CellPress

Methods to study the tumor microenvironment under controlled oxygen conditions

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The tumor microenvironment (TME) is a complex heterogeneous assembly composed of a variety of cell types and physical features. One such feature, hypoxia, is associated with metabolic reprogramming, the epithelial–mesenchymal transition, and therapeutic resistance. Many questions remain regarding the effects of hypoxia on these outcomes; however, only a few experimental methods enable both precise control over oxygen concentration and real-time imaging of cell behavior. Recent efforts with microfluidic platforms offer a promising solution to these limitations. In this review, we discuss conventional methods and tools used to control oxygen concentration for cell studies, and then highlight recent advances in microfluidic-based approaches for controlling oxygen in engineered platforms.

Studying hypoxia in the tumor microenvironment

Molecular oxygen is used as a terminal electron acceptor in cellular respiration by most heterotrophic eukaryotes. Due to a variety of factors, including the transport of oxygen via diffusion, oxygen concentration gradients often exist within tissues [1]. When the transport of oxygen is unable to meet demand, hypoxia (sub-physiologic tissue oxygenation) occurs [2]. The partial pressure of oxygen in one such location where hypoxia is often found, the TME, ranges from 2 mm Hg (0.3% O_2) to 15 mm Hg (2.1% O_2) depending on tumor type (Table 1) [3,4]. Comparatively, arterial blood has a partial pressure of oxygen of 100 mm Hg (13.1% O_2), and the partial pressure introduced into traditional cell culture is 152 mm Hg (20% O_2) [5]. Therefore, to support the transition between *in vitro* knowledge and clinical successes, and to more accurately mimic oxygen conditions seen *in vivo*, advanced platforms of study are needed to more closely recapitulate hypoxia in the TME (Box 1) and tissue in general.

Controlling oxygen conditions in a laboratory setting is challenging due to sample/reagent handling and constant diffusion of oxygen from ambient air ($\sim21\%$). For example, each step during an experiment must be performed under identical oxygen conditions to prevent bias and to ensure reproducibility. A standard laboratory practice, such as changing media for cells, requires the cell culture flask to be removed from a gas-controlled incubator and placed in a chamber with the same desired oxygen concentration. Further, fresh media must be pre-equilibrated to the same oxygen concentration. The dissolved oxygen concentration in the reagent, in this case media, should also be measured prior to addition. Hence, executing a series of relatively simple laboratory techniques quickly becomes arduous under controlled oxygen concentrations.

To control the gaseous environment for cell studies, a number of conventional methods are used. The two most common methods (Figure 1), enable *in vitro* cell assays under hypoxia by (i) having a chamber with an air-tight seal and introducing specific gas concentrations, or by (ii) biochemically inducing a state of pseudo-hypoxia within the cell. These two methods provide unique benefits, as well as limitations, for cell assays under hypoxia.

Perhaps the most prevalent method to control the oxygen concentration is modulation of the gas mixtures entering an incubator (Figure 1A). In this method, cells are grown and conditioned in an incubator with the desired oxygen concentration [6]. However, long oxygen equilibration periods and the burdensome measures taken to sustain hypoxia throughout all aspects of experimentation limit the effectiveness of this method. Furthermore, gas-controlled incubators require an additional system for manual handling of reagents such as a glove box. Similar tools, such as hypoxia chambers, have equivalent limitations when requiring real-time imaging or reagent manipulation [7]. To enable live imaging, perfusion chambers have been used in conjunction with microscopy to enable analysis of real-time data [8]. These perfusion chambers work by limiting diffusion of ambient air into the cell channel. The oxygen conditions are modulated by introducing liquids with a pre-equilibrated dissolved oxygen concentration into the chamber. However, handling and use of the reagents after equilibration of oxygen concentration is imprecise and challenging due to diffusion of oxygen from ambient air. Furthermore, current studies

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Table 1. Partial oxygen pressure (pO₂) of solid tumors and the associated surrounding healthy tissue[a]

Tumor type	Median diseased pO_2	Median Health pO_2
Glioblastoma	4.9–5.6	–
Head and neck carcinoma	12–15	40–51
Lung cancer	7.5	38
Breast cancer	10	–
Pancreatic cancer	2.7	52
Cervical cancer	3.0–5.0	51
Prostate cancer	2.7	52
Soft-tissue sarcoma	6.2–18	–

[a]All pO_2 values are combined medians of individual values that were measured in mmHg using an oxygen electrode (adapted from [4]).

using this system have required cells to adhere to a microscope slide and, thus, the cells were not grown in a three-dimensional (3D) environment. While providing cells a 3D environment may be possible when using perfusion chambers, current studies have relied on 2D cultures. As such, this limitation may affect cell behavior, as 3D cell cultures are more physiologically relevant and have been found to differ significantly from 2D cultures in the proliferation, migration, and expression of cell-surface receptors [9–11].

A second approach used to study cellular responses to hypoxia is by biochemically inducing a pseudo-hypoxic state in cells (Figure 1B). This method is distinct from others as the aqueous solution remains oxygenated while investigators rely upon chemical treatments to induce signaling events associated with hypoxia. A number of chemicals such as prolyl hydroxylase inhibitors, nickel chloride, and, the most widely used chemical inducer of hypoxia, cobalt chloride, stabilize the transcription factor hypoxia inducible factor 1-α (HIF-1α) (Box 2) and thus mimic hypoxia [6,12–16]. The two proposed mechanisms by which cobalt chloride stabilizes HIF-1α include inactivating prolyl hydroxylases by chelating their iron core and replacing it with cobalt [17], or by taking up the VHL-binding domain of HIF-1α, thus rescuing it from degradation [18]. In either case, this state of pseudo-hypoxia has proven to be useful for many biochemical analyses, as oxygen can be present in samples without affecting the

Box 1. Hypoxia in the tumor microenvironment

The tumor microenvironment consists of populations of transformed cells, stromal cells (e.g., fibroblasts, chondrocytes), vascular cells (e.g., endothelial cells, pericytes), and inflammatory cells (e.g., neutrophils, macrophages), along with acidity (lactic acid from enhanced glycolysis), increased interstitial pressure, and low oxygen partial pressures [57,73,74]. As tumors grow outward away from the local vascular architecture, and become increasingly metabolically active, formation of variable hypoxic regions throughout the solid mass is inevitable [75]. Neoplastic angiogenesis occurs in an attempt to restore oxygenation, but this neo-vasculature is often insufficient and poorly arranged. This leads to the development of even further gradients of oxygen, nutrients, and waste materials [76,77]. This poses as a therapeutic challenge, as tumor hypoxia is associated with resistance to radiotherapy and chemotherapy and is a negative prognostic indicator for a more aggressive phenotype [78,79]. Furthermore, tumor cells associated with hypoxia are thought to be more malignant and capable of metastasis through avoidance of apoptosis, expression of invasion genes such as matrix metalloproteases, and downregulation of homeostatic genes such as E-cadherin [80–83].

Two aspects of the TME, lower pH and hypoxia, are functionally linked via aerobic glycolysis (Warburg effect), the production of lactate from glucose despite the presence of ample oxygen [84]. When compared to the complete oxidation of glucose to pyruvate through the citric acid cycle and electron transport chain, this metabolic pathway is markedly inefficient [85]. However, aerobic glycolysis likely represents an adaptation to the needs of hyper-proliferative and/or hypoxic cells [74]. Rapidly dividing cells have anabolic needs greater than ATP; glucose can be shuttled away from ATP production to form other macromolecular precursors such as acetyl-CoA, glycolytic intermediates for amino acids, and ribose for nucleotides [86]. Furthermore, other metabolic alterations in cancer cells, such as increased fatty acid synthesis and glutaminolysis, have been suggested as adaptive changes to the constraints of rapid growth in hypoxia. While the molecular switches governing these responses are still being unraveled, tumorigenesis and metabolism are clearly intertwined as all prominent oncogenic alterations have been experimentally linked to changes in metabolism [87]. Ultimately, however, the bioenergetic rearrangement towards aerobic glycolysis emerges from a number of factors including oncogene upregulation, tumor suppressor loss, mtDNA mutation, and stabilization of hypoxia inducible factor 1-α (HIF-1α) in the hypoxic microenvironment [88].
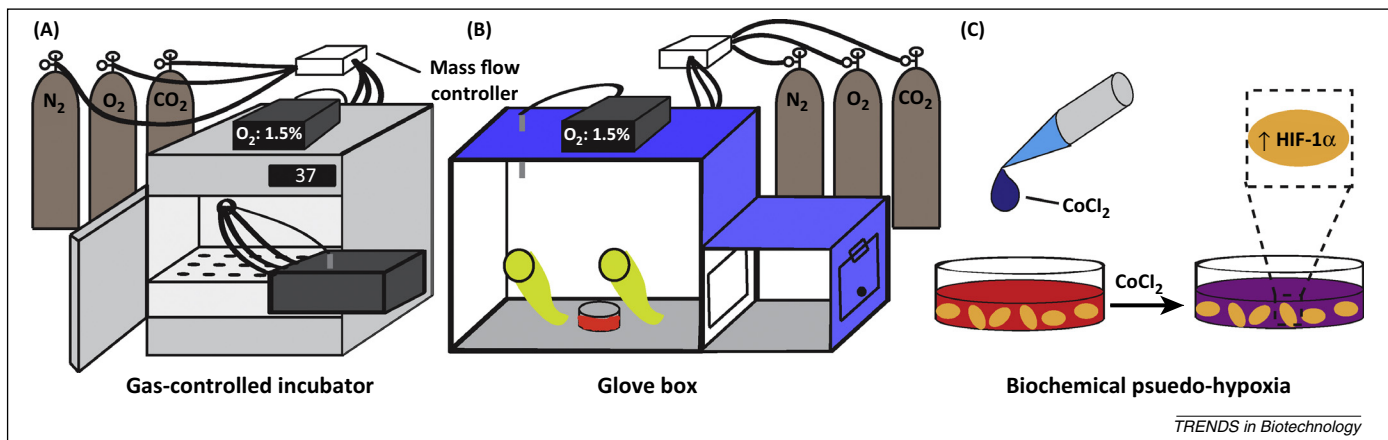

Figure 1. Illustrations of three conventional methods that enable control of oxygen concentration during cell studies: **(A)** a gas-controlled incubator; **(B)** a glove box; and **(C)** biochemical induction of a pseudo-hypoxic state. Abbreviation: HIF-1α, hypoxia inducible factor 1-α.