Video Article

Use of Time Lapse Microscopy to Visualize Anoxia-induced Suspended Animation in *C. elegans* Embryos

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Abstract

Caenorhabdits elegans has been used extensively in the study of stress resistance, which is facilitated by the transparency of the adult and embryo stages as well as by the availability of genetic mutants and transgenic strains expressing a myriad of fusion proteins[1-4]. In addition, dynamic processes such as cell division can be viewed using fluorescently labeled reporter proteins. The study of mitosis can be facilitated through the use of time-lapse experiments in various systems including intact organisms; thus the early *C. elegans* embryo is well suited for this study. Presented here is a technique by which *in vivo* imaging of sub-cellular structures in response to anoxic (99.999% N_2; <2 ppm O_2) stress is possible using a simple gas flow through setup on a high-powered microscope. A microincubation chamber is used in conjunction with nitrogen gas flow through and a spinning disc confocal microscope to create a controlled environment in which animals can be imaged *in vivo*. Using GFP-tagged gamma tubulin and histone, the dynamics and arrest of cell division can be monitored before, during and after exposure to an oxygen-deprived environment. The results of this technique are high resolution, detailed videos and images of cellular structures within blastomeres of embryos exposed to oxygen deprivation.

Video Link

The video component of this article can be found at http://www.jove.com/video/4319/

Protocol

1. Sample Preparation

1. Produce or obtain appropriate transgenic *C. elegans* strain of interest using transgenic methodologies or from *Caenorhabditis* Genetics Stock Center (CGC) or colleague. In this case we are using strain TH32 (*pie-1::tbg-1::GFP; pie-1::GFP::H2B*)[5] to visualize chromosomes and centrosomes as markers for cell division.
2. Generate a synchronized population by using one of three methods: 1) disintegrate gravid adults in hypochlorite solution and use remaining embryos[6], 2) pick gravid adults onto a seeded plate and let them lay embryos for 1-2 hr, or 3) pick L4 larvae from a mixed population and move to a new seeded plate.
3. Grow nematodes at 20 °C to gravid young adulthood, which will take approximately 96 hr from hatching, 72 hr from L1 larvae or 24 hr post L4 molt. Embryos (2-20 cell) *in utero* contain large blastomeres and nuclei that are optimal for imaging sub-cellular and sub-nuclear changes, respectively. This methodology provides a means to generate a population of young adults that contain an abundant number of early embryos.

2. Slide Preparation and Anoxia Chamber Set Up

1. Make a humid chamber to hold prepared slides by placing a damp paper towel inside a large Petri dish or bin covered with a lid of sufficient size.
2. Prepare molten 2% agarose in deionized H_2O by heating mixture in glassware via microwave or water bath.
3. Place 1-3 drops of warm agarose on a clean glass microscope slide. Immediately place a second slide inverted perpendicularly on top of the agarose drop(s), press slightly so that the resulting pad will be thin and free of air bubbles.
4. Allow time to cool and slowly take apart the two microscope slides leaving the agarose pad intact on one of the slides. It is important to ensure the agarose pad is thin enough to not interfere with the ability to focus the microscope later. Ability to visualize sample is also dependent upon the specific optical distance of the objective in use, which can vary between microscopes.
5. Use a clean razor blade to shape agarose pad into a small square. Store in the prepared humid chamber until ready to use.
6. Carefully, using the same clean razor blade, take the edge of the agarose pad and transfer it from the slide onto a round 25 mm micro coverglass, avoiding the accumulation of air bubbles underneath the pad. The round coverglass selected to use fits appropriately in the microincubator.

7. Add a drop of anesthetic (0.5% tricaine, 0.05% tetramisole in M9 buffer) onto the agarose pad for use as an anesthetic. Pick 5-10 worms and place into the drop of anesthetic. Allow 1-3 min for worms to cease movement.
8. The rationale for observing embryos within the adult uterus, instead of dissected embryos, is to minimize the potential of embryo desiccation and movement due to the flow of nitrogen gas across the embryo.
9. Immediately add a drop of halocarbon oil on top of the worms to prevent the worms from dehydration as gas is flowed through the chamber. Since the halocarbon oil is viscous one may use a pipet tip or tip of a worm pick to collect oil and drop onto the worms.
10. Once the circular coverglass containing the worms is ready, the two halves of the Leiden closed perfusion microincubator are separated, and the coverglass is then carefully placed upright onto the bottom ring. The two sides of the chamber are then closed tightly together.
11. The chamber is then connected to the nitrogen gas tank via flexible plastic tubing and placed into the appropriate spot on the microscope stage (**Figure 1C**). At this time the tubing and microincubator should be carefully checked for any leaks by ensuring secure attachment of the tubing and of the port plugs along the side of the chamber. One can also lightly spread a small amount of soap water over the tubing to look for bubbles, which will form if a leak is present.

3. Microscopy in Anoxia

1. Locate animals at lower magnification, then, move to the appropriate higher magnification (64x for this application) to image tissue or cells of interest such as embryonic blastomeres or oocytes in adults. Turn on the 488 nm laser to view GFP signal and locate GFP fusion protein in the cell(s) of interest.
2. To visualize the arrest at specific stage of cell cycle arrest (*e.g.* late prophase) identify a blastomere at a prior stage of cell division (*e.g.* late interphase or early prophase). Cellular markers such as centriole position, initiation of chromosome condensation, chromosome location and presence or absence of the nuclear envelope will help identify cell cycle stage (**Figure 2A**).
3. The chamber is then perfused with nitrogen gas (99.999% N_2; <2 ppm O_2). In this case we use a pressure of 6 psi, however pressure may need to be adjusted and optimized depending on size of each microincubator and diameter of tubing in use. Continue to monitor the blastomere(s) as the nitrogen fills the chamber, adjusting the focal plane as necessary.
4. At this point, time-lapse imaging has begun. Imaging is conducted for as long as necessary to capture phenomenon of interest, in this case prophase arrest and docking of chromosomes to the inner nuclear membrane. Images are taken once every 10 sec for no longer than 30 min. Frequencies of image capture and settings such as gain and laser power should be adjusted as necessary.
5. The time in which the embryos respond to the anoxic environment can vary depending on cell cycle stage upon anoxia exposure. Typically anoxia-induced prophase arrest occurs within 20-30 min of beginning nitrogen gas flow. We have observed anoxia-induced cell cycle arrest occurring in <20 min. Images can be obtained during this time frame, or specific images can be isolated later from a time-lapse series. An additional option is to use video microscopy to visualize sub-cellular structures in embryos.
6. To document recovery from the anoxia-induced arrested state, the gas is turned off and the chamber is allowed to return to normoxia by diffusion while recording a time-lapse series. Resumption of cell cycle progression and undocking of chromosomes typically occurs within 5-20 min of turning the nitrogen gas off.
7. Note that in separate experiments an anoxia indicator, resazurin, was placed in the flow-through microchamber; it was determined that the chamber becomes anoxic on average of approximately 19 min after nitrogen gas flow has started.
8. Images and videos are processed using Imaris, Image J or Photoshop and imported into QuickTime for display.

Representative Results

C. elegans embryos exposed to severe oxygen deprivation (anoxia) are able to survive by arresting biological processes including development and cell division[7]. The anoxia-induced arrest of cell division can be monitored, at a sub-cellular level, by using a microincubation chamber in conjunction with nitrogen gas flow through and a spinning disc confocal microscope to create a controlled environment in which animals can be imaged *in vivo* (**Figure 1**). Cellular structures of interest can be monitored in the embryo exposed to various gas environments; in this case we used GFP-tagged gamma tubulin and histone H2B to monitor chromosome structure in an embryo exposed to anoxia.

Within the embryo exposed to anoxia, the chromosomes of prophase blastomeres will condense and align with the inner nuclear periphery; a phenomenon referred to as "chromosome docking" (**Figure 2, Movie 1**). Upon re-oxygenation cell cycle progression will resume and chromosomes of prophase blastomeres will move from the nuclear periphery to the equatorial plate indicating progression to metaphase (**Movie 2**). This technique can also been used to visualize arrest at other stages of cell division or bivalent chromosomes of oocytes in hermaphrodites exposed to anoxia[8].

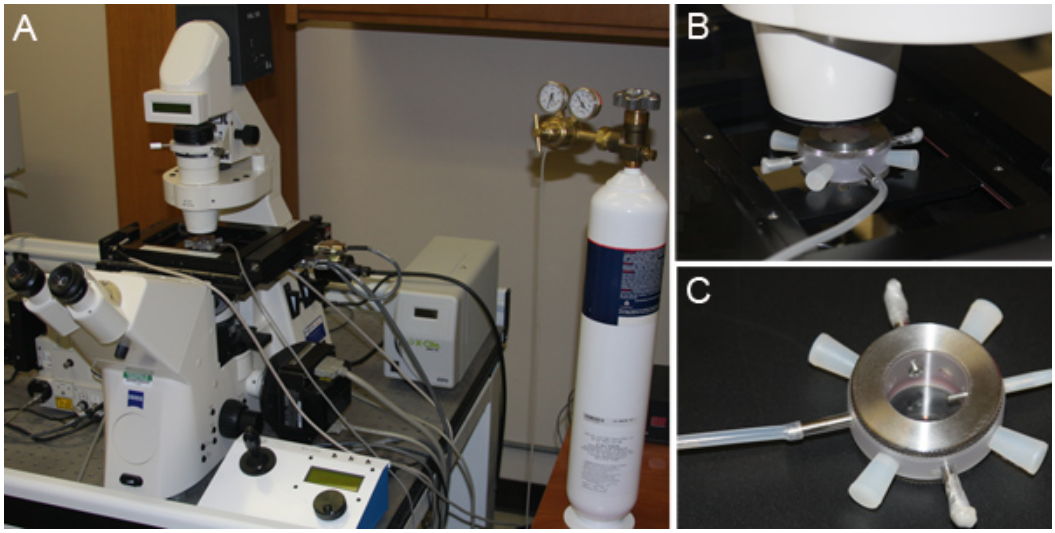

Figure 1. Example of microscope and chamber setup used for *in vivo* analysis. Zeiss inverted confocal microscope with Leiden Closed Perfusion Microincubator and attached nitrogen gas tank (A); microscope stage and microincubator (B); and enlarged view of microincubator (C).

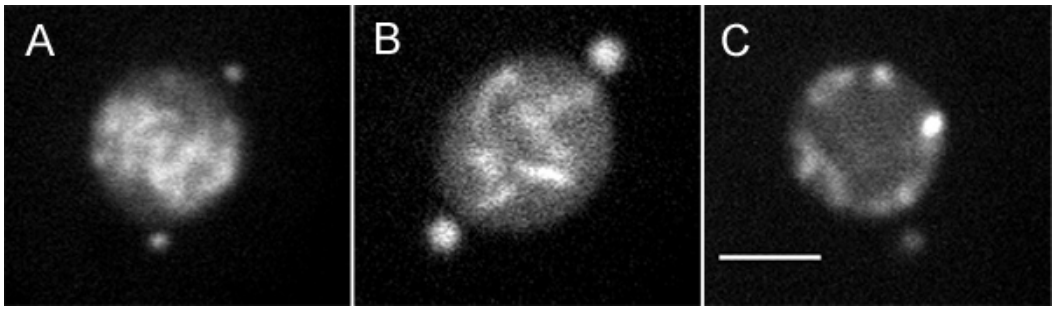

Figure 2. Hallmark of anoxia-induced prophase arrested blastomere. Shown are representative images of a prophase blastomere at the start of time-lapse imaging (A) blastomere of an embryo exposed to normoxia (B) or a blastomere of an embryo after 30 min of anoxia exposure (C). The anoxia-induced prophase arrested blastomere displays chromosomes docked near the inner nuclear periphery and NEBD is arrested. Scale bar = 5 µm.

Movie 1. Time-lapse analysis of a blastomere in transition from normoxia to anoxia. Note chromosomes aligning to the nuclear periphery. Images were captured 28 min after gas flow through began. Click here to view movie.

Movie 2. Time-lapse analysis of a blastomere recovering from anoxia. As oxygen returns to the environment cell cycle resumes. Images were captured 10 min after gas flow through was ceased. Click here to view movie.

Discussion

Oxygen Deprivation and Suspended Animation

Although exposure to severe oxygen deprivation can be fatal for some organisms, some organisms are able to survive exposure to anoxia. In the case of *C. elegans*, survival of anoxia exposure depends on developmental stage and response to anoxia is entry into a reversible state of suspended animation in which a number of observable biological processes are arrested. Cell division, development, movement, eating and reproductive processes cease until oxygen is reintroduced into the environment[7,9]. Cell cycle progression, of anoxia-exposed embryos, reversibly arrests at interphase, late prophase or metaphase. The use of cell biological techniques such as immunostaining in fixed samples and *in vivo* GFP fusion protein assays, which allows imaging of specific sub-cellular structures in an oxygen deprived environment, have been instrumental in identifying and characterizing specific hallmarks of anoxia-induced suspended animation in *C. elegans* [8,10,11].

Both embryos and oocytes display marked characteristics of anoxia-induced suspended animation that are easily visible using fluorescent microscopy in combination with fluorescently labeled proteins or antibodies against specific proteins to analyze fixed embryos. Interphase blastomeres, of embryos exposed to anoxia, are characterized by partial condensation of chromatin and arrest of cell cycle progression with some chromatin aligning at the nuclear periphery[9]. While nuclear envelope breakdown (NEBD) signifies a final commitment to mitosis in anoxia, prophase arrest is characterized by arrest of NEBD, and alignment of fully condensed chromosomes at the nuclear periphery[10,12]. Metaphase arrest, on the other hand, is characterized by arrest of the chromosomes at the metaphase plate and partial depolymerization of microtubules, among other things. Anoxia-induced cell cycle arrest at all stages is reversible and upon reoxygenation, cell-cycle progression resumes. In

anoxia exposures of 24 hr or less, control and experimental animals are indistinguishable upon recovery[7]. Using the methods described here the animal is able to survive anoxia exposure.

Imaging Analysis and Considerations

The method presented here utilizes the TH32 (*unc-119* (*ed3*) ruIs32 III; ddIs6) strain of *C. elegans*. This strain expresses two fluorescently labeled proteins that facilitate the visualization of centrosomes and chromosomes. Centrosomes are marked by ddIs6 [*pie-1::tbg-1::GFP + unc-119* (+)], which encodes GFP-tagged gamma tubulin, and localizes to centrosomes throughout the cell cycle. The position of the centrosomes is indicative of the stage of the cell cycle and is helpful in timing anoxia experiments. Chromosomes are marked by ruIs32 [*unc-119* (+) *pie-1::GFP::H2B*], which encodes a GFP labeled histone protein and is driven by a germ-line specific promoter[5]. Thus, the movement and localization of chromosomes within blastomeres or oocytes can be followed. Within a developing embryo, prior to anoxia-induced cell cycle arrest, a blastomere, and thus the nucleus, can move out of the focal plane making it necessary to adjust the focal plane while imaging. It is possible to follow more than one blastomere or nucleus but manual focusing on the cellular object of interest may need to be done to optimize visualization of the multiple cellular components of interest. This may limit the ability to automate the process unless one is using software to track a specific region and follow it. We have not tried to automate the process.

To optimize the protocol for one's specific assay one may consider various aspects of microscopy and genetic background of interest[13]. Transgenic animals have been successfully imaged for up to 2 hr in anoxia using this methodology (data not shown) and longer times have not been tested. We have analyzed embryos that are sensitive to anoxia (*npp-16(ok1839)*) using the same technique (*e.g.* same anoxia exposure time) as controls and found that the phenotype (in this case abnormal arrest of prophase blastomeres) can be captured within 30 min of exposure to anoxia[10]. Embryos can survive over three days of anoxia using other methodologies; we have not identified mutations that result in a resistance to anoxia exposure (survival extended past 3 days of exposure) in the embryo.

For our purposes, we are imaging a highly dynamic cellular process, and are using a spinning disk confocal microscope for rapid image acquisition. Depending upon the particular needs of an experiment, it will need to be determined whether a different kind of microscopy technique may be more appropriate.

When choosing or developing a transgenic strain for this method, it is important to consider the sensitivity of the microscope that will be used as well as the photobleaching tendency of the particular fluorophore. The use of time lapse imaging in this methodology may lead to significant photo bleaching in strains that do not strongly express a fluorescent protein.

There are several technical challenges and limitations to be considered when collecting images *in vivo* using this methodology. Because of the size of the chamber, and the potentially time consuming nature of the experiments, it may be difficult to analyze a large number of samples in a short amount of time. Experiments requiring large sample sizes can often be paired with other methods of anoxia exposure to generate large data sets, and *in vivo* imaging can be used to document, verify or more closely examine a phenotype of interest. Additionally, if the experiment requires examination at specific time points during a long-term exposure, issues with sample desiccation may arise. Despite being covered with halocarbon oil, animals may begin to desiccate due to continuous gas flow; therefore limiting the experimental time could alleviate this issue. Additionally, one can consider using hydrated gas or a chamber that allows humidity control to alleviate desiccation issues.

One advantage of this technique is that hypoxia experiments are also feasible. Thus, for additional oxygen deprivation studies one can simply use a gas tank with a specified oxygen concentration (*e.g.* 1% O_2 balanced with N_2) to examine responses to hypoxia rather than a completely anoxic environment. Additionally, another simple and flexible modification is the use of other gasses including O_2 levels above 21%, H_2S or CO; however, the safety of using these gas mixes is a major consideration and additional precautions must be taken[14].

Significance of the Technique

While we have outlined this method to expose *C. elegans* to anoxia and visualize hallmarks of anoxia-induced suspended animation within an embryo using very specific sub-cellular markers, these representative results are merely a single assay in a wide variety of experimental possibilities using the chamber system we describe here. Oxygen deprivation has been shown to affect a number of cellular processes in a wide range of organisms, and by exploiting this technique, specific effects of anoxia, hypoxia, and a variety of other environments can be visualized and captured *in vivo*. For example, using *C. elegans* this system could be used for analyzing other phenotypes induced by oxygen deprivation, as permitted with the use of the tricaine/tetramizole anesthetic (*i.e.* pharyngeal pumping and reproductive processes).

The application of this methodology is not limited to *C. elegans*. This gas flow through chamber setup provides a simple method for exposing cells and whole organisms to low concentrations of oxygen while simultaneously capturing cellular changes and visualizing activity of fluorescent protein fusions. For example, this method is readily adaptable to yeast, cell culture or small invertebrate or vertebrate embryos whose sizes are not too large for the chamber.

Disclosures

No conflicts of interest declared.

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References

1. Powell-Coffman, J.A. Hypoxia signaling and resistance in *C. elegans. Trends Endocrinol. Metab.* **21**, 435-440, [pii] S1043-2760(10)00041-X doi:10.1016/j.tem.2010.02.006 (2010).
2. Padilla, P.A. & Ladage, M.L. Suspended animation, diapause and quiescence: Arresting the cell cycle in *C. elegans*. *Cell Cycle.* **11**, [pii] 19444 (2012).
3. Hu, P.J. Dauer. In: *WormBook*, 1-19, doi:10.1895/wormbook.1.144.1 (2007).
4. Zhou, K.I., Pincus, Z., & Slack, F.J. Longevity and stress in Caenorhabditis elegans. *Aging (Albany NY).* **3**, 733-753, [pii] 100367 (2011).
5. Schmidt, D.J., Rose, D.J., Saxton, W.M., & Strome, S. Functional analysis of cytoplasmic dynein heavy chain in *Caenorhabditis elegans* with fast-acting temperature-sensitive mutations. *Mol. Biol. Cell.* **16**, 1200-1212 (2005).
6. Sulston, J. & Hodgkin, J. In: *The Nematode Caenorhabditis elegans.*, Wood, W.B., ed., Cold Spring Harbor Laboratory Press, 587-606 (1988).
7. Padilla, P.A., Nystul, T.G., Zager, R.A., Johnson, A.C., & Roth, M.B. Dephosphorylation of Cell Cycle-regulated Proteins Correlates with Anoxia-induced Suspended Animation in *Caenorhabditis elegans. Mol. Biol Cell.* **13**, 1473-1483 (2002).
8. Hajeri, V.A., Trejo, J., & Padilla, P.A. Characterization of sub-nuclear changes in *Caenorhabditis elegans* embryos exposed to brief, intermediate and long-term anoxia to analyze anoxia-induced cell cycle arrest. *BMC Cell Biol.* **6**, 47, [pii] 1471-2121-6-47 doi:10.1186/1471-2121-6-47 (2005).
9. Padilla, P.A., Goy, J.M., & Hajeri, V.A. In: *Anoxia.*, Padilla, P.A., ed., InTech, (2012).
10. Hajeri, V.A., Little, B.A., Ladage, M.L., & Padilla, P.A. NPP-16/Nup50 function and CDK-1 inactivation are associated with anoxia-induced prophase arrest in *Caenorhabditis elegans*. *Mol. Biol. Cell.* **21**, 712-724, [pii] E09-09-0787 doi:10.1091/mbc.E09-09-0787 (2010).
11. Nystul, T.G., Goldmark, J.P., Padilla, P.A., & Roth, M.B. Suspended animation in *C. elegans* requires the spindle checkpoint. *Science.* **302**, 1038-1041, [pii] 302/5647/1038 doi:10.1126/science.1089705 (2003).
12. Margalit, A., Vlcek, S., Gruenbaum, Y., & Foisner, R. Breaking and making of the nuclear envelope. *J. Cell Biochem.* **95**, 454-465 (2005).
13. Maddox, A.S. & Maddox, P.S. High-resolution imaging of cellular processes in *Caenorhabditis elegans. Methods Cell Biol.* **107**, 1-34, [pii] B978-0-12-394620-1.00001-1 doi:10.1016/B978-0-12-394620-1.00001-1 (2012).
14. Miller, D.L. & Roth, M.B. *C. elegans* are protected from lethal hypoxia by an embryonic diapause. *Curr. Biol.* **19**, 1233-1237, [pii] S0960-9822(09)01237-8 doi:10.1016/j.cub.2009.05.066 (2009).

