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1 **An innovative intermittent hypoxia model in cell cultures allowing fast PO₂**
2 **oscillations with minimal gas consumption**

3

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24 **Running title:** A new cellular intermittent hypoxia model

25 **Abstract**

26 Performing hypoxia-reoxygenation cycles in cell culture with a cycle duration accurately
27 reflecting what occurs in obstructive sleep apnea (OSA) patients is a difficult but crucial
28 technical challenge. Our goal was to develop a novel device to expose multiple cell culture
29 dishes to intermittent hypoxia (IH) cycles relevant to OSA with limited gas consumption.

30 With gas flows as low as 200 mL/min, our combination of plate holders with gas-permeable
31 cultureware generates rapid normoxia-hypoxia cycles. Cycles alternating 1 minute at 20% O₂
32 followed by 1 minute at 2% O₂ result in pO₂ values ranging from 124 to 44mmHg. Extending
33 hypoxic and normoxic phases to 10 minutes allows pO₂ variations from 120 to 25mmHg. The
34 volume of culture medium or the presence of cells only modestly affected the pO₂ variations.
35 In contrast, the nadir of the hypoxia phase increased when measured at different heights
36 above the membrane. We validated the physiological relevance of this model by showing
37 that HIF-1 α expression was significantly increased by IH exposure in human aortic
38 endothelial cells (HAoEC), murine breast carcinoma (4T1) cells as well as in a blood-brain
39 barrier model (2.5, 1.5 and 6-fold increases, respectively).

40 In conclusion, we have established a new device to perform rapid intermittent hypoxia
41 cycles in cell cultures, with minimal gas consumption and the possibility to expose several
42 culture dishes simultaneously. This device will allow functional studies of the consequences
43 of IH and deciphering of the molecular biology of IH at the cellular level using oxygen cycles
44 that are clinically relevant to OSA.

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51 INTRODUCTION

52 Intermittent hypoxia (IH) is the hallmark of obstructive sleep apnea (OSA), a common
53 chronic disease affecting 5-20% of the general population and characterized by recurrent
54 collapses of the upper airway leading to the repetitive occurrence of oxygen
55 desaturation/reoxygenation sequences (14). OSA is recognized as an important and
56 independent risk factor for hypertension, coronary heart disease and stroke, and could also
57 be associated with mild cognitive dysfunction (16). Moreover, recent studies suggest that
58 the excess mortality in OSA could be at least partly due to an increased risk of cancer (5, 19).
59 The molecular pathways underlying the deleterious consequences of OSA are under
60 investigation (8, 10, 26) but cellular and molecular mechanisms remain poorly understood.
61 Moreover, clinical research is limited by confounding factors that make it difficult to
62 distinguish between the respective effects of intermittent hypoxia and comorbidities. Since
63 stroke and coronary heart disease are common OSA-associated comorbidities, increased
64 knowledge of the effects of IH exposure of endothelial cells and blood brain barrier appears
65 to be crucial. Finally, IH is also observed in tumors and exposure of tumor microenvironment
66 to IH might promote tumor growth and metastatic activity (1).

67 In this context, significant efforts were recently made in several leading laboratories in the
68 field to obtain relevant models of IH in cell cultures. The achievement of rapid oxygen cycles
69 in standard culture dishes is flawed by the very slow oxygen diffusion in culture medium in
70 the absence of mixing (2). Moreover, thermally-induced convective mixing of the media is
71 not sufficient to ensure rapid oxygen equilibration across the height of medium (2, 17, 20)
72 thus limiting the development of relevant IH systems. For instance, one of the first cellular IH
73 model, based on air flushing in a Lucite chamber, generated cycles alternating 15 seconds of
74 1% O₂ and 3 minutes of 21% O₂ that allowed only limited (between 50 and 70 mmHg)
75 fluctuations in pO₂ in the culture medium (12). This highlights the challenge of oxygen
76 diffusion for effective cell exposure to IH. Longer cycles have allowed cycling between 2% O₂
77 and 15% O₂, but with cycle durations of one (11) and 1.5 (28) hour, that are not clinically
78 relevant in the context of OSA-related IH. Another strategy has consisted in using pre-
79 equilibrated culture medium (2, 24, 27). In these systems, the use of preconditioned medium
80 does ensure instantaneous oxygen changes at the cell level. However, repeated changes in
81 medium complicate the measurement of soluble factor secretion and can induce an

82 important shear stress that could impact cell activity and metabolism. More recently, a team
83 has proposed a system in which gas was directly injected in the culture flask (23). They
84 alternated 5 minutes of 16% O₂ and 5 minutes with 0% O₂ resulting in 6 cycles per hour
85 which was the best compromise to achieve sufficient oxygen variation amplitude.
86 Finally, the last type of system described in the literature uses gas permeable dishes in order
87 to obtain rapid and accurate cycles at the cell level (18, 22). In this system, variations
88 between 16% and 1% O₂ lead to similar oxygen variations in the culture medium within
89 minutes, allowing a frequency of 6 cycles per hour without any change in medium or
90 bubbling. The major disadvantage of this elegant setting is the high gas consumption
91 necessary to replace the air volume of the cabinet incubator hosting the dishes.
92 Achievement of the control normoxic exposure also relies on expensive pre-mixed gas
93 bottles (16% O₂, 5% CO₂ and 79% N₂). Recently, a variant of this system, based on air
94 circulation underneath highly-permeable PDMS (polydimethylsiloxane) membranes for cell
95 culture, achieved very fast oxygen cycles at the cell level in a system adapted for direct
96 microscopy imaging (4). The major limitation of this model relies on its small size (4-mm
97 diameter dishes) and thus in the low number of exposed cells, preventing large scale studies
98 or the collection of cells for biochemical or molecular biology studies.

99

100 Therefore, our objective was to set up a cost-effective and rapid-cycling model producing IH
101 cycles able to mimic the tissue oxygenation characteristics of OSA. We aimed at developing
102 and characterizing a device that would allow rapid oxygen cycling, minimal gas consumption,
103 avoiding the use of expensive premixed gas bottles, and allowing exposure of multiwell
104 plates and larger culture plates. The originality of this system is that it generates rapid cycles
105 in culture media while minimizing gas consumption by using gas-permeable cell cultureware
106 and custom made holders in which the air is renewed only below the dishes.

107

108 **MATERIALS AND METHODS**

109 *Gas-permeable dishes and plate holders.*

110 Gas-permeable dishes were either from Sarstedt (Lumox® 55-mm dishes, 24- and 96-well
111 plates, Sarstedt, Germany) or from Zell-Kontakt (Fluorocarbon Imaging plates, 24- and 96-

112 well plates, Zell-Kontakt GmbH, Germany). The thickness of the membrane is 25 μm for Zell-
113 Kontakt plates and Sarstedt dishes, while it is 50 μm for Sarstedt plates. Since poor cell
114 adhesion was observed with both types of membranes, they were coated with type I
115 collagen (0.2 mg/ml for 1 h at 37°C) before seeding cells.

116 Transwells with either 0.4 μm or 8 μm pores (Corning) were placed in 24-well Zell-Kontakt
117 plates. Transwells and plate wells were filled with 200 μL and 600 μL of culture medium,
118 respectively.

119 The plate holders were custom-made (SMTEC, Nyon, Switzerland) to accommodate either
120 55-mm dishes or 24- or 96-well plates (Figure 1). The dishes or plates are attached to the
121 holders with clips in order to minimize air leakage. The air volume under the plate or dish is
122 as low as 15-20 ml thus allowing very rapid flushing, even with flows as low as 200 ml/min,
123 and very low gas consumption.

124

125 *Gas supply.*

126 Compressed air and >98% nitrogen are provided by a nitrogen extractor and a gas
127 compressor and carbon dioxide is supplied by a gas bottle. The three gas inputs are
128 connected to a gas blender (Gas Blender 100, MCQ Instruments, Rome, Italy) that can mix
129 gases in any desired fraction with a maximum output flow of 250 ml/minute. The gas
130 mixture is heated in a water bath set a 37°C and travels through a 5-meter long plastic tube
131 to allow temperature equilibration before being transmitted to culture plate holders hosted
132 in a standard cell culture incubator. Pressure equilibration is possible with air above the
133 culture medium, thus the system operates under normobaric conditions

134

135 *Oxygen measurements.*

136 Oxygen in air was measured with a gas analyzer (ADInstruments, Oxford, United Kingdom).
137 Dissolved oxygen in culture medium was measured with pre-calibrated fluorescent oxygen
138 sensors (pO₂ E-Series Sensor) coupled to an Oxylite device (Oxford Optronix, Oxford, United
139 Kingdom). The sensors have a 90% response time of 20 seconds and a precision of 0.1
140 mmHg. The pO₂ sensors were maintained by a holder equipped with a micrometer screw
141 and a binocular microscope, allowing the direct observation of the contact between the
142 probe and the membrane, and the vertical movement of the sensor by 60- μm steps from the

143 membrane level (pericellular environment) up to the top of the culture medium. In sealed
144 dishes, the probe was inserted through a needle hole in the lid before sealing. Data were
145 recorded using an acquisition system (Powerlab, ADInstruments, Oxford, United Kingdom).

146

147 *Experimental intermittent hypoxia protocols.*

148 Different pO₂ cycling protocols were programmed to characterize the behavior of the model
149 (Table 1). The IH protocols alternated a 20% or 16% normoxic phase and a 5% or 2% hypoxic
150 phase. The 16% oxygen value of normoxic phase was chosen according to the mean arterial
151 pO₂ value of healthy humans, as in other IH models (22, 23). We programmed IH protocols
152 with 5% (5% 2'-2' and 3'-3') and 2% (2% 30''-30'', 1'-1', 2'-2', 3'-3', 3'-5' and 5'-5') hypoxic
153 phases named according to the duration (in seconds or minutes) of the normoxic and
154 hypoxic phases, respectively. Following hypoxia, a brief 20% O₂ phase was used to accelerate
155 the return towards the normoxic 16% plateau. Similarly, a 1-minute burst of 3% pO₂ was
156 added to rapidly attain a stable 5% hypoxic plateau. No burst was used to attain the 2%
157 hypoxic plateau, since 2% is the minimum oxygen contain of our gas supply (98% pure
158 nitrogen). Carbon dioxide was always set at 5% in the gas mixture.

159

160 *Cell culture and HIF-1 α quantification.*

161 Human aortic endothelial cells (HAoEC, Cascade Biologics) were cultured in M200 medium,
162 supplemented with large vessel endothelial supplement (LVES) as recommended by the
163 manufacturer, and used after 6-8 population doubling. Human breast carcinoma cells (4T1,
164 graciously provided by V. Josserand, IAB, Grenoble) were cultured in RPMI medium
165 supplemented with 10% fetal bovine serum and antibiotics. The blood-brain barrier (BBB)
166 model is composed of bEnd.3 endothelial cells (ATCC, USA) combined to C6 astrocytes
167 (ATCC, USA) cultured in transwells with 0.4 μ m pores, as previously described (6).

168 HAoEC and 4T1 cells were exposed to 6 hours of IH (protocol 2% 5'-5'). BBB model was
169 exposed to 2h of IH (protocol 2% 5'-5') followed by 6 hours of normoxia (16% O₂), repeated 3
170 times for a total of 24 hours.

171 At the end of IH exposure, supernatants were collected and frozen (-80°C), while the cells
172 were fixed in 4% paraformaldehyde (PFA) for 10 minutes and kept at 4°C until use. HIF-1 α
173 expression was measured with a cell-based Elisa assay (R&D Systems) on PFA-fixed cells.

174

175

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177

178 *Statistical analysis.*

179 Data were analyzed using GraphPad Prism 6 Software (San Diego, California). The various

180 statistical tests performed are detailed in figure legends. Normality was determined using

181 D'Agostino-Pearson normality tests as recommended by the GraphPad Prism software.

182 Normal data were expressed as means \pm SEM. Statistical significance was set at $P < 0.05$.

183

184

185 **RESULTS**

186 *Rapid intermittent hypoxia cycles with appropriate amplitude were achieved with the model.*

187 Fig. 2 and Table 2 depict typical oxygen cycles observed in 55-mm dishes, filled with 5 ml of
188 PBS, with the various protocols tested (see Table 1 for details of gas settings).

189 Short cycles composed of 30 seconds at 20% followed by 30 seconds at 2% resulted in
190 oxygen cycling between 105.8 ± 1.3 and 58.5 ± 2.2 mmHg in the culture medium (Fig. 2A).

191 Increasing the duration of the normoxic and hypoxic phases enhanced the nadir-to-peak

192 amplitude, with cycles between 124.3 ± 2.4 and 44.3 ± 1.5 mmHg (nadir-to-peak amplitude:

193 79.9 ± 3.6 mmHg) with protocol 2% 1'-1' (Fig. 2B), and between 118.8 ± 2.4 and 33.8 ± 0.3

194 mmHg (nadir-to-peak amplitude: 84.4 ± 1.9 mmHg) with protocol 2% 2'-2' (Fig. 2C).

195 Protocols with a 2-min normoxic phase resulted in a pO₂ plateau around 120 mmHg and pO₂

196 values > 110 mmHg were achieved within a mean time of 52.1 ± 2.2 seconds. Protocols with

197 5% O₂ generated a hypoxic plateau around 40 mmHg (Fig. 2D and Table 2). For protocols with

198 2% O₂, a hypoxic plateau was more difficult to achieve, even with a 5-minute hypoxia

199 duration (Fig. 2E and 2F) and time to reach pO₂ values < 50 mmHg and < 40 mmHg was 47.2

200 ± 4.5 and 89.0 ± 9.1 seconds, respectively. pO₂ values < 30 mmHg were reached after 3

201 minutes of hypoxia with protocols 2% 3'-3' and 2% 5'-5' while the minimum value of 25

202 mmHg could only be attained after 5 minutes of hypoxia (Fig. 2F, 2G and Table 2). On the

203 other hand, decreasing the duration of normoxia from 5 to 3 minutes resulted in identical

204 peak and nadir pO₂ values (Fig. 2F and Table 2).

205 Since our 55-mm dish holder is designed for 3 dishes, we measured the cycles in the 3 dishes
206 and did not observe any difference in cycle pattern or pO₂ range (Fig. 2H).

207

208

209 *Effects of cultureware type and membrane thickness on intermittent hypoxia cycles.*

210 We compared the impact of support type and membrane thickness on the nadir-to-peak
211 amplitude of the cycles generated by protocols 2% 3'-3' and 2% 5'-5'. Whatever the
212 protocol, we did not observe any difference on nadir-to-peak cycle amplitude between 25
213 µm-thick 24-well or 96-well plates and 55-mm dishes. Hence, typical cycles generated by
214 protocol 2% 5'-5' in 24-well plates were between 26 and 113 mmHg (Fig. 3A), similar to
215 those observed in 96-well plates and 55-mm dishes. We serially connected three 96-well
216 plates in order to compare the typical aspect of the pO₂ cycles. We did not observe a
217 difference in pO₂ cycles between the three plates (Fig. 3B).

218 However, there was a significant difference in nadir values and nadir-to-peak amplitude of
219 the cycles measured in 25 µm-thick and 50 µm-thick 24-well plates (Fig. 3C). Hence, nadir
220 values were significantly lower in 25 µm-thick plates compared to 50 µm-thick plates (27.0 ±
221 1.9 vs. 55.4 ± 2.3 mmHg, respectively, $P = 0.008$) while peak pO₂ values were not affected
222 (118.6 and 114.6 mmHg, respectively). Therefore, nadir-to-peak amplitude was 91.8 ± 2.9
223 mmHg in 25 µm-thick plates but only 59.2 ± 2.9 mmHg in 50 µm-thick plates ($P = 0.008$).

224

225 *Culture medium volume modestly alters the intermittent hypoxia cycles.*

226 Overall, culture medium volume had a significant impact on the cycles ($P = 0.037$) (Table 3).
227 In particular, the lowest nadir value was obtained with a volume of 5 ml (27.2 ± 2.4 mmHg
228 vs. 36.7 ± 2.9 mmHg with a 3 ml volume, $P = 0.019$). However, the nadir-to-peak amplitude
229 was not significantly affected by culture medium volume (101.3 ± 2.9, 103.2 ± 1.6 and
230 104.7 ± 0.3 mmHg for 3, 5 and 7 ml, respectively).

231

232 *Presence of cells decreases the amplitude of intermittent hypoxia cycles.*

233 The presence of cells induced a significant decrease in the nadir-to-peak amplitude of the IH
234 cycles (from 95.6 ± 3.0 to 86.5 ± 1.5 mmHg, $P = 0.004$) (Table 4). More precisely, the

235 presence of cells decreased the maximum pO₂ value (from 124.4 ± 0.9 to 113.1 ± 3.5 mmHg,
236 *P* < 0.01) without affecting the minimum value.

237

238

239

240 *Amplitude of intermittent hypoxia cycles decreases from bottom to top of culture medium.*

241 To test the homogeneity of the cycles throughout the culture medium, we recorded oxygen
242 cycles from the membrane up to the top of the liquid layer using protocols 2% 3'-3' and 2%
243 5'-5'. To estimate the effect of gas exchange with ambient air we also measured pO₂
244 gradient with protocol 2% 5'-5' in dishes sealed with tape. Whatever the distance from the
245 membrane and the IH protocol, the maximum pO₂ value remained stable. However, as
246 expected, the minimum pO₂ value linearly increased when the O₂ probe was moved away
247 from the membrane and the nadir-to-peak amplitude therefore concomitantly decreased
248 (Fig. 4A). Moreover, AUC obtained with sealed-plates was significantly higher than that
249 recorded in unsealed plates (*P* = 0.012) (Fig. 4B).

250 Finally, to evaluate the possibility of using transwells for co-cultures or for permeability or
251 migration assays under IH, we measured cycles in transwell inserts placed in 24-well
252 permeable plates. The nadir-to-peak amplitude of IH cycles measured in transwells increased
253 with pore diameter. Indeed, amplitudes of 49 mmHg (nadir and peak pO₂ values of 78 and
254 127 mmHg) and 59 mmHg (nadir and peak pO₂ values of 66 and 125 mmHg values) (Fig. 4C)
255 were measured in transwells with 0.4 μm and 8 μm pores, respectively.

256

257 *Gas permeable dishes allow optimal cell oxygenation under normoxic conditions.*

258 Baseline culture medium pO₂ values measured in plastic dishes with 4T1 cells were
259 significantly lower than those measured in permeable dishes independently of cell
260 confluence (Fig. 5). At 70% confluence, median pO₂ values of 120 and 137 mmHg were
261 measured in plastic and permeable dishes, respectively (*P* = 0.016). At 100% confluence,
262 median pO₂ values of 115 and 150 mmHg were measured in plastic and permeable dishes,
263 respectively (*P* = 0.008). Interestingly, no difference between plastic and permeable dishes
264 was observed with 100% confluent HAoEC cells.

265

266

267

268 *Intermittent hypoxia exposure increases HIF-1 α expression in cells.*

269 Six hours of cell exposure to IH with protocol 2% 5'-5' resulted in a significant increase in HIF-
270 1 α expression compared to normoxia (2.4 and 1.4 fold-increase in HAEC and 4T1 cells,
271 respectively, $P < 0.05$) (Fig. 6A).

272 Moreover, repeated IH exposure over 24h of a cell BBB model resulted in a very significant
273 increase in HIF-1 α expression compared to normoxia (6.1 fold-increase, $P < 0.01$) (Fig. 6B).

274

275 **DISCUSSION**

276 In this paper, we describe an innovative system aiming to perform exposure of cells cultures
277 to intermittent hypoxia with a pattern accurately mimicking that occurring in sleep apnea
278 patients. This system is adapted from commercial gas-permeable dishes and uses custom-
279 made plate holders. The small volume of air in the holders is easily renewed even with low
280 gas flow, thus allowing low gas consumption and fast cycling. Our system is working with gas
281 sources (air, CO₂ and N₂) either from commercial bottles (CO₂) or produced by gas extractor
282 and compressor, thus avoiding expensive premixed gas bottles described elsewhere (22). We
283 were able to connect up to 3 holders in series, thus allowing the simultaneous exposure of
284 three multiwell plates (24-well or 96-well) or up to nine 55-mm dishes, or combinations of
285 multiwell plates and 55-mm dishes. This low cost system effectively produced short IH cycles
286 in culture medium and allowed the exposure of a large number of cells. This should help the
287 field to address molecular biology of IH exposure with a relevant physiological model.

288 We characterized the oxygen cycles obtained with various settings, with normoxic phases
289 between 20 and 12% oxygen and hypoxic phases between 5% and 2%. With 16%-2% cycling
290 at a frequency of 6 to 8 cycles per hour, we obtained nadir-to-peak amplitudes of about 95
291 mmHg, cycling between 25-30 mmHg and 120 mmHg. Although a nadir value of 25-30
292 mmHg is not as low as those attained with other systems (4, 23), it is relevant to OSA
293 physiopathology since 30 mmHg roughly corresponds to the lowest arterial blood saturation
294 level (60% oxygen saturation) recorded in severe sleep apnea patients during hypoxic
295 episodes (15). Similarly, the peak can be chosen at any value up to 125 mmHg, which was

306 chosen to be slightly higher than the mean value of arterial pO₂ in healthy humans, as done
307 in other IH models (22, 23). Therefore, our system can reproduce patterns of hypoxia-
308 reoxygenation relevant to severe human OSA by cycling between 30 and 100-120mmHg or
309 to moderate OSA by cycling between 40-45mmHg and 100-120mmHg. This is a major
310 advantage to appropriately assess the dose-response relationship between the amount of IH
311 and its deleterious or preconditioning outcomes (13, 14).

312 Although similar cycles were observed in Lumox® dishes and in fluorocarbon plates which
313 have 25µm-thick membranes, cycles measured in plates with 50µm-thick Lumox®
314 membranes presented high nadir values and decreased nadir-to-peak amplitudes. This
315 suggests that Lumox® membrane thickness can affect gas diffusion. In contrast, similar
316 cycles were recorded with 10µm- and 37.5µm- thick PDMS membranes in another IH system
317 (4). This discrepancy could be due to different chemical composition and/or thickness of the
318 membranes.

319 Uncoated gas-permeable membranes result in poor cell adhesion and were recently shown
320 to profoundly affect cell morphology and function (21). We thus established that fibronectin,
321 collagen or Matrigel® coating did not significantly alter the cycles measured in the culture
322 medium (data not shown). We also observed that the presence of cells modestly decreased
323 peak values and amplitude of the cycles, whereas nadir values were unchanged. The
324 difference in cycles in the presence of cells could be due to oxygen consumption and/or to
325 impaired gas diffusion through the cell layer. Nevertheless, since the impact of the presence
of cells was modest (<10% decrease in cycle amplitude), we can conclude that the
membrane maintains good gas permeability even in the presence of a confluent cell
monolayer.

319 Variations in culture medium volume also modestly affected the IH cycles. While peak values
320 and nadir-to-peak amplitude were not significantly altered, nadir values measured in 55-mm
321 dishes were lower at high (5mL) compared to low (3mL) medium volume. This could be
322 explained by oxygen retrodiffusion from air above the medium since low volumes result in
323 thin thickness (approximately 1 mm with 3ml vs. 2 mm with 5ml in 55-mm dishes). Thus, it
324 appears mandatory to always use the same amount of culture medium in order to ensure
325 reproducibility of the IH cycles.

326 A drawback of some IH systems is the limited number of dishes or plates that can be
327 exposed simultaneously, resulting in insufficient amount of cells for biochemical analysis or
328 molecular biology. In order to minimize gas consumption, we did not choose to perform IH
329 cycles directly in the cell incubator as in other systems (22), but we wanted to be able to
330 expose at least several 55-mm dishes or multiwell plates. Our use of custom-designed plate
331 holders branched in series enabled us to obtain similar cycles in up to 3 multiwell plates or
332 nine 55-mm dishes (or combinations of multiwell plates and 55-mm dishes) simultaneously
333 exposed to IH. This setup ensures that several experiments can be performed simultaneously
334 and that a sufficient quantity of cells can be collected for biochemical analysis or molecular
335 biology.

336 However, since gases are diffusing from below the plate, we expected that the cycles would
337 perform well at the membrane level only. This is perfectly suitable for adherent cells but
338 represents a limitation for non-adherent cell cultures, such as monocytes or PBMC, or for co-
339 culture systems such as transwell inserts. Indeed, while the peak value was unchanged, we
340 observed that the nadir of the IH cycles gradually increased when measured away from the
341 membrane so that the cycles almost disappeared at the top of the liquid layer. However,
342 adequate IH cycles oscillating between 50 and 110 mmHg were still measured 500 μm above
343 the membrane. Moreover, cycles measured inside 8 μm -pore transwells, commonly used for
344 cell migration or permeability assays, oscillated between 65 and 125 mmHg, suggesting that
345 our system can be used to study the effects of moderate IH on cell migration. Furthermore,
346 our system was successfully used to induce a clear increase of HIF-1 expression in cells
347 cultured in 0.4 μm -pore transwell.

348 Indeed, HIF-1 α expression was increased by more than 6 fold in a BBB model composed of
349 endothelial cells and astrocytes cultured in transwells and exposed to IH. In addition to
350 transwells, we observed that HIF-1 α expression was increased by 2.6 and 1.5 fold, in primary
351 endothelial and in tumor cells, respectively, after 6h of IH exposure. It should be stressed
352 that HIF-1 α expression might have been underestimated in our experimental conditions
353 since it was assessed in PFA-fixed samples, in which PFA could have interfered with antigen
354 reactivity. In accordance with various other studies, we chose to use HIF-1 α expression as a
355 marker of hypoxia sensing to validate IH exposure of our cells. Hence, Yuan et al. reported an
356 increase in HIF-1 protein level in nuclear extracts together with an increase in HIF-1 reporter

357 genes in PC12 cells after 60 cycles of IH (4min-30sec, 20%-1.5%) (31), and more recently
358 Campillo et al. observed a 1.5 fold increase in HIF-1 α nuclear translocation in mesenchymal
359 stem cells exposed to 4h of IH (30sec-30sec, 20%-1%) (4). In a very different system,
360 exposure of endothelial cells to long IH cycles (1h-30 min, 20%-1%,) did not increase HIF-1
361 gene expression but increased its nuclear translocation and DNA binding activity (29).
362 Nevertheless, other studies have failed to observe changes in HIF-1 expression or
363 transcriptional activity with different paradigms of IH exposure (22-24). Thus, despite
364 numerous studies in animal models showing that HIF-1 is activated by intermittent hypoxia
365 in various organs (3, 7, 30), the determinants of HIF-1 activation in cells exposed to IH
366 remain to be assessed, with particular emphasis on the characteristics of the IH cycles
367 (minimal and maximal pO₂, duration of normoxic and hypoxic phases, total duration of
368 exposure or number of cycles) and on the metabolic activity of the cells exposed.

369 Finally, we compared standard plastic dishes and permeable dishes under normoxic
370 condition. We measured a difference of up to 50 mmHg between standard plastic and semi-
371 permeable dishes, depending on cell type and confluence rate. Indeed the biggest difference
372 was observed for metabolically active cancer cells. These results are consistent with previous
373 data showing different levels of culture medium hypoxia with more or less metabolically
374 active cells (17, 22). In particular, Polak et al. showed that HIF-1 activity was much higher in
375 standard than in permeable plates, probably due to poor oxygenation of culture medium in
376 plastic plates along with O₂ consumption by cells (22). Despite the fact that oxygen tension
377 during standard cell culture is often a neglected factor, these results suggest that it could be
378 of interest to use gas permeable plates even in routine use under normoxic conditions, in
379 order to ensure real and reproducible normoxia at different stages of cell culture and
380 confluence levels.

381 It should be pointed that the pO₂ measurements performed in the present study were
382 recorded with probes having a 90% response time of 20 seconds (data provided by the
383 manufacturer and experimentally verified in our lab, data not shown), which is not negligible
384 compared to the rise and decay duration of our IH cycles. Therefore, one can assume that
385 the oxygen cycles performed by our system are actually faster than those measured and that
386 the rise and decay durations are overestimated. Moreover, pO₂ values measured for short
387 cycles that did not reach plateau values might be inaccurate so that the nadir-to-peak

388 amplitude might actually be larger than reported. Nevertheless, we can suggest that the
389 peak and nadir pO₂ values measured for longer IH cycles (3'-3' or 5'-5') were estimated with
390 a good accuracy since we were able to attain plateau phases at in both cases. Carbon dioxide
391 was set at 5% in all gas mixtures, and since CO₂ has a very high diffusion coefficient it was
392 theoretically stable in the culture medium throughout intermittent hypoxia exposure.

393 In summary, we have developed a new low-cost device to perform rapid intermittent
394 hypoxia cycles at the cellular level characterized by very low gas consumption and good cell
395 throughput for biochemistry or molecular biology analysis. This device can be used to
396 mimick the cellular impact of OSA but also that of other pathological conditions in which
397 rapid hypoxia-reoxygenation cycles are observed, such as ischemia-reperfusion events in
398 heart, brain or other organs, or tumor development where oxygen variations can be
399 observed independently of OSA, due to structural abnormalities of the vascular network (9,
400 25). This device thus presents a new alternative to existing techniques for laboratories that
401 plan to perform cellular IH or simply want to precisely control oxygen tension in their cell
402 cultures.

403

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409

410 **DISCLOSURES**

411 No conflicts of interest, financial or otherwise, are declared by the author(s).

412

413 **AUTHOR CONTRIBUTIONS**

414 M.M., J.P., D.G-R. and A.B-M. conceived and designed the model; M.M., J.M., F.P., M.C.,
415 B.G., E.L., J-B.M. and A.B-M. performed experiments, analyzed data and interpreted results
416 of experiments; A.B-M. and M.M. prepared figures; M.M., J.M., and A.B-M. drafted

417 manuscript; J.P., J-L.P. and D.G-R. edited and revised manuscript; all authors approved the
418 final version of the manuscript.

419

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425

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509 intermittent hypoxia. *J Biol Chem* **280**: 4321-4328, 2005.

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512 **Table 1.**

513 Gas settings and duration (in seconds or minutes) of the normoxic and hypoxic phases of the
 514 various protocols tested. In all the protocols except for 2% 30"-30" and 2% 1'-1', a 20% O₂
 515 normoxic burst was used before stabilization at 16% O₂ in order to decrease the duration of
 516 the ascending phase. Similarly, in 5% O₂ protocols, a 3% O₂ hypoxic burst was used before
 517 stabilization at 5% O₂ in order to decrease the duration of the descending phase.

Protocol	20%	16%	3%	5%	2%
2% 30"-30"	0.5				0.5
2% 1'-1'	1				1
2% 2'-2'	1	1			2
2% 3'-3'	1	2			3
2% 3'-5'	1	2			5
2% 5'-5'	1	4			5
5% 2'-2'	1	1	1	1	
5% 3'-3'	1	2	1	2	

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521 **Table 2.**

522 Nadir and peak pO₂ values and cycle amplitude (calculated delta value) measured in 55-mm
 523 dishes submitted to the intermittent hypoxia protocols depicted in *Table 1*. Data (in mmHg)
 524 are means ± SEM. * *P* < 0.05 and ***P* < 0.01 vs. 2% 5'-5' protocol, two-way ANOVA. *p*=0.08
 525 for Delta 2% 2'-2' vs 2% 5'-5'.

Protocol	Nadir	Peak	Delta
2% 30"-30"	58.5 ± 2.2 **	105.8 ± 1.3 **	47.3 ± 3.0 **
2% 1'-1'	44.3 ± 1.5 **	124.3 ± 2.4	79.9 ± 3.6 *
2% 2'-2'	33.8 ± 0.3	118.2 ± 2.4	84.4 ± 1.9
2% 3'-3'	30.3 ± 1.4	115.4 ± 4.4	85.1 ± 3.7
2% 3'-5'	24.9 ± 2.0	120.4 ± 4.5	95.5 ± 3.3
2% 5'-5'	25.5 ± 1.2	120.6 ± 1.9	95.1 ± 1.6
5% 2'-2'	38.0 ± 3.1 *	123.3 ± 3.3	85.3 ± 6.4
5% 3'-3'	39.7 ± 5.5 *	126.0 ± 8.0	86.3 ± 4.5

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533 **Table 3.**

534 Impact of culture medium volume on intermittent hypoxia cycles. Nadir and peak pO₂ values
535 and cycle amplitude (calculated delta value) measured in 55-mm dishes filled with different
536 volumes of culture medium and submitted to protocol 2% 5'-5'. Data (in mmHg) are means ±
537 SEM. *n* = 3 independent experiments. Medium volume significantly alters the cycles (*P* =
538 0.037, two-way ANOVA). * *P* = 0.019 vs. nadir at 5ml, two-way ANOVA. *p*=0.07 for nadir 3mL
539 vs 7mL, *p*=0.06 for peak 3mL vs 5mL.

Volume	Nadir	Peak	Delta
3 ml	36.7 ± 2.9 *	138.0 ± 0.1	101.3 ± 2.9
5 ml	27.2 ± 2.4	130.3 ± 0.9	103.2 ± 1.6
7 ml	29.3 ± 3.0	134.0 ± 3.1	104.7 ± 0.3

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543 **Table 4.**

544 Impact of the presence of cells on intermittent hypoxia cycles. Nadir and peak pO₂ values
545 and cycle amplitude (calculated delta value) measured in 55-mm dishes filled with 4ml of
546 culture medium with or without a confluent layer of 4T1 cells, and exposed to protocol 2%
547 5'-5'. The presence of cells does not change nadir values but lowers peak and delta values.
548 Data (in mmHg) are means ± SEM. *n* = 3 independent experiments performed in duplicates.
549 ** *P* < 0.01 vs. corresponding values without cells, two-way ANOVA.

	Nadir	Peak	Delta
Without cells	28.8 ± 2.4	124.4 ± 0.9	95.6 ± 2.9
With cells	26.7 ± 2.7	113.1 ± 3.5 **	86.5 ± 1.5 **

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555 **Figure legends**

556 **Fig. 1.**

557 Pictures showing the holders for 55-mm dishes (first lane) and for 24-well and 96-well plates
558 (second lane). The dishes or plates are maintained with forceps in order to avoid air leakage.

559

560 **Fig. 2.**

561 Characterization of pO₂ cycles at the membrane level in 55-mm dishes containing 5ml of
562 culture medium without cells. *A-F* show typical cycles measured with the following
563 intermittent hypoxia protocols: 2% 30''-30'' (*A*), 2% 1'-1' (*B*), 2% 2'-2' (*C*), 5% 2'-2' (*D*), 2% 3'-
564 3' (*E*) and 2% 5'-5' (*F*). *G* shows peak and nadir pO₂ values and amplitude of pO₂ variation for
565 each protocol. Data are from at least 3 independent measurements and for over 10
566 independent experiments for protocol 2% 5'-5'. *H* shows cycles measured in three 55-mm
567 dishes exposed simultaneously in holder to protocol 5% 2'-2'.

568

569 **Fig. 3.**

570 Characterization of pO₂ cycles in 24-well plates. *A*: Typical cycles measured at the membrane
571 level in 24-well plates (filled with 500 µl PBS) with protocol 2% 5'-5'. Cycles measured in 96-
572 well plates were similar. *B*: Typical measurements in 3 plate holders serially connected
573 (protocol 2% 5'-5'), nadir and peak pO₂ values and cycle amplitude (calculated delta value)
574 for the 3 consecutive plate holders. Cycles were only very slightly attenuated, with no
575 significant difference. *C*: Nadir and peak pO₂ values and cycle amplitude (calculated delta
576 value) in gas-permeable 24-well plates with 50 µm-thick (Sarstedt) or 25 µm-thick (Zell-
577 kontakt) membranes using protocol 2% 5'-5'. *n* = at least 5 independent measurements. ** *P*
578 < 0.01 for nadir and cycle amplitude vs. 25 µm-thick membranes, Mann-Whitney rank sum
579 tests.

580

581 **Fig. 4.**

582 Evolution of the cycles throughout the culture medium and in transwells. A: Typical nadir
583 and peak pO₂ values and cycle amplitude (calculated delta value) at different heights above
584 the membrane. Measurements were performed with protocol 2% 5'-5' in 55-mm dishes.
585 Similar results were observed in 96-well dishes. Data (in mmHg) are means ± SEM. *n* = at
586 least 3 independent experiments. B: Cycle amplitude (calculated delta value) at different
587 heights above the membrane in unsealed and sealed 55-mm dishes, and corresponding
588 calculated Area Under the Curve (AUC). Measurements were performed with protocols 2%
589 3'-3' (diamonds) and 2% 5'-5' (squares) in unsealed dishes and with protocol 2% 5'-5'
590 (triangles) in dishes sealed with tape to limit gas exchanges with incubator air. Data are
591 means ± SEM. *n* = at least 3 independent experiments. * Area under the curve vs. protocol
592 2% 3'-3' in unsealed dishes, *P* = 0.012 (one-way ANOVA). There was no significant difference
593 in cycle amplitude between unsealed and sealed plates at membrane level (probe height =
594 0). C: Cycles measured with protocol 2% 5'-5' inside a 8µm-pore transwell with 100 µl of
595 medium placed in a 24-well plate (Zell-kontakt) filled with 600 µL of culture medium.

596

597 **Fig. 5.**

598 Comparison of pO₂ values in standard and permeable dishes under normoxia. pO₂ values
599 were measured at the level of the cell layer in standard plastic or gas-permeable 55-mm
600 dishes plated with HAoEC or 4T1 cells at different cell confluences and incubated under
601 normoxia. Cultures were pre-equilibrated at 21% O₂ in a standard incubator, placed under
602 normoxia (21% O₂ + 5% CO₂) in the intermittent hypoxia device and allowed to equilibrate
603 for at least 30 minutes before pO₂ measurements. Individual data and median are plotted,
604 *n*=5 measurements from 3 independent experiments. * *P* < 0.05 and ** *P* < 0.01 vs. gas
605 permeable plates, Mann-Whitney rank sum test. *p*=0.09 for HAoEC cells, permeable vs
606 plastic dish.

607

608 **Fig. 6.**

609 Intermittent hypoxia increases HIF-1α expression. A: Expression of HIF-1α in HAoEC and 4T1
610 cells after 6 hours of exposure to protocol 2% 5'-5', expressed as a ratio of HIF-1α expression
611 in cells exposed to 6 hours of normoxia (16% O₂). * *P* < 0.05 vs. normoxia, Mann-Whitney
612 rank sum test. B: Expression of HIF-1α in a blood brain barrier model composed of b. End3
613 endothelial cells and C6 astrocytes after 24 hours of IH (2h of 2% 5'-5' and 6h of normoxia,

614 repeated 3 times). Individual data and median are plotted. $n = 3$ independent experiments,
615 ** $P < 0.01$ vs. normoxia, Mann-Whitney rank sum test.

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Fig. 1

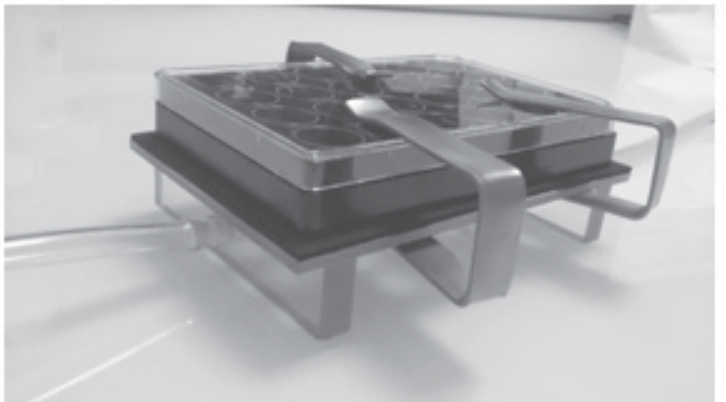
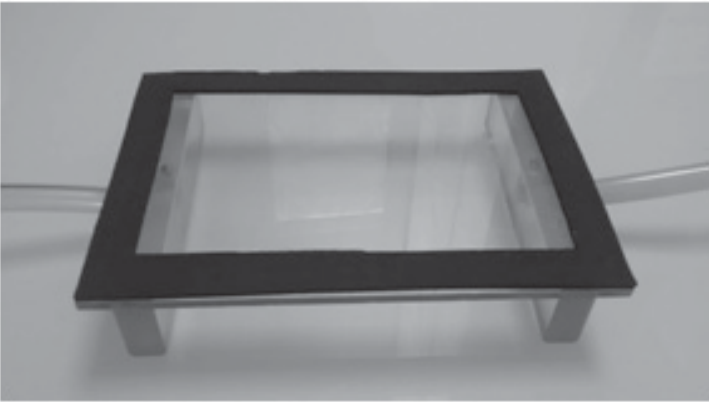
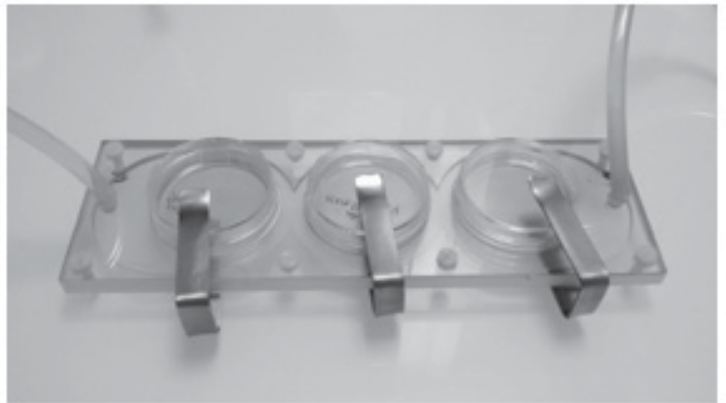


Fig. 2

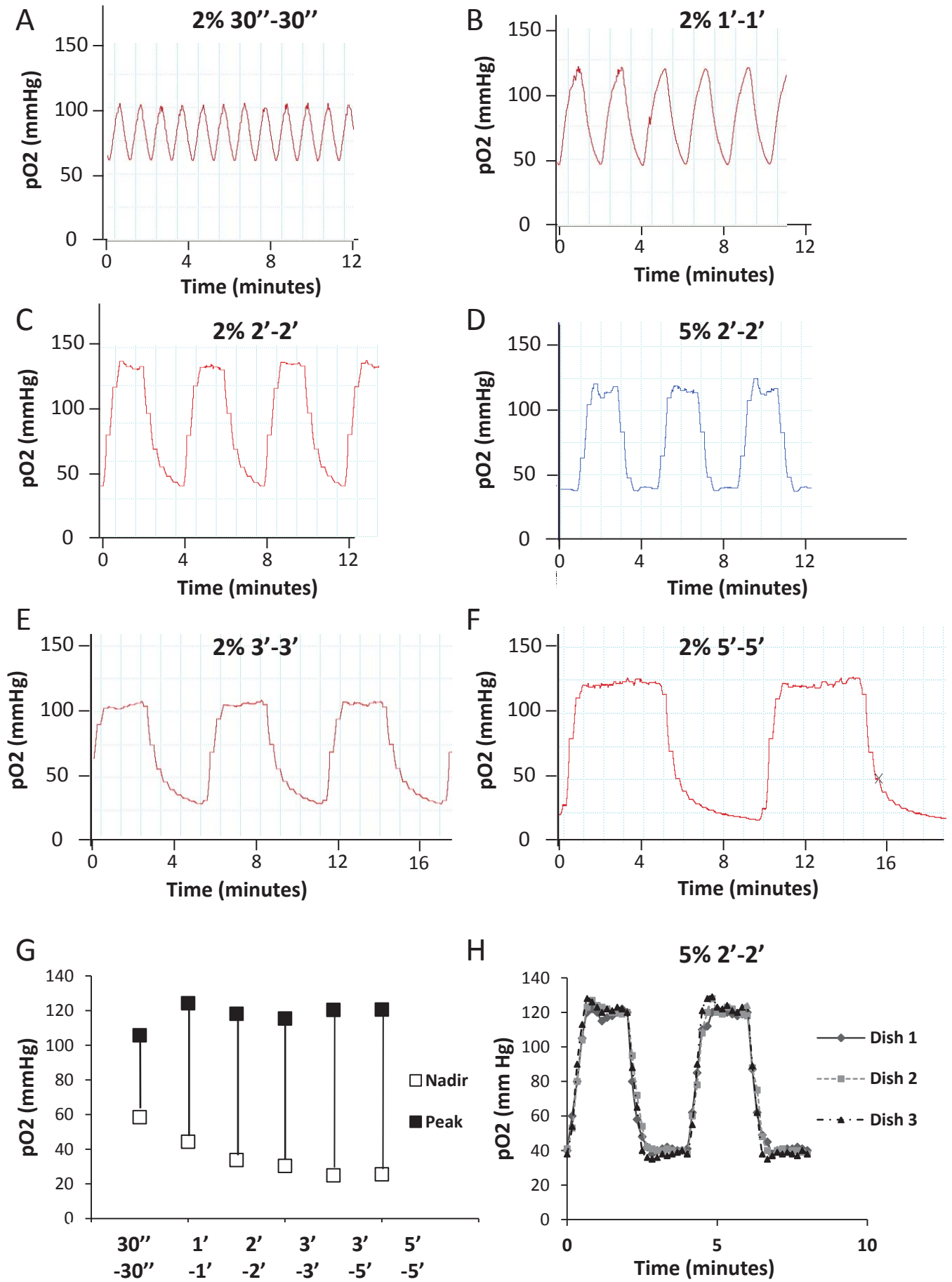


Fig. 3

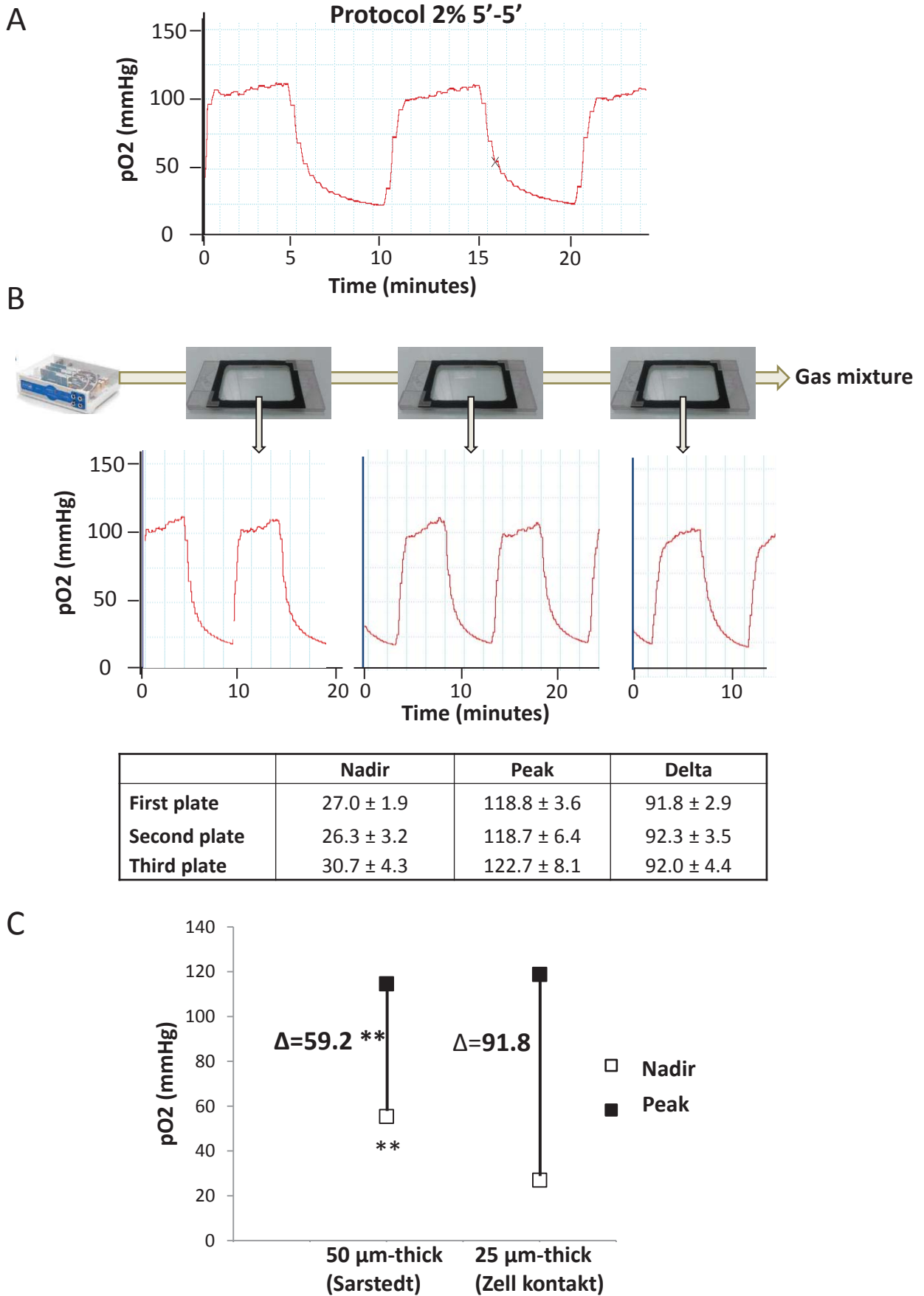
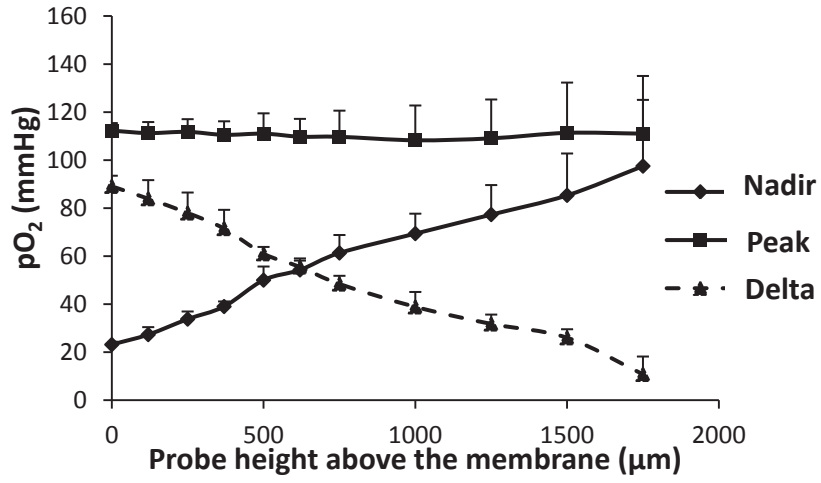
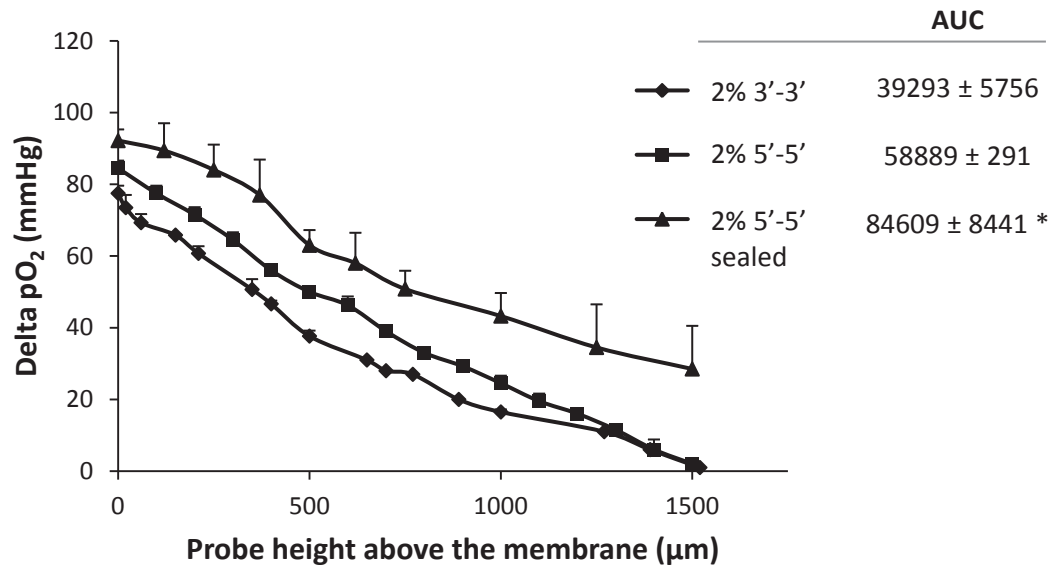


Fig. 4

A



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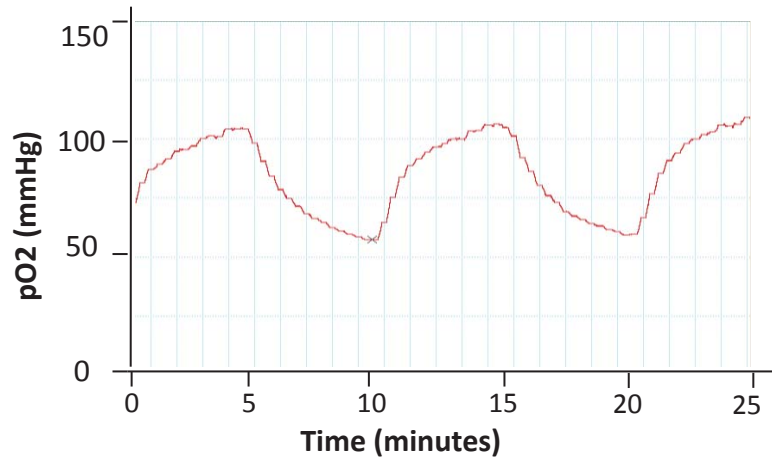


Fig. 5

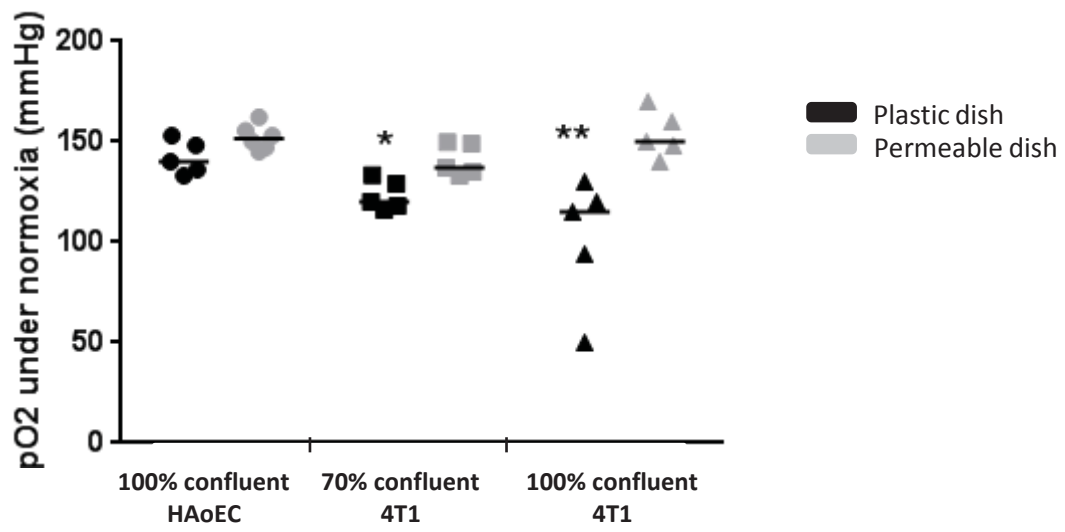


Fig. 6

