

# Frequently asked questions in hypoxia research

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**Abstract:** "What is the O<sub>2</sub> concentration in a normoxic cell culture incubator?" This and other frequently asked questions in hypoxia research will be answered in this review. Our intention is to give a simple introduction to the physics of gases that would be helpful for newcomers to the field of hypoxia research. We will provide background knowledge about questions often asked, but without straightforward answers. What is O<sub>2</sub> concentration, and what is O<sub>2</sub> partial pressure? What is normoxia, and what is hypoxia? How much O<sub>2</sub> is experienced by a cell residing in a culture dish in vitro vs in a tissue in vivo? By the way, the O<sub>2</sub> concentration in a normoxic incubator is 18.6%, rather than 20.9% or 20%, as commonly stated in research publications. And this is strictly only valid for incubators at sea level.

**Keywords:** gas laws, hypoxia-inducible factor, Krogh tissue cylinder, oxygen diffusion, partial pressure, tissue oxygen levels

## Introduction

A criticism often heard in hypoxia research is that the setting "1% O<sub>2</sub>" in a cell culture incubator does not match any physiological situation in vivo. So, what is a physiological O<sub>2</sub> concentration in the body? What is normoxia, and what is hypoxia? With the exponential rise in our knowledge on hypoxia-inducible signaling pathways, it has become increasingly clear to every scientist cultivating cells in vitro that not only temperature, humidity, and CO<sub>2</sub> but also O<sub>2</sub> needs to be controlled. Corresponding incubators are on the way to becoming standard equipment for cell culture, just like it has been standard for decades to control CO<sub>2</sub>. It appears obvious that the precise O<sub>2</sub> concentration cells are exposed to in these incubators must be disclosed in scientific publications. But, quite remarkably, in contrast to the measured hypoxic O<sub>2</sub> concentrations, the actual normoxic O<sub>2</sub> concentrations are almost never correctly indicated but rather given as "21%", "20.9%", or "20%" O<sub>2</sub>, which corresponds to the O<sub>2</sub> concentration of dry room air rather than incubator air. This review is addressed to newcomers to the hypoxia research field and explains the simple but not always intuitive properties of gases required for the daily work in cell culture. Herein, we will also discuss the actual O<sub>2</sub> concentration, or better O<sub>2</sub> partial pressure, inside tissues and cultured cells, a point that has all too often been subjected to over-simplifications. Using the example of the normoxic O<sub>2</sub> concentration in a cell culture incubator, a simple introduction to the physics of gases will be given.

## What is the O<sub>2</sub> concentration in the gas phase?

Whether at sea level or Mount Everest, whether on a pole or the equator, the O<sub>2</sub> concentration is always the same! The value of sufficient precision for biological

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considerations is 20.9% (volume/volume or v/v). However, this value is for dry air only, ignoring the fact that there is usually also water in its gaseous form in the atmosphere. The other gases in the air, mostly nitrogen, are not really relevant for cellular processes under physiological conditions.

## What is the O<sub>2</sub> partial pressure?

What changes at high altitude is not the concentration of any given gas but the total pressure of the air. Air pressure at a given altitude is built up by the height of the air column above. This air column has a certain mass that exerts force onto the gas below it under the influence of gravity. Because in contrast to liquids gases are compressible, the density of the air increases exponentially rather than linearly with the height/weight of the overlaying air column. Vice versa, air pressure falls exponentially with increasing altitude. The corresponding physical law allows the calculation of the decrease in atmospheric pressure with increasing altitude (a) expressed in kilometers (km), assuming that earth's gravity is equal on the entire surface of the planet (which is a simplification, of course):  $P_a = P_0 \times e^{-(0.127 \times a)}$ .  $P_0$  is the atmospheric pressure at sea level. For this calculation, it does not matter which pressure unit is chosen. The official unit is Newton (the unit of force) per square meter (N/m<sup>2</sup>), also called Pascal (Pa). At sea level, the atmospheric pressure is 101.3 kPa. However, biologists still prefer the old unit millimeter mercury (mmHg), also called Torr (torr). At sea level, a manometer filled with mercury shows a column height of 760 mm (ie, 101.3 kPa = 760 mmHg). This is an average value that is only theoretically constant, since both minor changes in gravity as well as, more importantly, the actual weather condition can slightly affect this value. The 20.9% of this total atmospheric pressure will result in the O<sub>2</sub> partial pressure (pO<sub>2</sub>), that is, 159 mmHg. According to the formula mentioned earlier, at 0.5 km altitude, for instance, the atmospheric pressure is 713.2 mmHg, and the pO<sub>2</sub> is 149.1 mmHg.

## Why must humidity be considered?

Cultured cells must be kept in 100% (relative to saturation) humidified incubators. Otherwise, the medium evaporates, and cell metabolism is compromised by changes in osmolarity, eventually resulting in cell death. Evaporated water molecules in the gas phase also generate a partial gas pressure, p<sub>H<sub>2</sub>O</sub>. This pressure is even built up if the gas is in equilibrium with its frozen aggregation state (ie, ice) by a process called sublimation. The p<sub>H<sub>2</sub>O</sub> increases with increasing temperature of the liquid source of the evaporated water, assuming that liquid and gas phases have the

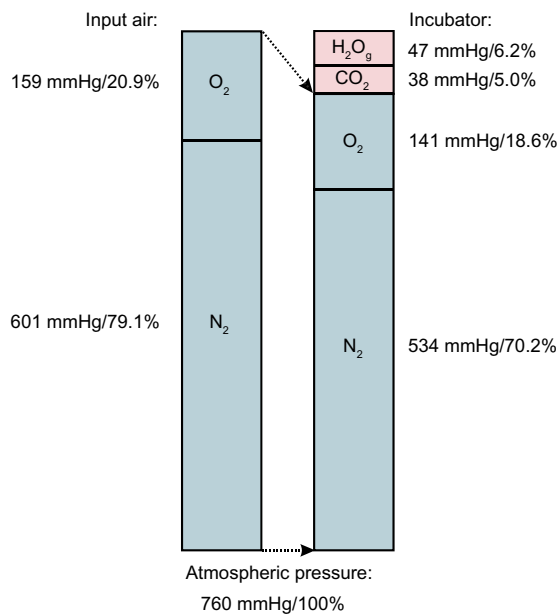
same temperature. Because tissue culture incubators usually mimic the human core body temperature, their temperature is set to 37°C, resulting in a p<sub>H<sub>2</sub>O</sub> of 47 mmHg. Remarkably, this partial pressure is independent of the atmospheric pressure. As long as there is a balance between the liquid and gas phases, that is, the gas phase is water saturated, there is always a p<sub>H<sub>2</sub>O</sub> of 47 mmHg at 37°C, whether we are at sea level, on Mount Everest, or in a vacuum chamber. That is also one of the reasons why cosmonauts cannot leave their spaceships without pressure suits: their body fluids of 37°C temperature would start to boil if exposed to environmental atmospheric pressure <47 mmHg (eg, the water of the lung alveolar surface), which according to the formula mentioned earlier happens at >22 km altitude.

## What is the O<sub>2</sub> concentration in a normoxic incubator?

In order to understand how all relevant gases in a cell culture incubator, that is, N<sub>2</sub>, O<sub>2</sub>, H<sub>2</sub>O, and CO<sub>2</sub>, sum up to the total atmospheric pressure, which is the same inside and outside normobaric incubators, we need a simple physical law, also called Dalton's law. It says that gas partial pressures are additive. This means that the partial pressures of all relevant gases together must be equal to the atmospheric pressure. The p<sub>H<sub>2</sub>O</sub> is 47 mmHg if we culture the cells at 37°C. The CO<sub>2</sub> concentration is usually set (and measured) at 5% (v/v), resulting in a pCO<sub>2</sub> of 5% of 760 mmHg, that is, 38 mmHg. Therefore, the remaining dry room air in the incubator has only 760–47–38 = 675 mmHg at its disposal. The 20.9% thereof is required for O<sub>2</sub>, resulting in a pO<sub>2</sub> of 141 mmHg. This partial pressure corresponds to an O<sub>2</sub> concentration of 18.6%, the "true" normoxic oxygen condition in every day's cell culture (Figure 1). However, this is correct only at sea level. At 0.5 km altitude, for example, the pO<sub>2</sub> would be 20.9% of 713.2–47–35.7 = 630.5 mmHg, that is, 131.8 mmHg, corresponding to an O<sub>2</sub> concentration of 131.8/713.2×100% = 18.5%. Thus, the relative effect of the constant p<sub>H<sub>2</sub>O</sub> on the final O<sub>2</sub> concentration increases with increasing altitude.

## What is the O<sub>2</sub> concentration in the liquid phase?

As nice as it is to know the O<sub>2</sub> concentration in the gas phase, it will never be what the (adherent) cells in a tissue culture dish actually experience, since they are attached to the bottom of the dish. To understand how oxygen actually reaches the cells, another simple physical law is required, also called Henry's law. It says that the partial pressure of



**Figure 1** Composition of the gas phase in a tissue culture incubator.  
**Notes:** Input room air (left) is mixed with gaseous water and CO<sub>2</sub> to form the incubator's gas mixture (right).

a gas in the liquid phase is equal to its partial pressure in the gas phase. Whereas this law is neither dependent on the nature of the gas nor of the liquid, the actual gas solubility is highly variable between different gases and liquids. At least, the dissolved gas concentration can easily be calculated as it is directly proportional to the partial pressure. The solubility constant, also called Bunsen's constant, is a specific number for each gas, depending on the nature and composition of the liquid as well as on the temperature. At 37°C, 1.32 μM O<sub>2</sub> dissolves in pure water per 1 mmHg O<sub>2</sub> partial pressure. However, the presence of dissolved salts lowers O<sub>2</sub> solubility. If we take as a likely approximation that typical cell culture media have properties similar to blood plasma, the plasma O<sub>2</sub> solubility of 1.26 μM O<sub>2</sub> per 1 mmHg at 37°C<sup>1</sup> would result in 1.26 μM/mmHg × 141 mmHg = 177.66 μM O<sub>2</sub> concentration under normoxic incubator conditions. This value increases in a nonlinear manner with decreasing temperature and vice versa. Importantly, O<sub>2</sub> solubility in the aqueous phase is rather low, and other biologically relevant gases have clearly distinct solubility constants. CO<sub>2</sub>, for instance, dissolves in blood plasma at 30 μM per 1 mmHg CO<sub>2</sub> partial pressure,<sup>1</sup> that is, in a 5% CO<sub>2</sub> incubator, this would result in 30 μM/mmHg × 38 mmHg = 1,140 μM CO<sub>2</sub> concentration.

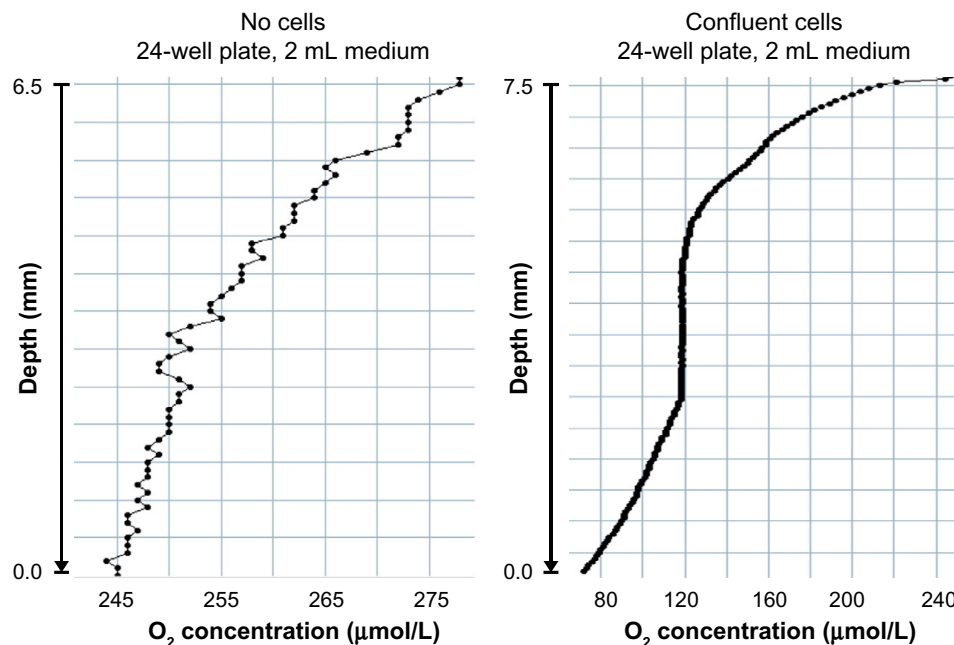
## How is O<sub>2</sub> distributed in the liquid phase?

Strictly speaking, Henry's law is only valid for stirred liquids or for the liquid phase just below the surface in resting liquids.

At least for adherent cell culture, the medium is usually not stirred. Unfortunately, under these typical cell culture conditions, O<sub>2</sub> will not reach the cells at the same partial pressures (or concentrations) as calculated earlier. The mechanism by which gases reach the bottom of the tissue culture dish or flask is by diffusion, which is almost always “the” limiting factor for cellular oxygenation. This is also called diffusion limitation. As described by Fick's law, diffusion is directly proportional to the partial pressure difference (ie, the driving force of diffusion), directly proportional to solubility, and inversely proportional to the diffusion distance. As a rule of thumb, O<sub>2</sub> diffusion in tissues becomes limited at ~100–200 μm.<sup>2–4</sup> This is not a problem for our lungs, where the diffusion distance from the alveolar surface to the hemoglobin inside the erythrocytes is only ~2 μm.<sup>1</sup> However, in a “10 cm” petri dish, ~10 mL medium is required, resulting in a medium height of 1.72 mm (assuming an inner diameter of 8.6 cm and a culture area of 58 cm<sup>2</sup>). This exceeds the O<sub>2</sub> diffusion limit by an order of magnitude and will inevitably result in an (unknown) low pericellular pO<sub>2</sub> and poor cellular oxygenation, eventually resulting in hypoxic cells even under normoxic incubator conditions. In contrast, because the solubility of CO<sub>2</sub> is ~24-fold higher than that of O<sub>2</sub> (as explained in the section “What is the O<sub>2</sub> concentration in the liquid phase?”), CO<sub>2</sub> diffusion is usually not limited in cell culture. As the usual bicarbonate buffer system used in cell culture determines the actual pH in equilibrium with the CO<sub>2</sub> concentration, equal CO<sub>2</sub> distribution also ensures equal pH values.

Taken together, O<sub>2</sub> diffusion is dependent on the driving force (the delta pO<sub>2</sub> or ΔpO<sub>2</sub>) and several matter constants that cannot be altered in cell culture such as the poor O<sub>2</sub> solubility. The ΔpO<sub>2</sub> is the difference between the incubator's pO<sub>2</sub> and the pericellular pO<sub>2</sub>, that is, the difference between O<sub>2</sub> supply and O<sub>2</sub> sink. In principle, the ΔpO<sub>2</sub> can be decreased by lowering the pO<sub>2</sub> in the incubator (eg, by experimental hypoxic conditions) and/or by elevating the pericellular pO<sub>2</sub> (eg, by lowered cell density and/or lowered O<sub>2</sub> consumption). To make the situation even more complex, an important physiological mechanism of cellular adaptation to hypoxia is lowered mitochondrial O<sub>2</sub> consumption. Thus, the pericellular pO<sub>2</sub> is also a function of time since these adaptive processes can take hours to days.

Figure 2 shows exemplary results of pO<sub>2</sub> measurements as a function of the distance from the surface of the medium toward the bottom of a cell culture dish. After moving the dish out of an normoxic incubator and exposing it to room air conditions, the environmental O<sub>2</sub> supply acutely increases



**Figure 2** O<sub>2</sub> concentration gradients in cell culture medium.

**Notes:** Normal 24-well tissue culture plates without (left) or with (right) confluent HeLa cell layers were removed from a normoxic 37°C incubator, and O<sub>2</sub> concentration profiles were determined under room air conditions at 25°C using a needle-type O<sub>2</sub> sensor (PreSens, Regensburg, Germany). Note that the change from incubator air to room air results in a higher pO<sub>2</sub> (no gaseous water, no pCO<sub>2</sub>) and a better O<sub>2</sub> solubility (temperature change from 37°C to 25°C).

and a shallow pO<sub>2</sub> gradient forms due to the poor O<sub>2</sub> diffusion in unstirred medium, even in the absence of cells (left panel). When cells are present at high density attached to the bottom of the dish (right panel), they consume considerable amounts of O<sub>2</sub> and form an O<sub>2</sub> sink. The resulting steep pO<sub>2</sub> gradient leads, on the one hand, to a strong  $\Delta pO_2$  as driving force for the O<sub>2</sub> flux from the gas phase toward the cells. On the other hand, this O<sub>2</sub> sink creates its own pericellular hypoxic microenvironment, even if the incubator's gas phase was set to "normoxic" conditions.<sup>5</sup> Note that the O<sub>2</sub> concentration profile is non-steady. This is the result of increased O<sub>2</sub> solubility due to decreased culture medium temperature profiles combined with uncontrolled convection at different medium heights when the culture dish is taken out of the incubator. Altogether, these hardly controllable variables result in nonpredictable O<sub>2</sub> concentration profiles.

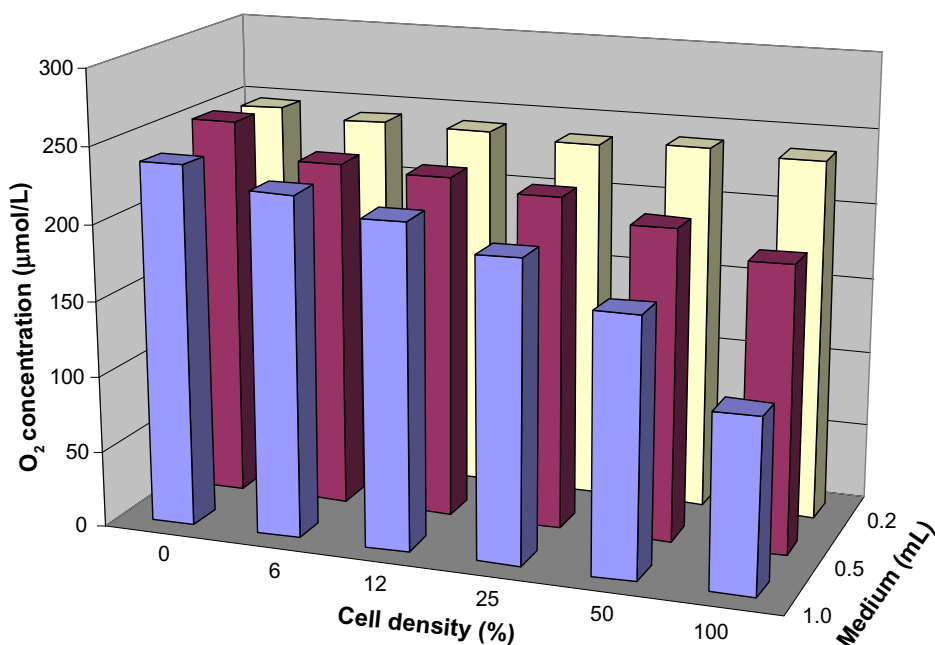
### What is the influence of the geometry of tissue culture flasks and dishes?

One should be aware that the culture medium pO<sub>2</sub> gradient also leads to differing pO<sub>2</sub> levels at the bottom of uneven medium heights, such as in the tilted neck region of tissue culture flasks or below the meniscus region of tissue culture dishes. The relative proportion of these areas becomes higher when the flasks and dishes are smaller. Especially in

96-well dishes, a large proportion of cells are located along the outer rim, that is, below higher fluid levels, due to the adhesive forces that "lift" the water along the plastic walls of the dish. Thus, the actual average pO<sub>2</sub> level can be different depending on the geometry of the used plasticware, even in the same hypoxia chamber. This might also explain variabilities between research groups, underlying the need for clear statements about this issue in the "Methods" section of a publication. Obviously, it is important to keep all tissue culture dishes absolutely horizontal, especially regarding the minimal medium volume that must be used in hypoxic experiments. A water level may be required to adjust the horizontal orientation of the dishes and to prevent uneven medium heights.

### What is the pericellular pO<sub>2</sub> in cultured cells?

As discussed earlier, only pericellular "on-line" pO<sub>2</sub> measurements would allow for accurate monitoring of the actual O<sub>2</sub> availability of cultured cells. Figure 3 shows an exemplary result of pericellular pO<sub>2</sub> measurements as function of medium height and cell density. As expected, based on theoretical considerations,<sup>6</sup> the pericellular pO<sub>2</sub> drops with increasing medium height and cell density. Somewhat frustratingly, these results clearly demonstrate that the knowledge of the precise O<sub>2</sub> concentration in the incubator air is



**Figure 3** Pericellular O<sub>2</sub> concentration as a function of medium height and cell density.

**Notes:** 24-Well SensorDish tissue culture plates were filled with different medium volumes and seeded with HeLa cells at various densities, resulting in 0%–100% confluency as indicated. The SensorDishes were removed from the 37°C incubator and the pericellular O<sub>2</sub> concentration determined under room air conditions at 25°C (resulting in a higher pO<sub>2</sub> and better O<sub>2</sub> solubility than within the incubator) using a SensorDish Reader (PreSens, Regensburg, Germany).

quite useless for the prediction of the pericellular pO<sub>2</sub>. So, how can this problem be solved? The usual approach is to ignore it and to simply compare “normoxic” with “hypoxic” exposure under otherwise identical conditions, knowing that these expressions refer to the incubator’s air composition only and have nothing to do with the physiological tissue situation. More precisely, but rarely done, the pericellular pO<sub>2</sub> could be measured just below the cells, using oxygen-sensitive phosphorescent dyes (as used in Figure 3). Another approach would be the use of O<sub>2</sub> permeable cell culture dishes, where O<sub>2</sub> reaches the cells by diffusion through the bottom plastics and where the pO<sub>2</sub> can hence be assumed to be identical to the gas phase.<sup>7</sup> Unfortunately, many cell lines poorly adhere to such dishes, which are hence rarely used.

### How long does it take to reach hypoxic conditions?

The onset of hypoxic exposure is usually defined as the moment when the doors of the hypoxic incubator are closed. However, it will take several minutes to several hours until the medium O<sub>2</sub> concentration asymptotically approximates the desired value, even if the incubator would change the gas phase composition rapidly.<sup>8,9</sup> A theoretical calculation with 1.72 mm medium height (refer to section “How is O<sub>2</sub> distributed in the liquid phase?”) in the absence of cells reveals a duration of 38 minutes, 45 minutes, and 60 minutes to fall

below a pO<sub>2</sub> value of 1.2-fold of the input value if a cell culture dish is acutely switched from 20% O<sub>2</sub> to 2%, 1%, or 0.2% O<sub>2</sub> concentration, respectively.

One possibility to circumvent this problem is to pre-equilibrate the medium in the hypoxic incubator by removing the cap of the medium bottle. However, without stirring, this will result in little change in the overall O<sub>2</sub> content since only the surface region actually releases O<sub>2</sub>. A better solution to this problem would be to bubble nitrogen through the medium, to shake it vigorously, or to use large petri dishes with small medium heights for pre-equilibration. Somehow counterintuitively, bulk medium pre-equilibration works more efficiently if the medium is cooled while removing O<sub>2</sub> and then warmed up again under the desired hypoxic conditions before use. A more or less immediate O<sub>2</sub> equilibration of the cells can be expected if O<sub>2</sub> permeable cell culture dishes are used. Finally, for suspension cells, so-called tonometers have been applied, allowing a tight control of the culture medium oxygenation by using spinning cups that generate very thin liquid layers along the cups’ walls while simultaneously exposing these liquid layers to high gas flow rates.<sup>10,11</sup>

### How long does it take to lose hypoxic conditions?

Unfortunately, even the briefest opening of an incubator’s door will ruin a hypoxic experiment. Gas exchange with room air



occurs almost instantaneously, and it will take up to 1 hour until hypoxic conditions in the incubator's gas phase are reestablished (the theoretical considerations outlined earlier are valid in both directions). There is little tolerance toward reoxygenation because this immediately generates reactive oxygen species, which are well known to have signaling, as well as toxic, properties. To prevent such reoxygenation artifacts, the incubator is allowed to be opened only at the time of cell collection, and all harvesting must be performed as quickly as possible, replacing the medium immediately with precooled washing or lysis solutions. It is always better to culture, harvest, and lyse the cells within hypoxic workstations. However, one should be aware that certain biological reactions, such as  $O_2$  sensing by hypoxia-inducible factor  $\alpha$  (HIF $\alpha$ ) prolyl-4-hydroxylation, will continue even in (non-denatured) cell lysates whenever  $O_2$  is available.<sup>12,13</sup>

## What is the $O_2$ concentration in biological fluids?

For biological purposes, it is often more important to know the  $pO_2$  than the  $O_2$  concentration, that is, the total  $O_2$  present in a certain volume of the fluid phase. In fact, the  $O_2$  concentration is the sum of dissolved  $O_2$  plus  $O_2$  bound to proteins. The dissolved  $O_2$  is proportional to the  $pO_2$  (as discussed earlier). Bound  $O_2$  depends, in addition to  $pO_2$ , on the  $O_2$  affinity, concentration, and composition of  $O_2$ -binding proteins. For example, in arterial blood, only a small part of  $O_2$  is dissolved and >98% of  $O_2$  is bound to hemoglobin, resulting in an  $O_2$  concentration of 20% (v/v) (ie, 200 mL  $O_2$  per 1 L of blood with a hemoglobin concentration of 150 g/L), assuming normal inspiratory  $O_2$  and lung function. Coincidentally, 20% is the same  $O_2$  concentration as in the atmosphere. However, within cells, neither the ratio between dissolved and bound  $O_2$  nor the relative concentrations and affinity curves of  $O_2$ -binding proteins are known. Anyway, this is not a problem because it is the  $pO_2$  and not the  $O_2$  concentration that drives the diffusion of  $O_2$  molecules to their targets, such as  $O_2$ -sensing dioxygenases or  $O_2$ -reducing cytochrome *c* oxidase in mitochondria.  $O_2$ -binding proteins only experience the  $pO_2$  and not the  $O_2$  concentration. Therefore, life scientists should use  $pO_2$  rather than  $O_2$  concentration as the preferred unit for biological tissue  $O_2$  availability.

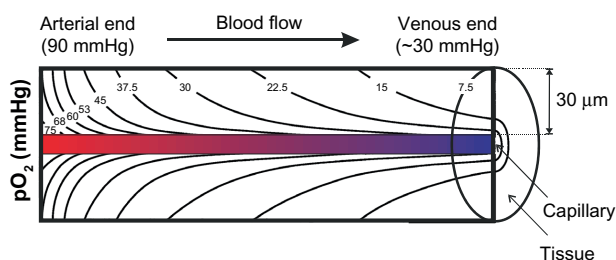
## What is the $pO_2$ in biological tissues?

Unfortunately, most of the publications provide single values for the tissue  $pO_2$  in different organs, not seldom – and even worse regarding what has been said so far – % $O_2$

concentrations are given. However, life would not be possible if  $O_2$  was equally spread throughout the tissue, that is, if neither supply nor sinks existed. Obviously,  $O_2$  is unevenly distributed in tissues, forming  $pO_2$  gradients. One gradient is found longitudinally along the small blood capillaries (ie, the  $O_2$  exchange segments of the blood vessel system) from the arterial to the venous ends. This gradient ranges from ~90 mmHg in arterial blood to 40 mmHg in mixed venous blood (corresponding to 75%  $O_2$  saturation of hemoglobin), but it can also be much lower at the venous end of a capillary if the corresponding tissue has a high  $O_2$  extraction capacity such as the heart. Another gradient is formed radially from the  $O_2$ -delivering hemoglobin to the actual  $O_2$  sinks in the mitochondria of  $O_2$ -consuming cells. Therefore, normal  $pO_2$  values distal to the venous end of a capillary can readily be <10 mmHg. The resulting  $pO_2$  profiles can be estimated within a cylinder of ~30  $\mu$ m radius (ie, half of the average distance between two capillaries) around each blood vessel, the so-called Krogh tissue cylinder (Figure 4).

## How can the tissue $pO_2$ be visualized?

No imaging/measurement technique is currently available to directly assess  $pO_2$  profiles within tissues. Infrared (pulse oximetry) and magnetic resonance (blood oxygenation level-dependent [BOLD]) techniques rely on hemoglobin  $O_2$  saturation rather than tissue  $pO_2$  levels. Polarographic and optical detection methods involve tiny electrodes and glass fibers, respectively, which are pierced into the tissues. Their diameters are minimally ~20  $\mu$ m but usually ~100  $\mu$ m; obviously still far too large to reliably detect biologically relevant  $pO_2$  profiles, not to mention the tissue damage they cause, leading to tissue compression, bleeding, edema, and  $O_2$  diffusion/convection along the penetration canal. It is



**Figure 4** Krogh's tissue cylinder.

**Notes:** Overlapping longitudinal (convective) and radial (diffusive)  $pO_2$  gradients form the physiological tissue  $O_2$  distribution (calculated isobaric  $pO_2$  profiles assuming constant tissue  $O_2$  consumption). All cells located within this  $pO_2$  profile are considered to be physiologically "normoxic", despite the highly variable absolute  $pO_2$  levels.

mandatory that histogram distributions over several hundred measurement sites are provided rather than single mean or median tissue  $pO_2$  values.<sup>14,15</sup>

A very popular method to visualize tissue hypoxia, especially in cancer research, is the IV injection of nitroimidazole compounds briefly before the (tumor) tissue is resected.<sup>16</sup> A large variety of such compounds exists, including derivatives bearing antibody epitopes (eg, pimonidazole or EF5), positron emission tomography tracers (eg, <sup>18</sup>F-fluoromisonidazole), and hypoxia-activated pro-drugs (eg, TH-302).<sup>17</sup> A four-electron reduction of these compounds by cellular nitroreductases is required to convert them into reactive species that covalently bind to macromolecules such as proteins and DNA. At  $pO_2$  levels above ~10 mmHg, the first of these four-electron reduction steps – forming a nitro radical anion ( $RNO_2^-$ ) – is reversed.<sup>17</sup> Therefore, nitroimidazole compounds cannot deliver a detailed map of different  $pO_2$  levels but only a “yes-or-no” picture of tissue regions with a  $pO_2 < 10$  mmHg, which is then often called “hypoxic” even if this represents an oversimplification. Moreover, one should be aware that two-electron nitroreductases, such as DT-diaphorase, can circumvent the  $O_2$ -sensitive step, leading to false-positive results.

Another emerging technique relies on heme-based probes whose phosphorescent lifetime is quenched by physiological ranges of  $pO_2$ , that is, the signal is not dependent on probe concentration. While in theory such probes should provide graded maps of  $pO_2$  variability, their limited tissue concentrations (they are not enriched in hypoxia areas), considerable costs, and the requirement for specialized microscopy equipment have prevented so far a more widespread application of these probes.<sup>18,19</sup>

Because of the lack of more appropriate methods, biology-based techniques, such as antibody-mediated detection of the  $O_2$ -sensitive HIF $\alpha$  subunits and their downstream target genes, are still commonly used to detect “hypoxic” tissue areas. For carbonic anhydrase IX, at least in cancer tissues, probably the most strongly induced HIF target gene, a non-antibody-mediated fluorescent in vivo probe (called HypoxiSense 680) has been developed.<sup>20,21</sup> However, at best, these techniques provide only indirect evidence for tissue hypoxia due to self-adaptation,<sup>22</sup> “normoxic” regulation, and cell type-specific expression.<sup>23</sup> At least the latter point has been circumvented by the generation of transgenic mice ubiquitously and constitutively expressing a luciferase reporter gene fused to the  $O_2$ -dependent degradation domain of HIF-1 $\alpha$ .<sup>24</sup> Following the injection of luciferin, hypoxia-dependent

bioluminescence can be imaged, which at least partially overlaps with pimonidazole and HIF $\alpha$  immunodetection.<sup>25</sup>

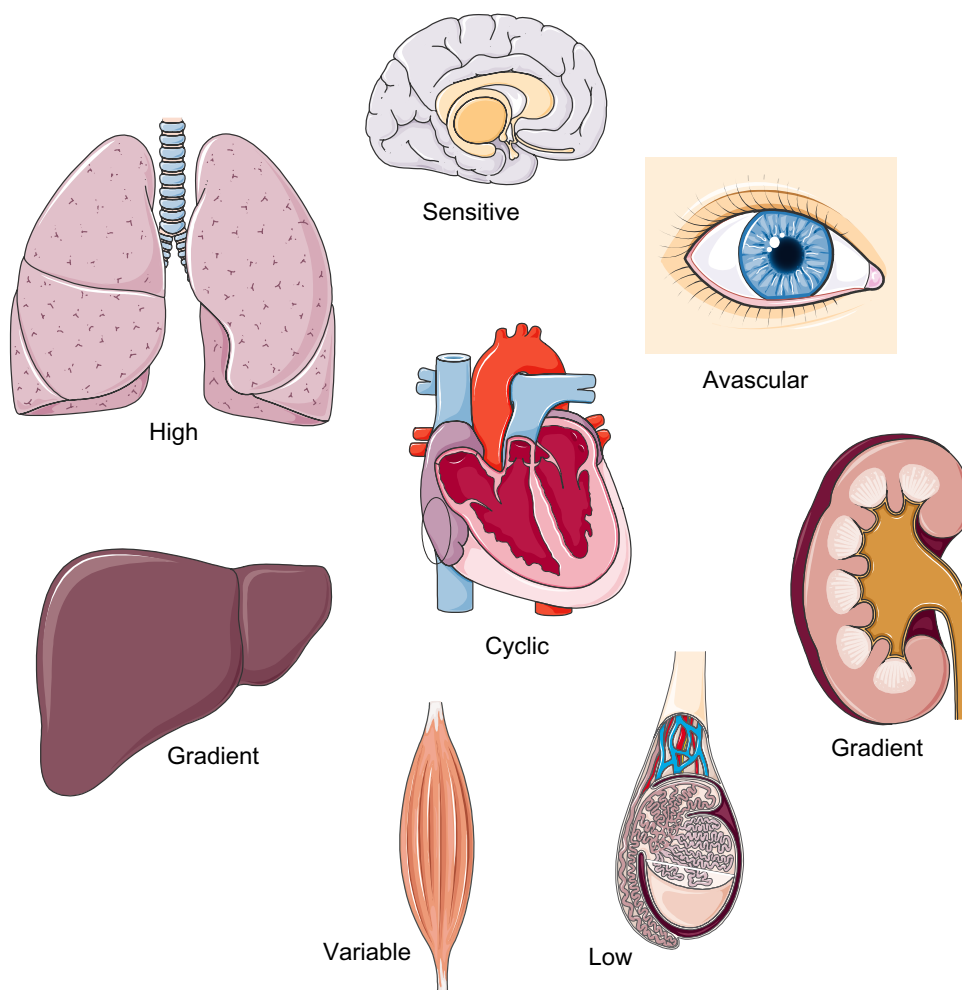
## What is the $pO_2$ in organs?

In addition to the general features of tissue  $pO_2$  distribution discussed earlier, several organotypic and cell type-specific characteristics must be considered (Figure 5).<sup>26,27</sup> Liver and kidney, for instance, display pronounced physiological  $pO_2$  gradients,<sup>28,29</sup> which can even be visualized by using EF5 or VEGF expression as HIF-1-dependent surrogate marker.<sup>30</sup> Lung alveolar epithelium contains the highest  $pO_2$  levels as it is oxygenated directly by the inspiratory air. Heavily working skeletal muscle has a large  $O_2$  extraction capacity and hence a huge variety of  $pO_2$  levels. Cardiomyocytes experience cyclic hypoxia with each heartbeat. Some tissues, such as the avascular cornea of the eye and nucleus pulposus of the intervertebral discs, have a very low  $pO_2$  but still must remain blood vessel free. Also central luminal cells of the testicular seminiferous tubuli reside within a very low  $pO_2$ .<sup>31</sup> Finally, some cell types, such as neurons, are strikingly hypoxia-intolerant,<sup>32</sup> whereas others, such as certain stem cells, need a hypoxic niche to remain in an undifferentiated stage.<sup>33</sup>

## What is normoxia, and what is hypoxia?

It may seem peculiar, but nobody has a precise answer to this apparently simple question. Physiological  $O_2$  availability is a continuum from lung alveolar  $pO_2$  of ~100 mmHg to functional anoxia at  $pO_2$  levels that are below the  $O_2$  affinity of mitochondrial cytochrome *c* oxidase. Mitochondrial  $P_{50}$  values from 0.06 mmHg to 0.45 mmHg  $pO_2$  have been reported, that is, 10–100-fold below the typical intracellular  $pO_2$ .<sup>34</sup> However, the  $O_2$ -sensing PHD-HIF system ensures that mitochondrial respiration is adapted to decreased oxygenation long before limiting  $pO_2$  levels are reached.<sup>35</sup> Many cell types do not even need mitochondria for their energy (ATP) production and solely rely on anaerobic glycolysis. Cancer cells usually maintain glycolytic energy metabolism even under high  $pO_2$  levels, the so-called aerobic glycolysis or Warburg effect. Therefore, no threshold  $pO_2$  level exists, which would define “hypoxia” based on limited mitochondrial respiration.

As outlined in Figure 4, tissue  $O_2$  is distributed along the  $pO_2$  profiles according to Krogh’s tissue cylinder. All cells residing within this  $pO_2$  profile are physiologically “normoxic”. Thus, it does not make sense to define a single  $pO_2$  value below which cells are called “hypoxic”.



**Figure 5** Organotypic characteristics in tissue oxygenation and O<sub>2</sub> metabolism.

**Notes:** Combined spatial and temporal processes result in a broad spectrum of physiologically “normoxic” tissue pO<sub>2</sub> values. For details see section: What is the pO<sub>2</sub> in organs?

Although 20.9% incubator O<sub>2</sub> conditions are usually referred to as “normoxic”, in physiological terms, they are rather “hyperoxic” because not even lung alveolar cells are ever exposed to 20.9% O<sub>2</sub>. Since the cellular O<sub>2</sub>-sensing system is self-adaptive,<sup>22</sup> cells do not “know” the absolute pO<sub>2</sub> levels in their microenvironment. In fact, “hypoxia” is a temporal rather than a spatial term. Every decrease in pO<sub>2</sub> that causes a biological effect, for example, a (transient) increase in HIF $\alpha$  protein stability, can be called “hypoxia”.

## Conclusion

Considering the discussed principles of biological O<sub>2</sub> distribution in vitro and in vivo, it becomes evident that it is quite useless to ask for the “correct” O<sub>2</sub> concentration in an incubator to mimic a certain cellular pO<sub>2</sub> corresponding to a specific tissue location. For routine experimental work, it is usually acceptable to compare at least two O<sub>2</sub> concentrations that are sufficiently different to cause specific biological effects while

not affecting general cell viability. In cases where absolute pO<sub>2</sub> levels need to be compared, for example, between different laboratories, only the actually measured pericellular pO<sub>2</sub> levels but not the adjusted gas phase O<sub>2</sub> concentrations in the incubator are relevant.

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## Disclosure

The authors declare no conflicts of interest in this work.

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