



Title	Subtle changes in host cell density cause a serious error in monitoring of the intracellular growth of <i>Chlamydia trachomatis</i> in a low-oxygen environment: Proposal for a standardized culture method
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30 **Abstract**

31 We monitored *Chlamydia trachomatis* growth in HeLa cells cultured with either DMEM or
32 RPMI medium containing 10% FCS under 2% or 21% O₂ conditions for 2 days. Bacterial
33 numbers, host cell numbers, and fibrosis-related gene expression in the host cells were
34 estimated by an inclusion forming unit assay, a cell counting assay, and a PCR array,
35 respectively. In contrast to RPMI, bacterial growth under low oxygen conditions in DMEM
36 rapidly decreased with increasing host cell density. The addition of supplements (glucose,
37 glutamine, vitamin B12, D-biotin, non-essential amino acids, glutathione) to the media had
38 no effect. The growth of host cells in DMEM under low oxygen conditions rapidly
39 decreased, although the cells remained healthy morphologically. Furthermore, the
40 downregulation of 17 genes was observed under low oxygen in DMEM. Whereas no effect
41 on bacterial growth was observed when culturing in RPMI medium at low oxygen, and the
42 downregulation of three genes (*CTGF*, *SERPINE1*, *JUN*) was observed following bacterial
43 infection compared with the uninfected control cells. Thus, our findings indicate the need
44 for carefully selected culture conditions when performing experiments with *C. trachomatis*
45 under low-oxygen environments, and RPMI (rather than DMEM) is recommended when a
46 low host cell density is to be used, proposing the major modification of cell culturing
47 method of *C. trachomatis* in a low-oxygen environment.

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50 **Keywords:** *Chlamydia trachomatis*, low-oxygen environment, host cell density, RPMI,

51 DMEM, CTGF

52

53 **1. Introduction**

54 Obligate intracellular bacterium *Chlamydia trachomatis* is the leading cause of
55 bacterial sexually transmitted diseases worldwide, with an estimated 100 million
56 chlamydial infections detected annually (Ziklo et al., 2016; World Health Organization
57 2011). Such infections are often asymptomatic in women (Imai et al., 2010; Haggerty et al.,
58 2010; Qayum et al., 2000) and can therefore be left untreated, resulting in ascending
59 infection with fibrosis, which is responsible for ductal obstruction, pelvic inflammatory
60 disease, and infertility (Vodstreil et al., 2015; Siracusano et al., 2014; Da Ros and Schmitt,
61 2008). It is well known that a low-oxygen environment is required for the induction of
62 fibrosis (Menon et al., 2015), although whether infection is involved in the induction
63 remains unknown. However, accumulating evidence suggests that a low-oxygen
64 environment plays a central role in stimulating host cell signaling pathways involved in
65 metabolism and inflammation, which are responsible for initiating fibrosis via stabilizing
66 hypoxia-inducible factor 1 α (HIF-1 α), an oxygen sensor in mammalian cells (Semenza et
67 al., 2010; Rupp et al., 2007; Kim et al., 2006). It is therefore reasonable to suggest that the
68 intracellular growth of *C. trachomatis* may modulate the signal pathway governed by
69 HIF-1 α in human fallopian tube cells, facilitating fibrosis.

70 *C. trachomatis* possesses a unique developmental cycle, consisting of the elementary
71 (EB) and reticulate body (RB) forms, differentiated from EB to RB (or re-differentiated
72 from RB to EB) in inclusion bodies surrounded by the membrane vesicle (Rockey and
73 Matsumoto, 2000). After infection, the maturation process in infected host cells is known to

74 require actin remodeling (Dunn and Valdivia, 2010; Jewett et al., 2006; Balañá et al., 2005),
75 lipid metabolism (Stehr et al., 2013; Elwell and Engel, 2012; Saka and Valdivia, 2012), and
76 inflammatory responses (Ziklo et al., 2016; Entrican et al., 2014; Cochrane et al., 2010).
77 However, chlamydial studies in a low-oxygen environment are limited and difficulties are
78 encountered in even assessing its intracellular growth. However, a few studies have shown
79 that *C. trachomatis* adapts well to a low-oxygen environment, such as that found in the
80 urogenital tract of women (Jerchel et al., 2014; Shima et al., 2011; Juul et al., 2007; Peters
81 et al., 2005). Meanwhile, it is well recognized in low-oxygen environment that cellular
82 metabolism such as glycolysis prompting ATP generation required for chlamydial growth
83 can be up-regulated by stabilizing HIF-1 α , which is a master transcriptional factor in the
84 environment (Palmer and Clegg, 2014; Papandreou et al., 2006). Therefore, the cells left
85 into culture under low-oxygen condition may rapidly consume substances in the cultures,
86 inevitably responsible for deteriorating chlamydia growth into limited culture medium.
87 Thus, accurate evaluation of chlamydial growth in a low-oxygen environment is a crucial
88 issue for understanding chlamydial dynamics in the urogenital tract, although it has not
89 been standardized among researchers.

90 In the present study, we carefully monitored *C. trachomatis* growth in immortal
91 human epithelial HeLa cells and fibrosis-related host responses in a low-oxygen
92 environment. We showed that in contrast to normal atmospheric conditions, subtle changes
93 in host cell density can lead to serious errors in the monitoring of the intracellular growth of
94 *C. trachomatis* in a low-oxygen environment, proposing the major modification of cell

95 culturing method of *C. trachomatis* in a low-oxygen environment.

96

97

98 **2. Materials and methods**

99 The epithelial cell line HeLa was purchased from ATCC (Manassas, VA, USA). The
100 cell line was maintained at 37°C in 5% CO₂ in DMEM (Sigma) containing 10%
101 heat-inactivated fetal calf serum (FCS), 10 µg/ml gentamycin, 10 µg/ml vancomycin, and 1
102 µg/ml amphotericin B (Sigma), according to a previously published protocol (Roblin et al,
103 2007). *C. trachomatis* D/UW3 Cx strain (VR-885) was purchased from ATCC, and
104 propagated as described previously (Kubo et al., 2012). The number of infectious progeny
105 for *C. trachomatis* was determined as inclusion forming units (IFU) by counting chlamydial
106 inclusions formed in HeLa-2 cells using a fluorescein isothiocyanate (FITC)-conjugated
107 monoclonal anti-*Chlamydia* antibody specific for *Chlamydia* lipopolysaccharide (with
108 Evans Blue) (Denka Seiken) (Kubo et al., 2012).

109 HeLa cells were adjusted to a concentration of 2×10^5 cells/well and simultaneously
110 infected with *C. trachomatis* at a multiplicity of infection (MOI) of 2 by centrifugation for
111 450×g at room temperature. After washing to remove non-infecting bacteria, the cells at a
112 concentration of 1 to 20×10^4 cells/well were incubated with DMEM (D6046) or RPMI
113 (R8758) containing 10% FCS, gentamycin (10 µg/ml), vancomycin (10 µg/ml), and
114 amphotericin B (0.5 µg/ml) for 2 days at 37°C under conditions of either 21% or 2% O₂.
115 For some experiments, cells were cultured in DMEM plus supplements (vitamin B12,

116 D-biotin, non-essential amino acids, glutamine, and glucose) (See Table S1) or
117 cycloheximide (final concentration 2 $\mu\text{g/ml}$). Cells were harvested and viability was
118 determined by a cell counting assay (see below) and EB numbers were determined by an
119 IFU assay. A low-oxygen environment (2% O_2) was created using a dedicated chamber
120 MIC-101 (Billups-Rothenberg). The mixed gas containing 2% and 21% O_2 used as a
121 control consisted of 2% O_2 , 5% CO_2 , and 93% N_2 or 21% O_2 , 5% CO_2 , and 74% N_2 ,
122 respectively. In addition, pH values in culture mediums were also monitored in some
123 experiments with a hand-type's pH meter, twinpH (Horiba Ltd.); the values was measured
124 less than 5 min after removed from the chamber to minimize affect of atmosphere.

125 To determine the morphology of chlamydial inclusions, cells stained with specific
126 antibodies were observed using a conventional [IX71 (Olympus)] or a confocal laser
127 microscope [LSM510 (Carl Zeiss Japan Group)] (Kubo et al., 2012). Cell Counting Kit-8
128 (Dojindo) was used to determine cell viability according to the protocol described in the
129 manufacturer's instruction. Briefly, water-soluble tetrazolium salt, WST-8, is reduced by
130 dehydrogenase activity in cells to give a yellow colored formazan dye, which is soluble in
131 tissue culture media. The intensity of the color is measured at $\text{OD}_{450\text{ nm}}$ as an indicator of
132 cell viability.

133 The Human Fibrosis RT² Profiler PCR Array kit equipped with 84 genes (Table S2)
134 was used to determine the expression of fibrosis-related genes (Qiagen), according to the
135 protocol described in the manufacturer's instructions. In brief, cells (20×10^4 cells/well in
136 DMEM; 5×10^4 cells/well in RPMI) with or without *C. trachomatis* infection were

137 incubated for 24 h under 2% or 21% O₂ conditions. After incubation, the cells were
138 collected and then total RNA was extracted using the High Pure RNA Isolation Kit (Roche).
139 An RT reaction was performed on 500 ng of RNA and the synthesized cDNA was analyzed
140 by Applied Biosystems StepOne Plus real-time PCR system to determine the changes in
141 fibrosis-related genes. The values were expressed as relative fold changes compared with
142 the control array. According to the protocol, more than or less than two-fold changes
143 compared with the control were defined as meaningful changes. Each of the values was
144 expressed as the average of two independent experiments.

145 Comparison of the total IFU numbers between oxygen conditions and between
146 experimental groups was conducted using a Bonferroni/Dunn test and Student's *t* test,
147 respectively. A *p*-value of <0.01 was considered significant.

148

149

150 **3. Results**

151 First, by assessing bacterial numbers using an IFU assay and confocal fluorescence
152 microscopy observations, we compared the growth of *C. trachomatis* (MOI 2) in HeLa
153 cells (5×10^4 cells/well of a 24-well plate) cultured either in DMEM or RPMI medium in a
154 21% or 2% oxygen environment (Fig. 1A). The growth of *C. trachomatis* was gradually
155 dampened in DMEM compared with RPMI medium, in particular in the 2% oxygen
156 environment 48 h after infection (Fig. 1B). However, even when cultured in DMEM, there
157 was no significant difference in inclusion size between the 21% and 2% oxygen

158 environments (Fig. 1C). Furthermore, all of the cells appeared healthy morphologically,
159 although the growth of HeLa cells was slowed under hypoxic conditions compared with
160 conditions of 21% O₂ (Fig. S1). Thus, although bacterial growth initially appeared the same,
161 there was a significant difference in terms of bacterial numbers, with numbers being
162 decreased particularly in DMEM under low-oxygen conditions.

163 In contrast to RPMI medium, DMEM has a low glucose concentration and lacks
164 certain components such as glutamine, non-essential amino acids, and some vitamins
165 (Table S1). We therefore assessed whether supplementation of these components into
166 DMEM could restore bacterial growth to the levels observed in RPMI medium under low
167 oxygen (2% O₂) conditions (Fig. 2A). However, regardless of supplementation, bacterial
168 growth did not recover to the levels observed under 21% O₂ conditions (Fig. 2B). Despite
169 this, it was noted that in contrast to supplementation of DMEM with vitamin B12, D-biotin,
170 and non-essential amino acids or glutamine alone, the addition of glucose, which is the
171 most effective energy source, had an impact on bacterial growth (Fig. 2C). This result
172 indicated that the lack of bacterial growth in DMEM under low oxygen was likely due to
173 the rapid starvation of glucose arresting host cell growth, presumably in turn resulting in a
174 decrease in bacterial intracellular growth because of the requirement for ATP energy from
175 the host cells (Rockey and Matsumoto, 2000). This implied that changing the host cell
176 density may have an impact on bacterial growth in HeLa cells in low oxygen environments.

177 To investigate the effect of host cell density changes, we compared the growth of *C.*
178 *trachomatis* when cultured with host cells at a range of seeding concentrations (from 1×10^4

179 to 20×10^4 cells/well in a 24-well plate) under conditions of 21% or 2% O₂ (Fig. 3A). In
180 contrast to growth in RPMI medium, bacterial growth in DMEM under low oxygen (2%
181 O₂) conditions was significantly decreased (by up to 10,000 times) with increasing host cell
182 density (Fig. 3B). Meanwhile, morphological observations and cell counts revealed that
183 even under low-oxygen conditions at high-cell density, the viability of host cells was
184 maintained during culturing despite the decrease in growth speed (Fig. 3C, Fig. S2). The
185 addition of cycloheximide, a protein synthesis inhibitor specific to mammalian cells that
186 inhibits cellular replication, recovered the inclusion size of the bacteria back to normal
187 levels (Fig. 3C, with cycloheximide), supporting host cell density as a critical factor for
188 bacterial intracellular growth under low-oxygen conditions. These results indicated that
189 unlike under normal oxygen conditions, subtle changes to host cell density under
190 low-oxygen conditions may be detrimental to the intracellular growth of *C. trachomatis*
191 particularly in DMEM, presumably causing serious errors potentially affecting host
192 responses; however, this has been overlooked when monitoring the intracellular growth of
193 *C. trachomatis* in low-oxygen environments. In addition, although pH values in cultures
194 were monitored during 48h after incubation, no significant changes was observed
195 regardless of either distinct medium or O₂ concentration (Fig. S3). The results indicated
196 effect of pH changes on *C. trachomatis* growth was minimal in either case.

197 As mentioned above, although several studies had shown decreased growth of *C.*
198 *trachomatis* in immortalized human epithelial cells (HeLa or HEP-2 cells) cultured in
199 DMEM under low-oxygen conditions (Jerchel et al., 2014; Shima et al., 2011; Juul et al.,

200 2007), subtle changes in host cell density seeding with *C. trachomatis* in these experiments
201 may have been overlooked and may lead to serious errors in the monitoring the bacterial
202 intracellular growth or the evaluation of gene expression changes in host cells. To
203 investigate this, we employed a PCR array of 84 genes (Table S2) to evaluate the
204 expression of genes associated with fibrosis in HeLa cells cultured in DMEM at high host
205 cell density (20×10^4 cells/well) and RPMI medium at optimal host cell density (5×10^4
206 cells/well) under conditions of either 21% or 2% O₂ (Fig. 4A). Compared with gene
207 expression under 21% O₂ conditions (HeLa cells: 20×10^4 cells/well) (without *C.*
208 *trachomatis* infection), 17 genes were downregulated under 2% O₂ conditions (HeLa cells:
209 20×10^4 cells/well) (without *C. trachomatis* infection) in DMEM (Fig. 4B). However, no
210 changes in gene expression were observed when cells were cultured in RPMI medium
211 (HeLa cells: 5×10^4 cells/well) (with or without *C. trachomatis* infection) (Fig. 4C–E).
212 These results clearly indicated the DMEM containing a high host cell density caused the
213 artificial downregulation of host cell genes, resulting in a serious mistake in translation. By
214 contrast, when cells were cultured in RPMI medium, no effect on bacterial growth at low
215 oxygen was observed and we found that bacterial infection caused the downregulation of
216 three genes, *CTGF*, *SERPINE1*, and *JUN*, all of which are critical factors regulating fibrosis,
217 when compared with uninfected control cells (Fig. 4E). These data revealed that when
218 performing *C. trachomatis* experiments under low-oxygen conditions, medium selection
219 and host cell density are critical factors for the correct monitoring of chlamydial dynamics.
220

221

222 **4. Discussion**

223 *C. trachomatis* is the most common pathogen causing sexually transmitted infections
224 worldwide (Qayum et al., 2013; World Health Organization 2011; Haggerty et al., 2010;
225 Imai et al., 2010). The symptoms of infection are often minimal in females and therefore
226 remain untreated, resulting in chronic inflammation responsible for pelvic inflammatory
227 disease or infertility with fibrosis (Vodstrcil et al., 2015; Siracusano et al., 2014; Da Ros
228 and Schmitt, 2008). *C. trachomatis* adapts well to a low-oxygen environment, such as that
229 found in the urogenital tract of women (Entrican et al., 2014; Jerchel et al., 2014; Shima et
230 al., 2011; Juul et al., 2007; Sommer et al., 2001). Therefore, accurate evaluation of
231 chlamydial growth in a low-oxygen environment is a crucial issue for understanding
232 chlamydial dynamics in the urogenital tract. Our findings showed that subtle changes in
233 host cell density can cause serious errors in the monitoring of the intracellular growth of *C.*
234 *trachomatis* in a low-oxygen environment.

235 It was clear that during a 2-day culture, the viability of HeLa cells was
236 morphologically maintained under low-oxygen conditions regardless of the bacterial
237 infection. However, the speed of host cell growth decreased compared with cells cultured in
238 a normal oxygen environment. Recent studies have revealed that HIF-1, which is a critical
239 factor controlling cellular homeostasis in low-oxygen environments (Palmer and Clegg,
240 2014), reduces the induction of pyruvate dehydrogenase kinase-1 (PDK-1) in response to
241 low-oxygen conditions (Kroening et al., 2009; Papandreou et al., 2006). Specifically,

242 PDK-1 phosphorylates and then inactivates pyruvate dehydrogenase E1 α , which converts
243 pyruvate to acetyl-coenzyme A, resulting in blocking the entry of pyruvate into the
244 tricarboxylic acid cycle (Kroening et al., 2009; Papandreou et al., 2006). It has also been
245 established that HIF-1 induction suppresses mitochondrial ROS production under hypoxic
246 conditions, preventing cell death caused by apoptosis (Minet et al., 2000). Thus, although
247 detrimental, the influence of low-oxygen on host cell viability is unexpectedly minimal, as
248 the cells subtly adjust to the shortage in energy source (ATP) by slowing their growth. It is
249 therefore inevitable that the bacterial growth rate would decrease in response to a subtle
250 increase in the number of seeded cells as a result of the consumption of more ATP.

251 Studies to evaluate chlamydial dynamics in low-oxygen environments have
252 concluded that *C. trachomatis* growth is similar to that under normal atmospheric
253 conditions (Entrican et al., 2014; Jerchel et al., 2014; Shima et al., 2011; Juul et al., 2007;
254 Sommer et al., 2001). Whether subtle changes in host cell density seeding and media type
255 can influence chlamydial dynamics under low-oxygen conditions remains to be investigated.
256 While host cells remain healthy even under low-oxygen conditions, chlamydial growth is
257 gradually blocked when the number of seeded host cells is increased. Surprisingly, this
258 decrease in bacterial growth rate was even more pronounced during culturing with DMEM
259 compared with RPMI medium. In contrast to RPMI medium, DMEM has a low glucose
260 concentration and lacks certain components such as glutamine, non-essential amino acids,
261 and some vitamins (See Table S1). Supplementation of DMEM with these components
262 partially rescued bacterial intracellular growth (although this was not statistically

263 significant), indicating that seeding host cell density is a crucial factor for propagating *C.*
264 *trachomatis* under low-oxygen conditions.

265 As mentioned above, during the 2-day culture required for *C. trachomatis* maturation,
266 the HeLa cells appeared healthy morphologically regardless of the oxygen conditions and
267 the deterioration of bacterial growth at a host cell density of 5 to 20×10⁴ cells per well
268 (24-well plate) may therefore have gone unnoticed. However, regarding gene expression
269 associated with fibrosis, a serious error was detected due to the downregulation of 17 genes
270 only under conditions of 2% oxygen in DMEM. Thus, our findings emphasize the need to
271 carefully select appropriate culture conditions for chlamydial experiments under
272 low-oxygen conditions and we recommend the use of RPMI (rather than DMEM) media
273 accompanied by a low host cell density.

274 It has been reported that accumulation of CTGF, a factor responsible for connective
275 tissue growth (Kroening et al., 2009; Ito et al., 1999), occurred in low-oxygen tissues
276 activating matrix proteases such as TIMPS, which in turn resulted in an increase in
277 fibroblasts producing extracellular matrix proteins (Kroening et al., 2009; Ito et al., 1999).
278 By performing PCR array experiments under optimal culture conditions using RPMI, we
279 found that *C. trachomatis* infection caused the downregulation of three genes (*CTGF*,
280 *SERPNE1*, and *JUN*). Since it is well known that cancer-associated fibroblasts play a key
281 role in cancer progression by contributing to invasion, metastasis, and angiogenesis in a
282 CTGF-dependent manner (Ren et al., 2015), the downregulation of these three genes may
283 lead to the accumulation of *C. trachomatis*-infected cells in tissues such as the fallopian

284 tube, likely resulting in the exacerbation of inflammation. A previous study revealed that in
285 models of persistence under normal oxygen conditions, chlamydial infection induced
286 expression of the *CTGF*, *EGR-1*, and *ETV4* genes associated with fibrosis (Peters et al.,
287 2005), which supports our data.

288 Taken together, we conclude that in contrast to normal atmospheric conditions, subtle
289 changes in host cell density cause serious errors in the monitoring the intracellular growth
290 of *C. trachomatis* in low-oxygen environments, recommending the use of RPMI (rather
291 than DMEM) and low host cell densities in such experiments. Our findings highlight an
292 important issue that must be addressed when accurately monitoring the intracellular growth
293 of *C. trachomatis* in low-oxygen environments, proposing the major modification of cell
294 culturing method of *C. trachomatis* in a low-oxygen environment.

295

296

297 **Abbreviations**

298 DMEM: Dulbecco's modified Eagle's medium; RPMI: Roswell Park Memorial Institute
299 medium; IFUs: inclusion-forming units; Glu: glucose; Gln: glutamine; GG: glucose +
300 glutamine; PCR: polymerase chain reaction; RT: reverse transcriptase

301

302 **Ethics**

303 The study reported in this manuscript did not involve any human participants, human data,
304 human tissue, data on specific individuals, or animal experiments.

305

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311

312 **Competing interests**

313 The authors declare that they have no competing interests.

314

315 **Author contributions**

316 Conceived and designed the project: HY. Analysis and confirmation: ST, JM, HY. Imaging:

317 SN. Critical editing: JM, TO, HY. Writing of the manuscript: HY. All authors read and
318 approved the final manuscript.

319

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324

325 **Conflicts of interest:** none.

326

327

328

329 **Supplementary data**

330 **Table S1.** Comparison of the medium formulation between DMEM(D6046) and
331 RPMI(R8758)

332

333 **Table S2.** List of the genes loaded onto the Human Fibrosis RT² Profiler PCR Array

334

335 **Fig. S1.** Comparison of the viability and morphological features of HeLa cells infected with
336 *C. trachomatis* cultured under hypoxic (2% O₂) and normal (21% O₂) conditions. The
337 bacteria were used to infect cells at a MOI of 2 (see the text). Cell viability was determined
338 using a Cell-counting assay kit (see the text). Phase-contrast images were captured at 600×.
339 White asterisks show representative inclusion bodies formed by *C. trachomatis* growth.
340 Comparisons of the cell numbers between 2% and 21% O₂ conditions were conducted
341 using a Student's *t* test. Black asterisks show statistical significance at $p < 0.05$. Experiments
342 were performed at least three times independently.

343

344 **Fig. S2.** Changes in the viability of HeLa cells grown under hypoxic (2% O₂) and normal
345 (21% O₂) conditions depending on the number of cells seeded. Cell viability was
346 determined using a Cell-counting assay kit (see the text). The experiments were performed
347 in 96-well plates (not 24-well plates) as this was more time efficient. Cell numbers
348 following seeding in 96-well plates were almost identical to those in 24-well plates.
349 Comparison of the cell numbers between 2% and 21% O₂ conditions were conducted using

350 a Student's *t* test. Asterisks indicate statistical significance at $p < 0.05$. Experiments were
351 performed at least three times independently.

352

353 **Fig. S3.** Changes of pH values in culture of HeLa cells with or without *C. trachomatis*
354 infection into DMEM and RPMI under hypoxic (2%O₂) and normal (21%O₂) conditions.
355 The bacteria were infected to HeLa cells (5×10^4 cells) at MOI 0.2. Each of the values were
356 expressed as average values plus SD into three experiments. Also, phase contrast images
357 (magnification, $\times 400$) show representative inclusion formations at 48h after infection
358 (White arrows).
359

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466 **Legends to figures**

467

468 **Fig 1. Dampening effect of DMEM on *C. trachomatis* growth in HeLa cells,**
469 **particularly under low-oxygen conditions.** Panel (A) shows the experimental design and
470 time course. Panel (B) shows the comparison of IFU values between 2% and 21% O₂
471 conditions in DMEM or RPMI media. Statistical analysis was conducted by a Student's *t*
472 test. Black stars indicate a significant difference ($p < 0.05$) between the two oxygen
473 conditions. Experiments were performed at least three times independently. Panel (C)
474 shows representative images of the inclusion bodies formed in *C. trachomatis*-infected
475 HeLa cells. White arrows show inclusion bodies. Scale bar: 5 μm.

476

477 **Fig 2. Addition of supplements recovered bacterial growth in HeLa cells cultured in**
478 **DMEM under low-oxygen conditions.** Panel (A) shows the experimental design and time
479 course. Panel (B) shows the comparison of IFU values between 2% and 21% O₂ conditions
480 following the addition of supplements (glucose, glutamine, vitamin B12, D-biotin,
481 non-essential amino acids, glutathione) lacking in DMEM but not RPMI medium.
482 Statistical analysis was conducted by a Student's *t* test. Black stars indicate a significant
483 difference ($p < 0.05$) between the two oxygen conditions. Experiments were performed at
484 least three times independently. Panel (C) shows a comparison of all sets of data. Statistical
485 analysis was conducted by a Bonferroni/Dunn test. Blue color indicates a significant
486 difference ($p < 0.05$) between the two sets.

487

488 **Fig. 3. In contrast to normal oxygen conditions, subtle changes in the host cell density**
489 **were detrimental to the intracellular growth of *C. trachomatis* in a low-oxygen**
490 **environment.** Panel (A) shows the experimental design and time course. Panel (B) shows
491 the comparison of IFU values between 2% and 21% O₂ conditions in DMEM or RPMI
492 media depending on changes in the number of cells seeded. Statistical analysis was
493 conducted by a Student's *t* test. Black stars indicate a significant difference ($p < 0.05$)
494 between two oxygen conditions. Panel (C) shows the phase-contrast images. White stars
495 show inclusion bodies. Squares surrounded by dashed lines are enlarged versions of the
496 images connected by arrows.

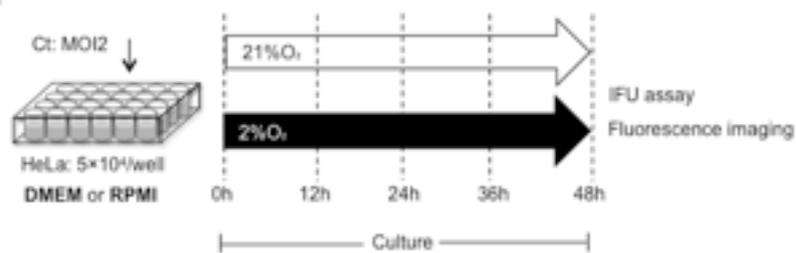
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498 **Fig. 4. PCR array data revealed that in contrast to RPMI medium, the artificial**
499 **downregulation of 17 fibrosis-associated genes occurred in DMEM under low oxygen**
500 **conditions.** Human Fibrosis RT² Profiler PCR Array kit equipped with 84 genes (Table S2)
501 was used to determine the expression of fibrosis-related genes (Qiagen), according to the
502 protocol described in the manufacturer's instructions (see the text). Panel (A) shows the
503 experimental design and time course. Panel (B) shows the changes in gene expression in
504 the HeLa cells (20×10^4 cells/well) (without infection) under 2% O₂ conditions compared
505 with 21% O₂ conditions in DMEM. Panel (C) shows the changes in gene expression in
506 HeLa cells (5×10^4 cells/well) (with infection) under 2% O₂ conditions compared with 21%
507 O₂ conditions in DMEM. Panel (D) shows the changes in gene expression in HeLa cells

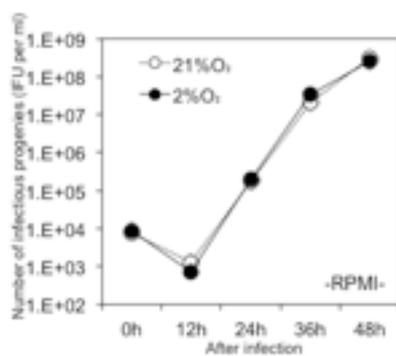
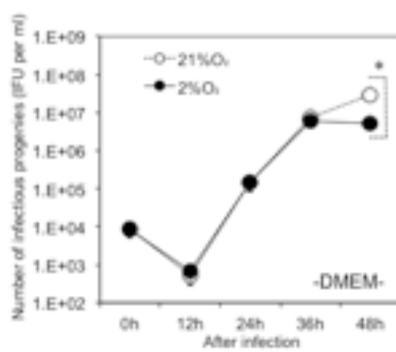
508 (5×10⁴ cells/well) (without infection) under 2% O₂ conditions compared with 21% O₂
509 conditions in RPMI medium. Panel (E) shows the changes in gene expression in HeLa cells
510 (5×10⁴ cells/well) (with infection) under 2% O₂ conditions compared with 21% O₂
511 conditions in RPMI medium. Genes names in red and green indicate upregulated and
512 downregulated genes, respectively. Each of the values is expressed as the average of two
513 independent experiments.
514

Fig. 1

A



B



C

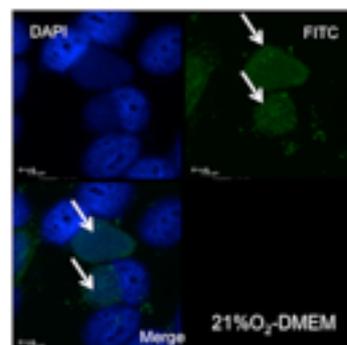
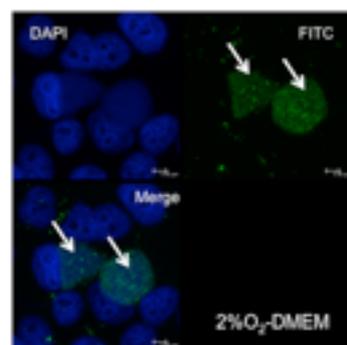


Fig. 3

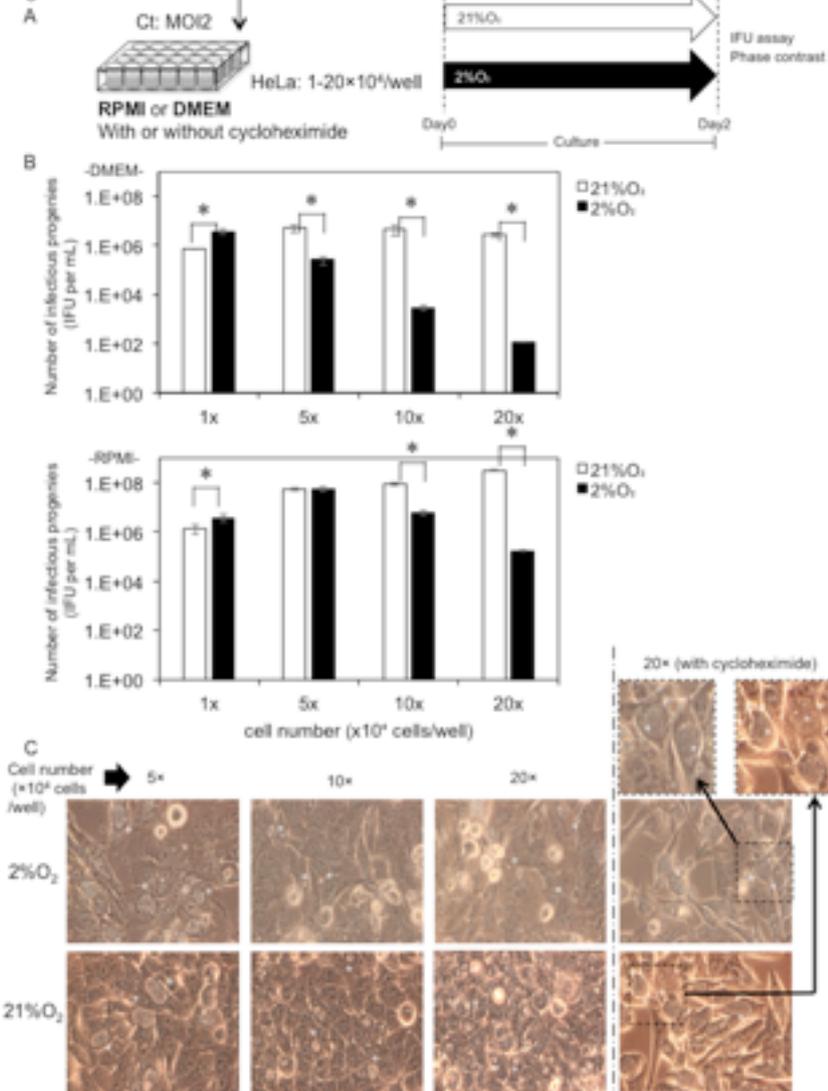


Fig. 4

