Video Article

Anaerobic Growth and Maintenance of Mammalian Cell Lines

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URL: https://www.jove.com/video/58049
DOI: doi:10.3791/58049

Keywords: Biology, Issue 137, anoxia, anaerobiosis, phenotypic variation, mammalian cell lines, HeLa cells, Vero cells, long-term culture

Date Published: 7/21/2018

Citation: Plotkin, B.J., Sigar, I.M., Swartzendruber, J.A., Kaminski, A. Anaerobic Growth and Maintenance of Mammalian Cell Lines. *J. Vis. Exp.* (137), e58049, doi:10.3791/58049 (2018).

Abstract

Most mucosal surfaces along with the midpoints in tumors and stem cell niches are geographic areas of the body that are anoxic. Previous studies show that the incubation in normoxic (5% CO_2 in air) or hypoxic (low oxygen levels) conditions followed by an anoxic incubation (an absence of free oxygen) results in limited viability (2–3 days). A novel methodology was developed that enables an anoxic cell cultivation (for at least 17 days; the maximum time tested). The most critical aspect of this methodology is to ensure that no oxygen is introduced into the system. This is obtained by the degassing of media, and by flushing tubes, dishes, flasks, and pipettes with an anaerobic gas mixture (H_2, CO_2, N_2) followed by permitting the materials to equilibrate to the anoxic (non-oxygen) environment prior to usage. Additional care must be exercised when acquiring photomicrographs to ensure that the micrographs obtained do not contain artifacts. In the absence of oxygen, cell morphology is significantly altered. Two distinct morphotypes are noted for all anaerobically-grown cells. The ability to grow and maintain mammalian cells in the absence of oxygen can be applied to the analysis of cell physiology, polymicrobial interactions, and the characterization of biosynthetic pathways for novel cancer drug development.

Video Link

The video component of this article can be found at https://www.jove.com/video/58049/

Introduction

Cells from solid tumors, stem cell niches, and those lining the mucosal surfaces exist in environments that experience reduced oxygen levels, including anoxia[1,2,3,4]. In normal physiologic states, oxygenation varies beyond that of hypoxia to anoxia (the complete absence of oxygen)[5,6].The realization that atmospheric oxygen adversely affects mammalian cell replication and that *in vitro* cell growth can be optimized under depleted oxygen conditions was reported in the early 1970s. Richter *et al.*[7] showed that 1–3% of oxygen levels (hypoxia) enhanced plating efficiency as compared to atmospheric oxygen (20%). The human diploid cell lifespan is also extended in the hypoxic culture conditions[8].

In vivo, hypoxic conditions occur when oxygen stores are depleted (*e.g.*, during intense exercise), wherein the ATP production is switched in the skeletal muscle from aerobic respiration to fermentation (anaerobic respiration) with the end-product of lactic acid[9]. Pathologically, in cancerous tumors, the interior of the tumor mass is hypoxic to anoxic due to poor vascularization[10]. The effect of the limited perfusion on tumor interiors is independently validated by tumor interiors colonized by obligate anaerobes[1]. Mechanistically, tumor cell survival in hypoxia is thought to be solely dependent on the expression of the hypoxia-inducible factor 1-alpha gene (*HIF1*-alpha), which is the initial spontaneous response to hypoxia[4,11,12]. *HIF1*-alpha is induced under hypoxic conditions by heat shock proteins that bind the *HIF1*-alpha promoter and upregulate the gene transcription[12]. These heat shock proteins are believed to aid in the induction of the various phenotypes seen in the tumor hypoxic microenvironment. These phenotypes exhibit an increased expression of the cell membrane glucose transporters and the rate of glycolysis (the Warburg effect)[13]. The result is a switch from mitochondrial oxidative phosphorylation to lactate fermentation.

Anoxic survival can also utilize alternatives to glucose to support the survival phenomenon[14,15]. The best studied mammalian example is the mole rat, which can survive for nearly 20 min without oxygen through a fructose-driven glycolytic fermentation pathway[14]. An alternative adaptation occurs in certain fish (*e.g.*, carp [*Carassius* sp.], which can survive for significantly longer time periods using glycolysis with ethanol as the terminal by-product)[15]. In both cases, fermentation drives the metabolism enabling the survival in the absence of oxygen. The current hypothesis for anoxic survival is that so long as *HIF1*-alpha is activated during hypoxia, mitochondrial respiration, without the need for oxygen, occurs under anaerobic conditions[16]. Furthermore, it is postulated that the use of a fermentative pathway for hypoxic/anoxic survival enhances tumor survival since the cells avoid oxidative stress which could prove to be detrimental to the cell survival[17]. This postulate is supported by a recent study which shows that in cardiomyocytes, hypoxia reduces the oxidative stress placed on the tumor cell[17].

To date, the essential nature of a fermentative pathway for anoxic mammalian cell survival has been ingrained in the literature, due, in large part, to an inability to culture mammalian cells in the complete absence of oxygen for more than 3 days. However, an alternative to glycolysis for the anaerobic survival occurs in bacteria. In certain bacteria, nitrogen or sulfate (among other compounds) can serve as terminal electron acceptors for the cytochrome oxidase system in the absence of oxygen[18]. Although the bacterial and eukaryotic evolution occurred in parallel since diverging from the last universal common ancestor, it is estimated that mitochondria were providing energy to cells for 1.542 million years before the oxygenation of the oceans[19]. Since researchers have shown that the isolated mitochondria can produce ATP in the absence of

oxygen, with nitrite as the terminal electron acceptor, it is reasonable to assume that cells could function for periods of time longer than 3 days in the absence of oxygen[20,21,22,23]. The methodology described in this study has a utility for the anaerobic mammalian cell growth of numerous cell lines.

Protocol

1. Prepare Media for Anaerobic Culture of Various Mammalian Cell Lines

1. **Make the complete PS-74656 medium for an anoxic cell culture[25].**
    1. Make the sterile nitrite stock solution (5 M; 100x) by dissolving 17.25 g of nitrite in 50 mL of distilled deionized water, then filter to sterilize it.
    2. Add 0.5 mL of the nitrite stock solution per 50 mL of low glucose DMEM (1 g/L of glucose) with 110 mg/L of L-glutamine, 584 mg/L of sodium pyruvate, and 10% fetal bovine serum (FBS; heat-inactivated).
    NOTE: The use of FBS concentrations greater than 10% are toxic. Of note, one cancer cell line tested had a low tolerance for media with nitrite (MDA-MB-231, breast cancer cell line) and, thus, was grown in the absence of nitrite. In addition, although PS-74656 medium supported the growth of all cell lines that were tested (*n* = 9), certain cell lines (*e.g.*, Vero cells) exhibited higher cell concentrations and a higher viability in PS-74656 with high glucose (4.5 g/L) DMEM[25].
    3. Place 20 mL of medium in a sterile 50 mL conical centrifuge tube. Do not tighten the cap; it should be loose.
    4. De-gas the medium by placing the tube in a vacuum bell jar at an approximately 45° angle, to maximize the medium surface area.
    5. Apply a vacuum using a vacuum pump, or its equivalent, at room temperature. Maintain the vacuum until bubbles are no longer observed (24–48 h).
    6. Remove the tube from the jar and immediately close the cap, sealing the tube to minimize the introduction of air into the medium.
    7. Place the tube in the commercial anaerobic chamber.
    8. Loosen the tube cap and allow the atmosphere in the tube to equilibrate with that of the anaerobic gas mixture in the chamber for at least 24 h.
    NOTE: The medium should remain in the chamber until it is used up. For speeding up the process, flush the tube at least 3x with the anoxic gas mixture using a sterile transfer pipette filled with anoxic gas, which consists of H_2, CO_2, and N_2.
    9. Degas all tubes (10–15 min) placed in the anaerobic chamber as described above before sealing the tube and placing in the chamber. Before use, flush it with the anaerobic gas mixture using 1.5–2 mL transfer pipettes or pipette tips that have been flushed with anoxic gas at least 3x.
    10. While in the anaerobic chamber, remove 100 µL of the medium to a sterile degassed micro-centrifuge tube and test it for the dissolved oxygen using an oxygen probe within the chamber.
    NOTE: The oxygen electrode is calibrated per the manufacturer's protocol prior to use. Readings of the properly degassed and equilibrated anaerobic gas mixture media are 0.
    CAUTION: If the readings are above 0.3 ppm of oxygen, continue to incubate the media in the anaerobic chamber since more time to equilibrate the medium is required.

2. Anoxic Cultivation of Various Mammalian Cell Lines

1. **Designated day -1**
    1. Grow cells (37 °C, 5% CO_2) in a T150 flask to 90% confluence for the anoxic and normoxic culture control to provide a sufficient number of cells for three 24-well plates (80% confluence).
    NOTE: For this study, HeLa 229 and Vero cells were used. This protocol was also tested and proved successful for the growth and maintenance of seven other cell lines[25].
    2. Remove the medium from the flask by aspiration and wash it with 10 mL of HBSS.
    3. Replace the medium with 5 mL of trypsin. Agitate the flask until the cells visually detach from the plastic. Aspirate most of the trypsin, then tap the flask to dislodge the adherent cells. Add 10–15 mL of high glucose DMEM (4.5 g/L of glucose, 110 mg/L of L-glutamine, 10% FBS, 50 µg/mL of gentamicin) to inactivate the trypsin.
    4. Triturate the cells using a 25 mL pipette until all cell clumps are disrupted.
    5. Count the cells and determine the viability using the standard hemocytometer and the trypan blue dye exclusion method or the automated equivalent.
    6. Suspend the cells in the high glucose DMEM as mentioned above to a cell density of 2.24×10^5 cells/mL.
    7. Add 1 mL of the cell suspension to the wells of a 24-well tissue culture plate (2.24×10^5 cells/well; 80% confluence). Seed 1 plate for the anoxic culture and another for a control normoxic culture.
    8. Incubate the cells at 37 °C in normoxic conditions (5% CO_2) for 24 h.
    NOTE: The cells can be plated in various well-sized plates. However, seeding the cells for anoxic growth in flasks runs the risk of the introduction of oxygen into the system, unless care is exercised to completely flush out atmospheric gas.

2. **Designated day 0**
    1. After 24 h of the normoxic incubation, place the plates for the anoxic growth into the anaerobic chamber (a commercial anaerobic chamber; **Figure 1**).
    2. Immediately change the medium to antibiotic-free de-gassed PS-74656 medium which has been acclimated for 24 h to the anaerobic chamber. Place the plate in a container with a damp towel and loosely close it to maintain the moisture.
    NOTE: The plates for normoxic growth are handled identically with the exception that all the treatments are done under atmospheric conditions.

3. **Designated Day 1**

1. Day 1 of the anaerobic incubation starts after 24 h of the incubation in the commercial anaerobic chamber which contains the standard anaerobic gas mixture of 10% H_2, 10% CO_2, and 80% N_2.
2. Incubate the cells for various time periods.
3. Monitor the anaerobic medium for the color change over time.
   NOTE: A color shift from red-orange to magenta signals the time to change the medium. This can take up to 2 weeks to occur. A color shift in the medium from red-orange to yellow indicates the presence of a bacterial contamination.
4. When the medium changes from red to magenta, remove the runagate cells by aspiration.
5. Place the aspirated spent media with the runagate cells in degassed and anaerobic gas equilibrated microfuge tubes, close the tubes while making sure that the seal is secure, and then centrifuge them (326 x *g*; at room temperature for 10 min). Immediately replace the aspirated media in the well with 1 mL of new degassed PS-74656 medium.
6. Place the microfuge tube with pelleted cells in the anaerobic chamber prior to opening it. Aspirate the spent medium and replace it with fresh degassed PS-74656 medium to a total volume of 1 ml in the microfuge tube.
7. Return the cells from the tube to a well in the 24-well tissue culture plate.

3. Assessment of Phenotypic Cell Differentiation by Microscopy of Anaerobically Cultured Cells

1. **While in the anaerobic chamber, place the plate in a resealable box flushed with the anaerobic gas mixture and close the box.**
   NOTE: The box must contain a damp paper towel to provide moisture and prevent the plate from drying out. For microscopy, sandwich bags work the best since they are thin.
   1. To observe the cells under the microscope, completely seal the bag and remove it from the chamber.
   2. Carefully stretch the plastic bag over the plate, examine cells using an inverted phase contrast microscope with the camera attachment and take photomicrographs at various magnifications.
   3. Return the plate in the sealed bag to the chamber, and continue the incubation as described in step 2.3.3.
   4. To separately analyze the runagate cells that are in suspension, follow the steps outlined in step 2.3.5.
      NOTE: These cells can then be used for aerobic testing or can be returned to the anaerobic chamber for the testing of this specific population.

Representative Results

The strength of this protocol lies in its support of the longevity and the growth of multiple cell lines and in the recognition that there are a profound alteration and divergence in the cell morphology[25]. The most critical element of this study is the transfer and maintenance of the cells under strict anaerobiosis. This requires an anaerobic chamber organized to maximize the protocol (**Figure 1**) and the assurance that the cells removed for either microscopy or other testing are kept in a sealed environment. To date, the range of anoxic survival was 15–17 days for immortal cancer cell lines and up to 17 days for transformed cell lines (nine cell lines screened for anoxic growth). The levels of viable cells, once these cells were moved to anoxic conditions, declined at least 10%, to a maximum of 90%, before they stabilized. All cell lines reverted to the dual phenotypes (tethered and runagate) with the proportion of cells in the suspension increasing over time. Even after an extended incubation of several weeks, there remained some tethered cells, but the majority of the cells were in the rounded runagate morphology (**Figure 2**). As reported, these runagate cells are viable and proceeded through their cell cycle. It should be noted that the variables that may need to be adjusted include the glucose levels and the nitrite concentration. That said, from the over 60 different media formulations that were tested for their ability to support both normoxic and anoxic incubation, the PS-74656 medium was able to support the survival and replication of nearly all the cell lines tested.

An essential observation was the differentiation of anoxic cells, regardless of the cell tested, into two distinct phenotypes, tethered and runagate (**Figure 2**). Analogous to the normoxic monolayer cells, the tethered cells were attached to the tissue culture plastic. However, the attachment was weaker, easier to trypsinize or dislodge by scraping, than the attachment that occurred under the normoxic conditions. In addition, the cell morphology was smaller with more spindle (dendritic) extensions[26]. The second cellular morphotype observed were cells in suspension (*i.e.*, runagate cells). These cells spontaneously converted into free-floating single cell suspensions in the presence of tissue culture-treated plastic, which were rounded with a smaller cell size and less cytoplasm. They differed from the classical suspension culture cells, which typically require an enzymatic adaptation, grow in aggregates, require non-tissue culture-treated containers, and are larger than their normoxic counterparts[27]. Over time, the anoxic cell populations could be observed to shift from a predominantly tethered monolayer to that of the runagate cells in suspension. These cells in suspension were viable and replicating; thus, care had to be exercised should the supernatant be removed. Also, of note is that the medium did not require changing over the entire anaerobic incubation period. The medium pH indicator under the anoxic incubation, in contrast to its normoxic control, was also unchanged (*i.e.*, remained red). Should the investigator wish to harvest the runagate cells, or change the medium, the cells can be obtained using anaerobic gas-flushed micro-centrifuge tubes that are closed tightly within the anaerobic chamber. For the cell washing of tethered cells, the procedure must also be performed in the chamber to eliminate any oxygen exposure.

The final cell viability for a long-term cultivation was, to a great extent, dependent on the cell being used and the confluence to which the wells or the flask were initially seeded (**Figures 3** and **4**). That said, a good starting point is 80%, which had the greatest chance of optimal growth. In addition, a cell line-dependent loss of 10% to 60% of viability can occur in the initial 2–4 days. However, during the performance of this experiment, the remaining cells cycled through the cell cycle and replicated[28]. The seeding confluence effect on the longevity of the cell cultivation can be seen in **Figures 3** and **4**. Both HeLa 229 and Vero cells were seeded in 24-well plates to 40%, 60%, and 80% of initial confluence (100% confluence = 2.8 x 10^5 cells/well) and then incubated in the absence of oxygen for 17 days. The HeLa 229 cell anoxic growth was optimized at an initial seeding confluence of 80%. In contrast, the Vero cells optimal seeding confluence was 60%. This demonstrates the need to initially optimize the seeding density dependent on the cell line used.

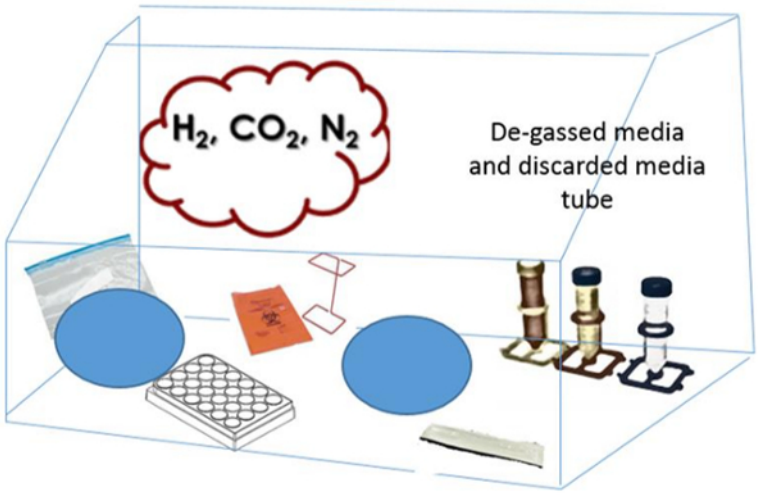

Figure 1: Model of anaerobic chamber set-up for culture of mammalian cells. The minimal materials required include tissue culture plates, sterile transfer pipets, biohazard bags, de-gassed media, a tube for the discarding of media or other liquids, and resealable bags for the transfer of the plates to the microscope while maintaining anaerobic conditions.

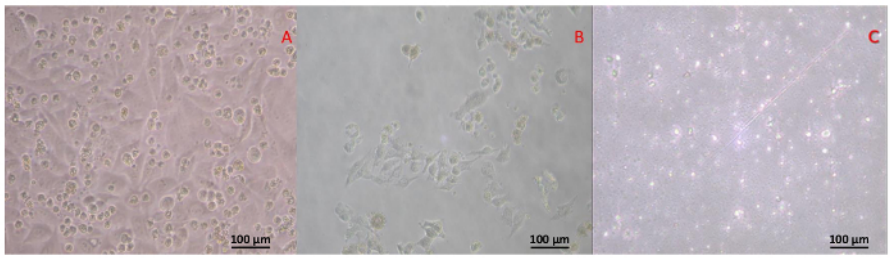

Figure 2: Anoxic HeLa cells morphologic differentiation. (**A**) This panel shows the phase contrast microscopy of the normoxic HeLa cell controls 4 days post-inoculation, with a 20X magnification. **(B)** This panel shows the anoxic HeLa cells 17 days post-inoculation (with the unchanged from day 1 PS-74656 medium, 80% seeded confluence, and a 20X magnification). (**C**) This panel shows microscopy artifacts of water droplets on the interior of a sealed plastic bag containing a 24-well anoxic plate. Please click here to view a larger version of this figure.

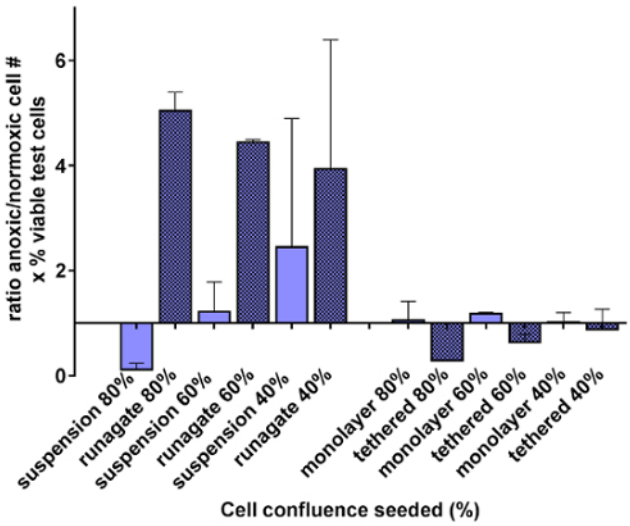

Figure 3: Ratio of anoxic to normoxic HeLa 229 viable cells post-17 days incubation. This panel shows the effect of the seeding density on the HeLa 229 cell viability in the PS-74656 medium as compared to the normoxic control. The results are shown as the mean ± SEM.

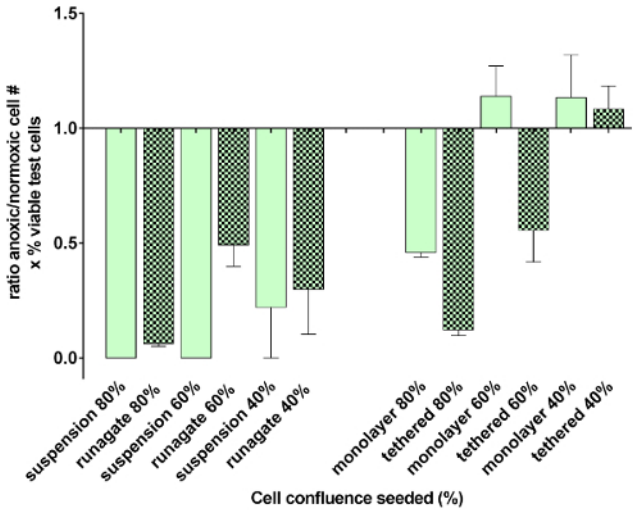

Figure 4: Ratio of anoxic to normoxic Vero viable cells 17 days post-incubation. This panel shows the effect of the seeding density on the Vero cell viability in the PS-74656 medium as compared to the normoxic control. The results are shown as the mean ± SEM.

Discussion

This method represents first-time mammalian cells that were cultured for extended periods of time in the absence of oxygen. Based on current observations, the anoxic growth capability *via* a non-fermentative pathway appears to be universal amongst mammalian cells lines[28], where the anaerobic growth resulted in phenotype divergence. This was observed for all cell lines tested. With the anaerobic cultivation, increasing proportions of the cells became rounded, developed a suspension-like population, and were able to replicate. The secondary cell type remained attached to the substrate; however, the cell morphology reverted to a spindle (dendritic) shape. These findings point out a common error in analyses and observations with regard to anoxic tissue culture cultivation. It is generally accepted that when mammalian cells grown under normoxic conditions (5% CO_2) round up and detach, they are dying. The contrary appears to be correct for cells grown in the absence of oxygen, in which case, cells that have rounded up and detached, are simply a morphologically divergent cell subpopulation.

The optimization of anoxic growth conditions is cell line dependent. Medium PS-74656 overall supported the growth of all cell lines under normoxic and anoxic growth conditions, with the exception of MDA-MB-231, a breast cancer cell line which preferred growth in the absence of nitrite. An encouraging observation is that even with a 98% die-off of cells per well, the remainder replicates, resulting in a highly adapted population. The critical action is to maintain cultures even when only a few cells are directly observed by microscopy. Thus, care must be exercised to ensure that the detached cells are dead, either by trypan blue dye exclusion or by an alternative cell viability detection method. It should be noted that with such a large decrease in cell numbers, sufficient wells must be seeded to have adequate total cell numbers for subsequent experiments. The expected loss of viable cells is both cell line and seeding confluence dependent; thus, optimal cell numbers, over time, must be determined experimentally and cannot be extrapolated from findings with other cell lines. That said, a good starting point is an 80% confluence, which has the greatest chance of optimal growth. It must be noted that any exposure to oxygen during the anaerobic steps in the procedure, no matter how brief, will compromise the findings due to the reported spontaneous cellular *HIF-1*-alpha response[11,12].

For this procedure, the most critical step is that of the de-gassing of the medium, tubes, and all other apparatuses and materials used. The introduction of even very low levels of oxygen could stop the transition from the fermentation metabolism to the anaerobic respiration. Alternatively, it could prolong the fermentative metabolism so that any experiments result in the measurement of hypoxia, not anoxia. The use of this novel procedure to measure cytotoxicity and chemotherapeutic activity could help to explain drug failure in the treatment of solid tumors.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors thank the Midwestern University Office of Research and Sponsored Programs for their support.

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