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HAL Authorization

1 **TITLE:**

2 Determination of S-Phase Duration Using 5-Ethynyl-2'-deoxyuridine Incorporation in
3 *Saccharomyces cerevisiae*

4
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18 **SUMMARY:**

19 We describe two complementary protocols to accurately determine S-phase duration in *S.*
20 *cerevisiae* using EdU, a thymidine analog, which is incorporated *in vivo* and detected using Click
21 chemistry by microscopy and flow cytometry. It allows for the easy characterization of the
22 duration of DNA replication and overlooked replication defects in mutants.

23

24 **ABSTRACT:**

25 Eukaryotic DNA replication is a highly regulated process that ensures that the genetic blueprint
26 of a cell is correctly duplicated prior to chromosome segregation. As DNA synthesis defects
27 underlie chromosome rearrangements, monitoring DNA replication has become essential to
28 understand the basis of genome instability. *Saccharomyces cerevisiae* is a classical model to study
29 cell cycle regulation, but key DNA replication parameters, such as the fraction of cells in the S
30 phase or the S-phase duration, are still difficult to determine. This protocol uses short and non-
31 toxic pulses of 5-ethynyl-2'-deoxyuridine (EdU), a thymidine analog, in engineered TK-hENT1
32 yeast cells, followed by its detection by Click reaction to allow the visualization and quantification
33 of DNA replication with high spatial and temporal resolution at both the single-cell and
34 population levels by microscopy and flow cytometry. This method may identify previously
35 overlooked defects in the S phase and cell cycle progression of yeast mutants, thereby allowing
36 the characterization of new players essential for ensuring genome stability.

37

38 **INTRODUCTION:**

39 Genome stability through mitotic division is ensured by the transmission of a complete and equal
40 set of chromosomes to the two produced cell progenies. This relies on the accurate completion
41 of a series of events occurring in a given time in each phase of the cell cycle. In G₁, the replication
42 origins are licensed upon the recruitment of several licensing factors, including Cdc6¹. In the S
43 phase, whole-genome duplication is initiated from multiple active replication origins and
44 performed by replication machineries that gather in microscopically visible foci named

45 replication factories². In the M phase, duplicated sister chromatids are attached and bioriented
46 on the mitotic spindle to allow their segregation to the opposite poles of the mitotic cell³. The
47 regulation, proper completion, and duration of each phase are key to ensure genome stability.
48 Indeed, premature exit from any of these phases leads to genome instability. For instance, a
49 shorter G₁ induced by deletion of the budding yeast CDK inhibitor Sic1 or by the overexpression
50 of G₁ cyclins will alter the subsequent S phase⁴⁻⁶. Consequently, these deregulations, associated
51 or not with replication stress, result in chromosome breaks, rearrangements, and mis-
52 segregation⁴⁻⁶. Therefore, monitoring the duration of the S phase and, more broadly, the
53 duration of the other phases of the cell cycle may be crucial to identify the defects occurring in
54 different mutants and in different stressful conditions.

55
56 A traditional method for measuring cell cycle phase duration includes simple DNA content flow
57 cytometry (**Figure 1A**) and relies on a fitting algorithm (available in most cytometry software)
58 used to separate the population into G₁, S, and G₂ + M phase fractions from the 1C and 2C peaks.
59 The fractions are then multiplied by the population doubling time⁷. However, this method gives
60 only estimated values, requires a homogeneous cell size distribution within a given fraction, and
61 is not applicable to synchronized cultures. To study the S-phase duration in mammalian cells,
62 several thymidine analogs have been developed and widely used, including EdU. Their uptake
63 from the extracellular medium and phosphorylation by thymidine kinase (hereafter referred to
64 as TK) make them available for DNA polymerases to incorporate them at sites of DNA synthesis
65 (replication, recombination, repair). To bypass the absence of the TK gene in *Saccharomyces*
66 *cerevisiae* cells, yeast strains have been engineered to allow stable and constitutive expression
67 of the herpes simplex virus TK⁸ and the human equilibrative nucleoside transporter (hENT1)⁹.
68 Once incorporated into DNA, EdU is detected *via* the selective Click reaction, which chemically
69 couples its alkyne moiety to azide-modified fluorochromes¹⁰.

70
71 This paper provides two optimized comprehensive protocols to pulse-label asynchronous and
72 synchronous TK-hENT1 engineered cells with EdU in order to precisely visualize and measure DNA
73 replication duration and dynamics, as well as the duration of the other phases of the cell cycle,
74 with high spatial and temporal resolution at both the single-cell and population levels by
75 microscopy and flow cytometry.

76
77 **PROTOCOL:**

78
79 **1. *S. cerevisiae* cell culture**

80
81 NOTE: The yeast strains used are listed in **Table 1**

82
83 NOTE: The S-phase duration can be monitored in different ways. Depending on the question to
84 be addressed, the cells can be grown asynchronously or synchronously following G₁ arrest.

85
86 **1.1. From asynchronously growing *S. cerevisiae* cells**

87
88 NOTE: This method allows for the determination of the percentage of cells in the S phase in an

89 asynchronously growing cell population. By determining the doubling time, the duration of the S
90 phase (and the other phases) can be extrapolated.

91
92 1.1.1. Inoculate *S. cerevisiae* cells in 10 mL of synthetic complete (SC) medium at a low cell
93 concentration (5×10^4 cells/mL) for an overnight culture at 30 °C with orbital agitation at 130
94 rpm.

95
96 NOTE: The concentration is measured with a cell counter. To efficiently calculate the doubling
97 times, it is recommended to inoculate the culture from an overnight culture that is still in the
98 exponential phase (i.e., ideally below 2×10^7 cells/mL). Growing cells in rich medium (YPD) is not
99 recommended, as EdU detection is not efficient.

100
101 1.1.2. The following day, dilute the cells in 20 mL of fresh SC medium at the final concentration
102 of 5×10^5 cells/mL.

103
104 1.1.3. Culture the cells at 30 °C in a shaking water bath with horizontal shaking at 120 rpm.

105
106 1.1.4. Measure the cell concentration every hour until it reaches 1×10^7 cells/mL.

107
108 NOTE: This step allows for making a graphical representation of the cell concentration increase
109 over time. The formula used to calculate the doubling times is explained in the legend of **Table**
110 **2**.

111
112 1.1.5. Proceed in parallel to step 2 for EdU labeling when the cell concentration is about 2×10^6 –
113 5×10^6 cells/mL.

114
115 1.2. From G₁-synchronized *S. cerevisiae* cells

116
117 NOTE: This method allows for the determination of when the S phase starts and finishes by means
118 of flow cytometry and/or microscopy analyses.

119
120 1.2.1. Inoculate *S. cerevisiae* cells in 10 mL of SC medium at a low cell concentration (5×10^4
121 cells/mL) for an overnight culture at 30 °C with orbital agitation at 130 rpm.

122
123 NOTE: See the notes after step 1.1.1.

124
125 1.2.2. The following day, dilute the cells in 20 mL of fresh SC medium at a final concentration of
126 2×10^6 – 3×10^6 cells/mL.

127
128 1.2.3. Add 40 µL of 1 mg/mL α-factor diluted in water.

129
130 1.2.4. Culture the cells at 30 °C with orbital agitation at 130 rpm for 1 h.

131
132 1.2.5. Add again 40 µL of 1 mg/mL α-factor diluted in water.

133
134 1.2.6. Culture the cells at 30 °C with orbital agitation at 130 rpm for 1 h.
135
136 1.2.7. Visualize the cells under a light microscope to monitor G₁ arrest. Proceed if more than 90%
137 of the cells display a shmoo and the others are rounded, unbudded cells.
138
139 NOTE: Depending on the background used, it is recommended to sonicate the cells before shmoo
140 visualization. For the W303 background, sonicate 2x, for 2 s each time, at an amplitude of 40–50.
141
142 1.2.8. Centrifuge for 3 min at 1,500 × *g*. Discard the supernatant with a vacuum pipette.
143
144 1.2.9. Resuspend the cells in 20 mL of SC medium.
145
146 1.2.10. Repeat steps 1.2.8–1.2.9 once.
147
148 NOTE: The α-factor is washed away with these steps, and the cells are released in the cell cycle.
149 Alternatively, the α-factor may be washed away by filtering the yeast cells with a 1.2 μm
150 nitrocellulose filter using a funnel set on a side-arm flask connected to a vacuum pump.
151
152 1.2.11. Collect 1 mL of cells 2x every 5 min and proceed to step 2 for EdU labeling.
153
154 NOTE: Pulse-label the cells from only one of the two tubes with EdU. The non-pulse-labeled cells
155 are used to distinguish EdU-positive from EdU-negative cells on a bivariate propidium iodide (PI)-
156 EdU graph.
157
158 1.2.12. Add 400 μL of 1 mg/mL α-factor diluted in water 30 min after the release.
159
160 NOTE: This high dose of α-factor is required to arrest the cells in the G₁ phase of the next cell
161 cycle and to prevent the cells from re-entering the subsequent S phase.
162
163 **2. EdU labeling**
164
165 2.1. Transfer 1 mL of cell culture to a 2.0 mL microfuge tube containing 1 μL of 10 mM EdU. Mix
166 well by inversion.
167
168 NOTE: To discriminate the EdU-positive cells from the EdU-negative cells on a PI-EdU bivariate
169 FACS, transfer another 1 mL of cell culture to a 2.0 mL microfuge tube containing 1 μL of DMSO.
170
171 2.2. Incubate for 3–5 min at 30 °C under agitation in a shaking water bath.
172
173 NOTE: Three minutes are sufficient for EdU detection with a microscope; 5 min are required for
174 optimal EdU detection on a flow cytometer.
175
176 2.3. Stop the reaction with the addition of 100 μL of 100% ethanol.

177
178 2.3.1. Stop the reaction with the addition of 100 μ L of 20% paraformaldehyde if cell size
179 measurement is required.

180
181 NOTE: If the nuclear architecture of the mitotic cells is to be kept intact for further analyses
182 following the Click reaction, we recommend fixing cells with 2% paraformaldehyde at room
183 temperature (RT) rather than putting the cells on ice, since the latter causes microtubule
184 depolymerization.

185
186 2.3.2. Leave the cells for 20 min at RT under mild agitation on a variable speed rocker at 20
187 tilts/min to fix the cells before the addition of 100 μ L of 100% ethanol.

188
189 **3. Cell fixation and permeabilization**

190
191 3.1. Pellet the cells for 2 min at 10,000 $\times g$ in a microfuge. Remove the supernatant using a
192 vacuum pipette.

193
194 3.2. Resuspend the cell pellet in 500 μ L of 70% ethanol. Mix well by vortexing.

195
196 3.3. Leave for ≥ 1 h at RT at 20 tilts/min on a variable speed rocker to permeabilize the cells.

197
198 NOTE: Cells grown in SC medium do not pellet well as they tend to stick to the microfuge walls.
199 The addition of ethanol improves pelleting and reduces cell loss. The cells may be stored at 4 $^{\circ}$ C
200 overnight or for longer periods at -20° C.

201
202 3.4. Pellet the cells for 2 min at 10,000 $\times g$ in a microcentrifuge. Discard the supernatant with a
203 vacuum pipette.

204
205 3.5. Wash the cells 2x with 500 μ L of 10% ethanol in PBS.

206
207 NOTE: The washes are crucial to remove unincorporated EdU from the cells.

208
209 **4. Click-it reaction**

210
211 4.1. For cytometry analysis

212
213 4.1.1. Pellet the cells for 2 min at 10,000 $\times g$ in a microfuge. Discard the supernatant with a
214 vacuum pipette.

215
216 4.1.2. Resuspend the pellet in 200 μ L of PBS containing 0.1 mg/mL RNase A and 0.2 mg/mL
217 proteinase K.

218
219 4.1.3. Incubate for 1–2 h at 50 $^{\circ}$ C with occasional shaking (or overnight at 37 $^{\circ}$ C).

220

221 4.1.4. Pellet the cells for 2 min at 10,000 × *g* in a microcentrifuge. Discard the supernatant with a
222 vacuum pipette.

223
224 4.1.5. Wash the cells with 500 μL of PBS.

225
226 4.1.6. Pellet the cells for 2 min at 10,000 × *g* in a microfuge. Discard the supernatant with a
227 vacuum pipette.

228
229 4.1.7. Resuspend the cell pellet in 200 μL of PBS + 1% bovine serum albumin (BSA). Incubate for
230 30 min at RT.

231
232 NOTE: Longer times are not necessary and are even detrimental to the efficiency of Click
233 reactions.

234
235 4.1.8. Pellet the cells for 2 min at 10,000 × *g* in a microcentrifuge. Discard the supernatant with a
236 vacuum pipette.

237
238 4.1.9. Resuspend the pellet in 300 μL of PBS + 1% BSA.

239
240 4.1.10. Distribute the cells between two tubes: 200 μL in a 1.5 mL microcentrifuge tube for the
241 Click reaction and 100 μL in another 1.5 mL microcentrifuge tube for Sytox Green staining.

242
243 4.1.11. Pellet the cells for 2 min at 10,000 × *g* in a microcentrifuge. Discard the supernatant with
244 a vacuum pipette.

245
246 4.1.12. For Sytox Green staining

247
248 NOTE: We highly recommend taking an aliquot for Sytox Green staining (without Click) in order
249 to obtain high-quality DNA content reference profiles. Indeed, the Click reaction strongly
250 quenches intercalant fluorescence, including the Sytox Green and PI fluorescence. Consequently,
251 the Click reaction can distort the reading of DNA content.

252
253 4.1.12.1. Resuspend the cell pellet in 100 μL of PBS.

254
255 4.1.12.2. Transfer 10–30 μL (depending on the cell concentration) to a flow cytometer tube
256 containing 300 μL of 50 mM Tris-HCl, pH 7.5, and 0.5 μM Sytox Green.

257
258 4.1.12.3. Sonicate 2x, for 2 s each time, at an amplitude of 40–50.

259
260 4.1.12.4. Leave in the dark until processing the samples on a flow cytometer.

261
262 NOTE: The cells can be kept at this stage at 4 °C for a few days.

263
264 4.1.13. For the Click reaction

265
266 4.1.13.1. Prepare fresh Azide Dye buffer by mixing the reagents in the following order (quantity
267 for one tube): 36 μL of PBS, 2 μL of 0.2 M CuSO_4 , 0.2 μL of 2 mM Cy5 Azide, and 2 μL of 1 M
268 ascorbic acid.

269
270 NOTE: It is possible to prepare a master mix of the Azide buffer. The reagents have to be mixed
271 in the same order as that mentioned above.

272
273 4.1.13.2. Resuspend the cell pellet with 40 μL of freshly made Azide Dye mix. Incubate at RT in
274 the dark for 60 min.

275
276 4.1.13.3. Pellet the cells for 2 min at $10,000 \times g$ in a microcentrifuge. Discard the supernatant
277 with a vacuum pipette.

278
279 4.1.13.4. Wash the cells 3x with 300 μL of 10% ethanol in PBS.

280
281 NOTE: The washes are crucial to eliminate all the soluble EdU-Cy5 azide.

282
283 4.1.13.5. Resuspend the cells in 100 μL of 50 $\mu\text{g}/\text{mL}$ PI in PBS. Leave for 10 min in the dark.

284
285 4.1.13.6. Transfer 10–30 μL of the cell suspension (depending on the cell concentration) to a flow
286 cytometer tube containing 300 μL of 50 mM Tris-HCl, pH 7.5.

287
288 4.1.13.7. Sonicate 2x, for 2 s each time, at an amplitude of 40–50.

289
290 4.1.13.8. Leave in the dark until processing the samples on a cytometer.

291
292 NOTE: The cells can be kept at this stage at 4 $^{\circ}\text{C}$ for a few days.

293
294 4.1.14. Read the Sytox Green samples using an excitation blue laser at 488 nm and a 530/30 BP
295 filter. See **Figure 1A** for typical results. Read the bivariate PI-EdU samples on a dot plot using an
296 excitation blue laser at 488 nm and a 615/20 BP filter for the PI (x-axis) and an excitation red laser
297 at 640 nm and a 660/20 BP filter (y-axis). See **Figure 1B** for typical results.

298
299 NOTE: **Figure 1C** represents the typical PI-EdU bivariate FACS result for EdU-negative cells. It
300 allows for the discrimination of the EdU-negative cells from the EdU-positive cells.

301
302 4.2. For microscopy analysis

303
304 4.2.1. Pellet the cells for 2 min at $10,000 \times g$ in a microcentrifuge. Discard the supernatant with a
305 vacuum pipette.

306
307 4.2.2. Resuspend the pellet in 200 μL of PBS + 1% BSA. Incubate for 30 min at RT.

308

309 4.2.3. Pellet the cells for 2 min at 10,000 × *g* in a microfuge. Discard the supernatant with a
310 vacuum pipette.

311
312 4.2.4. Prepare fresh Azide Dye buffer by mixing the reagents in the following order (quantity for
313 one tube): 36 μL of PBS, 2 μL of 0.2 M CuSO₄, 0.2 μL of 2 mM Dy-530 azide, 2 μL of 1 M ascorbic
314 acid.

315
316 NOTE: A master mix of the Azide buffer can be prepared fresh by mixing the reagents in the
317 aforementioned order.

318
319 4.2.5. Resuspend the pellet with 40 μL of freshly made Azide Dye buffer. Incubate at RT in the
320 dark for 60 min.

321
322 4.2.6. Pellet the cells for 2 min at 10,000 × *g* in a microfuge. Discard the supernatant with a
323 vacuum pipette.

324
325 4.2.7. Wash the cells 2x with 300 μL of 10% ethanol in PBS.

326
327 NOTE: The washes are crucial to eliminate all soluble Dy-530 azide.

328
329 4.2.8. Pellet the cells for 2 min at 10,000 × *g* in a microcentrifuge. Discard the supernatant with a
330 vacuum pipette.

331
332 4.2.9. Resuspend the cells in 100 μL of 0.5 μg/mL 4',6-diamidino-2-phenylindole (DAPI) in PBS.
333 Leave for 30 min in the dark at room temperature.

334
335 4.2.10. Pellet the cells for 2 min at 10,000 × *g* in a microcentrifuge. Discard the supernatant with
336 a vacuum pipette.

337
338 4.2.11. Wash with 300 μL of PBS to remove excess DAPI.

339
340 4.2.12. Pellet the cells for 2 min at 10,000 × *g* in a microfuge. Discard the supernatant with a
341 vacuum pipette.

342
343 4.2.13. Resuspend the pellet with 10–50 μL of PBS depending on the cell concentration.

344
345 4.2.14. Sonicate 2x, for 2 s each time, at an amplitude of 40–50.

346
347 NOTE: The cells can be kept at this stage at 4 °C for a few days.

348
349 4.2.15. Pipette 1.7 μL of the cells onto a glass microscope slide and cover with a clean coverslip.

350
351 4.2.16. Immediately observe under a fluorescence microscope with DAPI and TexasRed or Cy3
352 filters.

353

354 **REPRESENTATIVE RESULTS:**

355 To determine the S-phase duration and, more broadly, the duration of G₁ and G₂ + M (protocol
356 step 1.1), *S. cerevisiae* W303 wild-type cells (WT, **Table 1**) were grown asynchronously in SC
357 medium for 7 h. Every hour, the cell concentration was monitored to determine the doubling
358 time (**Figure 2B**). In these growth conditions, the calculated doubling time was 120 min ± 13 min
359 at 25 °C (**Table 2**). When the cells were in the exponential phase (2×10^6 – 5×10^6 cells/mL), an
360 aliquot of cells was pulse-labeled with EdU (10 μM) for 5 min to single out the cells in the S phase
361 and determine the percentage of cells that were in the G₁, S, and G₂ + M phases. Three
362 populations of cells were observed in a bivariate EdU-PI cytometer analysis (**Figure 1B**). The
363 discrimination between the EdU-negative and EdU-positive populations was made using a control
364 experiment in which the cells were not pulse-labeled with EdU but the Click reaction was carried
365 out (**Figure 1C**). The two EdU-negative populations in the bottom-left and the bottom-right areas
366 that differed in PI intensity two-fold corresponded to G₁ and G₂ + M, respectively (**Figure 1B,C**).
367 Therefore, the upper population (with an intensity $>1 \times 10^4$ – 2×10^4) corresponded to the EdU-
368 positive cells that were in the S phase at the time of the pulse (**Figure 1B**). Hence, 27% ± 5% of
369 the cell population was in the G₁ phase, 29% ± 3% was in the S phase, and 44% ± 2% was in G₂ +
370 M. As the doubling time was 120 min ± 13 min in these conditions, we extrapolated that the G₁,
371 S, and G₂ + M phases lasted 32 min ± 4 min, 35 min ± 6 min, and 53 min ± 7 min, respectively, at
372 25 °C (**Table 2** and **Table 3**).

373

374 Next, we aimed to validate this method and show that it is sensitive enough to identify mutants
375 with DNA replication defects that have been overlooked so far. We reasoned that tunable loss-
376 of-function alleles of the factors involved in DNA replication would be ideal validation controls.
377 Hence, we used a yeast strain containing a temperature-sensitive *cdc6-1* mutant (**Table 1**)¹¹. Cdc6
378 is an essential licensing factor that is expressed in late M and G₁ to assemble prereplication
379 complexes (preRC) on ORC-bound chromosome sites that may later be used as replication origins.
380 Therefore, at a permissive temperature, its S-phase duration should be the same as that of the
381 WT, while at a restrictive temperature, no DNA replication should occur as no origin is licensed¹².
382 However, at a semi-permissive temperature, where fewer origins are licensed but there are
383 enough to confer cell viability (our unpublished data; Barba Tena et al., in preparation), we
384 anticipated different durations for each phase. As expected, based on drop tests, the *cdc6-1* cells
385 grew the same as WT cells at a permissive temperature (i.e., 25 °C, **Figure 2A**), displaying the
386 same doubling time (**Figure 2B** and **Table 2**), but were dead at or above 34 °C (**Figure 2A**). Of
387 interest, at a semi-permissive temperature (i.e., 28 °C), *cdc6-1* was viable (28 °C, **Figure 2A**).
388 However, the doubling time was longer than for WT (**Figure 2B** and **Table 2**), and the duration of
389 each phase was different. Indeed, in *cdc6-1*, G₁ was slightly shorter (12 min ± 1 min vs. 16 min ±
390 2 min), while the S phase was slightly extended (34 min ± 4 min vs. 29 min ± 5 min), and the G₂ +
391 M phase was significantly longer (77 min ± 4 min vs. 45 min ± 3 min) compared to WT (**Figure**
392 **2C,D** and **Table 3**). The S phase was, surprisingly, not extended very much, but the mean intensity
393 of the EdU signal was decreased by 25% (**Figure 2C,D**), which is consistent with an S phase
394 initiated from fewer origins. Moreover, while the EdU-positive WT cells were homogeneously
395 distributed between the early (S1) and late S (S2) phases (**Figure 2C**, **Supplemental Figure S1A**,
396 early [S1] and late S [S2] phases delimited with a vertical dashed line in the upper gate), 65% of

397 the EdU-positive *cdc6-1* cells accumulated in the late S phase (**Figure 2D, Supplemental Figure**
398 **S1B**). There was even no clear distinction between the S and G₂ + M populations (**Figure 2D**),
399 suggesting that the cells struggled to complete the S phase before entering G₂ phase. Therefore,
400 this method is suitable and sensitive to identify mutants with defects in the S phase (duration
401 and/or distribution) and in cell cycle phase duration.

402
403 A complementary method was devised to determine when the cells start and finish their DNA
404 replication and estimate the S-phase duration using synchronized cells (protocol step 1.2). To this
405 end, using α -factor with synchronized cells in G₁, cells were released in SC medium and collected
406 every 5 min. The duration of the S phase may be about 25 min based on the time when the DNA
407 content changed from 1C to 2C on the Sytox Green flow cytometer profile (**Figure 3A**). However,
408 this estimation depends on when a significant cell fraction of the population has incorporated
409 enough Sytox Green to be seen in the FACS profile. The early and late replication events cannot
410 be detected with this method. To define accurately when the S phase starts and finishes and how
411 long it lasts, S-phase cells were singled out from an aliquot of cells upon pulse-labeling with EdU
412 (10 μ M) for 5 min every 5 min after the G₁ release. As expected, within the first 10 min after the
413 release, all the cells were in the bottom-left area (i.e., in G₁, **Figure 3B, Supplemental Figure S2**).
414 Fifteen minutes after the release, a fraction of EdU-positive cells was already detected (compare
415 the first two rows in **Supplemental Figure S2** and **Supplemental Figure S3**, EdU-treated and EdU-
416 free cells, respectively), indicating that the S phase had started (**Figure 3C**). The progression
417 through the S phase was seen by the cell cloud first moving upward and then rightward in the
418 bivariate PI-EdU graph (**Figure 3D–F**). Finally, 35 min from the release, a fraction of cells was EdU-
419 negative but with twice the amount of DNA, indicating that those cells had completed the S phase
420 and were in the G₂ + M phase (**Figure 3G**). Thus, the S phase lasts 20 min in these conditions. Of
421 note, despite the high synchrony observed with the overlay of DNA content detected with Sytox
422 Green, suggesting that the S phase was over in the whole population 40 min after the release,
423 the bivariate analyses showed that some cells finished the S phase 60 min after the release and
424 that the S phase was complete for the whole population 65 min after the release (**Figure 3H,I** and
425 **Supplemental Figure S2**).

426
427 Additionally, the S phase and DNA synthesis can be monitored by microscopy. From each
428 activated replication origin, DNA synthesis is carried out by two tethered replisomes, forming a
429 nuclear replication focus that may be followed by microscopy by imaging a tagged version of a
430 replication factor and/or operators array and their corresponding repressor fused to a
431 fluorescent protein^{2,13}. Alternatively, DNA synthesis can be detected by microscopy using
432 thymidine analogues^{14,15}. We used EdU to monitor the subnuclear DNA regions that undergo DNA
433 synthesis. To this end, we pulse-labeled asynchronous WT and *cdc6-1* cells with EdU (10 μ M) for
434 3 min at 28 °C and imaged them after the Click reaction. We were expecting to detect variations
435 in the EdU signal intensities depending on the DNA synthesis rate and on the cell progression in
436 the cell cycle during the 3 min EdU pulse (i.e., cells spending the whole 3 min in the S phase will
437 display a stronger signal than those entering or exiting S phase during the pulse). Accordingly,
438 WT S-phase cells displayed an EdU signal in their nucleus that varied in intensity (**Figure 4A**). The
439 S-phase duration can be readily extrapolated from this analysis. Indeed, it was determined from
440 two biological replicates (at least 150 cells were counted), that 34% \pm 3% and 38% \pm 3% of WT

441 and *cdc6-1* cells were EdU positive, respectively. As in these growth conditions, the doubling time
442 was 90 min and 123 min for the WT and *cdc6-1* cells, respectively (**Table 2**), we extrapolated that
443 the S phase lasted 31 min and 47 min, respectively. The former result is in line with that obtained
444 from our FACS analyses (**Table 3**), indicating that the detection of EdU-positive cells by
445 microscopy allows the determination of the S-phase duration. Of note, the latter is higher than
446 the S-phase duration extrapolated from our FACS analyses because there was no clear distinction
447 between the S and G₂ + M populations (**Figure 2D**). The EdU foci were readily observed in the WT
448 cells (**Figure 4B**) but were dimmer and fewer in the *cdc6-1* cells (**Figure 4C**). To rule out the
449 possibility that the EdU signal intensity difference between the WT and *cdc6-1* cells depended on
450 the step of the S phase, the intensity was quantified from synchronized cells. As expected, the
451 mean EdU signal intensity was three-fold lower in the *cdc6-1* cells (**Figure 4D**), consistent with
452 DNA replication initiated from fewer replication origins. The EdU signal was restricted to the
453 nucleus, colocalizing with a strong DAPI signal, and organized in globular patterns, consistent with
454 the organization of DNA replication in nuclear regions or replication factories. Importantly, EdU
455 was only detected in unbudded or small-budded cells and never present in cells with large buds,
456 indicating that the WT yeast cells had finished replication by the time they entered mitosis. This
457 method is, therefore, sensitive for visualizing DNA replication at a high spatial resolution, as well
458 as for detecting and quantifying mild DNA replication defects.

459

460 **FIGURE AND TABLE LEGENDS:**

461 **Figure 1: Representative FACS analyses.** (A) Representative Sytox Green FACS analyses for WT
462 cells grown at 25 °C. (B,C) Representative EdU-PI bivariate FACS analyses for WT cells grown at
463 25 °C and pulse-labeled for 5 min with EdU (10 μM) or 1 μL of DMSO. The polygons were the
464 same in both analyses. C was used to delineate the EdU-negative from the EdU-positive cells
465 (generally, the limit is set at an intensity $>1 \times 10^4$ – 2×10^4). The top polygon gate delineated the
466 EdU-positive cells (S-phase fraction).

467

468 **Figure 2: Fraction of cells in the G₁, S, and G₂ + M cell cycle phases in asynchronous cell**
469 **populations.** (A) WT and *cdc6-1* strains were spotted at serial five-fold dilutions on rich medium
470 and grown either at 25 °C, 28 °C, or 34 °C. (B) Population doubling time. Asynchronous WT and
471 *cdc6-1* cells were grown at 25 °C or 28 °C, and the cell concentration was measured every hour
472 for 7 h. (C,D) EdU-PI bivariate FACS analysis of WT and *cdc6-1* cells grown at 28 °C and pulse-
473 labeled for 5 min with EdU (10 μM). Multiplying the population doubling time by the S-phase
474 fraction provides the S-phase duration. The mean intensities of the EdU-positive and EdU-
475 negative cells were calculated as the mean of the intensity of each value in the corresponding
476 polygon. The mean intensities of the WT and *cdc6-1* EdU-negative cells (5,077 and 4,454,
477 respectively) were normalized to 1. The mean intensities of the WT and *cdc6-1* EdU-positive cells
478 (52,604 and 36,141, respectively) were divided by the normalization factor used for each strain.
479 The obtained values were 10.4 and 8.1, respectively (i.e., a 25% decrease, as mentioned in the
480 text). Abbreviations: WT = wild-type; EdU = 5-ethynyl-2'-deoxyuridine; FACS = fluorescence-
481 activated cell sorting; PI = propidium iodide.

482

483 **Figure 3: Determination of the S-phase duration on synchronized cells.** (A) Time course analysis
484 of the DNA content of WT cells after α-factor release at 28 °C. The indicated time takes into

485 account the duration of the EdU pulse (5 min). (B–I) EdU-PI bivariate FACS analyses for
486 synchronized WT cells pulse-labeled for 5 min with EdU (10 μ M) and collected at the indicated
487 times after the release from G₁ arrest. Abbreviations: WT = wild-type; EdU = 5-ethynyl-2'-
488 deoxyuridine; FACS = fluorescence-activated cell sorting; PI = propidium iodide.

489

490 **Figure 4: EdU detection and quantification by microscopy.** Asynchronous WT and *cdc6-1* strains
491 were grown for 2 h at 28 °C, and then pulse-labeled for 3 min with EdU (10 μ M) and imaged by
492 wide-field microscopy using adequate excitation/emission filters. (A) Representative images
493 from wide-field microscopy for WT and (B,C) individual cells for WT and *cdc6-1* visualized by DIC
494 and stained with DAPI or for EdU, as indicated. The scale bars in (A) and (B,C) are 10 μ m and 2
495 μ m, respectively. (D) EdU intensity measurements on synchronous WT and *cdc6-1* cells grown at
496 28 °C 30 min after the release from G₁ arrest. The graph represents the pooling of three biological
497 replicates (at least 50 cells were counted in each biological replicate). Mean \pm SD are displayed
498 on the graph. An unpaired two-tailed t-test between WT and *cdc6-1* cells is indicated by **** (p
499 $<$ 0.0001). Abbreviations: WT = wild-type; EdU = 5-ethynyl-2'-deoxyuridine; DIC = differential
500 interference contrast; DAPI = 4',6-diamidino-2-phenylindole; A.U. = arbitrary units.

501

502 **Table 1: List of the strains used in this study.**

503

504 **Table 2: Mean doubling times of WT and *cdc6-1* strains grown at 25 °C and 28 °C.** The doubling
505 times were calculated from three biological replicates (four technical replicates for each
506 biological replicate) using the following formula: $\Delta t \times \ln(2)/\ln(C_f/C_i)$, where C_f and C_i correspond
507 to the final and initial cell concentrations, respectively, and Δt corresponds to the difference in
508 minutes between t_f and t_i , when C_f and C_i were measured, respectively.

509

510 **Table 3: Mean fractions of cells and duration of the G₁, S, and G₂ + M phases in WT and *cdc6-1***
511 **cells grown at 25 °C and 28 °C.** The fractions of cells were determined from three biological
512 replicates (two technical replicates for each biological replicate). The differences in G₁, S, and G₂
513 + M duration between the WT and *cdc6-1* cells grown at 28 °C were statistically significant
514 (unpaired two-tailed t-test, $p <$ 0.1).

515

516 **Supplemental Figure S1: Fraction of cells in the early and late S phases in WT and *cdc6-1* cells**
517 **grown at 28 °C.** Two identical polygons named S1 and S2 for the early and late S phases,
518 respectively, were drawn in the fraction of EdU-positive cells to split it into two halves on the
519 EdU-PI bivariate FACS analyses for (A) WT cells grown at 28 °C and (B) *cdc6-1* cells grown at 28
520 °C.

521

522 **Supplemental Figure S2: Cell cycle progression of EdU pulse-labeled cells after release from G₁**
523 **arrest visualized by EdU-PI bivariate FACS.** An aliquot of cells was pulse-labeled for 5 min with
524 10 μ M EdU every 5 min after release from α -factor arrest at 28 °C and until 80 min, as indicated.
525 Abbreviations: EdU = 5-ethynyl-2'-deoxyuridine; FACS = fluorescence-activated cell sorting; PI =
526 propidium iodide.

527

528 **Supplemental Figure S3: Cell cycle progression of DMSO-treated cells after release from G₁**

529 **arrest visualized by EdU-PI bivariate FACS.** This is the same as **Supplemental Figure 2**, but the
530 cells were incubated for 5 min with 1 μ L DMSO (dimethyl sulfoxide).

531
532 **Supplemental Figure S4: BrdU and EdU toxicity in TK hENT1 yeast cells.** Cells of the indicated
533 genotype were spotted in five-fold serial dilutions on YPD plates containing increasing
534 concentrations of BrdU or EdU and grown for 40 h at 30 °C. Abbreviations: DMSO = dimethyl
535 sulfoxide; BrdU = bromodeoxyuridine; Edu = 5-ethynyl-2'-deoxyuridine; TK = thymidine kinase;
536 hENT1 = human equilibrative nucleoside transporter.

537
538 **DISCUSSION:**

539 Yeast is a prime model organism for cell cycle studies, yet the characterization of its S phase has
540 long been hampered by its inability to incorporate exogenous nucleosides, such as BrdU, which
541 are used as tracers of DNA replication. Equipping yeast with a high expression of herpes simplex
542 thymidine kinase (TK) and the addition of a human nucleoside transporter (hENT) has largely
543 solved this problem^{15,16}. EdU is more versatile than BrdU as its detection with small fluorescent
544 azide and Click chemistry is amenable to permeabilized yeast cells and FACS analysis, unlike anti-
545 BrdU antibodies¹⁷. Here, we optimized the conditions of EdU labeling and detection in this TK-
546 hENT1 strain and showed that previously undetected replication defects can be quantified using
547 this protocol by FACS or microscopy.

548
549 Studying a biological mechanism using tools that may interfere with the same mechanism is a
550 common issue in biology. As EdU has a known cytotoxic effect, we searched for EdU
551 concentrations that are not toxic in chronic exposure to the engineered TK-hENT1 strain and
552 found 10 μ M to be a suitable dose. TK-hENT1 cells fail to proliferate at 25 μ M EdU, while TK-alone
553 or hENT1-alone cells easily withstand 100 μ M EdU (**Supplemental Figure S4**). Although yeast cells
554 are considerably more sensitive to EdU than BrdU, we found that proliferation decreased only
555 after the second cell cycle after EdU exposure, suggesting that it needs to be incorporated on
556 both strands to impact cell proliferation (data not shown). To remain on the safe side, we used
557 10 μ M EdU throughout this protocol, with short pulses only (3 min for microscopy; 5 min for
558 FACS), and found this was suitable for good detection using this optimized protocol.

559
560 Subtle defects in DNA replication may have a strong impact on chromosome rearrangements⁴.
561 Therefore, the development of techniques and protocols capable of detecting these subtle
562 defects is key for uncovering the etiology of chromosomal aberrations. Here, we show that this
563 optimized protocol of EdU incorporation and detection can measure up to a three-fold reduction
564 in signal intensity (**Figure 2** and **Figure 4**), indicating that the rate of DNA synthesis is reduced in
565 temperature-sensitive *cdc6-1* cells grown at 28 °C, which, as of yet, show no defects on plate
566 viability assays (**Figure 2A**). This protocol of EdU incorporation and quantification can, thus, be
567 used to screen other mutants for which replication defects are not suspected initially.
568 Additionally, it may be useful to detect mitotic DNA synthesis (MiDAS), meiotic recombination-
569 dependent DNA synthesis, or other long-tract DNA synthesis.

570
571 One drawback is that EdU detection can be performed on fixed cells only, precluding live analysis
572 as with PCNA-GFP or other fluorescent readouts of DNA replication, which suffer themselves

573 from the high phototoxicity of the laser beams used for imaging. Growing cells in a synthetic
574 medium is crucial for best detection, as YPD remnants appear to significantly quench the Click
575 reaction. Interestingly, singling out the S-phase cells using bivariate EdU-PI FACS analysis on
576 synchronized populations allows the estimation of the duration of the S phase to 20 min in single
577 cells (**Figure 3, Supplemental Figure S2, and Supplemental Figure S3**). However, even when
578 synchrony after α -factor release appears as near-perfect as in classical Sytox Green FACS analysis
579 (**Figure 3A**), it becomes clear from bivariate EdU-PI FACS analysis that cells enter the S phase
580 relatively asynchronously (**Figure 3B–I**).

581
582 Here, we describe three methods to determine S-phase duration using flow cytometry and
583 microscopy on synchronous and asynchronous cells at both the single-cell and population levels.
584 The average S-phase durations determined in asynchronous WT cells at the population level are
585 similar using flow cytometry and microscopy, at 29 min and 31 min, respectively, whereas our
586 synchronized single cell-based approach shows that the duration can be as short as 20 min
587 (**Figure 2, Figure 3, Figure 4, and Table 3**). The discrepancy between these values is surprising but
588 can be readily explained. Indeed, with our single cell-based approach, S-phase duration was
589 determined based on the earliest events (i.e., the first cells entering and exiting the S phase,
590 **Figure 3C,G**), but it does not imply that the S phase lasts 20 min in every cell within a population.
591 These data would support the unexpected notion that there is a certain level of heterogeneity in
592 S-phase duration between cells.

593
594 Improved protocols or new techniques can overthrow long-standing dogmas or novel ideas.
595 There are three that these data challenge. First, it is believed that DNA synthesis is concomitant
596 with bud emergence in *S. cerevisiae*. **Figure 4A–C** shows that EdU staining is strongest in
597 unbudded and small-budded cells, despite the 3 min labeling lag, indicating that the S phase
598 clearly starts before budding, as shown previously¹⁶. Second, it is reported that yeast cells do not
599 have a G₂ phase, with mitotic spindles forming at the same time as DNA synthesis. The very short
600 pulses combined with sensitive EdU detection by FACS (**Figure 3, Supplemental Figure S2, and**
601 **Supplemental Figure S3**) or microscopy (**Figure 4**) allows for determining precisely when the S
602 phase begins and finishes in single cells. By doing so, we found that bulk DNA synthesis finishes
603 40 min after α -factor release (**Figure 3, Supplemental Figure S2, and Supplemental Figure S3**),
604 while short mitotic spindles only form 20 min later (Magiera et al.¹⁶ and data not shown). We
605 conclude that yeast cells have a G₂ phase. Third, it was proposed recently that up to 30% of yeast
606 cells complete DNA synthesis in anaphase-telophase¹⁸. Using this optimized protocol, we did not
607 detect EdU incorporation in cells displaying an anaphase/telophase spindle (data not shown), but
608 we cannot rule out that this second wave of DNA synthesis is below the used threshold of
609 detection. Given the strong signal/noise ratio, we consider the latter option unlikely.

610
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615 microscopy were performed at the Montpellier MRI BioCampus imaging facility.

616

617 **DISCLOSURES:**

618 The authors declare that they have no competing financial interests.

619

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Table 1: Strains used in this study

Name	Genotype	Figures and tables
WT (E3087):	MATa, <i>ade2-1</i> , <i>trp1-1</i> , <i>can1-100</i> , <i>leu2-3,112</i> , <i>his3-11,15</i> , <i>RAD5</i> , <i>ura3 ::URA3 /GPD-TK(5x)</i> , <i>AUR1c ::ADH-hENT1</i>	Fig.1A-D, Fig.2, Fig3A,B,D, Supp Fig.1, Tables1,2
<i>cdc6-1</i> (E5956):	MATa, <i>ade2-1</i> , <i>trp1-1</i> , <i>can1-100</i> , <i>leu2-3,112</i> , <i>his3-11,15</i> , <i>cdc6-1</i> , <i>RAD5</i> , <i>ura3 ::URA3 /GPD-TK(5x)</i> , <i>AUR1c ::ADH-hENT1</i>	Fig.1A,C,E, Fig3C,D, Tables1,2
TK+ (E1000):	MATa, <i>ade2-1</i> , <i>trp1-1</i> , <i>can1-100</i> , <i>leu2-3,112</i> , <i>his3-11,15</i> , <i>RAD5</i> , <i>ura3 ::URA3 /GPD-TK(7x)</i>	Supp Fig.2
TK+ hENT+ (E2031):	MATa, <i>ade2-1</i> , <i>trp1-1</i> , <i>can1-100</i> , <i>leu2-3,112</i> , <i>his3-11,15</i> , <i>RAD5</i> , <i>ura3 ::URA3 /GPD-TK(7x)</i> , <i>AUR1c ::ADH-hENT1</i>	Supp Fig.2
hENT+ (E2031):	MATa, <i>ade2-1</i> , <i>trp1-1</i> , <i>can1-100</i> , <i>leu2-3,112</i> , <i>his3-11,15</i> , <i>RAD5</i> , <i>AUR1c ::ADH-hENT1</i> , <i>RAD52-GFP</i> , <i>URA3 ::mCherry-TUB1</i>	Supp Fig.2
<i>cdc6-1</i> TK+ hENT+ (E3968):	MATa, <i>ade2-1</i> , <i>trp1-1</i> , <i>can1-100</i> , <i>leu2-3,112</i> , <i>his3-11,15</i> , <i>cdc6-1</i> , <i>RAD5</i> , <i>ura3 ::URA3 /GPD-TK(7x)</i> , <i>AUR1c ::ADH-hENT1</i>	Supp Fig.2
<i>W303-1A</i> (E001):	MATa, <i>ade2-1</i> , <i>trp1-1</i> , <i>can1-100</i> , <i>leu2-3,112</i> , <i>his3-11,15</i> , <i>RAD5</i> , <i>ura3-1</i>	Supp Fig.2

Table 2: Measurements of doubling time of WT and *cdc6-1* strains grown at 25 °C and 28 °C.

	WT		<i>cdc6-1</i>	
	25 °C	28 °C	25 °C	28 °C
Doubling time (min)	120	90	118	123
SD	±13 min	±3 min	±10 min	±6 min

Table 3: Fractions of cells in G₁, S and G₂+M phases for WT and *cdc6-1* strains and their calculated duration at 25°C or 28°C

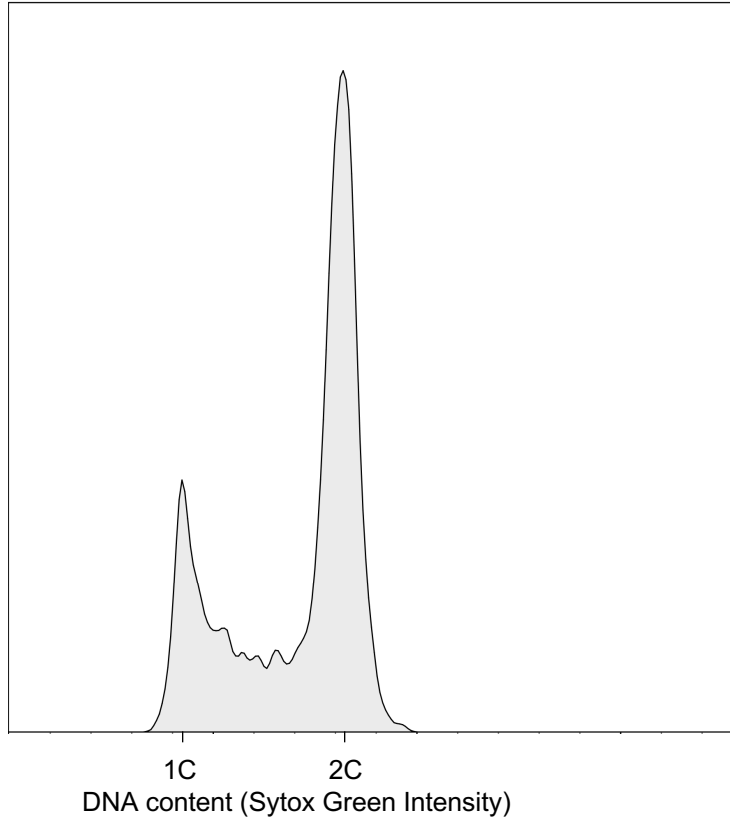
	WT				<i>cdc6-1</i>			
	25 °C		28 °C		25 °C		28 °C	
	Percent	Duration (min)	Percent	Duration (min)	Percent	Duration (min)	Percent	Duration (min)
G₁	27	32	18	16	13	16	10	12
S	29	35	32	29	28	34	28	34
G₂+M	44	53	50	45	59	71	62	77
SD G₁	±5	±4	±3	±2	±2	±1	±1	±1
SD S	±3	±6	±5	±5	±5	±7	±4	±4
SD G₂+M	±2	±7	±4	±3	±4	±4	±6	±4

Materials

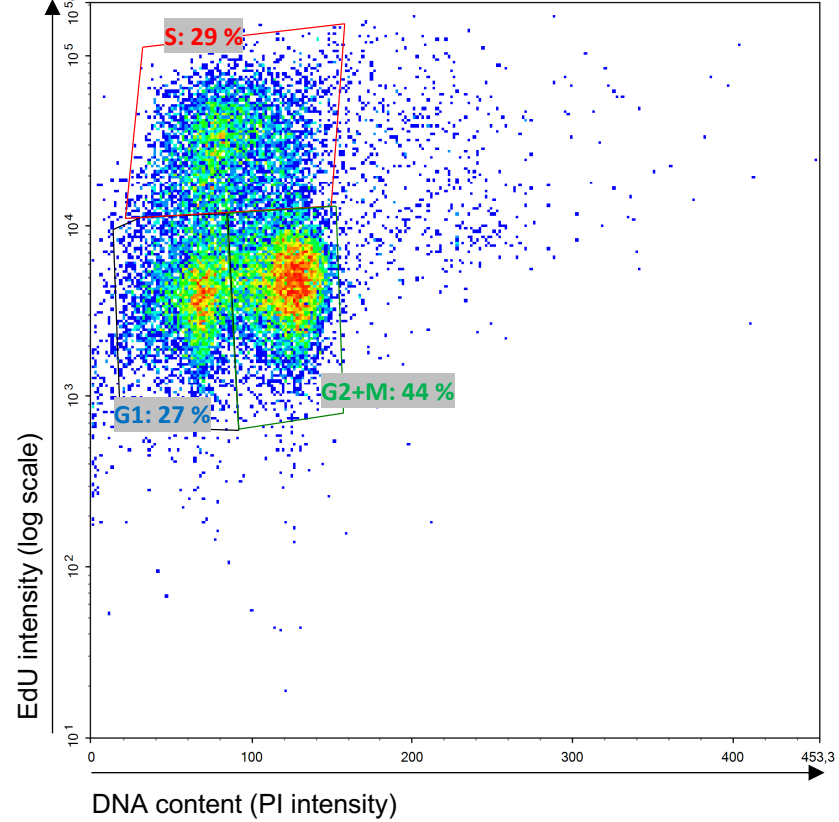
Name	Company	Catalog Number	Comments/Description
α -factor	Genescript	RP01002	
EdU (5-ethynyl-2'-deoxyuridine)	Carbosynth	NE08701	
Copper sulfate	Sigma	C1297	
L- ascorbic acide	Sigma	A4544	
Di-sulfo-Cyanine5 azide (Cy5 azide)	Interchim	FP-JV6320	Alternative to Alexa647-Azide
Dy-530 azide	Dyomics	530-10	
DAPI	Sigma	D9542	
Sytox Green	Invitrogen	S-7020	
Propidium iodide	Sigma	P4864	
Bovine Serum Albumin (BSA)	Euromedex	04-100--812-E	
Ethanol absolu	Carlo Erba reagents	P013A10D16	or equivalent
Proteinase K	Euromedex	EU0090	
Rnase	SIGMA	R5000	
Equipment	Company	Catalog Number	Comments/Description
Shaking water bath	Julabo	SW22	or equivalent
Shaking incubator	Infors	444-4230	or equivalent
Wide-field microscopy	Leica	THUNDER Imager	or equivalent
Flow cytometer	Agilent	NovoSampler Pro	
Cell counter	OLS	CASY	
Sonicator	Sonics	Vibra cell	

Fig.1: Representative FACS profiles for WT cells grown at 25 °C

A



B



C

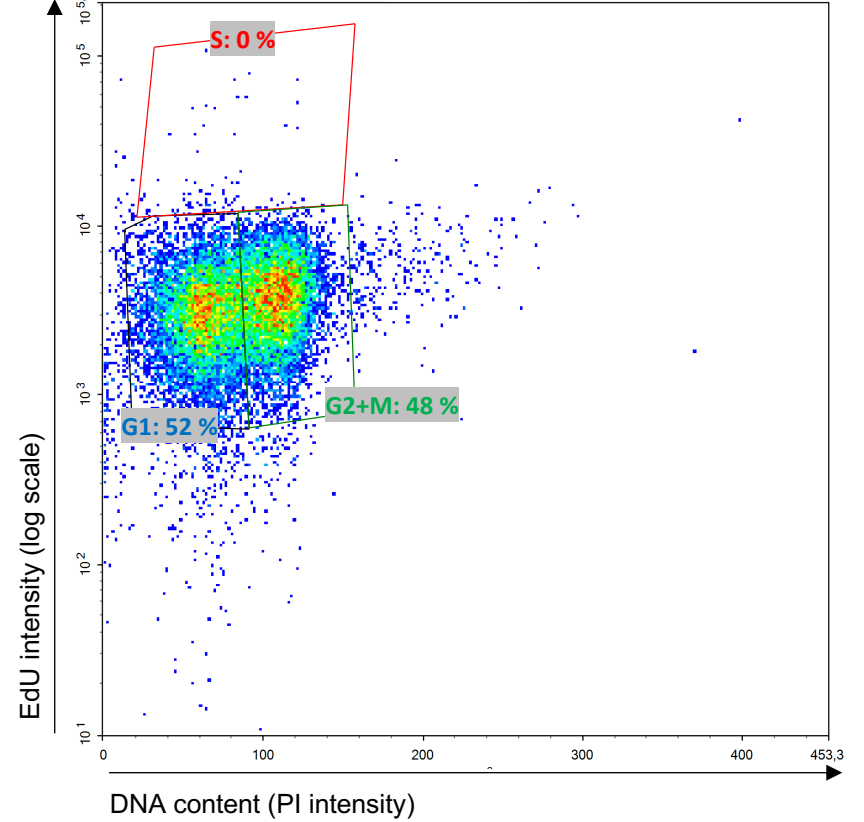
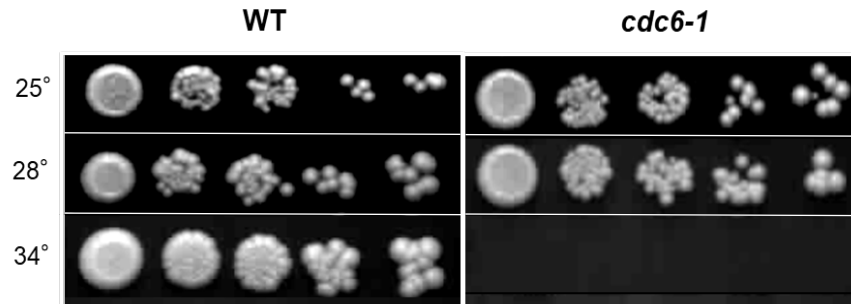
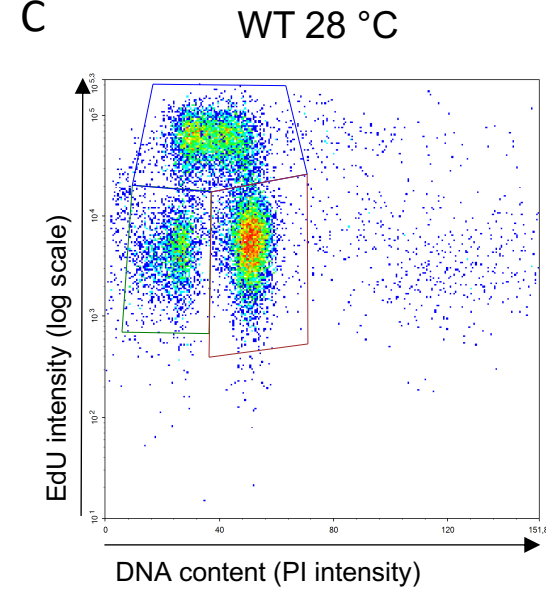


Fig.2: Cell cycle distribution in asynchronous WT and *cdc6-1* cell populations

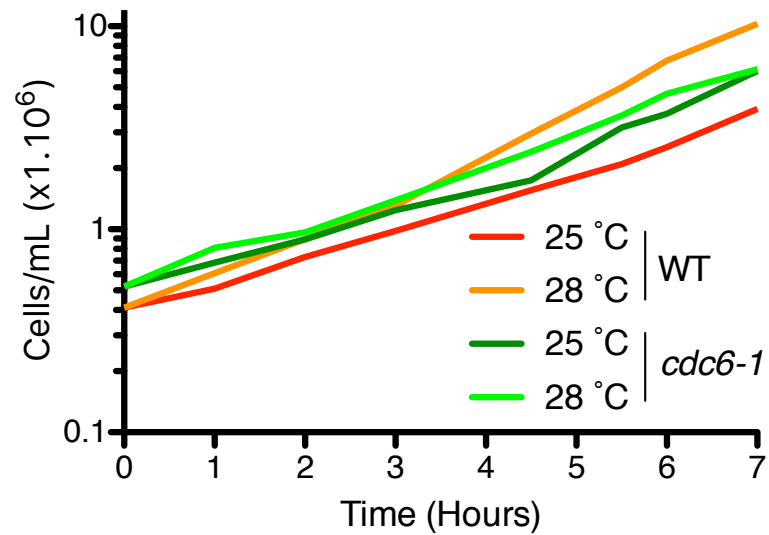
A



C



B



D

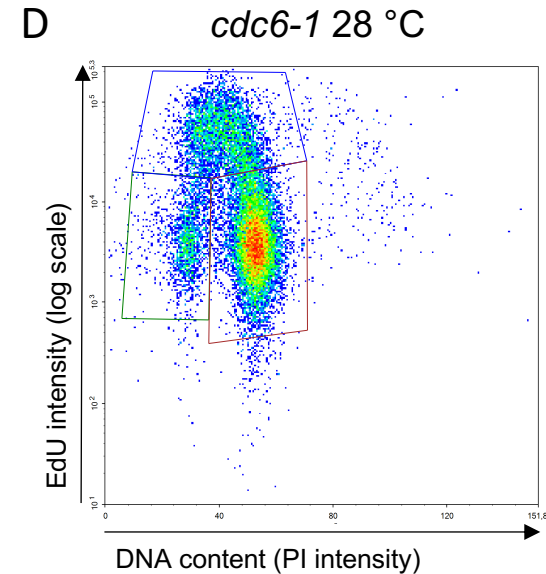


Figure 3: S-phase duration on synchronized WT cells

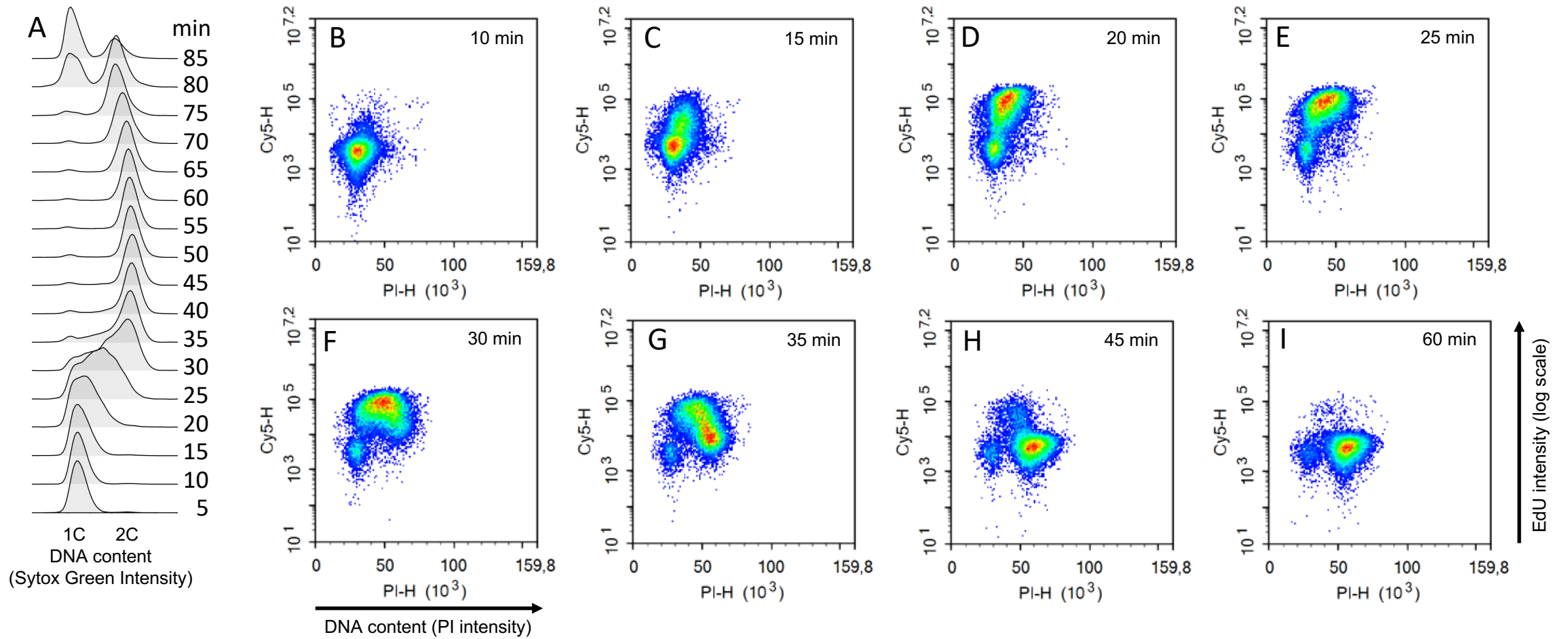
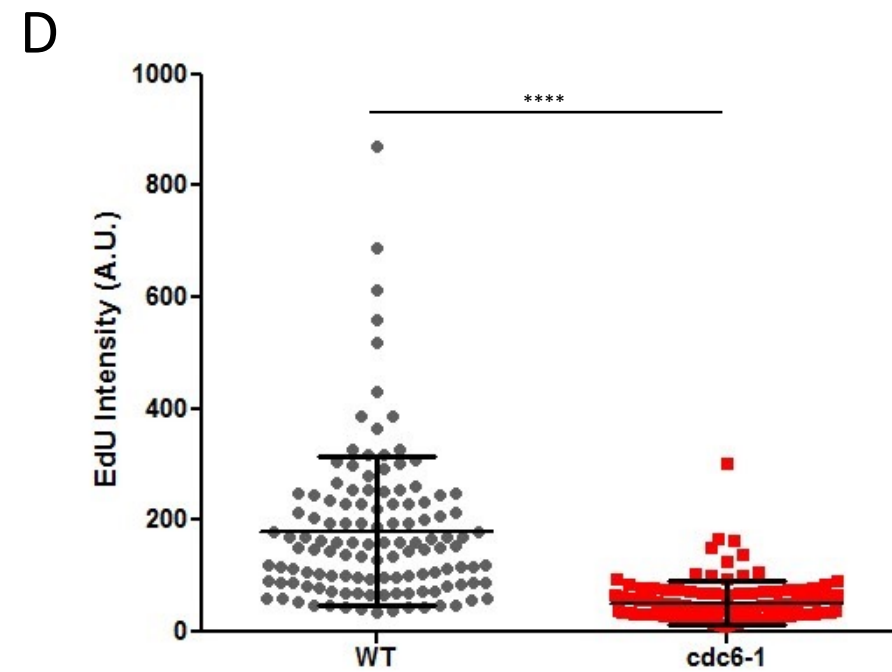
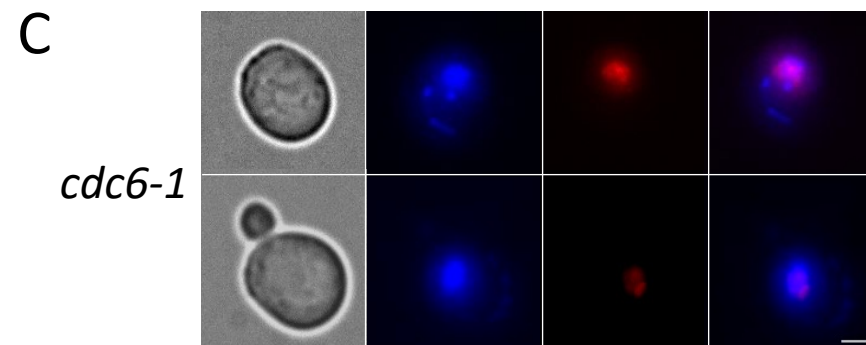
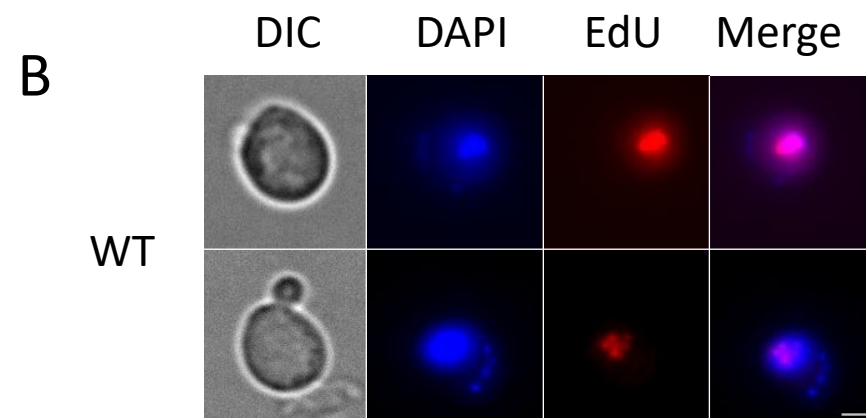
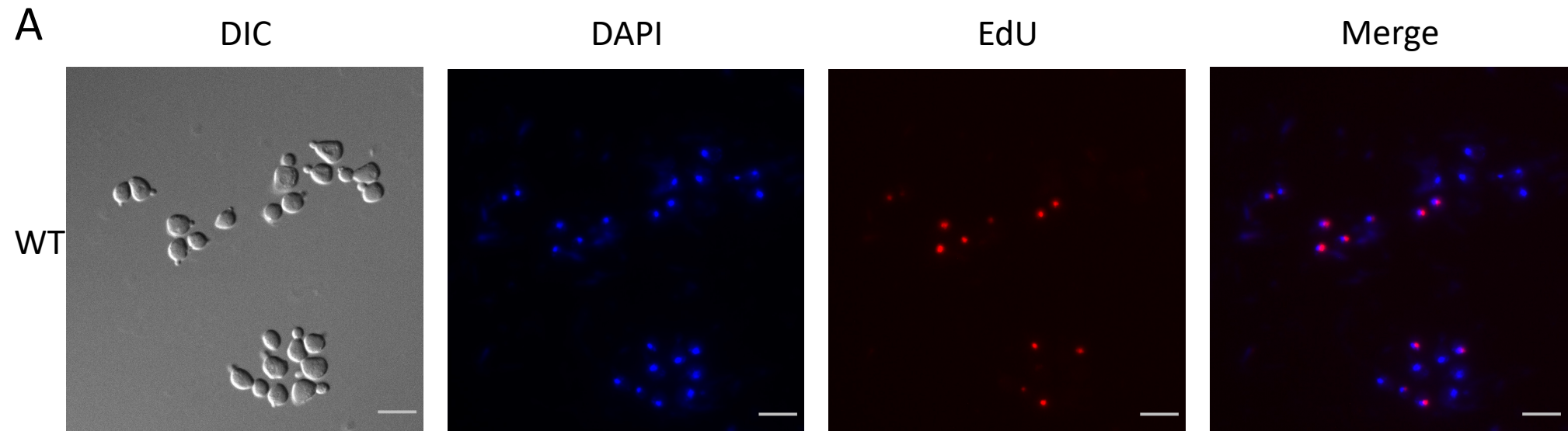
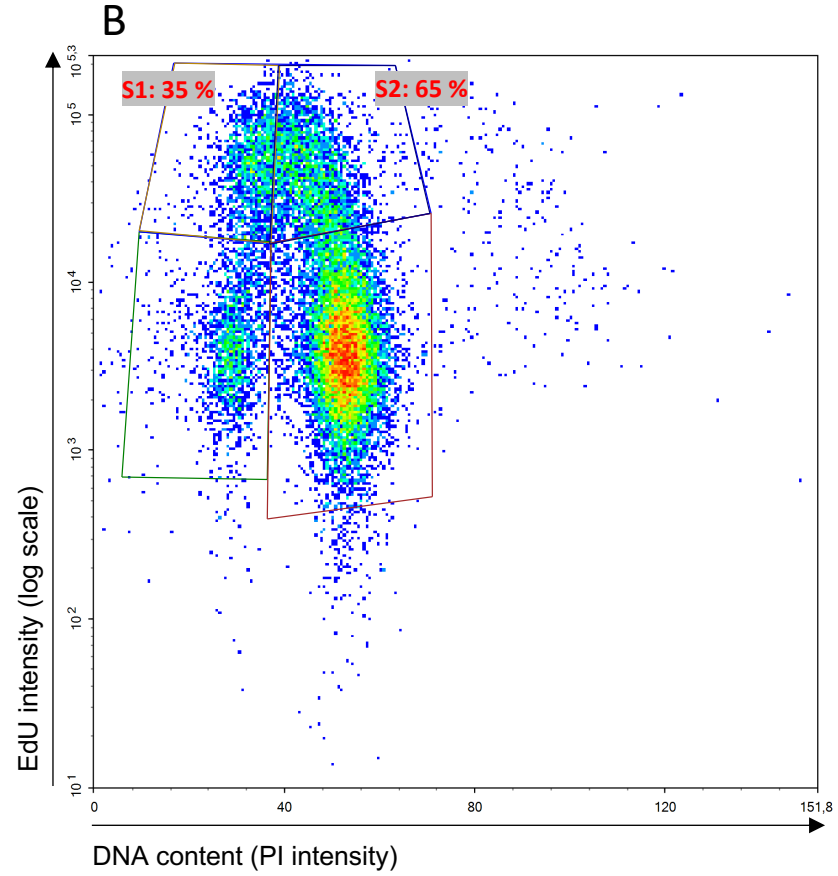
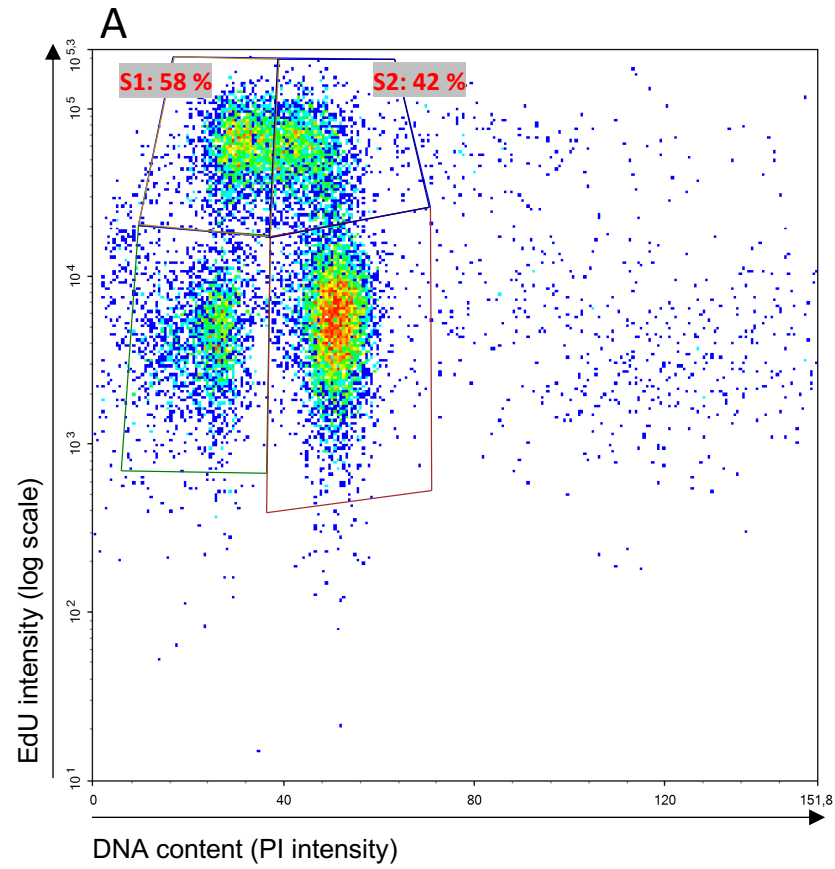


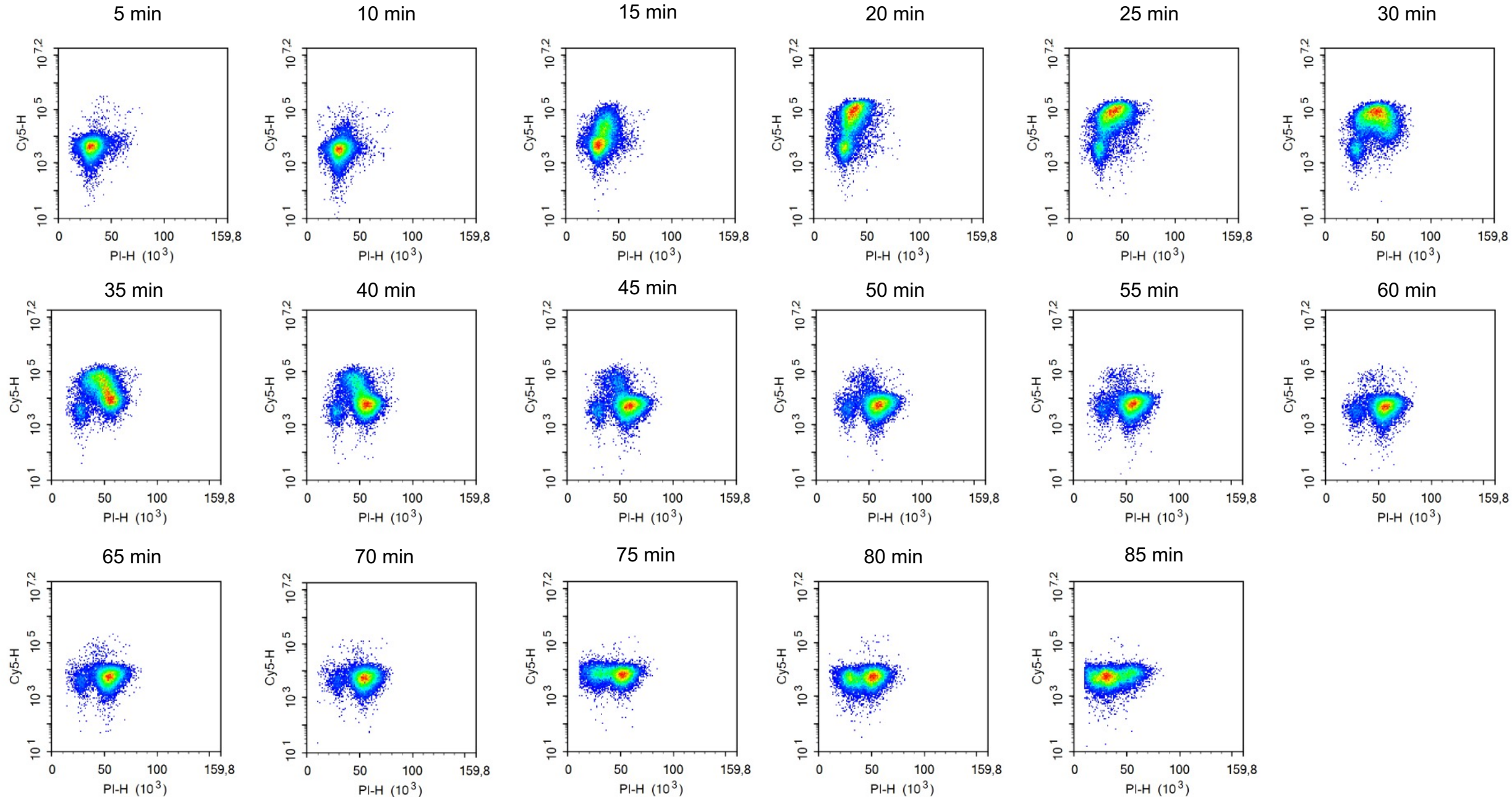
Fig.4: EdU detection and quantification by microscopy



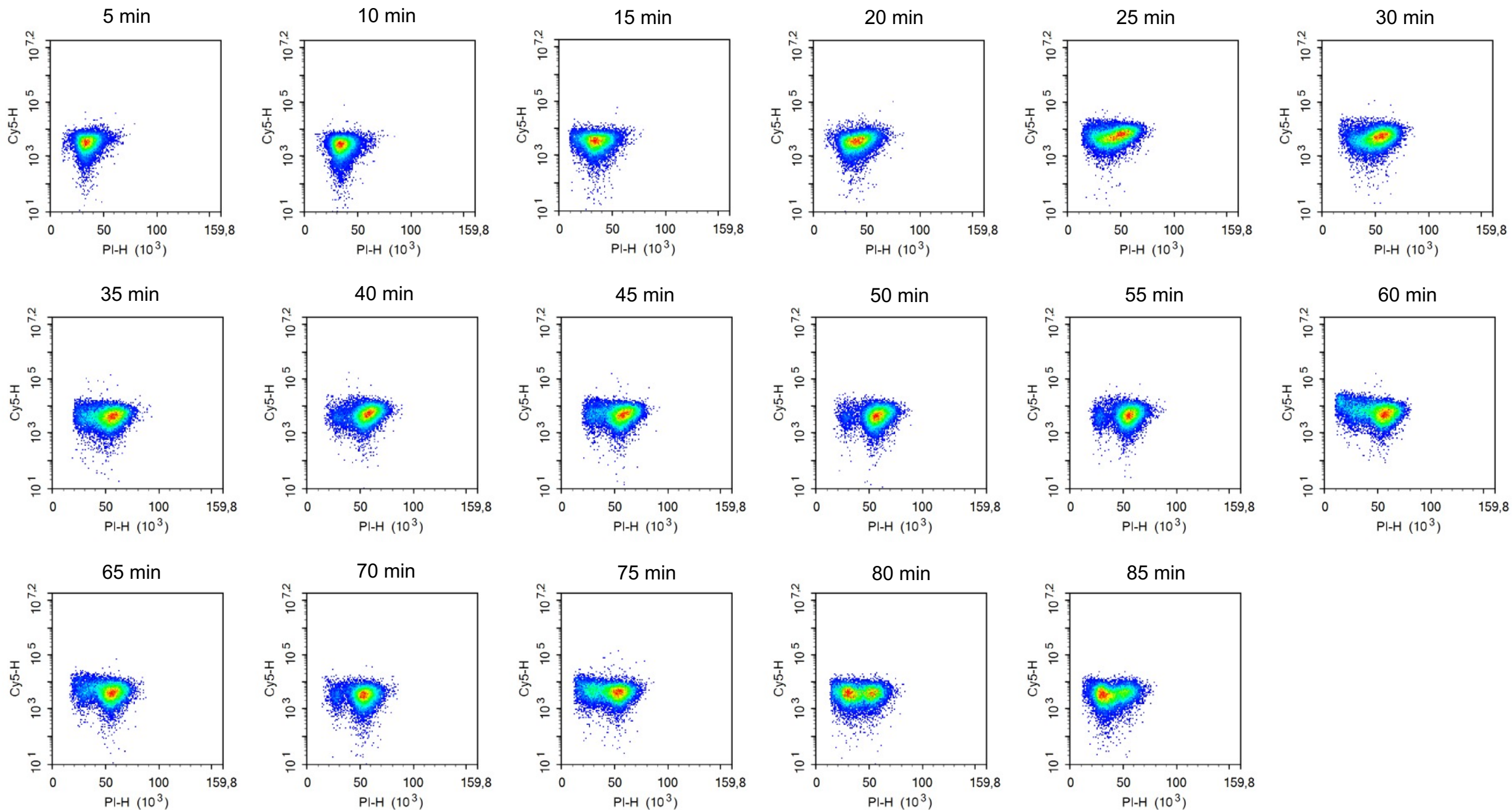
Supplemental figure 1: Fraction of cells in early and late S phase in WT and *cdc6-1* cells grown at 28 °C

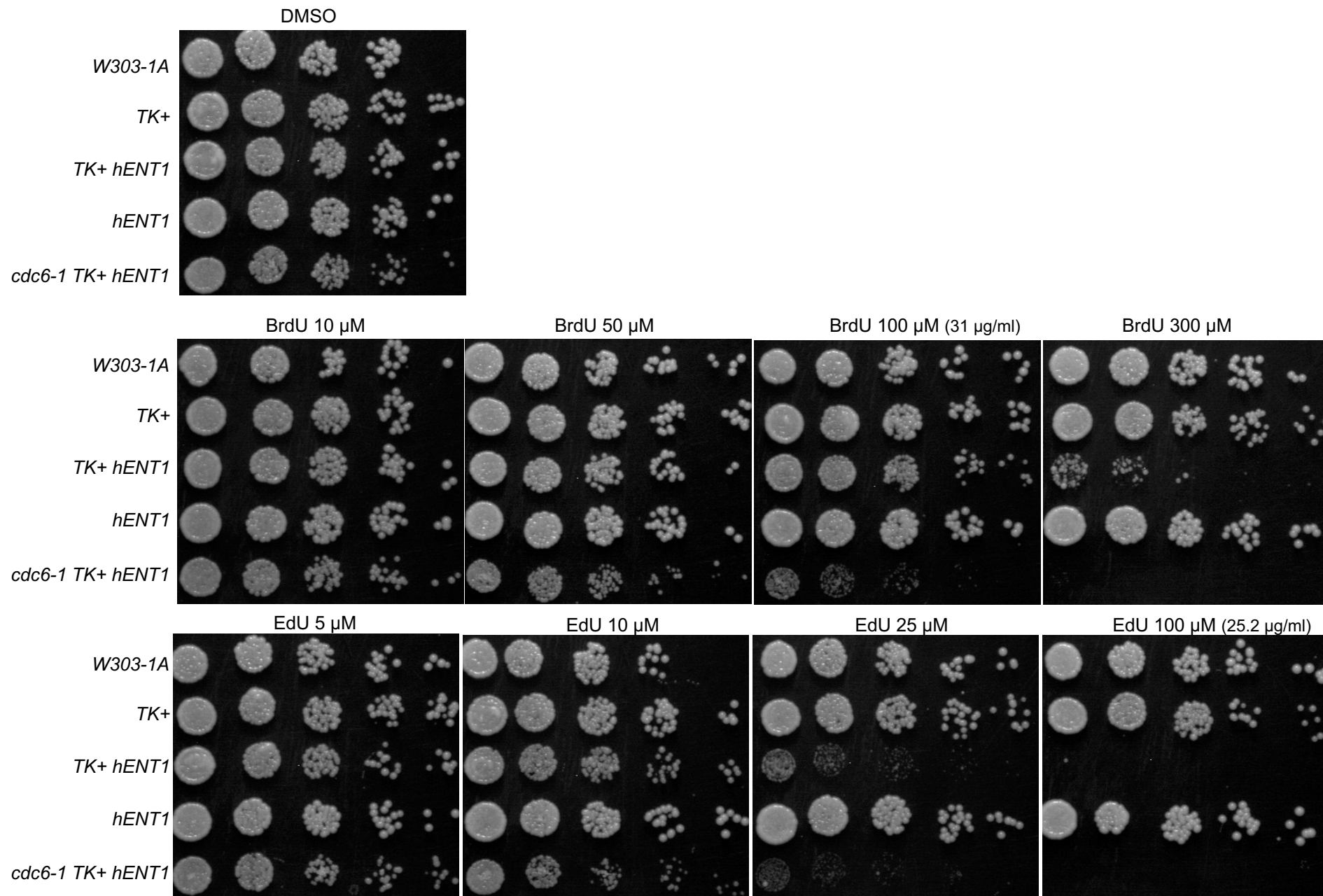


Supplemental Figure S2: Cell cycle progression of EdU pulse-labelled cells after release from G1 arrest visualized by EdU-PI bivariate FACS.



Supplemental Figure S3: Cell cycle progression of DMSO treated cells after release from G1 arrest visualized by EdU-PI bivariate FACS.





Supplemental Figure 4: BrdU and EdU toxicity in TK *hENT1* yeast cells.