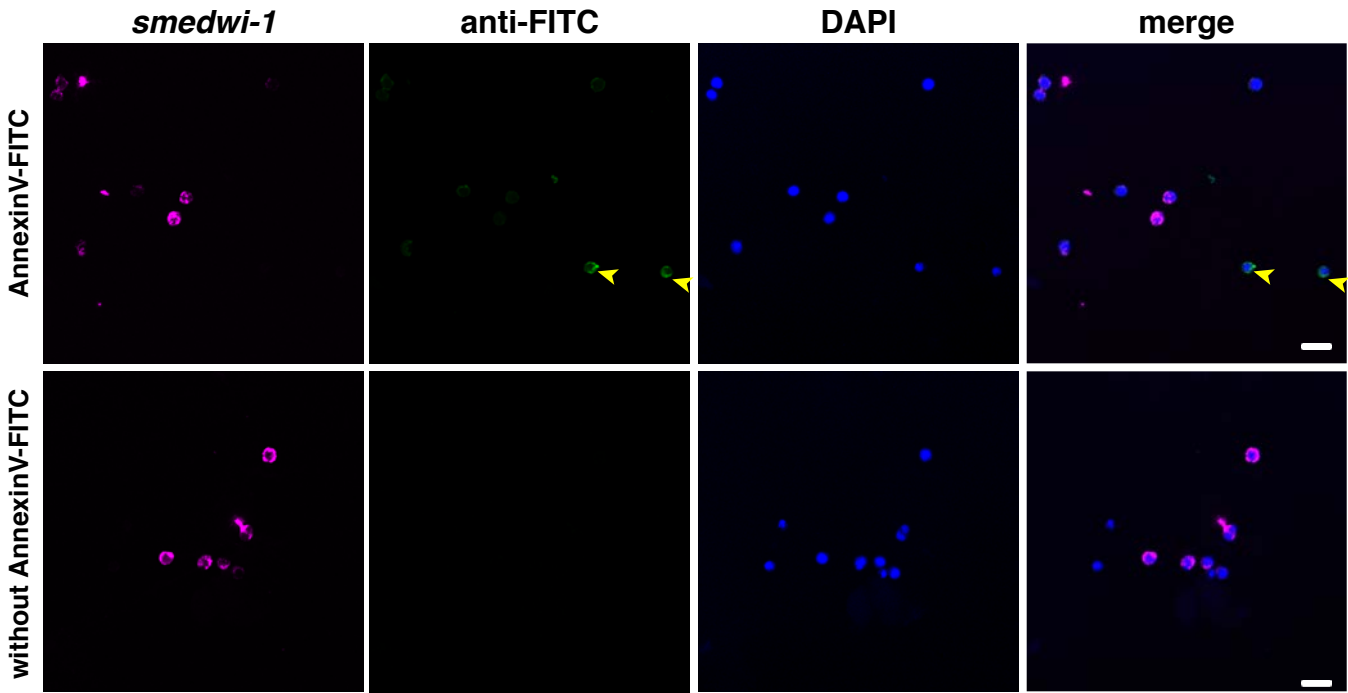
6 days

L15\_atmosphere



# Lei et al. Supplementary Figure 2

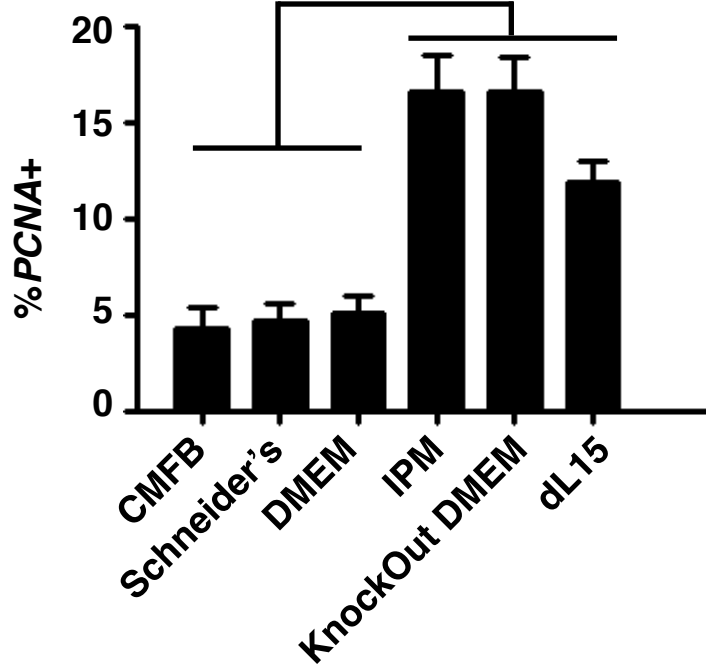
X1(FS) in IPM + 5% CO<sub>2</sub> for 2 days

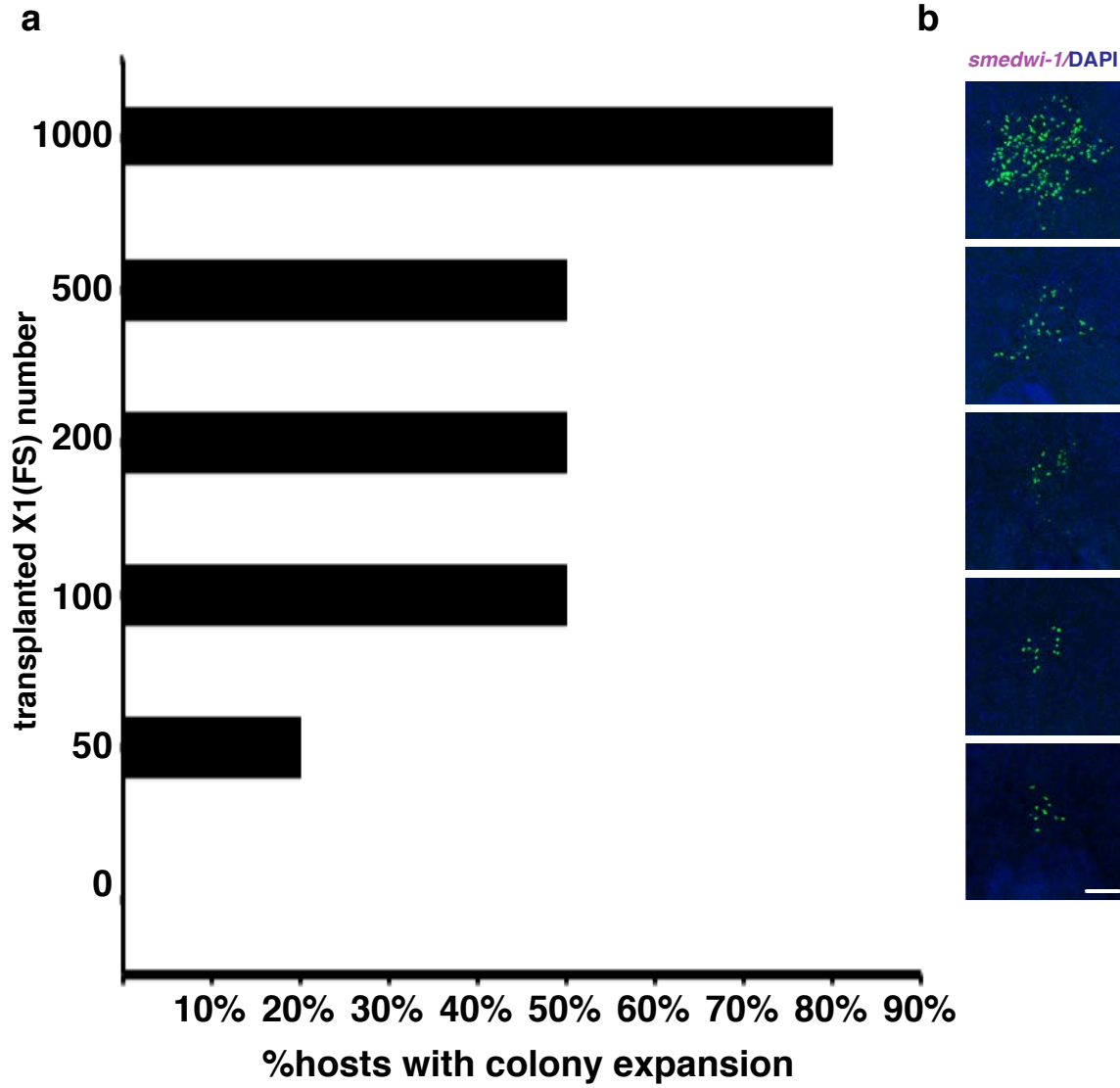


# Lei et al. Supplementary Figure 3

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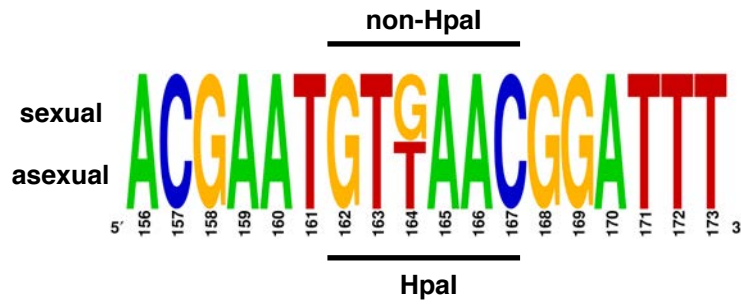
1 day in culture



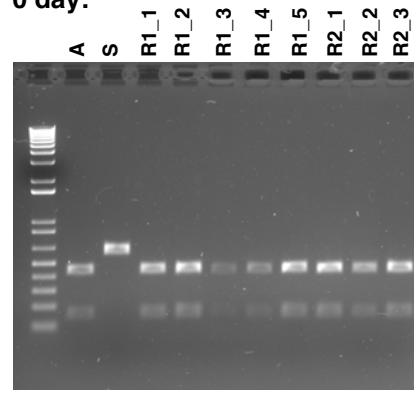




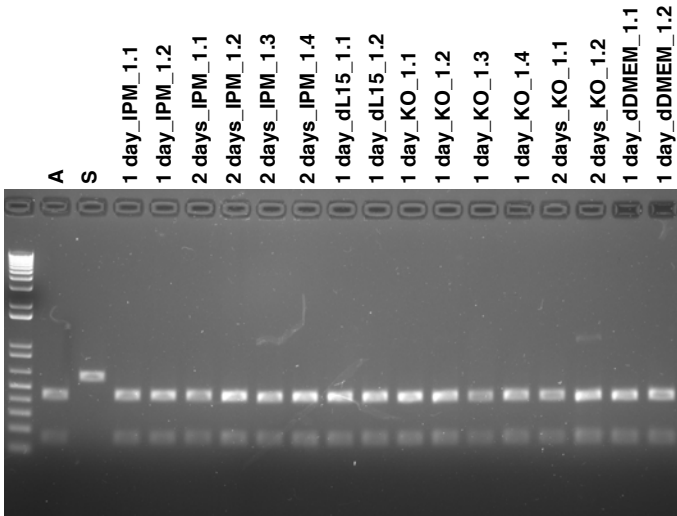
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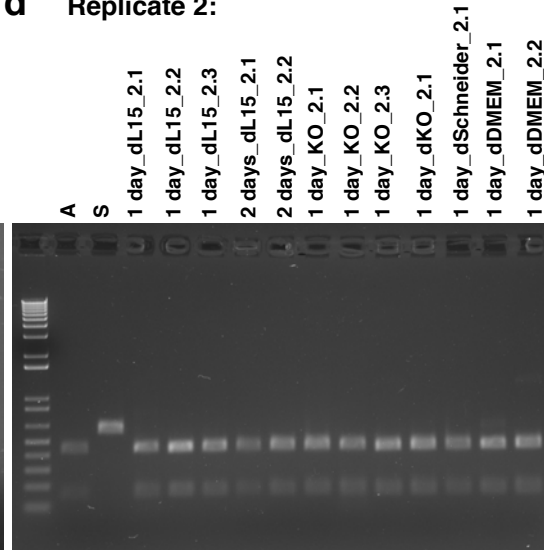
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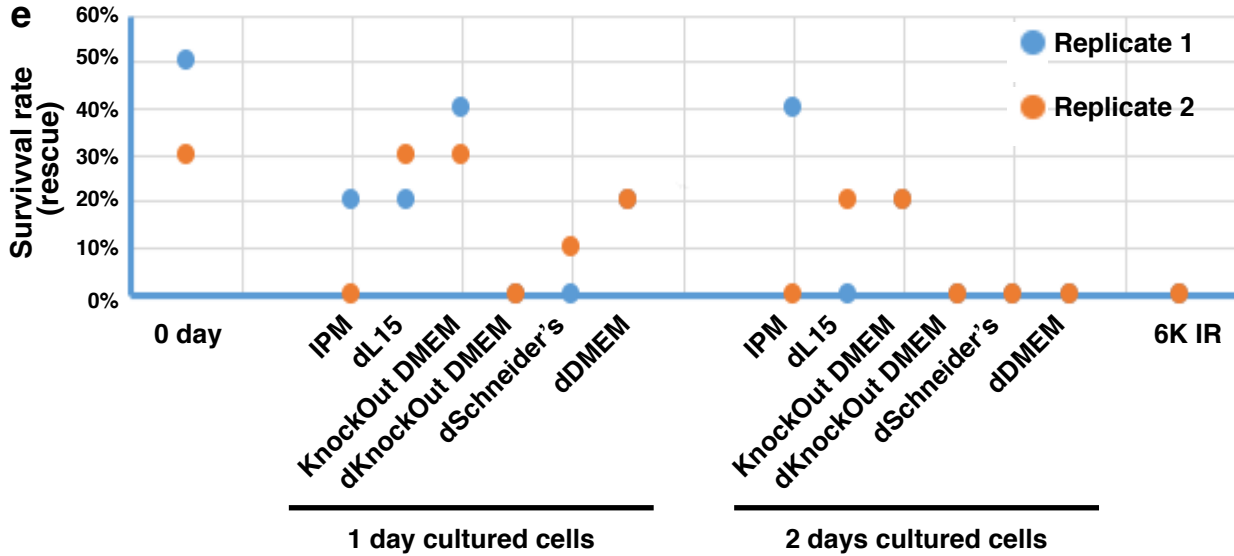
**c** Replicate 1:



**d** Replicate 2:

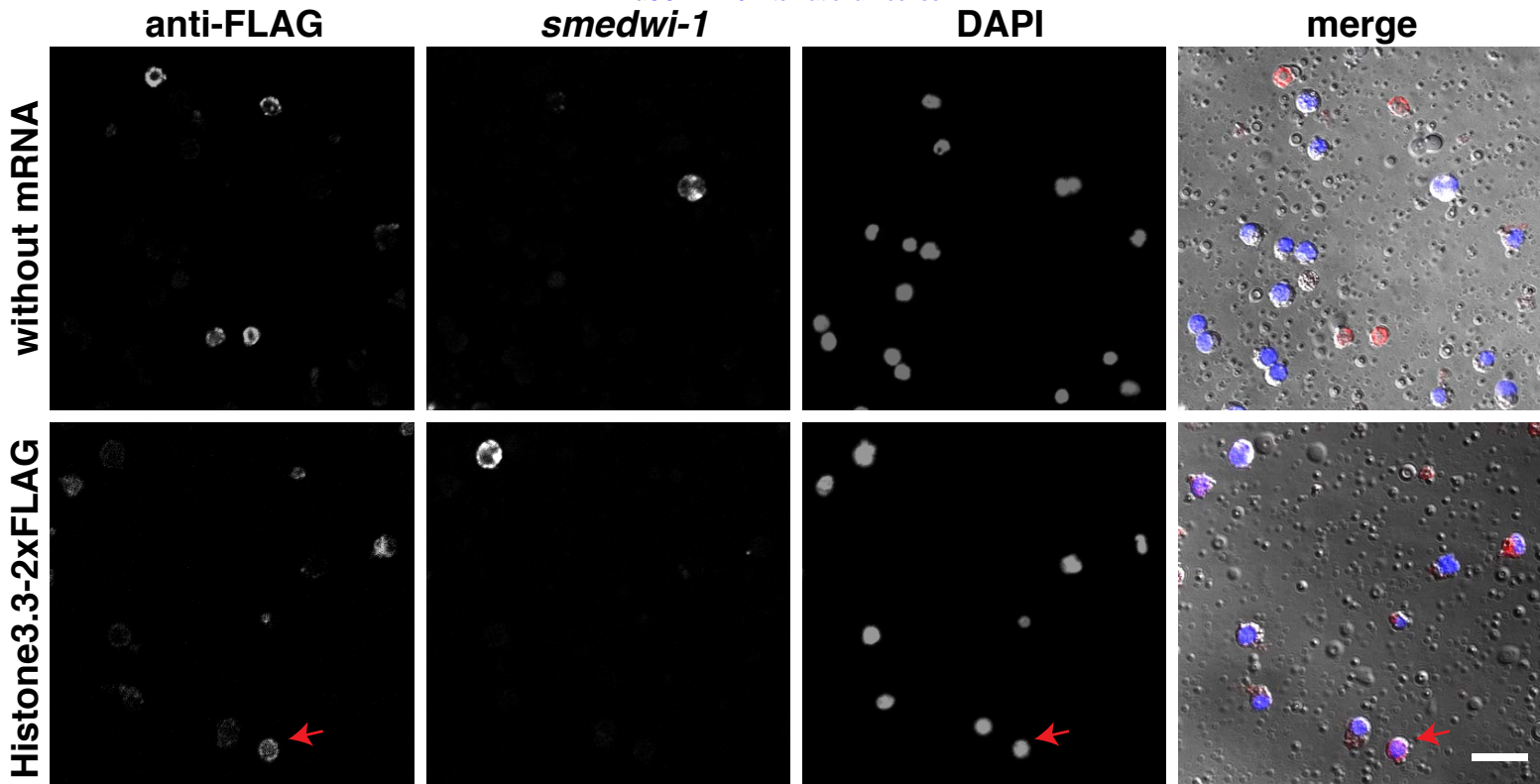


**e**



# Lei et al. Supplementary Figure 6

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# Lei et al. Supplementary Figure 7

