

Video Article

Measuring mRNA Levels Over Time During the Yeast *S. cerevisiae* Hypoxic Response

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URL: <https://www.jove.com/video/56226>

DOI: [doi:10.3791/56226](https://doi.org/10.3791/56226)

Keywords: Genetics, Issue 126, anaerobic, aerobic, transcriptome, next-generation, time course, yeast

Date Published: 8/10/2017

Citation: Willis, S.D., Hossian, A.K., Evans, N., Hickman, M.J. Measuring mRNA Levels Over Time During the Yeast *S. cerevisiae* Hypoxic Response. *J. Vis. Exp.* (126), e56226, doi:10.3791/56226 (2017).

Abstract

Complex changes in gene expression typically mediate a large portion of a cellular response. Each gene may change expression with unique kinetics as the gene is regulated by the particular timing of one of many stimuli, signaling pathways or secondary effects. In order to capture the entire gene expression response to hypoxia in the yeast *S. cerevisiae*, RNA-seq analysis was used to monitor the mRNA levels of all genes at specific times after exposure to hypoxia. Hypoxia was established by growing cells in ~100% N₂ gas. Importantly, unlike other hypoxic studies, ergosterol and unsaturated fatty acids were not added to the media because these metabolites affect gene expression. Time points were chosen in the range of 0 - 4 h after hypoxia because that period captures the major changes in gene expression. At each time point, mid-log hypoxic cells were quickly filtered and frozen, limiting exposure to O₂ and concomitant changes in gene expression. Total RNA was extracted from cells and used to enrich for mRNA, which was then converted to cDNA. From this cDNA, multiplex libraries were created and eight or more samples were sequenced in one lane of a next-generation sequencer. A post-sequencing pipeline is described, which includes quality base trimming, read mapping and determining the number of reads per gene. DESeq2 within the R statistical environment was used to identify genes that change significantly at any one of the hypoxic time points. Analysis of three biological replicates revealed high reproducibility, genes of differing kinetics and a large number of expected O₂-regulated genes. These methods can be used to study how the cells of various organisms respond to hypoxia over time and adapted to study gene expression during other cellular responses.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56226/>

Introduction

Many organisms respond to hypoxia, or low O₂, by altering gene expression^{1,2,3}. This response helps cells cope with the lack of a substrate critical for aerobic respiration and for several biosynthetic reactions, but also with a changing redox state⁴. Several microarray studies performed in *S. cerevisiae* show that the mRNA levels of hundreds of genes change in response to hypoxia^{5,6,7,8,9,10,11,12}. Recently, RNA-seq was used to characterize gene expression changes over time during hypoxia¹³. Here, the experimental details are presented and discussed.

Hypoxia can be achieved in various ways, each producing a different level of O₂. Here, hypoxia was established by continuously flowing ultra-high-purity N₂ into flasks, which lowers [O₂] dissolved immediately with reproducible kinetics¹⁰. It is possible that there are some O₂ molecules present that contribute to metabolism and gene expression but this environment is considered very close to anaerobic. In the absence of O₂, yeast cells cannot biosynthesize heme, ergosterol and unsaturated fatty acids^{4,12,14}. Thus, previous studies have included these metabolites when growing yeast without oxygen^{5,10,15}. However, many hypoxic responses are mediated by depletion of these metabolites and thus replenishing them reverses the hypoxic gene expression responses^{12,16}. In order to mimic natural hypoxia, these metabolites were not added to the media. In the short time that cells were exposed to hypoxia without the presence of these essential metabolites, there was no noticeable increase in cell death (data not shown) nor a prolonged stress response¹³.

The response is also dependent upon the strain and its genotype. Especially important are the alleles of the known regulators of hypoxic responses². The S288C strain background is highly desired so that results can be compared to the other genomic studies performed with this strain. However, S288C contains a partial loss-of-function allele of the *HAP1* gene¹⁷, a transcriptional regulator critical for the hypoxic response. This allele was repaired in S288C using a wildtype copy from the Σ 1278b strain background¹¹.

Gene expression is highly dependent upon the cellular environment. Thus, when performing genome-wide mRNA analysis, it is important to maintain a constant environment while varying another parameter such as time, stimulus, or genotype. To achieve highly reproducible results, consider these three practices for the study and all of its biological or technical replicates. First, the same experimenter(s) should carry out the study, since technical practices may vary across experimenters. Second, the same batch of ingredients should be used in the growth media

as each batch has a slightly different composition that can affect gene expression. Third, to minimize cell cycle effects, each time point should consist of asynchronous cells in the mid-log phase of growth ($1 - 2 \times 10^7$ cells/mL).

When characterizing a complex response like the gene expression response to hypoxia, a time course is advantageous for determining the kinetics of various events. Specific time points should be chosen that will capture the major changes of the response. In this study, time points between 0 and 4 h were observed, because past experiments revealed widespread changes in gene expression during this period¹³.

To measure global gene expression, RNA-seq was used^{18,19}. This method uses next-generation sequencing to determine the relative abundance of each gene's transcript. Compared to DNA microarray analysis, RNA-seq exhibits higher sensitivity (to detect less abundant transcripts), a greater dynamic range (to measure greater fold changes) and superior reproducibility (to accurately follow gene expression over time). Typically, most cellular RNA is ribosomal RNA so many methods have been developed to enrich for specific RNA species²⁰. Here, poly-T beads were used to purify poly-A-containing mRNA transcripts, though the various commercially-available rRNA depletion kits could also be effective in mRNA enrichment.

Here, the *S. cerevisiae* gene expression response to hypoxia was characterized. Cells were exposed to hypoxia and then sampled at eight time points (0, 5, 10, 30, 60, 120, 180 and 240 min). To confirm reproducibility and to identify statistically changed transcripts, three biological replicates were performed. RNA was extracted by mechanical disruption and column purification, and then processed for RNA-seq analysis. The post-sequencing pipeline is described and programming scripts are provided that allow exact replication of the analyses performed. Specifically, Trimmomatic²¹, TopHat2²², HTSeq²³, the R statistical environment²⁴, and the DESeq2 package²⁵ were used to process the RNA-seq data and to identify 607 genes that change significantly during hypoxia. Principal component analysis (PCA) and gene expression of the replicates indicated the reproducibility of the technique. Clustering and heatmaps revealed wide-ranging expression kinetics, while gene ontology (GO) analysis showed that many cellular processes, like aerobic respiration, are enriched in the set of oxygen-regulated genes.

Protocol

1. Inducing Hypoxia

- One day or more before the hypoxia time course: Prepare the incubator, cell filtering system, vacuum, gas tank, flasks, stoppers, glass tubing, and tubing, as in the **Materials Table**.
- Place the N₂ tank, incubator, vacuum and filtering system in close proximity, to enable quick processing of cells.
- Prepare sterile liquid YPD media (1% Yeast Extract, 2% peptone, 2% glucose) by mixing the components in a glass bottle and autoclaving.
- Plan layout of flasks in the incubator.
NOTE: From the N₂ tank, the first flask will be a water trap, the second flask will be the last time point, the third flask will be the second-to-last time point and so on until the last flask which is a final water trap. **Figure 1** shows the order and connections between the flasks.
- The day before the time course, inoculate a yeast colony into a culture of 5 mL liquid YPD within a sterile test tube. Specifically, pick up a full colony from a plate using a sterile applicator stick. Place the stick into the liquid YPD and shake the stick until most of the cells are in suspension.
- Rotate the tubes at 30 °C overnight (~16 h).
NOTE: After 16 h, wildtype cells will achieve saturation ($\sim 2 \times 10^8$ cells/mL), indicated by a very cloudy culture. Other strains may take longer to reach saturation and should be tested by measuring cell concentration with a spectrophotometer, as indicated in Step 1.9.4.
- On the day of the time course, after 16 h of incubation, dilute the saturated culture 1:50 by using a 5 mL pipet to place 4 mL of overnight culture into 196 mL of liquid YPD within a sterile 500 mL flask. Grow with shaking at ~200 rpm for 4 h at 30 °C.
NOTE: After 4 h, a wildtype culture will reach a mid-log concentration of $\sim 1-2 \times 10^7$ cells/mL.
- During the 4 h incubation, label the flasks (for hypoxia and water traps) and the 50 mL collection tubes. If the incubator in step 1.7 above is not used for the hypoxia time course, turn on the other incubator and set to 30 °C.
- Just before subjecting cells to hypoxia:**
 - Add ~50 mL water to two of the sterile 250 mL flasks which will serve as the water traps.
 - Fill $\frac{1}{3}$ of an ice bucket with liquid nitrogen and submerge a polystyrene foam rack to hold 50 mL centrifuge tubes.
 - Set up the filter system for collecting cells. Add a sterile filter disc onto the bottom unit of the filtration system using sterile tweezers, being careful to only touch the edges of the filter disc. Place the filter top unit onto the filter bottom unit and secure with the clamps provided with the system. Ensure that the bottom and top units are aligned so that there is a tight seal and no leakage.
 - Measure the cell concentration with a spectrophotometer. Dilute cells so that they can be measured in the linear range of the spectrophotometer – OD₆₀₀ between ~0.2 - 0.6, depending on the spectrophotometer.
NOTE: One OD₆₀₀ unit is $\sim 3 \times 10^7$ cells/mL, depending on cell morphology and spectrophotometer. A concentration of $\sim 1-2 \times 10^7$ cells/mL is expected, corresponding to OD₆₀₀ of ~0.33 - 0.66.
- Quickly obtain the time 0 sample.**
 - Connect the filter system to a strong (~10 mbar) vacuum via vacuum tubing and a 1,000 mL flask trap. Turn on the vacuum. Pour the culture (usually ~20 mL) into the top unit and wait for liquid to be pulled through the filter (~10 s, depending on the strength of the vacuum).
 - Carefully remove the filter disc with clean tweezers and place into a 50 mL centrifuge tube. Immediately place the centrifuge tube into the rack submerged in liquid nitrogen. Importantly, do not pre-chill the tubes before inserting the filter as the tubes will explode.
 - After >30 s, place the tube into a -80 °C freezer for later RNA extraction. After each filtration, clean and reassemble the filter system. Rinse the system by pulling water through for 10 s without a filter disc. Finally, wipe dry the system with a paper towel.
- Dilute the 4 h culture into different flasks for the various time points, so that each flask reaches mid-log concentration ($\sim 1-2 \times 10^7$ cells/mL) at the indicated time point of hypoxia.
NOTE: Table 1 shows dilutions that were used for the wild-type S288C *HAP1*⁺ haploid strain. It is recommended to test each strain in these hypoxic culture conditions and adjust the dilutions accordingly.

7. Once cells and media are added to flasks, replace the aluminum foil covering the flask opening with a stopper containing two glass tubes inserted.
8. Place the flasks in the incubator in the layout determined earlier.
9. Securely connect the tubing as shown in **Figure 1**.
10. Check that all the stoppers are tightly pushed into the flask openings.
10. At time 0, open the regulator valve. Then set the flow meter to 3 L/min.
NOTE: Bubbling will be observed in both water flasks, indicating proper gas flow. If this is not the case, check that all stoppers are tight and tubing is properly attached.
11. Close the incubator and set the shaking speed to ~200 rpm. Start a timer.
12. Before each time point, set up the filter system as describe above in step 1.9.3.
13. **At each time point (e.g., 5 min, 10 min, etc.), have two people quickly process the cells as follows:**
 1. First person: remove the appropriate flask from the holder and take out the stopper (with glass tubing attached).
NOTE: This will not break the flow of N₂ to any of the remaining culture flasks but will temporarily break the flow to the last water trap.
 2. Second person: Pipet 1 mL of culture and dispense into a cuvette. Reconnect the tubing from the culture flask that is now at the end of the line to the last water trap (One tube and one stopper will be removed in the process.). Then, measure cell concentration in the cuvette, as described above in step 1.9.4.
 3. First person: Pour the remainder of the culture into the vacuum filtration system, and then perform filtering and freezing as described above in step 1.9.5.
14. When finished with all the time points, turn off the N₂ gas. First close the regulator and wait for the pressure to release, and then turn off the flow meter. Finally, disassemble the system and clean all materials with water and then 70% ethanol.

2. RNA Extraction

1. Prepare RLT buffer for RNA column purification, as described in the **Materials Table**.
2. Prepare a working solution of DNase by adding 10 μ L of the DNase stock solution to 70 μ L of buffer RDD per sample (see **Materials Table**).
3. Remove the 50 mL tubes containing filters and cells from the -80 °C freezer and place on ice to thaw (~15 min). Perform the following steps with tubes on ice.
4. Label 2 mL screw-cap tubes and place them on ice, one tube for each sample. Add ~0.6 mL of acid-washed beads to each tube, using a 1.5 mL microcentrifuge tube to scoop and measure the beads.
5. Add 0.6 mL of cold RLT buffer to the 50 mL tubes.
6. Pipet up and down to remove cells from filter and suspend into solution. Avoid letting the filter remain in the solution as the filter will soak up the solution. Remove all solution absorbed into the filter by using the pipet tip to squeeze the filter against the wall of the tube.
7. Transfer all of the liquid from the 50 mL tube to the screw-cap tubes containing beads.
8. Place the screw-cap tubes into a bead mill homogenizer and run for one min.
Immediately place the tubes on ice for three min. Again, place the tubes into the homogenizer and run for one min.
9. Remove the tubes from the homogenizer and place on ice for 5 min. The beads will settle to the bottom of the tubes.
10. Perform the remainder of the steps at room temperature.
11. With a pipet, transfer only the lysate (~350 μ L), avoiding the beads, to a new 1.5 mL microcentrifuge tube.
12. Microcentrifuge for 2 min at max speed and then transfer the supernatant to a new 1.5 mL microcentrifuge tube.
13. Add 1 volume of 70% ethanol (made from 200 proof molecular biology-grade ethanol). Mix well by pipetting.
14. Transfer the solution and any precipitate to an RNA column that has been placed inside a 2 mL collection tube.
15. Centrifuge for 15 s at $\geq 12,000 \times g$ and discard the flow-through (the liquid in the tube).
16. Add 350 μ L of buffer RW1 to the column. Centrifuge for 15 s at $\geq 12,000 \times g$ and discard the flow-through.
17. Add 80 μ L DNase I incubation mix to the column membrane (do not get on sides of tube) and allow to sit for 15 min.
18. Add 350 μ L of buffer RW1 to column. Microcentrifuge for 15 s at $\geq 12,000 \times g$ and discard the flow-through.
19. Add 500 μ L of buffer RPE to the column. Microcentrifuge for 15 s at $\geq 12,000 \times g$ and discard flow-through.
20. Add 500 μ L of buffer RPE to the column. Microcentrifuge for 2 min at $\geq 12,000 \times g$.
21. Carefully remove the column and place into a new 2 mL collection tube. Microcentrifuge at full speed for 1 min (to completely dry the membrane).
22. Discard the collection tube and place the column into a 1.5 mL microcentrifuge tube. Add 30 μ L of RNase-free water to the column membrane.
23. Elute the RNA from the column by microcentrifuging for 1 min at $\geq 12,000 \times g$. When loading the columns into the microcentrifuge, make sure the lids of the collection tube are facing in the direction the centrifuge spins, to prevent the lids breaking off.
24. Add another 30 μ L of RNase-free water to the column membrane, while keeping the column in the same microcentrifuge tube.
25. Microcentrifuge for 1 min at $\geq 12,000 \times g$, so that the final eluate volume is 60 μ L.
26. Store each 1.5 mL tube containing 60 μ L of RNA in the -80 °C freezer.

3. Determining RNA Concentration and Quality

1. Measure the concentration of RNA and DNA in each RNA sample, using (a) a fluorometer, (b) DNA- and RNA-specific fluorescent dyes and (c) assay tubes, as listed in the **Materials Table**. Follow the instructions of the fluorometer and the dyes.
2. Alternatively, measure the nucleic acid concentration with a UV spectrophotometer set at 260 nm.
NOTE: An A₂₆₀ reading of 1.0 is equivalent to ~40 μ g/mL single-stranded RNA.
3. Test RNA quality by running the RNA on a commercial nucleic acid analyzer (see **Materials Table**) or on a standard formaldehyde/agarose gel²⁶.

4. RNA-seq Analysis

1. From the total RNA stock, make up 21 μL of 100 ng/ μL RNA in RNase-free water. Use 1 μL of this 21 μL to test the RNA concentration as above. Submit the remaining 20 μL (2 μg) total RNA to an external sequencing center for mRNA enrichment, strand-specific library preparation (with eight or more barcodes for multiplexing) and sequencing (see **Materials Table**). Alternatively, locally perform mRNA enrichment and library preparation, and then submit the library for sequencing.
2. Pool eight or more multiplexed samples and sequence in one lane of a next-generation sequencer.
3. Download the FASTQ file containing all of the sequence reads and the corresponding index reads. Also, download the text file containing the multiplex barcodes.
NOTE: The index reads may be present in a separate FASTQ file. Place all of these files into one directory.
4. Place the shell script provided here, "fastq_pipeline.sh", in the same directory. Edit this script as appropriate for the experiment and the computer directories.
NOTE: this script contains extensive annotations explaining the steps. In brief, the script quality trims the reads, maps the reads to the genome, and generates a tab-delimited file containing the number of reads per gene for each sample.
5. Deposit the FASTQ files and raw read count data into NCBI's Gene Expression Omnibus before publication²⁷. Follow the instructions for "Submitting high-throughput sequence data to GEO": <https://www.ncbi.nlm.nih.gov/geo/info/seq.html>.
6. Import the read count data into the R statistical environment and perform statistical analysis, using the R script provided here, "time_course_script.R". Edit this script as appropriate for the experiment and the computer directories.
NOTE: This script contains extensive annotations explaining the steps. In brief, this script imports the read data, normalizes the reads, identifies genes that change significantly during the time course, performs PCA analysis, and graphs the expression of selected genes.

Representative Results

The hypoxia time course and RNA-seq analysis were performed independently three times. To examine the reproducibility of the three replicates, gene expression data for all genes was analyzed using Principal Component Analysis (PCA). **Figure 2** shows how the samples change over the first two principal components, which together represent 58.9% of the variability. This analysis indicated that each time course exhibits similar changes (as depicted by the similar shape of each curve). In addition, the last two replicates were more similar to each other than to the first replicate. This is consistent with the fact that the last two replicates were performed by the same person using the same batches of media components, while the first replicate was performed by a different person and batches. This finding highlights the fact that consistency of technique and chemicals is an important factor in obtaining high reproducibility. Lastly, PCA analysis is useful in assessing whether the chosen time points capture the major changes that occur during the time course. Focusing on one replicate curve, there are larger distances between the earlier samples, indicating large changes in gene expression, and smaller distances between the later samples, indicating smaller changes and possibly the end of the switch from aerobic to hypoxic gene expression.

Next, the DESeq2 package in R²⁵ was used to identify genes showing a significant change compared to time 0 (p-values in **Supplementary Table 1**). Of these significant genes, 607 changed more than 4-fold (fold changes in **Supplementary Table 1**). The expression patterns of these genes were clustered, so that similarly-regulated genes were grouped together, and then displayed in a heatmap (**Figure 3**). This figure shows that expression changes are highly reproducible across replicates. Also, genes exhibit variable kinetics with both increases and decreases in expression. Many genes exhibit a peak increase or decrease at 30 min, likely due to a transient stress response¹³.

Figure 4 depicts the expression of two downregulated and two upregulated genes previously found to be O₂-regulated. The genes change expression reproducibly, with similar curves between the biological replicates. The gene with the least variability between replicates is *DAN1*, with overlapping curves. The gene with the most variability is *CYC1*, perhaps because expression of this gene is naturally variable. This figure also illustrates that large fold changes (up to 1,024-fold) were detected.

To identify the cellular processes enriched in the set of 607 O₂-regulated genes, GO Term enrichment was performed (**Supplementary Table 2**). As expected, many O₂-regulated genes play a role in cellular respiration, other aspects of metabolism, and in ribosomal processes¹³.

Sample#	Time in Hypoxia	mL culture + mL YPD
2	5 min	20 mL + 0 mL
3	10 min	20 mL + 0 mL
4	30 min	20 mL + 0 mL
5	60 min	10 mL + 10 mL
6	120 min	10 mL + 10 mL
7	180 min	5 mL + 15 mL
8	240 min	4 mL + 16 mL

Table 1. Cell Dilutions Performed at Time 0.

These dilutions have been experimentally determined to result in mid-log concentration ($1 - 2 \times 10^7$ cells/mL) after the indicated time in N₂.

Supplementary Table 1. Expression and Statistical Data for all Genes.

This table contains the systematic name, the adjusted p-values for seven DESeq2 tests (*i.e.*, 5 min vs. 0 min, 10 min vs. 0 min, *etc.*), the minimum adjusted p-value, and the log2 maximum fold-change. [Please click here to download this file.](#)

Supplementary Table 2. GO Term Enrichment Results.

This table shows GO Processes, their representation in the set of 607 O₂-regulated genes and their representation in the genome. Then, the chi-square test was used to identify GO Terms that were significantly over- or under-represented in the set of 607 genes. GO enrichment analysis was performed using GO Slim Mapper at SGD²⁸. [Please click here to download this file.](#)

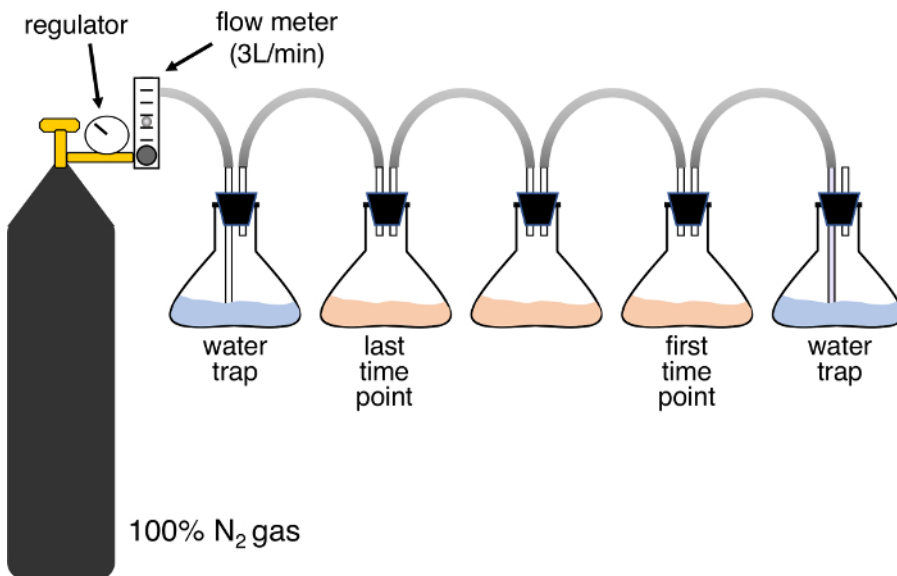


Figure 1. Hypoxia Set-up for Taking Time Points without Disrupting Gas Flow to Later Time Points.

It is important that all of the connections remain secure, as described in the protocol. Note the location of the short (9 cm) and long (17 cm) glass tubes. [Please click here to view a larger version of this figure.](#)

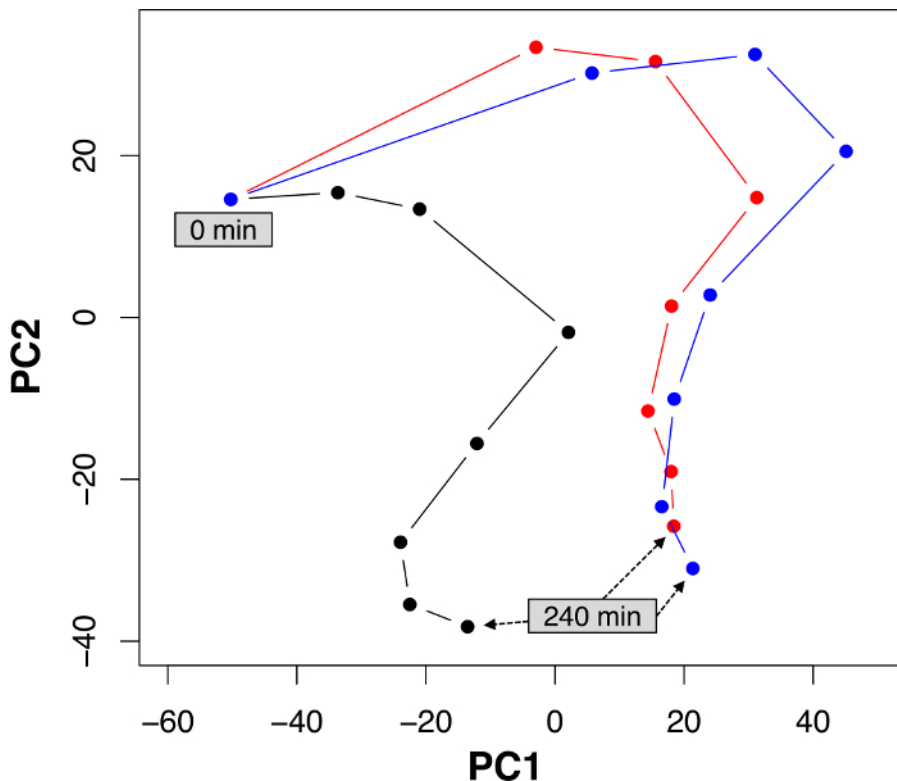


Figure 2. Principal Component Analysis (PCA) Shows the Relationship Between the Three Biological Replicates.

Each curve has the 0 min to 240 min samples in order. The log₂ normalized gene expression for all genes was used in the PCA. PC1 accounts for 34.7% of the total variance and PC2 accounts for 24.2%. The black line is the first replicate, the red line is the second, and the blue line is the third. Note that the arrows are being used to point out the 240 min time-point in different samples. Principal component analysis (PCA) was carried out in R by using the *prcomp* function (Q-mode, or singular value decomposition) on the log₂-transformed matrix containing genes in columns and time points in rows²⁹. [Please click here to view a larger version of this figure.](#)

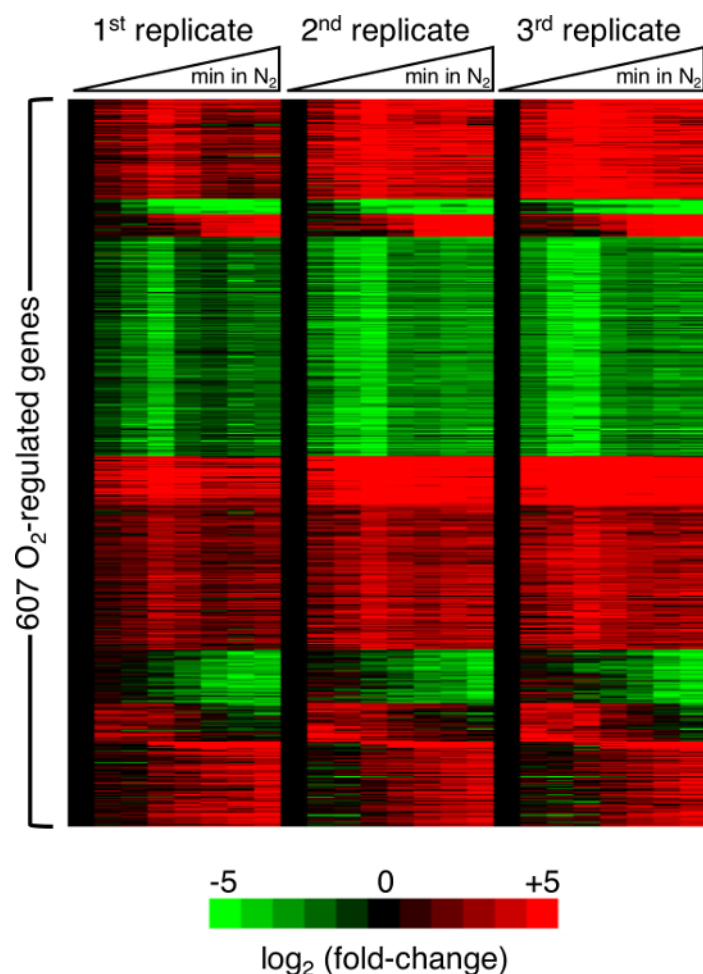


Figure 3. Heatmap Showing Expression of the 607 Genes Identified as O₂-regulated.

These genes were significant in at least one of the DESeq2 tests and changed expression by more than 4-fold. Shown is log₂(relative expression). Red = increased expression. Green = decreased expression. Intensity of color is proportional to degree of change. Before creating the heatmap in TreeView³⁰, gene expression was k-means clustered with k=10 in Cluster 3.0³¹. The key below the heatmap shows the scale. [Please click here to view a larger version of this figure.](#)

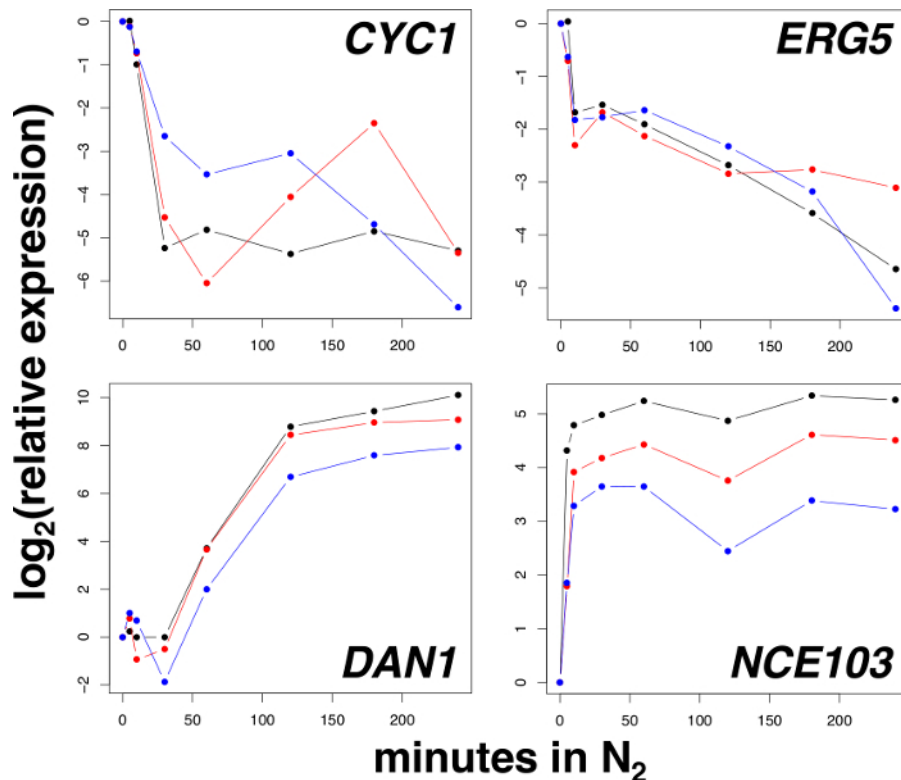


Figure 4. Relative mRNA Levels of Four Genes Over Time in the Three Replicates.

The black line is the first replicate, the red line is the second, and the blue line is the third. Common/systematic gene names are *CYC1/YJR048W*, *ERG5/YMR015C*, *DAN1/YJR150C*, and *NCE103/YNL036W*. [Please click here to view a larger version of this figure.](#)

Supplemental File 1. fastq_pipeline.sh

[Please click here to download this file.](#)

Supplemental File 2. time_course_script.R

[Please click here to download this file.](#)

Discussion

In this study, the mRNA levels for all genes was measured during hypoxia in the yeast *S. cerevisiae*. The goal was to analyze how global gene expression changes due to growth in a controlled near-anoxic environment. Several steps were taken to ensure that the method described here was carefully controlled and reproducible. First, cells were exposed to a precisely defined hypoxic environment: 99.999% N₂ in rich media (YPD). Other studies of hypoxia have closed off the flask or tube to air³², used the hypoxia-mimetic cobalt chloride³³, or employed hypoxia-inducing anaerobic chambers or bags³⁴. Each of these methods may result in varying levels of hypoxia or other unintended environmental changes. It would be useful to study gene expression while systematically varying the gas mixture (e.g., 90% N₂ and 10% O₂), as yeast in the wild are likely to experience less-severe hypoxic conditions. Second, ergosterol and unsaturated fatty acids (e.g., Tween 80) were not added to the culture media, since they influence gene expression as discussed in the Introduction. Third, to minimize gene expression changes caused by different phases of growth, empirically-determined dilutions were performed so that when a culture reached a time point, it was in the mid-log phase of growth (~1 - 2 x 10⁷ cells/mL). Fourth, the quick filtering and freezing (~15 s) of cells that have been removed from hypoxia is critical, as gene expression changes can occur in <5 min of exposure to O₂, as occurs when cells are collected by centrifugation (data not shown). The time that cells are exposed to O₂ could be reduced by performing the growth and harvesting of cells in an anoxic hood or chamber. Fifth, an appropriate strain was chosen for this study; to be consistent with other genomic studies, the common S288C lab strain background was employed. However, the S288C strains contains a mutant *hap1* allele and thus was repaired¹¹. This allowed the study of genes like *CYC1* that respond to O₂ levels via the Hap1 transcription factor¹¹.

Time points were chosen because they exhibited large gene expression changes in previous hypoxia experiments. The results shown here can inform future experiments. For example, it would be helpful to use a higher resolution of time points during intervals that show large changes (e.g., 0 to 30 min hypoxia) to more finely capture the diverse kinetics. Additionally, later time points, beyond the time period assayed here (i.e., 4 h of hypoxia) may reveal further expression changes.

RNA-seq was used to measure mRNA levels because its reproducibility and wide dynamic range allow measurement of large fold changes^{18,35}. RNA was extracted by mechanical disruption of the cells using vigorously shaken beads and purified on a column. Other RNA purification methods could be used, such as hot phenol³⁶. Ideally, the RNA concentration will be in the range of 200 ng/μL - 1000 ng/μL since the RNA concentration will be diluted to 100 ng/μL for reverse transcription and library preparation. The RNA concentration should be measured with a fluorometer and RNA-specific dye; a UV spectrophotometer is limited because it measures the total concentration of nucleic acids, consisting of both RNA and DNA. The quality of the RNA is determined by gel electrophoresis; ribosomal RNA bands should be well-defined on the gel and

smearing indicates RNA degradation. Here, mRNA transcripts were enriched, but there are diverse methods for isolating different RNA types²⁰ that may also be important in the response.

To save resources, between 8 and 12 samples were multiplexed and sequenced together in one lane, resulting in an average of at least 693 reads per gene for each sample, sufficient to reproducibly determine the number of reads per gene. In fact, more than 12 samples could be multiplexed in one lane, likely resulting in adequate sampling of most genes. In order to calculate the average reads per gene, the total number of reads that mapped to genes (in the HTSeq output file) was divided by the number of genes provided to HTSeq (in this study, 7140). When estimating the maximum number of samples that can be multiplexed in one lane, consider genes of interest that exhibit the lowest expression (*i.e.*, lowest normalized counts), using data from this and other RNA-seq studies. If the normalized read counts for a gene vary significantly across replicates, then the number of reads may be too low, suggesting that less samples should be multiplexed per sequencing lane.

Next-generation sequencing will generate FASTQ files containing the reads and other information. If multiplexing was performed, a barcode file and an additional FASTQ file may be generated. These files can be downloaded for local analysis, as in this protocol. Alternatively, there are several online next-gen sequence analysis tools for those not familiar with command-line functions or R. In this regard, we recommend Galaxy (<https://usegalaxy.org/>)³⁷ and GenomeSpace (<http://www.genomespace.org/>). In the present protocol, two scripts that have been written by the authors are used for FASTQ processing and expression analysis. The scripts are well-annotated and can be edited for different experimental designs and computer setups. Also, the two scripts and the "Materials" Table provide websites for downloading the tools used in these scripts. The first script, "fastq_pipeline.sh", is a shell script to be run at the command line in a UNIX/Linux terminal. This script de-multiplexes the original FASTQ file containing all of the reads obtained from one lane of the sequencer, thus generating one FASTQ file for each sample present in that lane. Next, the reads in each FASTQ file are quality trimmed using Trimmomatic with default settings²¹. The trimmed reads are then mapped to the *S. cerevisiae* S288C reference genome (SGD R64-1-1_20110203) using TopHat2 with default settings²². The script finally uses HTSeq to determine the number of reads that map to each annotated feature¹⁹.

The second script, "time_course_script.R", has also been written by the authors and is run within the R statistical environment²⁴. The script initially imports into R the read count data generated by HTSeq. Each sample is assumed to be a time point and thus is organized relative to the other time points. The raw read counts are then imported into the R package, DESeq2²⁵, in order to identify genes that are differentially expressed at any of the time points relative to the first (*i.e.*, aerobic, time 0). Because of its flexibility, DESeq2 can be used to perform other statistical tests, such as whether expression changes as a function of time¹³. Alternatively, other tools employing parametric and non-parametric statistics for RNA-seq read count data could be used²⁰. The script then normalizes the read counts to control for the degree of sequencing of each sample, and log2 transforms the counts. These processed reads are used to perform PCA analysis, to determine the maximum fold-change for each gene, and to create the expression graphs in **Figure 4**.

An important issue is how best to employ statistics to identify genes that respond significantly to hypoxia. At least three biological replicates of the time course are necessary to calculate the natural variability of each gene and thus determine genes that respond to hypoxia more significantly than expected by chance. Alternatively, it is possible to successfully identify genes that respond over time, without biological replicates^{13,25,38,39}. The main drawback to these methods is that they do not take into account the biological variability of each gene. However, if RNA-seq replicates are not performed, individual gene expression changes could be verified by performing RT-qPCR with multiple biological replicates¹³.

There are two primary benefits to assessing multiple time points of a response. First, as discussed earlier (especially in **Figure 2**), a time course identifies the intervals exhibiting large expression changes, informing whether additional time points are required to improve resolution of kinetics or to observe later events of the response. Second, a time course allows the differentiation of each gene's expression kinetics. This helps with identifying unique signaling pathways that act at specific times during the response, and gives insight into the timing of initiation and termination of signaling. Specifically, the clustering of genes by expression pattern that was performed here resulted in sets of co-regulated genes. The promoters of a gene set may share a transcription factor binding site, suggesting that the transcription factor becomes active when the genes change expression.

When studying a cellular response, the observed variability is ideally due to the stimulus (*e.g.*, hypoxia) and not to other experimental variables. However, as seen in **Figure 2**, the individual researcher and/or the batches of the media components make major contributions to the variability in gene expression. Thus, these parameters should be considered in order to achieve high reproducibility and minimal experimental variability. As little time as possible should separate each replicate, because experimental variables are more likely to change as time passes.

In conclusion, this method can be used to accurately monitor genome-wide alterations in mRNA levels (or other events such as histone modifications or protein levels) during a hypoxia time course. The method can be adapted for other organisms, especially microbial species that can be grown in liquid culture. Such genome-wide time-course analyses will complement studies of cell morphology, protein activity and metabolism. Together, these data will lead to a richer understanding of a cellular response.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We thank the Lewis-Sigler Institute for Integrative Genomics Sequencing Core Facility at Princeton University for technical advice and for RNA library preparation and sequencing. This work was supported by grants from Rowan University and NIH NIGMS R15GM113187 to M.J.H.

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